Scholars Academic Journal of Biosciences (SAJB) **ISSN 2321-6883 (Online)**
Sch. Acad. J. Biosci., 2016; 4(2):132-143 **ISSN 2347-9515 (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com DOI : 10.36347/sajb.2016.v04i02.010

Original Research Article

In vitro and in vivo toxicity study of Kariuppu mezhugu (NIS KM) a Siddha Herbo- mineral formulation

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Abstract: Kariuppu Mezhugu (NIS KM) is a Herbo mineral Siddha medicinal formulation featured in the treatise, Anuboga Vaithya Navaneetham. The objective of this study is **t**o carry out acute and repeated dose 28-day oral toxicity study of Kariuppu Mezhugu (NIS KM) in Wistar albino rats. In acute study (OECD 423), NIS KM was administered orally at 2000mg/kg/bw and animals were observed for toxic signs at 0.5, 1, 4, 24 hours and for the following 13 days. In repeated dose-28 day toxicity study (OECD 407), NIS KM was administered at 200, 400 and 800mg/kg body weight/day to 6 groups of rats, including vehicle control and satellite control. Mortality, toxic signs, body weight, food and water consumption, haematological, serum electrolytes and plasma biochemical parameters, gross necropsy, relative organ weights and histopathology were performed to substantiate No-Observed Adverse Effect Level (NOAEL) and lowest observed adverse effect level (LOAEL). A satellite group for NIS KM high dose (800 mg/kg) was also included in the study to determine the delayed occurrence of toxic effects. Students't' test was used for statistical analyses. NIS KM at single 2000mg/kg dose produced no treatment related toxic signs or mortality during study. In the repeated dose study, no significant differences in body weights, haematological, serum electrolyte and biochemical parameters were observed between Palm Jaggery solution control and NIS KM rats. Relative organs weights, gross necropsy and histopathological examination revealed no abnormalities with NIS KM treatment.: Results of the present study suggest that LD_{50} of NIS KM >2000mg/kg and NOAEL >800mg/kg/day in rats and hence 100 mg/kg/day administered in humans is validated as safe therapeutic dosage.
Keywords: *Kariuppu Mezhugu*, Dysmenorrhoea, acute oral toxicity, repeated oral toxicity, OECD guidelines.

INTRODUCTION

Siddha System of medicine is an ancient practice of medicine still surviving the test of time. Remedies found in this system are the oldest modalities for the treatment of non-communicable and infectious diseases as well. According to a WHO estimate about 80% of population in developing countries depend exclusively on traditional medicine[1]. Yet they are not encouraged at large in the developed countries because many traditional medicinal formulations have not been scientifically proved for their safety and efficacy. Many formulations mentioned in Siddha system of medicine are Herbo-mineral in the making with excellent efficacy observed in routine clinical practice confined to South India. Among the 32 types of pharmaceutical dosage forms of internal medicine mentioned in Siddha system 'Mezhugu' is an unique form with a waxy consistency and a shelf life of 5 years[2]. Active principle(s) and the chemical complexes present in this medicinal formulation exert significant physiological and pharmacological actions in the biological system. Therefore at the outset it is pertinent that the safety assessments should be conducted as a part of validation process on this Siddha formulation for which certain medicinal uses have been claimed traditionally.

Kariuppu Mezhugu (NIS KM), a Siddha Herbo-mineral formulation has its chief ingredient as 'Kariuppu' (Table salt-Sodium Chloride) which is the start up ingredient to be processed with Ripe, yellowish Calotropis leaves juice [3]. *NIS KM* is well known for its usage in the treatment of abdominal pain which includes dysmenorrhoeal pain or menstrual cramps. It is also indicated in the traditional Siddha text so as to correct the irregular menstrual cycle[3]. Another important herbal ingredient is *Calotropis* (Milk weed/Madar Plant) which is a medium sized hardy shrub grown as weed in the tropics and is used in traditional Indian medicine.

Preliminary X-Ray Fluorescence spectroscopy (Bruker S8 Tiger) analyses of NIS KM done at the Drug Testing Laboratory, SASTRA University, Thanjavur, Tamil Nadu, India showed the presence of Sodium

Oxide (30.61%), Chloride (41.95%), Potassium Oxide (11.22%), Sulphur trioxide, (6.36%) Calcium Oxide,(2.63%) aluminium trioxide and other trace elements. The heavy metal concentration of Mercury, lead, chromium and Arsenic were well within the WHO permissible limits[4,5]. Many Siddha formulations using salts of various types have been indicated for such conditions of cyclical menstrual pain[3]. Therefore, efforts are now being made in our laboratories to establish its anti dysmenorrhoeal activity by in vitro and in vivo methods including clinical trial. Before the initiation of in vivo therapeutic screening, we planned to ascertain the safety profile of *NIS KM* by acute and repeated dose 28-day studies in rats. This communication would be the first of its kind to summarize the toxicity/safety information on *NIS KM*.

MATERIALS AND METHODS

Drugs and reagents

NIS KM was prepared in the Gunapadam (Siddha Materia medica) Laboratory of National Institute of Siddha (NIS), Chennai an Autonomous Institute under Ministry of AYUSH, Govt of India as per the textual reference.[3] Erba® Mannheim XL system packs of Clinical Chemistry reagents were purchased for diagnostic biochemical tests of animal samples and run in the fully automated biochemistry Analyser - Erba® Mannheim, Germany at NIS. All other chemicals and reagents used were of analytical grade.

