Research Article

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ACUTE, SUB-ACUTE (28-DAYS), AND SUB-CHRONIC (90-DAYS) ORAL TOXICITY STUDIES OF NANDHI MEZHUGU

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ABSTRACT

Nandhi Mezhugu (NM) is a classical Siddha herbo-mineral formulation indicated for all types of Arthritides. Acute toxicity study was performed at 3 dose levels, i.e., 50, 300, and 2000 mg/kg body weight of rats. Sub-acute toxicity study was performed at doses of 9, 45, 90 mg/kg body weight and sub-chronic toxicity study was performed at doses of 9, 45, 110 mg/kg body weight of rats. Palm Jaggery solution was used as vehicle. In sub-acute and sub-chronic toxicity studies, the drug was administered twice for 7 days followed by 7 days of drug holiday, i.e., in sub-acute toxicity study, the period was 56 days and in the sub-chronic toxicity study, it was 180 days. In both studies, the high dose recovery and recovery control groups were used to study the safety of the drug. Biochemical, haematological parameters, gross pathology and histopathology of the rats were analysed in both studies. ICP-OES detection of heavy metal traces in animal tissue samples in sub-chronic toxicity study (high dose group) were analysed. Acute toxicity study showed no mortality and no treatment-related toxicity signs. Similarly, sub-acute and sub-chronic toxicity studies showed no treatment-related abnormalities at the high dose levels such as 90 and 110 mg/kg body weight in rats respectively. Hence, the scientific validation of the safety of Nandhimezhugu is proven by the toxicity studies.

Keywords: Nandhimezhugu, Mercury, Arsenic, Seraankottai, Toxicitystudies, OECD guidelines.

INTRODUCTION

Siddha system of medicine is a comprehensive ancient system having its roots in the Tamil- speaking land of South India. This system was founded by Siddhars who expounded the philosophy and theory of immortality. This system employs a holistic approach in its treatment methodology and has made enormous contributions towards the health care of Tamil people. The siddha system of medicine is based on the resources derived from herbs, metals, minerals and animal products. For global acceptance, this system of medicine should undergo scientific validation, i.e., upgrading the levels of quality, safety, reliability and efficacy. The siddha herbo-mineral formulation, Nandhi Mezhugu (NM), is one of the versatile classical siddha medicines recommended for a wide range of indications by siddha practitioners[1,2]. It is the need of the hour to do scientific validation related to quality and safety for this drug to enter the global platform.

MATERIALS AND METHODS

Preparation of Nandhi Mezhugu

Preparation as per The Siddha formulary of India Part 1 and Siddha Vaidhiya Thirattu[1,2,3]

Details of Trail Drug Nandhi Mezhugu

Batch No: S.11 – 015.
Mfg Date: 19.04.2014
Exp Date: 5 years from the date of manufacture.
Manufacturing Unit: IMPCOPS (Indian Medicine Practitioners Co-operative Society) Pharmacy, Chennai-41.

Animal Care and Husbandry

The study protocol involving animals was reviewed and accepted by Institutional Animal Ethical Committee (IAEC), National Institute of Siddha, Chennai-600047, with the experimental protocol IAEC approved Number: 1248/AC/09/CPCSEA-4/June2011/10 for acute toxicity study and 1248/AC/09/CPCSEA-9/Dec2013/5 for sub-acute and sub-chronic toxicity studies. Experiments were performed as per the guidelines prescribed by the Committee for the purpose of conduct and supervisions of experiments on animals (CPCSEA). Male and female Wistar albino rats (160–200 g) were obtained from Laboratory Animal Medicine, Centre for Animal Health Studies, Madhavaram Milk Colony, Chennai-600051 and housed in Animal House of National Institute of Siddha. Each group of rats was separately housed in polypropylene cages in a well-ventilated room under an ambient temperature of 22±3°C and 35-75% relative humidity, with a 12-h light/dark artificial light cycle. They were provided with rodent chow from VRK Nutritional Solutions, Sangli, Maharastra and reverse osmosis purified water *ad libitum*. All the animals were acclimatized to the laboratory conditions for 7 days, prior to experimentation.

