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RESEARCH ARTICLE

Acute and Subacute Toxicity Assessment of *Siddha* Formulation *Navauppu Mezhugu* (A Herbomineral Formulation) in Wistar Albino Rats

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Abstract

Background: *Navauppu Mezhugu* is a Herbo mineral *Siddha* formulation indicated for diseases such as *soolai, Magotharam, Neerkovai, Visha vatham, Mudaku vatham, Kunmam anda vatham, vali vatham, parisavayu*. **Aim:** This article elaborates on the acute and subacute toxicity profile of *Navauppu Mezhugu* Method: The study was conducted in Wistar Albino rats by administering the drug orally. **Results:** The acute toxicity study revealed *Navauppu Mezhugu* produced no toxicity in rats for a maximum dose of about 2000mg/kg b.wt. A sub-acute toxicity study revealed the investigational drug was safe for 28 days on a range of dosage forms 200mg/kg (low dose), 400mg/kg (middle dose) and 800mg/kg (High dose). Histopathological study revealed normal architecture maintenance of liver, kidney, heart and spleen. **Conclusion:** The results confirm the safety of *Navauppu Mezhugu* in dosage levels recommended in the literature.

Major Findings: From acute oral toxicity study the maximum tolerable dose identified as 2000 mg/kg body weight. no mortality and no abnormal signs were noted in subacute toxicity study. Weight gain was observed in all the animals and decrease in the plasma glucose level, other parameters of lipid profile except HDL in all the animals of test group. Liver function was found normal.

Keywords: Acute Toxicity, Herbo Mineral Formulation, *Navauppu Mezhugu*, *Siddha*, Subacute Toxicity

1. Introduction

Siddha medicine is an ancient medical system practiced in Tamil Nadu, a Southern region of India for more than five thousand years, serving mankind in combating diseases and maintaining health[1], with considerably extensive literature exploring treatment strategies for Communicable and Non Communicable diseases. According to a WHO estimate, about 80% of the population in developing countries depends on Traditional Medicines[2]. In *Siddha* Medicine, herbs, metals, minerals, and animal products are used as raw drugs to compound various formulations like *Choornam, Parpam, Chenduram, Kattu, Kazhangu, etc*. There are a total of 32 different forms of pharmaceutical dosage forms of *Ulmarunthu* (internal medicine) mentioned in the *Siddha* system. *Mezhugu* is one among the dosage forms waxy in consistency usually prepared by grinding mineral salts or Mercury compounds with leaf juices. *Karasaram* (salts) belongs to a variety of minerals used extensively in *Siddha* Medicine preparation[3].

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Navauppu Mezhugu (NM) is a herbo mineral formulation made from *Karasaram* (salts) and *Veeram* (mercuric chloride) and herbal juices. The preparation of NM is quoted in *Siddha Vaidhya Thiratu* indicated for *soolai, magotharam, neerkovai, vishavatham, mudaku vatham, kunmam, anda vatham, vali vatham, parisavayu*[4]. *Navauppu Mezhugu* preparation using nine salt varieties includes some ingredients that are irritants in nature such as mercuric chloride, camphor, *Croton tigilum,* and *Citullus colocynthis* producing gastrointestinal problems. Of these, mercuric chloride, one of the mercurial salts is known for its rapid solubility, absorption and lethality. Prior to clinical use, any medicine has to be evaluated for its toxic nature, which could be achieved by conducting acute and subacute toxicity tests in suitable animal models.

Considering this, though the ingredients were purified before preparation, the investigational drug *Navauppu Mezhugu* has to be subjected to a preclinical trial for the presence of a toxic nature if any. This scientific validation of NM will throw light on its safety while using it for its wide range of indications and global acceptance.

2. Materials and Methods

Preparation of *Navauppu Mezhugu*: Preparation of the drug as mentioned in *Siddha Vaidhiya Thirattu*[4,5]. The investigational drug was purchased from a *Siddha* drug store branch of The Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd., Chennai.