Animals and Husbandry

Male and female Wistar albino rats weighing 130-160g (initial weight) were used in the study. Animals were housed in groups (3-5/cage) in polypropylene cages in a well ventilated room (air cycles: 15/min; 70:30) under an ambient temperature of 23±2°C and 40–65% relative humidity, with a 12-h light/dark artificial light cycle. They were provided with food (Nutrilab Rodent, Tetragon Chemie Pvt Ltd, India) and purified water ad libitum. All the animals were acclimatized at least for 7 days to the laboratory conditions prior to experimentation. Guidelines of "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed all through the study. Institutional Animal Ethical Committee (IAEC), C.L.Baid Metha College of Pharmacy, Thoraipakkam Chennai, India approved the study (IAEC-XLIV/21/CLBMCP/2014)

Formulating procedure of NIS KM

Kariuppu (Table salt), Vediuppu (Salt Petre), Seenakkaram (Alum), Indhuppu (Rock Salt), and Vengaaram (Borax) were purchased from the Country Medicine shop, Broadway, Chennai and the herbs including ripe Calotropis leaves were collected from the local surroundings of Tambaram, Chennai and were authenticated by Dr.S.Ravikumar, Asst Professor, Department of Plant Biology and Plant Biotechnology, Presidency College (Autonomous), Chennai-600 005, India. Ripe leaves of Calotropis gigantea R.Br (Accession No. HPRKGJC2015006) were washed thoroughly with water to remove the dust and matter and freed from debris. Similarly other herbs *Citrullus colocynthis* (L.) Schrader (Accesion No.HPRKGJC2015003), *Moringa tinctoria* Roxb (HPRKGJC2015005), *Ferula asafoetida* Linn (HPRKGJC2015004), *Aegle marmelos* (L.) Correa (HPRKGJC2015001), Carica papaya Linn. (HPRKGJC2015002) *and Allium sativum* Linn (HPRKGJC2015007) were cleaned. Mineral salts like Alum, Potassium nitrate, Borax and Rock salt were authenticated at the Dept. of Geology, University of Madras. Then the salts were heated in an earthenware gradually been charged with the above herbal juices starting from Ripe leaves juice of *Calotropis gigantea* for 12 hours, *Citrullus colocynth* fruit juice, *Carica papaya* leaves juice, *Allium sativum* juice, *Morinda tinctoria* leaves juice, *Ferula asafoetida* decoction and *Aegle marmelos* leaves juice for a period of 5 hours altogether until the formulation NIS KM attained a waxy consistency before it was bottled up.

Standardization of NIS KM

The *NIS KM* extract was standardized for its fractional content by high performance thin layer chromatography (HPTLC) using Camag Linomat applicator V and TLC Scanner III. NIS KM (Track 1-5µl of Sample & Track 2-10µl of Sample) was dissolved 5g in 100ml of methanol, applied on pre-coated silica gel plates (Merck $60F_{254}$) and developed using the solvent system: Toluene: Ethyl acetate: Formic acid (5:4:1) up to 8 cm. Developed plates were dried and scanned at 254nm & 366 nm. Fractional contents in *NIS KM* were recorded from the peak areas for Finger printing. The particulate size of *NIS KM* was calculated by using Scanning Electron Microscope at Sophisticated Instrument Facility (SAIF), IIT Madras. Elemental analysis and screening for heavy metal analysis was done by Inductively Coupled Plasma with Optical Emission Spectroscopy at IIT Madras.

Invitro Toxicity of NIS KM by MTT Assay

Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Minimum Essential Medium (MEM) Dulbecco's modified eagle medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

Cell lines and Culture medium

MCF-7 (Human breast Carcinoma) and H_9C_2 (Rat Cardiac cell) cell line were procured from National Centre for Cell Sciences (NCCS), Pune, India. MCF-7

and H_9C_2 Stock cells were cultured in MEM and DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS) respectively, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For Cytotoxicity study, the weighed test drug NIS KM was separately dissolved in distilled DMSO and volume was made up with MEM and DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out Cytotoxicity studies.

Determination of cell viability by MTT Assay[6]

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using MEM/ DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of the test drug NIS KM were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilise the formed formazan. The absorbance was measured using a micro plate reader at the wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line. The tests were performed at Kovai Medical Centre College of Pharmacy, Coimbatore, India.

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

Acute Oral toxicity

Acute oral toxicity was conducted in accordance with Organization for Economic Cooperation and Development (OECD) TG 423 (adopted – December, 2001) with slight modification. Young adult female (non-pregnant and nulliparous) Wistar Albino rats of 140-160g body weight were used for the study. Seven days after acclimatization, the animals were randomized as (vehicle) control and treatment groups. Control group received 5% Palm Jaggery Solution with R.O. Water and treatment group was administered with NIS KM, 2000mg/kg body weight prepared in 0.3% Palm Jaggery solution at a single oral dose. Immediately after administration observations of mortality, morbidity and clinical signs of toxicity were started to be recorded at the 30 min, 1h, 2h, 4h and for the next 13 consecutive days. Body weight was recorded once in a week, at the end of 14th day animals were necropsied and organs were observed for gross pathological changes.

Repeated dose 28-day oral toxicity study

Repeated dose oral toxicity was conducted in accordance with Organization for Economic Cooperation and Development (OECD) TG 407(adopted on April 2006) with slight modification. Young adult WA rats weighing 140-160g of both the sex was used for study. Animals were divided into six groups of 10 animals (5 male and 5 female). Group I (n=10, 5males and 5 females) was treated as vehicle control, 5% Palm Jaggery solution with R.O Water; group II(n=10, 5males and 5 females) received 200 mg/kg, group III (n=10, 5males and 5 females) received 400mg/kg and group IV(n=10, 5males and 5 females) 800 mg/kg. Satellite groups of Group V and VI treated as vehicle control and NIS KM high dose (800 mg/kg) were included to determine the delayed occurrence, or persistence of, or recovery from toxic effects. All the animals in the control and treatment groups were treated once daily for 28 consecutive days and the satellite group was observed for another 14 days without the administration of drug and necropsied at the end of 42 days. Toxic manifestations such as signs of toxicity and mortality were monitored daily. Body-weight changes and feed and water intake was monitored once in a week. Animals were observed individually after drug administration for morbidity and mortality throughout the study period. Rats were monitored for mortality and clinical signs of toxicity. Body weight was measured at an interval of 7 days. Rats were over night fasted, blood samples were collected from the retro-orbital puncture under diethyl ether anaesthesia with and without anticoagulant and used for haematological and biochemical parameters.