Acute Toxicity Studies

The acute oral toxicity study was performed in accordance with OECD test guideline 423[4]. The limit test dose of 2000 mg/kg b.w of rat was used as stipulated in OECD guidelines. The test drug suspension was administered at three dose levels such as 50, 300 and 2000 mg/kg body weight once orally to the fasted rats. The following observations were made in all the 3 dose level groups.

a. Mortality and morbidity were noted.
b. Following the trail drug administration, weekly body weight was recorded.
c. Clinical signs such as lethality, convulsion, tremor, straub tail, motor coordination, abnormal gait (tiptoe, rolling), fore paw treading, sedation, excitation, jumps, loss of balance, writhes, piloerection, stereotypies (head movements, chewing), head twitches, scratching, respiration, aggressiveness, fear, reactivity to touch, muscle tone, loss of righting reflex, ptosis, exophthalmos, loss of grasping, akinesia, catalepsy, loss of traction, loss of corneal reflex, analgesia, lacrimation, salivation, defecation and other behavioural changes were observed at about 30 mins, 1 hr, 2 hr and 4 hr on day 1 and daily thereafter for 14 days.

At the end of 14th day, the experimental animals were necropsied and investigated for gross pathological examination.

Sub-acute Toxicity Study
Justification for Dose Selection

As per the result of acute toxicity study in Wistar rats, it was inferred that NM was non-toxic up to the maximum dose of 2000 mg/kg b.w. On the basis of these results and the therapeutic dose for humans as mentioned in Siddha Literature, which was extrapolated to the body surface area of rat, the following doses of 9 mg/kg, 45 mg/kg and 90 mg/kg b.w were selected for the sub-acute toxicity study and administered twice daily by oral route.

Preparation and Administration of Dose

Repeated-dose (28 days) oral toxicity study was carried out according to OECD guideline 407[5]. The animals were divided into six groups of 10 animals each (5 males and 5 females). Group 1 received Palm Jaggery solution and considered as Vehicle Control. Groups II, III and IV (Low dose, Mid Dose, and High Dose respectively) received test drug suspensions, (NM with Palm Jaggery solution) at doses of 9, 45 and 90 mg/kg b.w respectively. Group V (Recovery control) and Group VI (High dose recovery). The NM was administered by oral gavage for 28 days (4 weeks) of dosing duration at regular intervals twice daily as per the protocol mentioned in the Siddha literature where 7 days of test drug administration is followed by 7 days of drug holiday alternatively for four cycles of dosing to match 28 days of dosing period. Recovery groups were scheduled for follow-up observations for the next 14 days without NM administration. High dose recovery and recovery control were included in the study to determine the delayed occurrence, or persistence of, or recovery from toxic effects, if any. All groups were observed twice daily for morbidity and mortality, and clinical signs, once daily. The bodyweight and food intake of the animals were evaluated at weekly intervals. Any change in water consumption was observed and recorded.

On the 57th day, after an overnight fasting, the rats were anaesthetized with Thiopentone sodium and blood samples were collected from retro-orbital plexus under light ether anaesthesia by trained personnel. The blood samples for haematological and biochemical analysis were collected in tubes with and without EDTA, respectively. Total WBC, Differential Counts, RBC, Haemoglobin, HCT, MCV, MCH, MCHC, RDW, PLT, MPV, PDW and PCT were analysed. Biochemical analysis was performed on serum obtained after centrifugation of total blood (without EDTA) at 2500 rpm for 15 min. Blood Glucose, Urea, Creatinine, Cholesterol, Triglyceride, HDL, LDL, Total serum protein, ALT or SGPT, AST or SGOT were analysed.

Sub Chronic Toxicity Study
Justification for Dose Selection

As stated in results of acute toxicity studies in Wistar rats indicated that NM was nontoxic up to the maximum dose of 2000 mg/kg b.w. On the basis of these results and the therapeutic dose as mentioned in Siddha Literature, which was extrapolated to body surface area of rat, the following doses of 9 mg/kg, 45 mg/kg and 110 mg/kg b.w were selected for the study and administered orally twice daily.

Preparation and Administration of Dose

Repeated-dose oral toxicity study was carried out according to OECD test guideline 408[6]. The animals were divided into six groups of 10 animals each (5 males and 5 females). Group I received Palm Jaggery solution and considered as Vehicle control. Groups II, III and IV (Low Dose, Mid Dose, and High Dose respectively) received test drug suspensions, (NM with Palm Jaggery solution) at doses 9, 45 and 110 mg/kg b.w respectively. Group V (Recovery control) and Group VI (High dose recovery). The NM was administered twice daily by oral gavage for 7 days followed by 7 days' drug holiday alternatively for 180 days (90 days dosing and 90 days' drug holiday). This protocol is followed to mimic the treatment protocol in humans as mentioned in the traditional Siddha Literature.