Trail Drug details: *Navauppu Mezhugu*
Batch No/Product ID: S.11 – 006
Mfg Date: 06/2019
Date of Expiry: 36 months from manufacturing date

2.1 Acute Toxicity Study

2.1.1 Experimental Animals

Guidelines for the care and use of Laboratory Animals were strictly followed and an acute oral toxicity test was performed based on Organization for Economic Co-operation and Development (OECD) guidelines 423[6]. The protocol for these experiments was approved by the Institutional Animal Ethical Committee (IAEC) of K. M. College of Pharmacy, Madurai, Tamilnadu, India. The IAEC approval number is IAEC/RADHA S/TNMGRMU/M.D(S)/3355/KMCP/122/2021. Healthy non-pregnant female Wistar albino rats weighing 125-160 g were selected for the study. Female rats were found to have greater sensitivity to treatment so they were preferred[7].

2.1.2 Assigning of Animals

The rats were randomly divided into two groups, each consisting of five animals. One rat was unmarked, and the other four were marked with yellow stains on their heads for identity and ease of observation.

2.1.3 Housing and Diet

Cages measuring 55 x 32.7 x 19 cm made of polypropylene were used to house the animals, with sawdust on the floor, in 12 h light and 12 h dark for each day and under the controlled environmental temperature of 23±2°C. The cages were labelled with a card mentioning the cage number, the number of animals, their body weight, test substance code, route of administration, and dosage. Laboratory animal food pellets were fed to the animals and they had unrestricted access to water.

2.1.4 Administration Route and Dosage

The animals were fasted for 3 h and then the test substance was given as a single dose using a specially designed oral needle (only food was withheld, not water). Food restriction was maintained for 2 h after drug administration.

2.1.5 Test Substance Administration Volume

The dosage of the study drug was fixed at 1000mg and 2000mg/kg for Groups I and II respectively.

2.1.6 Observation Period

Each animal was observed at least once during the first half an hour and periodically for 1 day, with particular focus on the first 4 hrs, also daily observation for 2 weeks was done. Any signs of illness or changes in behaviour were monitored at least twice a day and recorded.

2.1.7 Observations during Acute Toxicity Assessments

Parameters observed directly included tremors, convulsions, salivation, diarrhea, lethargy, sleep and

coma, as well as in various body systems along with somatomotor activity and behaviour, were noted. In case of death, the time of occurrence was recorded. After administering *Navauppu Mezhugu*, rats were fasted for 1-2 h. The animals survived after 24 hours and were observed daily for an additional 14 days.

2.2 Repeated Dose Oral Toxicity Assessment

2.2.1 Experimental Animals and Grouping

Wistar Albino rats of both sexes, weighing 190±10 g, were employed for evaluating repeated dose oral toxicity. The rats were divided into four groups, each comprising six animals (n=6, comprising 3 males and 3 females) and based on the animals' body weight dosage was determined. A card indicating the cage number, number and weight of the rats, drug code, route of administration, and dosage was placed in each cage for easy identification.

2.2.2 Test Drug Administration

Group I animals received a daily oral dose of vehicle control, consisting of a 5% Palm jaggery solution in R.O. water, for 28 days.

Group II - 200 mg/kg body weight (low dose)
Group III- 400 mg/kg (middle dose)
Group IV- 800 mg/kg (high dose)[8-10]

2.2.3 Observation and Collection of Blood Sample

The experimental animals were weighed once a week starting from the beginning of treatment to monitor changes in body weight. At the end of treatment, after administering mild anaesthesia, blood was drawn from the retro-orbital plexus for assessing haematological parameters and biochemical analysis. Blood samples were collected, centrifuged within 5 min at 4000 x g for 10 min to obtain plasma and then analyzed for liver enzymes, lipid profile, creatinine, and urea.