Hematological parameters like WBC, RBC, Hb, HCT, MCV, MCH, MCHC, platelet and MPV were analysed using Sysmex Corporation Japan ® Fully automated Veterinary haematology analyser. Plasma was separated and used for the estimation of glucose, triglyceride, cholesterol, creatinine, urea and total proteins with System Packs using Erba Mannhein Germany ® Fully automated biochemical analyser. Enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and other constituents were also estimated in

plasma. Serum electrolytes like total calcium, potassium, sodium, chloride, calcium and pH were estimated using Roche ® Ion Specific Liquid membrane electrode electrolyte analyser.

Histopathology examinations of organs

Terminal necropsy was done on all animals on day 29 except the satellite which were done on day 42. After blood collection, animals were anesthetised with ether and necropsied. Vital organs like brain, heart, liver, kidneys and spleen were observed for gross lesions and organs were weighed for changes in relative organ weights. Tissues were fixed in 10% formalin and sections of 5–6 mm were routinely stained with haematoxylin and eosin (H & E) and examined under a light microscope (Olympus). Changes if any, in the treatment group were compared with the control group.

Statistical analysis

Data was presented as mean ± standard error of mean. Data were subjected to statistical analysis using Student't' test using Graph Pad prism 4.0 to determine significant difference between the means. $P \leq 0.05$ was considered as significant.

RESULTS

Standardization of Test Drug NIS KM

Preliminary physico-chemical analysis and quantitative analysis revealed the presence of elemental composition of Sodium, Chloride, Potassium, Sulphur, Aluminium, other trace elements and Flavonoids. (Detailed results not presented here). Heavy metals were within the WHO permissible limits. HPTLC chromatogram of NIS KM extract revealed the finger print pattern in the given chromatographic condition. The Scanning electron microscope image shows the nano sized structure of the particles in conglomeration **(Figure 3).**

***In vitro Cytotoxicity study* – MTT assay**

The results of In vitro Cytotoxicity concentration of DSMO, MEM and DMEM extract of NIS KM in MTT Assay method in MCF-7 (Human breast Carcinoma cell line) and H_9C_2 (Rat Cardiac myocyte) cell lines. CTC 50 values are furnished in table Nos. : 1 and 2).

Acute oral toxicity study

In this present study, acute oral toxicity of NIS KM was performed following the OECD test guidelines 423. NIS KM at a dose of 2000mg/kg, b.wt produced no mortality or signs of treatment related behavioural toxicity in the animals during 14 days of the study. There was no loss in body weight in the vehicle control or NIS KM administered rats and no significant difference in weight gain between the control and test groups. **(Figure 1)** . Furthermore, the gross necropsy showed no abnormalities in the internal viscera of the study animals.

Repeated oral dose 28 days study

In the repeated oral dose toxicity study, rats received NIS KM at 200, 400 and 800mg/kg body weight/day for 28 days. No sign of toxicity or mortality either was observed in the NIS KM administered groups throughout the study period. No abnormal home cage activities, behavioural responses or neurological symptoms were observed before and after the exposure of NIS KM. Body weight gain was found to be normal in NIS KM administered rats and was comparable with that of vehicle treated rats **(Figure 4)**. No significant difference in feed and water consumption was observed between the vehicle and NIS KM treated animals throughout the study. The faecal and urinary excretion patterns were also found to be normal in NIS KM administered rats in comparison with the vehicle treated rats. Similar results were observed in the NIS KM treated satellite rats.

Measurement of haematological, serum electrolytes, biochemical parameters and relative organ weights.

Haematological results were summarised in the **Table 3**. No significant difference in any of the tested haematological parameters was observed between the vehicle and the NIS KM treated rats. There were no significant changes in the serum Na, K, Ca**,** Cl, and pH between the vehicle and NIS KM treated animals (**Table 4**). Administration of NIS KM for a period of 28 days did not produce any significant changes in the serum biochemical parameters such as glucose, cholesterol, triglyceride, bilirubin and liver damage marker enzymes like aspartate amino transferase, alanine amino transferase, alkaline phosphatase, urea, creatinine, albumin and total protein at any of the tested dose levels when compared to the Palm Jaggery (vehicle) treated rats (**Table 5**). Effects of NIS KM on relative organ weights were shown in **Table 6**. No statistically significant changes in the relative organ weights were observed between the vehicle and NIS KM treated rats. Similar results were observed in the NIS KM treated satellite rats.

Histopathology

At necropsy, gross and histo-pathological examination of the organs did not reveal any abnormal changes. Histopathological examinations of the tissues revealed no abnormalities in control and high dose NIS KM treated experimental animals. Histopathological microphotographs of Lungs, Heart, Liver, Kidney and Spleen tissue samples of control group and NIS KM treated satellite group were shown in **Figures 5-9**. Histopathological examinations of organs of satellite control and high dose animals revealed no changes in the architecture and found to be normal.