Recovery groups were scheduled for follow-up observations for the next 14 days without NM administration. High dose recovery and recovery control were included in the recovery study to determine the delayed occurrence, or persistence of, or recovery from toxic effects. All groups were observed twice daily for morbidity and mortality, and clinical signs, once daily. Bodyweight and food intake of the animals were evaluated weekly. Any change in the water consumption was observed and recorded.

On the 181st day, after an overnight fasting, the rats were anaesthetized with Thiopentone sodium and blood sample for haematological and biochemical analysis were collected into tubes with and without EDTA, respectively. Total WBC, Differential Counts, RBC, Haemoglobin, HCT, MCV, MCH, MCHC, RDW, PLT, MPV, PDW and PCT. Biochemical analysis was performed on serum obtained after centrifugation of total blood (without anticoagulant) at 2500 rpm for 15 min. Blood Glucose, Urea, Creatinine, Cholesterol, Triglyceride, HDL, LDL, Total serum protein, ALT or SGPT, AST or SGOT were screened.

Histopathology

In subacute toxicity study, necropsy was done in all animals in Group I to Group IV on 57th day and Group V and Group VI (recovery groups) were done on 70th day. In sub chronic toxicity study, necropsy was done in all animals in Group I to IV on 181st day and the necropsy for Group V and Group VI (recovery groups) were performed on 195th day. Blood samples were collected from abdominal aorta following which all the animals were euthanized by excess dose of Thiopentone sodium administered through intraperitoneal route. The gross pathological examinations of all major internal organs were

performed. The organs such as brain, heart, lungs, liver, kidneys, and spleen were weighed and the relative weights of the organs were calculated as mentioned in both studies. The organs were fixed in 10% neutral buffered formalin, trimmed and 5µ thickness of tissue sections were stained with hematoxylin and eosin for histopathological investigation.

Statistical Analysis: All the values are expressed as Mean ± SEM. The data were statistically analyzed by one-way ANOVA if the number of groups to be compared is more than two followed by Post-hoc test, Dunnet-t test. If only two groups are to be compared as in the case of recovery groups, Student's t-test was used. It was assumed that the data were normally distributed and there was a homogeneity of variance. P value < 0.05 was considered as statistically significant.

Table 1: Clinical signs or behavioural observations of the rat in the acute toxicity study

S.No	Dose mg/kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	Control	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	NM (50mg/kg)	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	NM (300mg/kg)	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	NM (2000mg/kg)	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(+ indicates that the particular behaviour is present and – indicates absence of the behaviour in animals)

1.Alertness 2. Aggressiveness 3. Piloerection 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. Abnormal Respiration 20. Mortality.

Table 2: Effect of Nandhi mezhugu on hematological parameters of wistar rats in sub-acute toxicity study

Groups	WBC × 10^9/L	Lymph × 10^9/L	Mon× 10^9/L	Gran× 10^9/L	Lymph %	Mono %	Gran %	RBC× 10^{12}/L	HGB g/dL
Vehicle control	3.51 ± 1.1	2.56 ± 0.9	0.1 ± 0.03	0.8 ± 0.3	61.6 ± 12	2.95 ± 0.8	18.83 ± 4	5.34 ± 1.4	8.63 ± 2.9
Low dose 9 mg/kg	8.23 ± 1.3**	5.55 ± 0.8	0.4 ± 0.07**	2.3 ± 0.4	61.9 ± 7	3.91 ± 0.4	24.19 ± 3	7.35 ± 1.3	14.56 ± 1.7**
Mid dose 45 mg/kg	12.02 ± 1.5**	8.91 ± 1.3**	0.5 ± 0.06**	2.7 ± 0.32**	71.9 ± 3.5	4.25 ± 0.6	23.83 ± 3	9.83 ± 0.7*	15.80 ± 1.2**
High dose 90 mg/kg	3.0 ± 0.5	2.15 ± 0.3	0.1 ± 0.02	0.8 ± 0.13	71.4 ± 2	3.63 ± 0.2	24.93 ± 2	6.66 ± 0.8	10.76 ± 1.4