2.2.4 Postmortem Observations and Histopathological Analysis of Organs

All animals were examined carefully for changes in external body surface, all orifices, body cavities and their contents on postmorterm[11]. On day 29, a terminal necropsy was performed for all animals. Following blood collection, the rats were anaesthetized with ether for necropsy. Heart, liver, kidneys, and spleen, were looked for gross lesions. The tissues were fixed with 10% formalin, sliced 5-6 mm, stained with hematoxylin and eosin (H and E) dye, and examined under a light microscope. Any changes in the treatment group were compared with the control group[12].

2.2.5. Statistical Analysis

One-way Analysis Of Variance (ANOVA) was applied to compare the changes in biochemical and haematological parameters of treatment groups. All data analyses employed the Newman-Keuls multiple range tests, with statistical significance set at $p<0.05$, using Graph Pad Instat version 3.1.

3. Results

3.1 Acute Toxicity Study

This study demonstrated that *Navauppu Mezhugu* did not cause any mortality over 14 days, even at the maximum limit dose of 2000mg/kg body weight. LD50 could not be found out, as the dosage exceeded 2000mg/kg. As the formulation *Navauppu Mezhugu* was found practically non-toxic, it is not necessary to test the drug *Navauppu Mezhugu* at a higher dose. On observation up to 15 min after administration of *Navauppu Mezhugu*, the animals were found to be well alert and showed response to righting reflex, pinna, and corneal reflexes. They also had normal pupils, urination, salivation, lacrimation, and gripping strength with no apparent change in their skin colour. Hyperactivity, grooming, tremors and convulsions never occurred during the study period.

All the other parameters were observed to remain in the normal range up to a maximum dosage of 2000mg/kg body weight, indicating *Navauppu Mezhugu* does not possess any oral toxicity. Since the LD50 is more than 2000mg/kg body weight, the formulation produced no adverse effects on single-dose administration and with a significant value of 5% on statistical analysis.

3.2 Repeated Dose Oral Toxicity Study

3.2.1 Changes in Body Weight

Changes in body weight of animals in each group, including the control group, were recorded on Days 1, 14, 21, and 28 and tabulated in Table 1.

Table 1. Changes in the body weight of experimental animals

Treatment	Day 1	Day 14	Day 21	Day 28
Control	180.30±5.10	182.15 **±5.35**	185.45 ±5.60	190.2±5.85
Navauppu Mezhugu **200 mg.kg^{-1}**	186.95 ±5.70	192.45 **±5.90**	197.80 ±6.10	202.80±6.35*
Navauppu Mezhugu **400 mg.kg^{-1}**	189.60 ±6.30	194.40 **±6.50**	198.55 **±6.70**	204.75±6.90*
Navauppu Mezhugu **800 mg.kg^{-1}**	192.20 **±6.30**	195.35±6.55	199.60 **±6.80****	204.70±6.95**

Values expressed in terms of mean ± S.E.M. (n=6). Group I values were compared with those of Groups II, III and IV. A one-way ANOVA test was applied, with significance *p<0.01 and **p<0.05.

Table 2. Changes in blood glucose and lipid profile of experimental animals

Dosage	Glucose (mg.dl^{-1})	Cholesterol (mg.dl^{-1})	Triglyceride (mg.dl^{-1})	HDL (mg.dl^{-1})	LDL (mg.dl^{-1})
Control	97.35±2.60	47.30± 2.15	34.75±1.60	165.35±3.10	80.30±1.75
Navauppu Mezhugu **200 mg.kg^{-1}**	98.90±2.80	35.60±1.90*	19.45± 0.85*	171.15±3.30*	75.65±1.55
Navauppu Mezhugu **400 mg.kg^{-1}**	95.30±2.55	37.85±1.96*	21.55±1.15*	162.35±2.90*	78.50±1.62
Navauppu Mezhugu **800 mg.kg^{-1}**	94.90±2.40**	42.35±2.15*	23.75±1.30*	169.10±3.08*	74.10±1.30

Values expressed in terms of mean ± S.E.M. (n=6). Group I values were compared with those of Groups II, III and IV. A one-way ANOVA test was applied, with significance *p<0.01 and **p<0.05.