Table 1: Cytotoxicity properties of NIS KM against MCF-7 cell line

Test Conc. (µg/ml)	Absorbance (Mean ± SD)	% Cytotoxicity (Mean ± SEM)	CTC_{50} (µg/ml)
1000	0.611 ± 0.015	13.09 ± 1.27	> 1000
500	0.635 ± 0.009	9.67 ± 0.69	
250	0.644 ± 0.024	8.39 ± 1.90	
125	0.679 ± 0.020	3.46 ± 1.50	
62.5	0.691 ± 0.010	1.66 ± 0.75	
Control	0.696 ± 0.002	--	

n=3 ; values are expressed in mean ± SEM; Statistical analysis was performed using Student‘t’ test using Graph Pad prism 4.0

Table. 2: Cytotoxicity properties of NIS KM against H9C2 cell line

Test Conc. (µg/ml)	Absorbance (Mean ± SD)	% Cytotoxicity (Mean ± SEM)	CTC_{50} (µg/ml)
1000	0.561 ± 0.009	18.17 ± 0.75	> 1000
500	0.615 ± 0.009	10.30 ± 0.75	
250	0.632 ± 0.004	7.92 ± 0.35	
125	0.658 ± 0.004	3.98 ± 0.35	
62.5	0.681 ± 0.003	1.66 ± 0.23	
Control	0.686 ± 0.009	--	

n=3; Absorbance are expressed in Mean ± SD; % Cytotoxicity expressed in Mean ± SEM ; Statistical analysis was performed using Student‘t’ test using Graph Pad prism 4.0

Table. 3: Effect of Per Oral NIS KM on haematological parameters in WA rats

Treatment	Haematological Parameters								
	WBC (10^9/L)	**RBC (10^6/uL)**	**Hb (%)**	**HCT (%)**	**MCV (fL)**	**MCH (pg)**	**MCHC (g/dl)**	**PLT (10^9/L)**	**MPV (fL)**
Control (5% Palm Jaggery)	8.69±0.33	7.21±1.01	12.0±0.31	36.75±1.13	43.42±5.01	16.60±0.26	32.61±0.32	215.00±15.80	9.20±1.08
NIS KM (200mg/kg/day)	8.93±0.39	7.21±0.26	12.19±0.22	37.65±0.90	50.71±0.64	16.60±0.24	32.39±0.32	224.70±21.09	9.88±0.35
NIS KM (400mg/kg/day)	9.12±0.82	6.95±0.19	11.13±0.30	34.05±0.88	48.74±0.99	15.98±0.20	32.64±0.36	207.40±12.07	8.29±1.75
NIS KM (800mg/kg/day)	8.98±0.61	9.07±0.63	14.50±1.39	47.85±3.50	50.81±0.81	17.15±0.15	32.42±0.23	133.80±22.56	8.88±1.64
Satellite Group									
Control (5% Palm Jaggery)	8.40±0.56	7.46±0.80	12.20±0.22	32.53±3.38	48.65±1.45	16.50±0.28	33.84±0.79	224.80±15.37	7.20±1.55
NIS KM (800mg/kg/day)	9.12±0.82	6.22±0.97	16.43±0.83	34.59±3.50	45.26±5.66	18.0±0.16	34.28±0.53	216.40±13.05	7.27±1.56

n=10 (5 Male +5 Female); values are expressed in mean ± SEM; Statistical analysis was performed using Student‘t’ test using Graph Pad prism 4.0

Table. 4: Effect of NIS KM on serum electrolytes in WA rats

Treatment	Serum electrolytes (mmol/l)				
	Total calcium	**Potassium**	**Sodium**	**Chloride**	**pH**
Control (5% Palm Jaggery)	2.54±0.09	4.50±0.12	144.36±1.13	106.14±1.15	7.72±0.02
NIS KM (200mg/kg/day)	2.55±0.03	4.56±0.10	146.27±0.49	104.19±0.39	7.69±0.01
NIS KM (400mg/kg/day)	2.34±0.08	3.99±0.09	145.36±1.19	96.19±1.42	7.72±0.01
NIS KM (800mg/kg/day)	2.56±0.03	4.70±0.13	147.07±2.45	95.89±2.44	7.63±0.01
Satellite Group					
Control (5% Palm Jaggery)	2.95±0.05	5.42±0.12	152.98±2.36	97.47±1.43	7.54±0.01
NIS KM (800mg/kg/day)	2.73±0.11	5.15±0.22	146.08±1.65	103.53±4.42	7.62±0.02

n=10 (5 Male +5 Female); values are expressed in mean±SEM; Statistical analysis was performed using Student‘t’ test using GraphPad prism 4.0

Table. 5: Effect of Per Oral NIS KM on plasma biochemical parameters in WA rats

Treatment	Biochemical parameters										
	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Bilirubin (mg/dl)	AST (IU/l)	ALT (IU/l)	ALP (IU/L)	Urea (mg/dl)	Creatinine (mg/dl)	Total protein	Albumin (gm/dl)
Control (5% Palm jaggery solution)	109.79±5.59	81.68±8.08	233.30±27.20	0.62±0.04	133.30±9.51	87.20±5.22	335.20±21.83	32.62±0.77	0.81±0.03	12.60±1.40	1.85±0.09
NIS KM 200mg/kg/day)	116.40±9.47	93.10±7.54	162.20±24.90	0.59±0.08	136.30±9.71	66.50±4.71	380.60±10.29	42.96±2.45	0.81±0.03	11.70±1.60	2.13±0.18
NIS KM 400mg/kg/day)	124.20±10.28	85.80±7.37	258.60±30.36	0.77±0.05	128.00±6.85	97.50±7.66	352.40±25.04	36.40±1.40	0.92±0.04	12.30±1.17	1.98±0.11
NIS KM 800mg/kg/day)	85.80±3.44	90.00±6.42	147.70±14.20	0.79±0.05	131.80±5.55	77.30±6.20	359.30±29.18	33.30±1.61	0.85±0.03	12.15±1.43	2.04±0.16
Satellite Group											
Control (5% Palm jaggery solution)	109.94±7.53	100.27±9.39	69.24±5.14	0.68±0.03	132.43±6.40	87.88±5.16	332.20±21.40	37.40±1.33	0.84±0.02	13.10±1.20	1.92±0.08
NIS KM 800mg/kg/day)	97.001±5.62	73.66±11.92	77.36±8.48	0.74±0.05	134.80±5.45	76.29±6.40	388.92±29.50	34.40±1.20	0.88±0.03	12.20±1.34	1.89±0.18

n=10 (5 Male +5 Female); values are expressed in Mean±SEM; Statistical analysis was performed using Student‘t’ test using GraphPad prism 4.0