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Recovery Groups

Groups	WBC× 10^9/L	Lymph× 10^9/L	Mon× 10^9/L	Gran× 10^9/L	Lymph %	Mono %	Gran %	RBC× 10^{12}/L	HGB g/dL
Recovery Control	4.4 ± 1.6	3.1 ± 1.2	0.16 ± 0.1	1.2 ± 0.4	68.3 ± 1	3.66 ± 0.3	28.1 ± 1.5	8.42 ± 1.2	12.33 ± 2.4
High dose recovery 90 mg/kg	3.7 ± 1.1	2.5 ± 0.8	0.16 ± 0.2	1.0 ± 0.2	66.9 ± 3	4.26 ± 0.3	28.7 ± 2.7	6.14 ± 1.4	9.95 ± 2.4

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Groups	HCT %	MCV fL	MCH pg	MCHC g/dL	RDW %	PLT × 10^9/L	MPV Fl	PDW Fl	PCT %
Vehicle control	27.4 ± 7.4	51.09 ± 0.39	15.13 ± 0.0	29.78 ± 1.3	12.03 ± 0.3	185.1 ± 96	6.76 ± 0.2	15.45 ± 0.2	0.12 ± 0.06
Low dose 9 mg/kg	38.8 ± 5.8	51.38 ± 6.7	22.04 ± 4.5*	36.71 ± 5.6	18.20 ± 4*	530.5 ± 66*	5.89 ± 0.7	13.43 ± 1.5	0.35 ± 0.04
Mid dose 45 mg/kg	49.59 ± 3.9*	50.25 ± 0.69	16.07 ± 0.2	32.05 ± 0.2	12.51 ± 0.4	529.4 ± 87*	6.57 ± 0.1	14.9 ± 0.1	0.35 ± 0.05
High dose 90 mg/kg	33.25 ± 4.2	49.83 ± 0.83	15.93 ± 0.3	32.09 ± 0.3	11.45 ± 0.3	219.7 ± 67	6.34 ± 0.1	15.02 ± 0.1	0.13 ± 0.04

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Recovery Groups

Groups	HCT %	MCV fL	MCH pg	MCHC g/dL	RDW %	PLT × 10^9/L	MPV fL	PDW fl	PCT %
Recovery Control	42.5 ± 6.2	50.6 ± 1.2	14.23 ± 1.5	28.31 ± 3.1	12.16 ± 0.4	319.6 ± 78.5	6.4 ± 0.1	15.03 ± 0.09	0.2 ± 0.04
High dose recovery 90 mg/kg	31.15 ± 7.1	50.71 ± 0.85	15.75 ± 0.46	31.18 ± 0.7	12.43 ± 0.44	349.16 ± 115	6.58 ± 0.1	15.2 ± 0.19	0.22 ± 0.07

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Table 3: Effect of Nandhi mezhugu on biochemical parameters of experimental wistar rats in sub-acute toxicity study

Groups	Glucose mg/dl	Urea mg/dl	Creatinine mg/dl	Cholesterol mg/dl	TGL mg/dl	HDL mg/dl	LDL mg/dl
Vehicle control	85.0 ± 3.6	37.0 ± 2.2	0.9 ± 0.03	78.6 ± 0.3	137.5 ± 8.5	21.0 ± 0.0	26.5 ± 1.5
Low dose 9 mg/kg	53.6 ± 6.1	39.5 ± 1.8	0.7 ± 0.1	82.1 ± 4.6	105.2 ± 25.5	24.6 ± 4.1	36.6 ± 6.9
Mid dose 45 mg/kg	92.3 ± 13.6	38.7 ± 1	0.8 ± 0.1	78.6 ± 1.8	111.7 ± 10.4	24.0 ± 0.78	32.8 ± 1.8
High dose 90 mg/kg	137.3 ± 8.5***	42.2 ± 1.1*	0.7 ± 0.03	74.7 ± 3.8	84.9 ± 5.4*	25.6 ± 1.9	31.3 ± 2