Table 3. Changes in liver enzymes on repeated oral administration of *Navaupu Mezhugu*

Dosage	AST (IU.l^{-1})	ALT (IU.l^{-1})	ALP (IU.l^{-1})	TP (g.l^{-1})	ALBUMIN (g.l^{-1})
Control	315.6±6.65	72.3± 2.90	265.30±5.45	67.50± 2.95	41.30±2.15
Navauppu Mezhugu **200 mg.kg^{-1}**	306.3±6.30**	73.6±2.98**	260.15±5.20**	68.15±3.05	38.20±1.90
Navauppu Mezhugu **400 mg.kg^{-1}**	310.4±6.45**	76.4±3.15**	258.70±4.95**	72.40±3.25	37.40±1.96
Navauppu Mezhugu **800 mg.kg^{-1}**	325.5±6.40	70.6±3.30	268.70±5.60	75.50± 3.60	40.25±2.05

Values expressed in terms of mean ± S.E.M. (n=6). Group I values were compared with those of Groups II, III and IV. A one-way ANOVA test was applied, with significance *p<0.01 and **p<0.05.

Table 4. Changes in haematological parameters on repeated oral administration of *Navauppu Mezhugu*

Dosage	Haemoglobin (mg.dl^{-1})	RBC (10^6/mm^3)	WBC (10^6/mm^3)	Calcium (mg.dl^{-1})
Control	13.5±1.06	9.35± 0.85	10.6± 1.02	9.70 ±0.85
Navauppu Mezhugu **200 mg.kg^{-1}**	14.1±1.28*	9.12±0.80*	9.6± 0.72*	9.25 ±0.43
Navauppu Mezhugu **400 mg.kg^{-1}**	14.3±1.36*	9.45±0.93*	9.8± 0.84*	9.45 ±0.65
Navauppu Mezhugu **800 mg.kg^{-1}**	14.5±1.42*	8.90± 0.68*	9.9± 0.90*	9.80 ±0.85

Values expressed in terms of mean ± S.E.M. (n=6). Group I values were compared with those of Groups II, III and IV. A one-way ANOVA test was applied, with significance set at *p<0.01.

3.2.2 Effect of Navauppu Mezhugu on the Biochemical Profiles of Rats

The effect of *Navauppu Mezhugu* on various biochemical parameters, including plasma Total Cholesterol (TC), Triglycerides (TGL), HDL, and LDL cholesterol levels in the experimental rats, was assessed periodically; results are presented in Table 2.

3.2.3 Effect of Navauppu Mezhugu on Liver Function of Rats

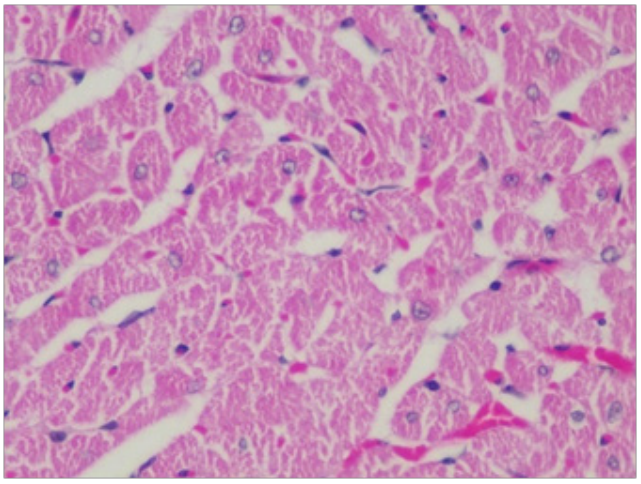
To assess impaired liver function and hepatotoxicity, an assay of liver enzymes was carried out. Therefore, liver enzymes such as AST, ALT, ALP, Total Protein (TP), and albumin were assessed periodically, and the results are presented in Table 3.