Table. 6: Effect of Per Oral NIS KM on relative organ weights in WA rats

Treatment	Relative organ weight (gm)							
	Brain	Heart	Liver	Kidney	Adrenal	Spleen	Testis	Ovaries
Vehicle Control (5% PJ)	0.99±0.07	0.48±0.02	4.31±0.28	0.90±0.04	0.02±0.001	0.59±0.07	1.25±0.09	0.06±0.01
NIS KM (200mg/kg/day)	1.06±0.05	0.45±0.03	4.38±0.36	0.66±0.10	0.02±0.006	0.53±0.11	1.36±0.18	0.06±0.01
NIS KM (400mg/kg/day)	1.01±0.03	0.42±0.01	4.28±0.15	0.78±0.02	0.02±0.103	0.62±0.21	1.31±0.09	0.05±0.01
NIS KM (800mg/kg/day)	1.02±0.03	0.44±0.01	4.48±0.40	0.90±0.02	0.03±0.001	0.53±0.05	1.33±0.05	0.07±0.01
Satellite Group								
Control (5% Palm Jaggery solution)	1.07±0.25	0.41±0.09	4.30±0.94	0.80±0.21	0.02±0.001	0.44±0.13	1.29±0.11	0.06±0.01
NIS KM (800mg/kg p.o/day)	1.04±0.20	0.42±0.08	4.97±0.72	0.85±0.16	0.02±0.001	0.40±0.08	0.91±0.02	0.08±0.02

n=10 (5 Male +5 Female); values are expressed in mean ± SEM; Statistical analysis was performed using Student‘t’ test using GraphPad prism 4.0

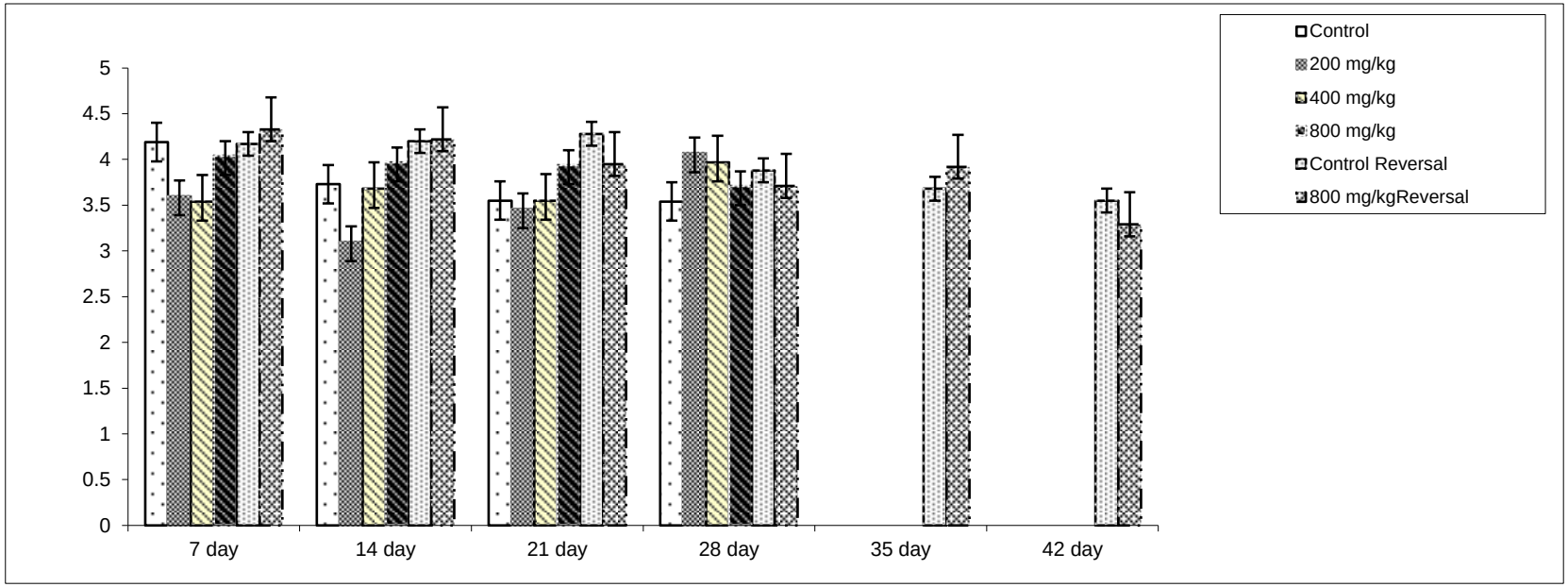
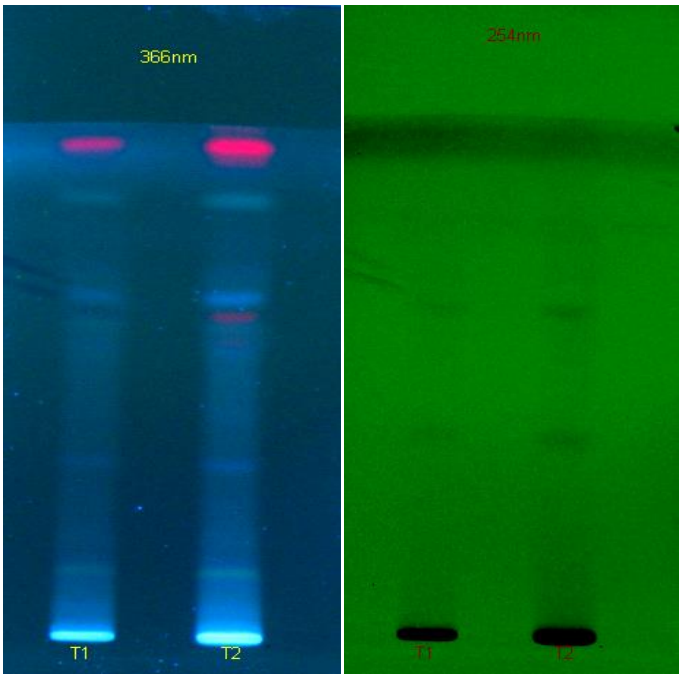