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Recovery Groups

Groups	Glucose mg/dl	Urea mg/dl	Creatinine mg/dl	Cholesterol mg/dl	TGL mg/dl	HDL mg/dl	LDL mg/dl
Control Recovery	124.0 ± 5.3	38.6 ± 1.8	0.55 ± 0.03	82.83 ± 5.3	134.3 ± 13.4	25.25 ± 2.7	30.83 ± 7.2
High dose Recovery 90 mg/kg	103.8 ± 9.6	36.6 ± 2	0.49 ± 0.03	79.16 ± 6.2	168.16 ± 20.6	27.48 ± 4	32.5 ± 10.8

Table 4: Effect of Nandhi mezhugu on liver markers of experimental wistar rats in sub-acute toxicity study

Groups	TRP g/dl	ALB g/dl	SBIL mg/dl	SGOT IU/L	SGPT IU/L	SAP IU/L
Vehicle control	7 ± 0.1	3.4 ± 0.4	0.25 ± 0.00	159.3 ± 19.6	55.4 ± 5.2	179.3 ± 21.2
Low dose 9 mg/kg b.w	7.5 ± 0.3	3.3 ± 0.2	0.20 ± 0.00	138.0 ± 7.4	65.4 ± 25.7	131.6 ± 25.3
Mid dose 45 mg/kg	7.8 ± 0.2	3.3 ± 0.16	0.25 ± 0.02	156.1 ± 15.6	61.1 ± 2.9	133.3 ± 3.2
High dose 90 mg/kg	6.8 ± 0.3	3.1 ± 0.24	0.23 ± 0.03	215.6 ± 15.6*	55.5 ± 2.6	116.0 ± 6.3

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Recovery group

Groups	TRP g/dl	ALB g/dl	SBIL mg/dl	SGOT IU/L	SGPT IU/L	SAP IU/L
Control Recovery	7.53 ± 0.52	2.9 ± 0.3	0.3 ± 0.1	193.1 ± 18	50 ± 4	111 ± 13.4
High dose Recovery	7.11 ± 0.19	2.8 ± 0.1	0.2 ± 1.2	211.1 ± 34	59 ± 4	109 ± 21.8

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Table 5: Effect of nandhi mezhugu on hematological parameters of experimental wistar rats in sub-chronic oral toxicity study

Groups	WBC× 10^9/L	Lymph × 10^9/L	Mon× 10^9/L	Gran × 10^9/L	Lymph %	Mono %	Gran %	RBC× 10^{12}/L	HGB g/dL
Vehicle control	6.24 ± 3.5	3.96 ± 2.8	0.24 ± 0.05	2.04 ± 0.8	60.2 ± 11.3	4.7 ± 1.4	35.14 ± 10	10.42 ± 3.6	16.98 ± 6
Low dose 9 mg/kg	5.71 ± 1.23	3.78 ± 0.8	0.25 ± 0.06	1.68 ± 0.39**	66.53 ± 1.3	4.05 ± 0.18	29.4 ± 1.3	7.7 ± 0.9	12.26 ± 1.4
Mid dose 45 mg/kg	6.62 ± 1.54	4.53 ± 1.03	0.27 ± 0.07	2.31 ± 0.57**	61.15 ± 6.8	4 ± 0.17	26.79 ± 1.63	8.54 ± 1.15	14.07 ± 1.9
High dose 110 mg/kg	6.36 ± 0.4	4.22 ± 1.03	0.29 ± 0.06	1.85 ± 0.2**	66.88 ± 2.7	4.28 ± 0.83	28.82 ± 1.63	9.05 ± 0.66	15.07 ± 1.4

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Recovery Group

Groups	WBC× 10^9/L	Lymph× 10^9/L	Mon× 10^9/L	Gran × 10^9/L	Lymph %	Mono %	Gran %	RBC× 10^{12}/L	HGB g/dL
Recovery Control	5.05 ± 1.4	3.16 ± 0.9	0.2 ± 0.07	1.66 ± 0.4	61.45 ± 1.7	4.65 ± 0.3	33.9 ± 1.6	5.9 ± 0.53	9.56 ± 0.9
High dose recovery 110 mg/kg	3.43 ± 1.7	2.18 ± 1.14	0.1 ± 0.06	1.15 ± 0.52	55.6 ± 5	4.5 ± 0.7	39.8 ± 4.5	4.2 ± 1.3	6.91 ± 2.2