3.2.4 Effect of Navauppu Mezhugu in Haematological Parameters in Rats

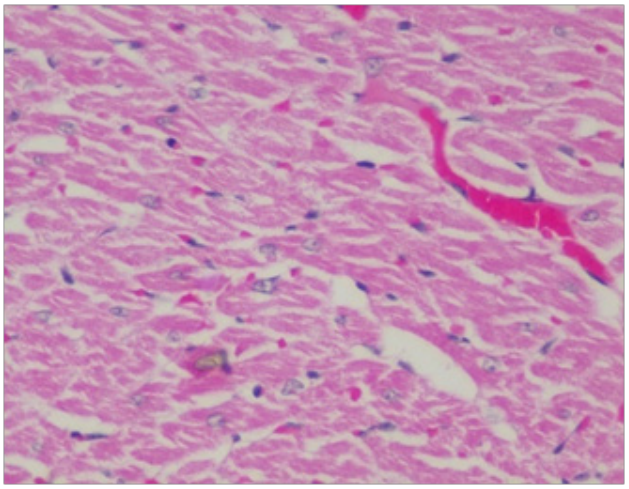
The effects of *Navauppu Mezhugu* on haematological parameters, including Hemoglobin (Hb), Red Blood Corpuscles (RBC), and White Blood Corpuscles (WBC) in the experimental rats, were observed. The mean values of these results are presented in Table 4.

3.2.5 Histopathological Analysis

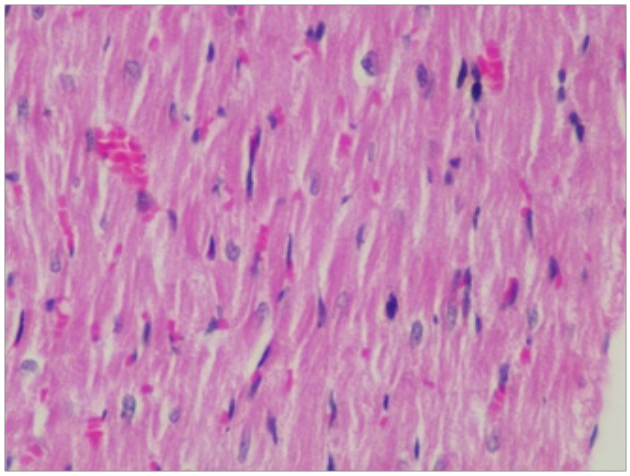
Histopathology of vital organs such as the heart, kidney, liver, and spleen are illustrated in Figures 1-4.