Fig-1: Body weight change at weekly intervals in repeated dose toxicity study (n=10; 5/sex)



(a) (b)

Fig-2: HPTLC FINGERPRINTING PROFILE OF NIS *KM(Based on Flavanoids)* PHOTO DOCUMENTATION UNDER UV *(a)*.At 254 nm b) At 366nm
TLC Details Track 1: 5µL of sample Track 2: 10µL of sample

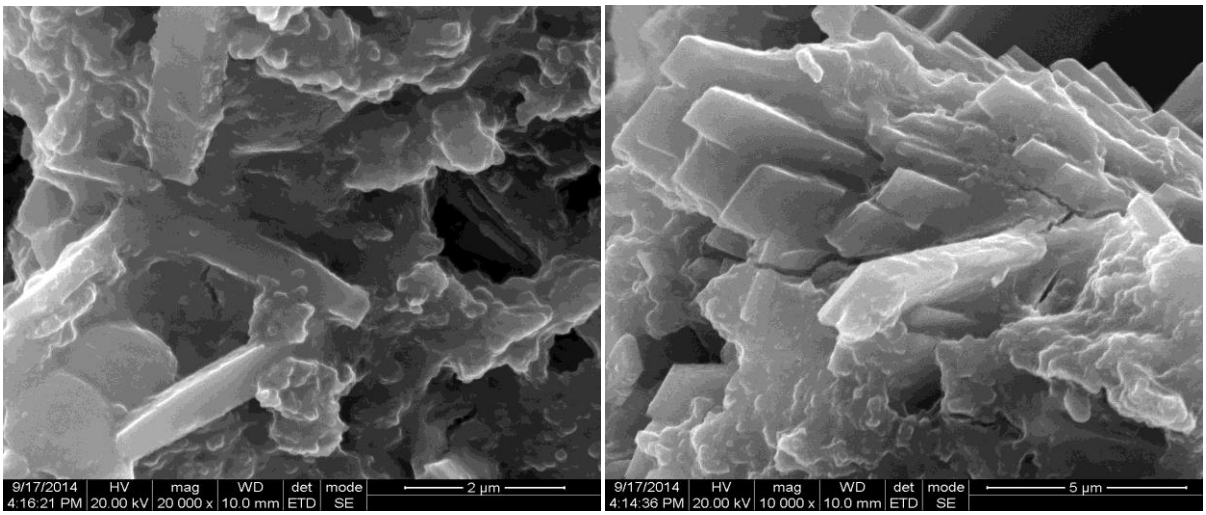


Fig-3: Scanning Electron Microscopic image of NIS KM particle size

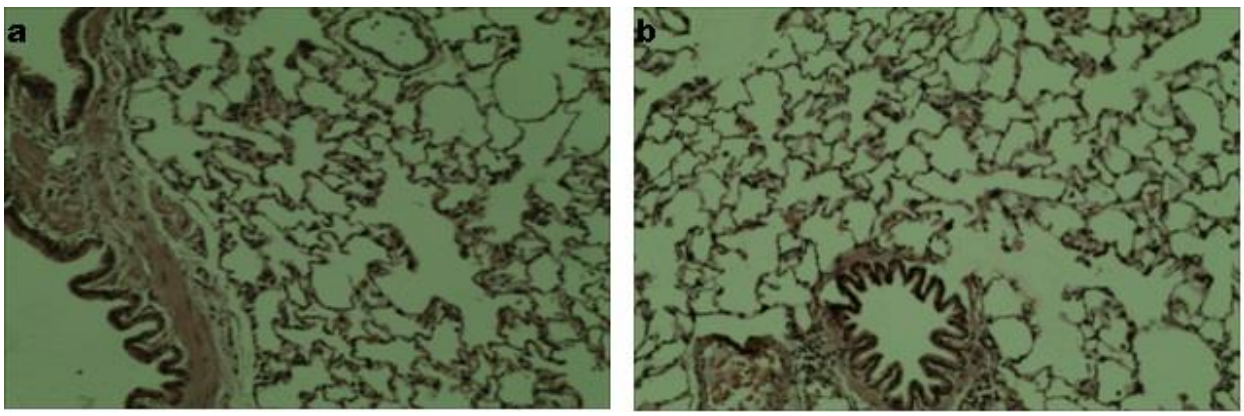


Fig-4: Histopathological examination of Lungs of (a) – control and (b) - satellite high dose