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Groups	HCT %	MCV fL	MCH pg	MCHC g/dL	RDW %	PLT× 10^9/L	MPV fL	PDW fL	PCT %
Vehicle control	51.9 ± 18	49.6 ± 2.5	16.2 ± 0.66	32.8 ± 0.8	12.4 ± 0.9	408.2 ± 198	6.92 ± 0.4	15.3 ± 0.3	0.28 ± 0.13
Low dose 9 mg/kg	37.5 ± 4.1	49.2 ± 1	15.9 ± 0.3	32.5 ± 0.33	12.24 ± 0.24	301.6 ± 55.2	6 ± 0.088	15.05 ± 0.09	0.19 ± 0.03
Mid dose 45 mg/kg	41.9 ± 5.7	48.9 ± 0.7	16.3 ± 0.2	33.6 ± 0.17	11.6 ± 0.35	319.2 ± 62.2	6.64 ± 0.15	15.09 ± 0.15	0.2 ± 0.03
High dose 110 mg/kg	45.4 ± 3.7	50.0 ± 0.9	16.5 ± 0.33	33.1 ± 0.4	12 ± 0.64	242.66 ± 37.5	6.88 ± 0.12	15.37 ± 0.11	0.16 ± 0.02

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Recovery Group

Groups	HCT %	MCV fL	MCH pg	MCHC g/dL	RDW %	PLT× 10^9/L	MPV fl	PDW fl	PCT %
Recovery Control	28.95 ± 2.6	48.7 ± 0.9	16.0 ± 0.3	33.0 ± 0.3	10.91 ± 0.5	338.3 ± 180	6.66 ± 0.1	15.36 ± 0.2	0.090 ± 0.03
High dose recovery 110 mg/kg	20.56 ± 6.4	48.5 ± 0.8	15.8 ± 0.4	32.7 ± 0.6	10.48 ± 0.2	170.5 ± 61	6.75 ± 0.2	15.76 ± 0.2	0.108 ± 0.03

All values are mean ± SEM. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Table 6: Effect of Nandhi mezhugu on serum biochemical parameters of experimental wistar rats sub-chronic oral toxicity study

Groups	Glucose mg/dl	Urea mg/dl	Creatinine mg/dl	Cholesterol mg/dl	TGL mg/dl	HDL mg/dl	LDL mg/dl
Vehicle control	172.4 ± 3.5	34.0 ± 2.4	0.7 ± 0.02	100.6 ± 5.3	131.4 ± 17	26.0 ± 2.0	48.4 ± 3.5
Low dose 9 mg/kg	149.2 ± 5.7	32.1 ± 3	0.7 ± 0.05	91.0 ± 7.5	100.5 ± 13	22.5 ± 1.0	48.5 ± 6.7
Mid dose 45 mg/kg	132.5 ± 11.3*	26.6 ± 2.7	0.7 ± 0.04	81.4 ± 3.2*	146.4 ± 13	22.2 ± 1.0	29.8 ± 3.0*
High dose 110 mg/kg	160.7 ± 6.6	27.3 ± 1.5	0.6 ± 0.02	85.7 ± 3.3	141 ± 18.0	20.79 ± 1.0*	36.7 ± 3.6

All values are mean ± SE. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Recovery Group

Groups	Glucose mg/dl	Urea mg/dl	Creatinine mg/dl	Cholesterol mg/dl	TGL mg/dl	HDL mg/dl	LDL mg/dl
Recovery Control	139 ± 5.9	35 ± 0.9	0.41 ± 0.01	75 ± 4	118 ± 15	24.8 ± 1.19	31 ± 1.88
High dose Recovery 110 mg/kg	146 ± 9.6	37 ± 0.73	0.31 ± 0.03	76 ± 3.9	131 ± 22	26.6 ± 1.9	31 ± 4.9

All values are mean ± SE. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Table 7: Effect of Nandhi mezhugu on liver markers of experimental wistar rats sub-chronic oral toxicity study

Groups	TRP g/dl	ALB g/dl	SBIL mg/dl	SGOT IU/L	SGPT IU/L	SAP IU/L
Vehicle control	7.04 ± 0.25	2.82 ± 0.19	0.12 ± 0.02	312.4 ± 30.7	96.0 ± 2.9	198.2 ± 18.3
Low dose 9 mg/kg	6.88 ± 0.28	2.83 ± 0.22	0.13 ± 0.02	236.3 ± 57.3	80.8 ± 10	170.7 ± 13.7
Mid dose 45 mg/kg	6.67 ± 0.11	2.84 ± 0.19	0.10 ± 0.0	238.8 ± 16.0	69.3 ± 3.2**	146.1 ± 16.2
High dose 110 mg/kg	6.89 ± 0.06	2.82 ± 0.09	0.11 ± 0.01	216.3 ± 13.5	67.6 ± 3.2**	114.4 ± 9.0**