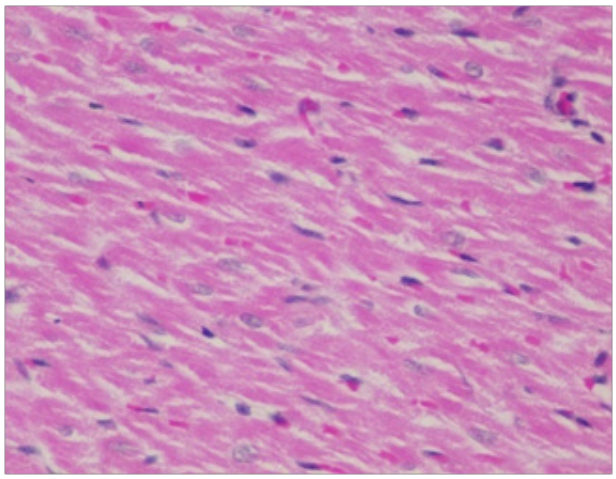
(a) Group-I Normal Control



(b) Group-II- 200 mg/Kg

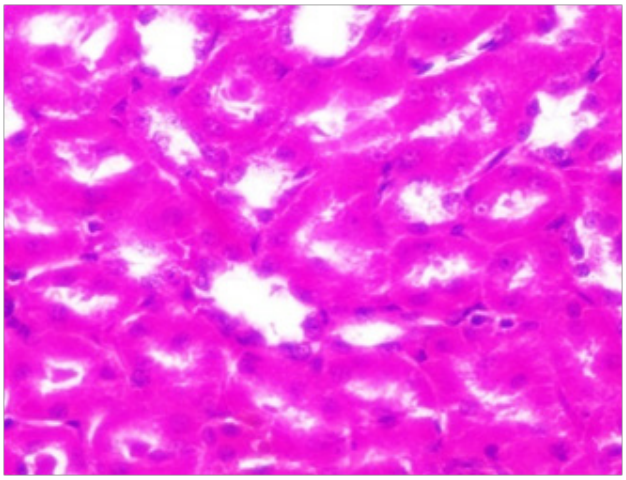


(c) Group-III- 400 mg/Kg

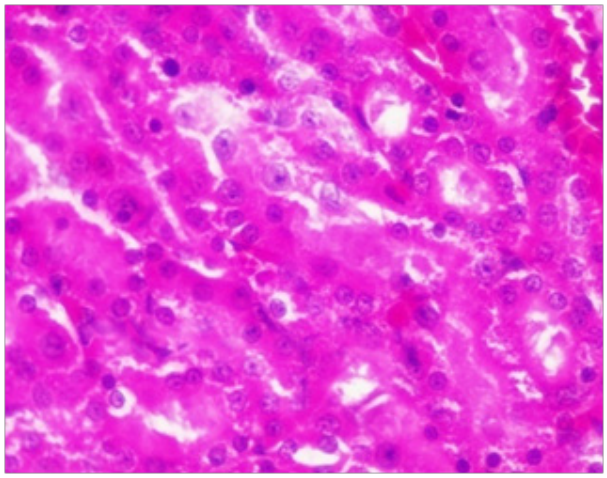


(d) Group-IV- 800 mg/Kg

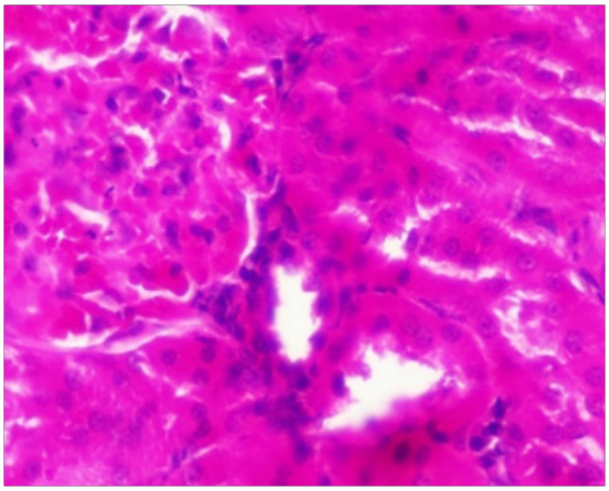
Figure 1. Heart. (**a-d).** Histopathological changes of heart in Group I - normal control, Group II - 200mg/kg, Group III - 400mg/kg and Group IV - 800mg/kg, respectively.