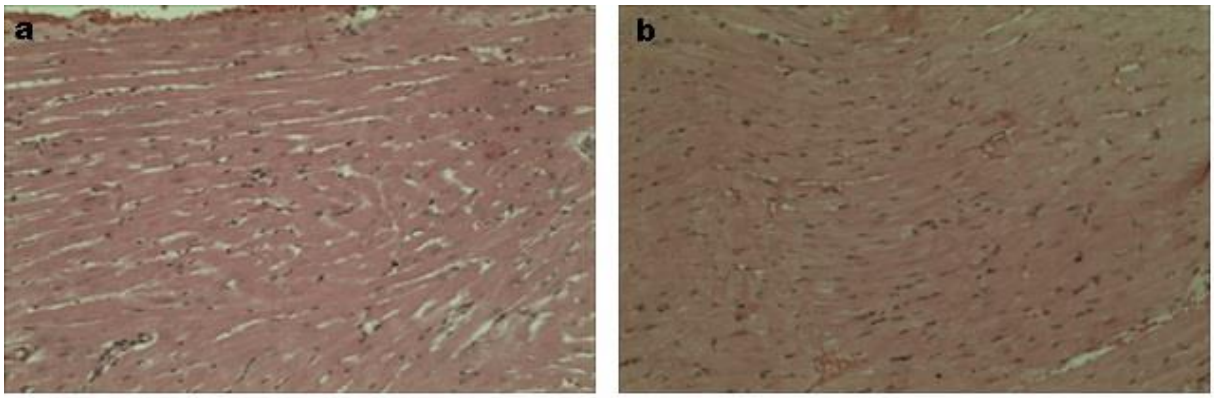


Fig-5: Histopathological examination of heart of (a) - control, and (b) - satellite high dose

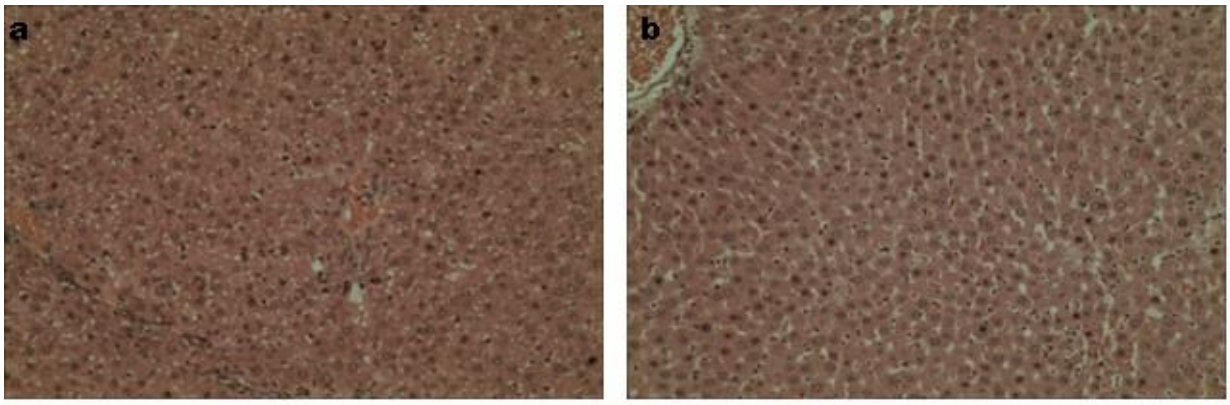


Fig-6: Histopathological examination of liver of (a) – control and (b) - satellite high dose

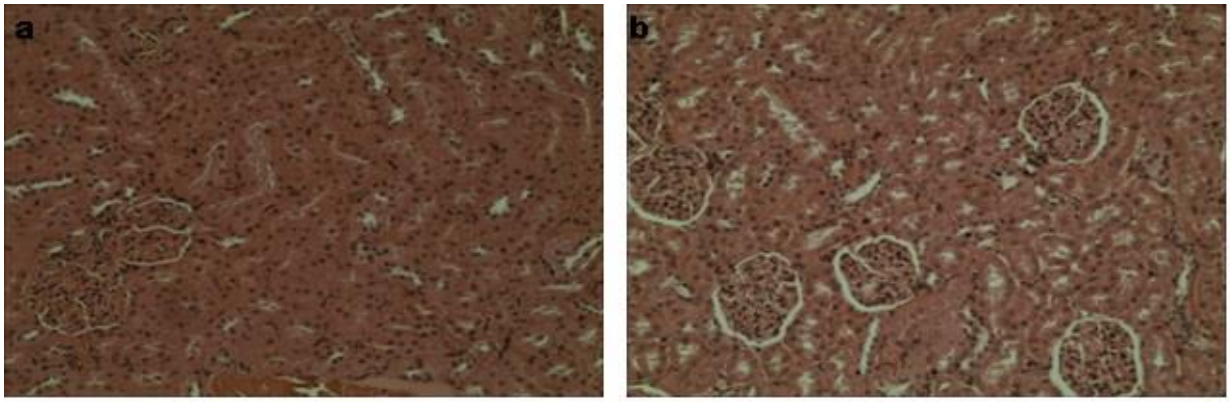


Fig-7: Histopathological examination of kidney of (a) – control and (b) - satellite high dose

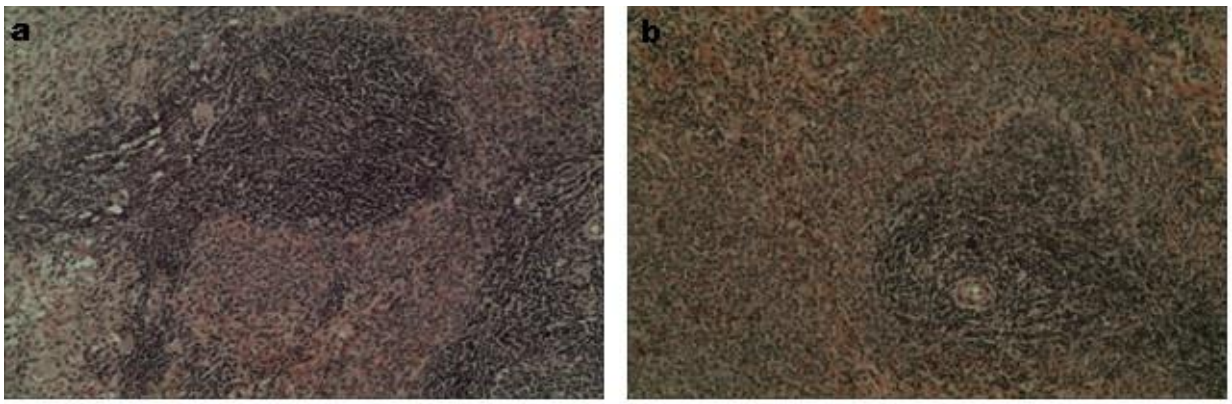


Fig-8: Histopathological examination of spleen of (a) – control and (b) - satellite high dose