All values are mean ± SE. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

Groups	TRP g/dl	ALB g/dl	SBIL mg/dl	SGOT IU/L	SGPT IU/L	SAP IU/L
Recovery Control	6.8 ± 0.22	2.3 ± 0.18	0.1 ± 0.02	191.5 ± 13.3	66 ± 7.6	181.16 ± 18.4
High dose Recovery 110 mg/kg	6.3 ± 0.18	2.3 ± 0.14	0.1 ± 0.02	192.33 ± 23.2	67 ± 5.2	178.66 ± 12.3

All values are mean ± SE. Statistical analysis by one-way ANOVA followed by Dunnett's Multiple Comparison. **p < 0.05. *p < 0.01

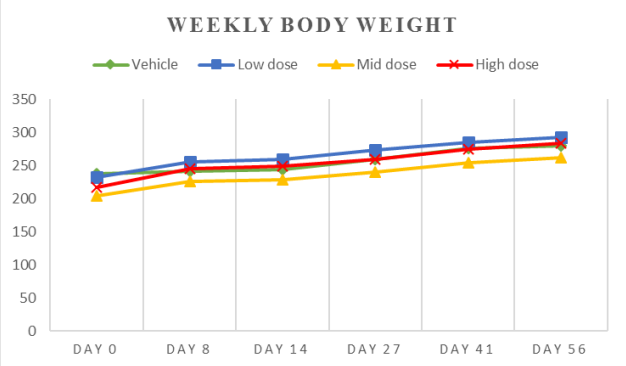

Figure: 1 weekly body weight of rat in sub-acute toxicity study

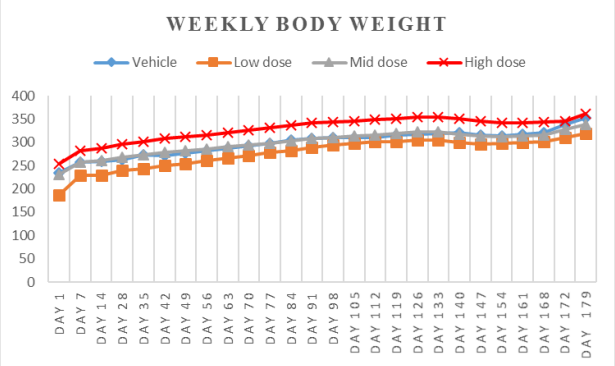

Figure: 2 weekly body weight of rat in sub-chronic toxicity study

RESULTS AND DISCUSSION

Acute Toxicity Study

The limit dose of 2000 mg/kg did not cause mortality or any signs of acute toxicity, in the three rats on single dosing by oral route, for a short period (48 h) and long period (14 days). No mortality or behavioural changes (see Table-1) were observed at the end of the treatment. Similarly, subsequent study with 50, 300 and 2000 mg/kg did not show any significant differences in bodyweight between control and treated groups during this period. It was observed that there was no mortality and toxic signs up to 2000 mg/kg. NM can be classified under category-5 since the LD50[7] value was greater than 2000 mg/kg in accordance with GHS [Globally Harmonized System of Classification and Labelling of Chemicals][8] and this provides us a direct relevance for protecting human and animal health. Therefore, it can be concluded that Nandhi Mezhugu, when administered at single dose, is non-toxic and can be used safely as oral formulation.

Sub-Acute Toxicity Study

There were no signs of toxicity or mortality observed in both sexes of rats treated at 9, 45 and 90 mg/kg orally for a dosing period of 28 days and in the recovery group of rats and did not show any significant differences in bodyweight between control and treated groups during this period (Figure 1). Haematological parameters such as haemoglobin, red blood cells, white blood cells, mean corpuscular haemoglobin, though found to be statistically significant at low and mid doses as compared to the control, they were found to be well within the normal range of

rats in experimental groups (Table 2). Hence the changes observed were not treatment-related effects. All the biochemical parameters such as glucose, total cholesterol, triglycerides, total protein, alkaline phosphatase, creatinine, blood urea were within the normal range (Table 3) observed between control and treated groups. The levels of liver marker enzymes like SGOT and SGPT were found to be well within the normal range for rats in NM treated groups (Table 4). In this study, histopathological examinations in control and high dose group revealed no abnormalities. There were no haematological, biochemical and histopathological alterations observed with NM administration even at 90 mg/kg twice daily in rats for a period of 28 days compared to control. The No Observed Adverse Effect Level (NOAEL) of NM was estimated to be greater than 90 mg/kg/day in rats. Hence, it is concluded that NM is safe for oral administration in rat up to 90 mg/kg/day.

Sub Chronic Toxicity Study

There were no signs of toxicity or mortality observed in both sexes of rats treated at 9, 45 and 110 mg/kg orally for a period of 90 days (intermittently with 7 days of dosing and 7 days of drug holiday) and in the recovery group of rats and did not show any significant differences in bodyweight between control and treated groups during this period (Figure 2). Haematological parameters such as haemoglobin, red blood cells, white blood cells, mean corpuscular haemoglobin were found to be well within the normal range for rats in experimental groups (Table 5). All the parameters in serum biochemical profile such as glucose, total cholesterol, triglycerides, total protein, alkaline phosphatase, creatinine, blood urea were within the normal range (Table 6). The levels of liver marker enzymes like SGOT and SGPT were found to be well within the clinical range for rats in NM treated groups (Table 7). In our study, histopathological examinations in control and high dose group revealed no abnormalities. There were no haematological, biochemical and histopathological alterations observed with NM administration even at 110 mg/kg twice daily in rats for a period of 90 days compared to control. The No Observed Adverse Effect Level (NOAEL) of NM was estimated to be greater than 110 mg/kg/day in rats. Hence, it can be concluded that NM is safe for oral administration.

ICP-OES – Detection of Heavy Metal Traces in Animal Tissue Samples (Brain, Liver, Kidney) in Sub-Chronic Toxicity Study (High dose group) of Nandhi Mezhugu

In sub chronic repeated oral 90 days toxicity study of Nandhi Mezhugu in Wistar rats (OECD 408) the tissues of brain, kidney, and liver of high dose group were subjected to ICP-OES study to screen for heavy metal content and it was found that there was absence of traces of heavy metals such as lead, cadmium, mercury, and arsenic.

The toxic effects of mercury were neutralized in the presence of sulphur, which is one of the ingredients in NM[9]. From other safety studies on herbo-metallo mineral formulations[9,10], it was evident that if the preparation of such formulations is carried out as per Standard Operating Procedures (they undergo thorough extensive purification and preparation methods involving distillation, calcination, heating, melting and extraction using earthen utensils at specified temperatures to make the minerals ready for human consumption) mentioned in classical literature, they do not produce any toxic effects. In the prepared NM, the essential elements such as Na, K, Ca, Mg, Cu, Fe and Zn had also been found in µg/g amounts and trace amounts of arsenic and mercury seemed to remain chelated with organic ligands derived from medicinal herbs by alchemic processes making these biologically assimilable. If the Siddha herbo-metallo mineral formulation containing biologically-produced nanoparticles is taken along with palm jiggery, honey, ghee, and milk, the latter makes these elements easily assimilable, eliminating their harmful effects and enhancing their biocompatibility. It is well-established that several metals play a vital role in the biochemical processes as well as in the cure of many diseases. It is believed that heavy metals such as Hg and As, which are widely used in the Siddha system of medicine act as catalysts, which exert their catalytic activity by virtue of their presence in the intestines without ever reaching the bloodstream. It is, probably, this property which renders many of the highly toxic metals, nontoxic. It is believed that the toxic effects of these medicines are neutralized by the medium of honey/ghee/milk and provide a natural and effective alternative to synthetic allopathic drugs[9].

CONCLUSION

At present, the world's focus has turned towards traditional systems of medicine because of the adverse effects reported from other systems of medicine in treating the debilitating diseases. The Siddha system of medicine is very powerful among the Indian systems of medicine. Nandhi mezhugu is a sasthric siddha herbo-metallo mineral formulation, which has gained a place in treating autoimmune diseases like rheumatoid arthritis and different types of cancers etc. The drug NM has been validated scientifically in the area of safety.

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