DISCUSSION

Siddha system of Medicine offers many effective medicinal formulations for the management of many diseases without many side effects. They are easily the choice of primary health care because of the affordability, natural sourcing and limitations and adverse/side effects of conventional drugs. Despite their wide use by native medical practitioners and local people, lack of scientific evidence for the safety and efficacy of Siddha formulations precludes their use in state healthcare schemes [7].

NIS KM (Kariuppu Mezhugu) is a text based Siddha herbo-mineral formulation which is said to have very good efficacy in alleviating the menstrual cramps and other abdominal pains [3]. Table salt (Sodium chloride) and the herb Calotropis ripe leaves juice are the predominant ingredients used in formulating this study drug[3]. NIS KM could possibly exert anti-dysmenorrhoeal activity by virtue of its impact over the hormonal axis. Therefore it is worth screening and validating the safety of the above formulation which may then be subjected to large scale administration considering the global burden of Primary dysmenorrhoea to be afflicting more than 50% of adolescent women[8]. The present data on NIS KM is a part of our ongoing anti-dysmenorrhoeal research on Siddha drugs at National Institute of Siddha, Chennai - 47. Our unpublished open clinical trial conducted at National Institute of Siddha, Chennai 47 (IEC clearance No: NIS/IEC/11/2/07) validated the literary indication and traditional physicians' experiences about the anti-dysmenorrhoeal efficacy of NIS KM. MTT assay which was performed implied that the drug did have a broader range of safety. Also, it is found in our in vitro research that NIS KM up-regulates the oestrogen mRNA expression and enhances the e- NOS in the myometrial uterine cell line through gene expression studies.

Juice of the ripe Calotropis is used in the formulation which is not considered to be edible and this reason necessitated the safety validation even though it is a textual and long used formulation by local healers. Also since no reports on toxicity of NIS KM are yet available, it was deemed necessary to generate information on safety of the formulation tested.

In the present study, single dose oral administration of NIS KM in female rats at 2000mg/kg did not produce mortality, toxicity signs, body weight alteration or visceral damage. This observation reveals that the LD_{50} of NIS KM is greater than 2000 mg/kg body weight. Thus, in reference to the Globally Harmonised System of Classification and Labelling of chemicals (OECD, 1998), NIS KM can be classified as Category-5 and non-toxic drug.

Observation of clinical signs plays a major role in toxicological testing[9]. There were no treatment-related toxicological changes observed at the tested dose levels for a period of 28 days to male and female rats. Also, no signs of toxicity or mortality were observed even in satellite group rats. No abnormalities on body weight, feed and water intake was observed at the administered doses. The determination of these parameters assumes more significance as nutrition and water plays a major role in proper maintenance of physiological status and any change in metabolism may be reflected due to the drug effects. The hematopoietic system serves as important susceptible targets for toxic substances and a very sensitive index of physiological and pathological states in both humans and animals[10]. In this study, haematological parameters of the animals treated with NIS KM remained within the normal range of the species used in the present study [11] which demonstrate that it has no adverse effects on the circulating blood cells or on their productions.

Some studies report that herbal drugs could cause biochemical alterations and damage to internal organs[12,13]. In the present study, hepato-renal function tests were performed to evaluate the toxicity potential of NIS KM. Elevation of bilirubin level found in serum or plasma indicates hepatocellular damage and dysfunction[14,15]. NIS KM did not produce any significant adverse effect in the liver and renal functions as elicited from the plasma levels of SGOT, SGPT, alkaline phosphatase, bilirubin, creatinine and urea and this was further reinforced by histopathological examination. Further, rats in the satellite group did not develop toxicological signs during the recovery period which shows that NIS KM does not produce delayed toxic effects.

Gross and histopathological examination of organs of animals administered with highest dose (800mg/kg) of NIS KM and control (5% Palm jaggery solution) showed normal histological architecture, indicating no detrimental effects on them. Based on these results, the no-observed adverse effect level (NOAEL) of NIS KM was found to be greater than 800mg/kg/day in Wistar albino rats. As the test drug NIS KM did not produce any toxicological sign at the experimental dose levels both in terms of physiological or biochemical and histological, the low observed adverse effect level (LOAEL) was not determined in the present study.

In conclusion, the Siddha herbo-mineral formulation Kariuppu Mezhugu (NIS KM) which is micronized during the Siddha formulating procedure as proved by the Scanning electron microscopic studies on particle size is found to be non toxic at the in vitro studies and on acute and repeated (28 days) oral administration in rats. This study validates the safety of NIS KM which is normally indicated in text and administered for only 3- 4 days in humans for the treatment of Primary dysmenorrhoea. However, chronic toxicity study is needed to further support the safe use

of this Siddha formulation if it were to be administered as a prolonged course in humans.

Conflict of interest

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article

Acknowledgment

The author wishes to express sincere thanks to Dr. M. Ramamurthy, Faculty, National Institute of Siddha, Chennai and Dr. Thanigaivelan, Sri Sai Ram Siddha Medical college and Research, Tambaram for their immense help in fine tuning the manuscript. Heartfelt gratitude to Dr. N. Sangeetha, PG Alumnus and Dr.S.Anusha, PG Scholar, National Institute of Siddha for assisting in the formulating process of Kariuppu Mezhugu.

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