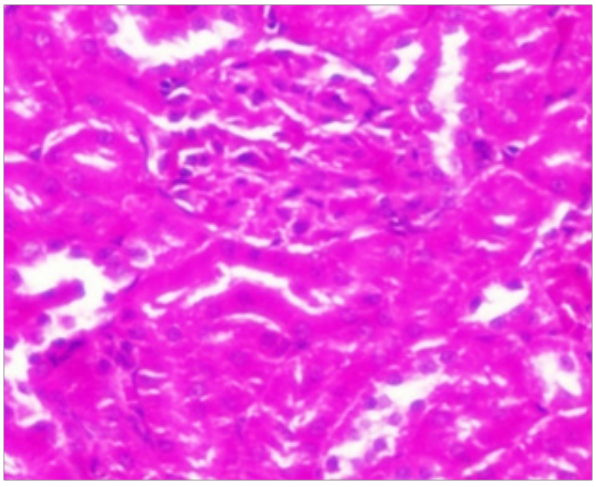
(a) Group-I Normal Control



(b) Group-II- 200 mg/Kg

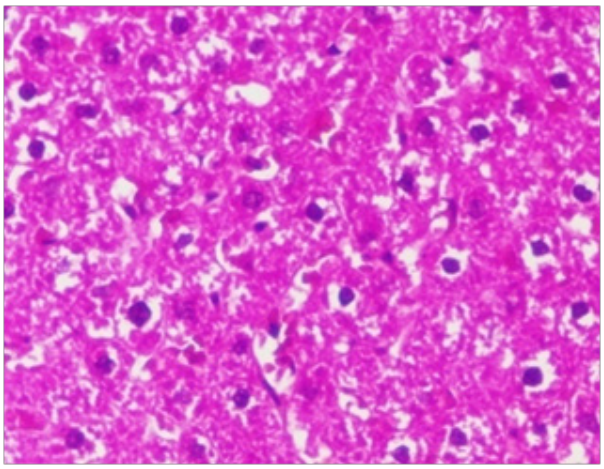


(c) Group-III- 400 mg/Kg

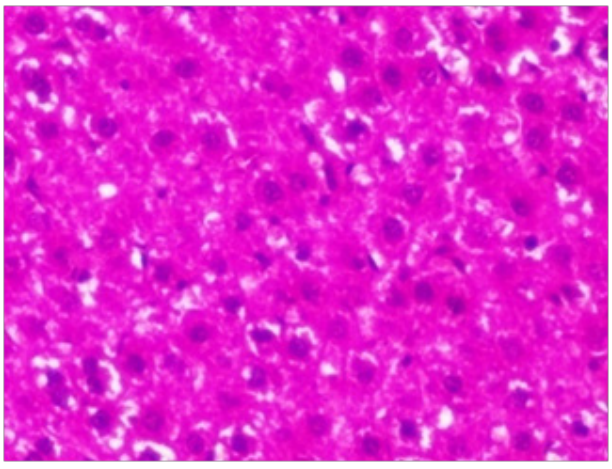


(d) Group-IV- 800 mg/Kg

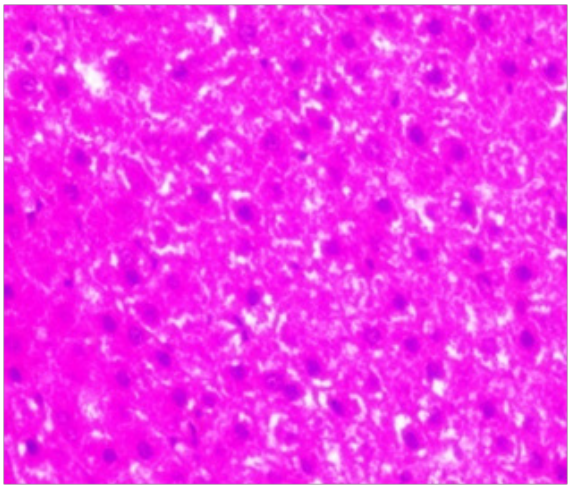
Figure 2. Kidney. **(a-d).** Histopathological changes of the kidney in Group I - normal control, Group II - 200mg/kg, Group III - 400mg/kg and Group IV - 800mg/kg, respectively.



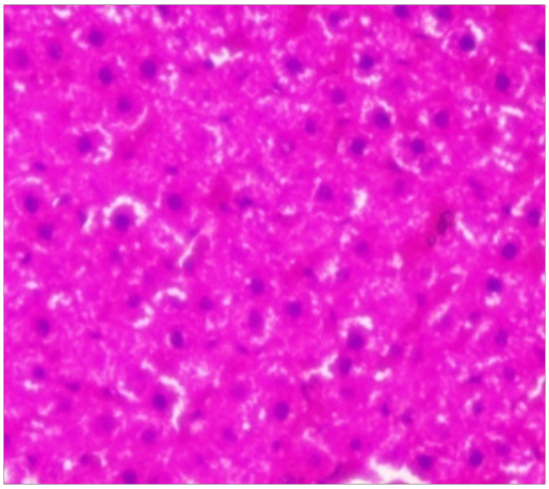
(a) Group-I Normal Control



(b) Group-II- 200 mg/Kg

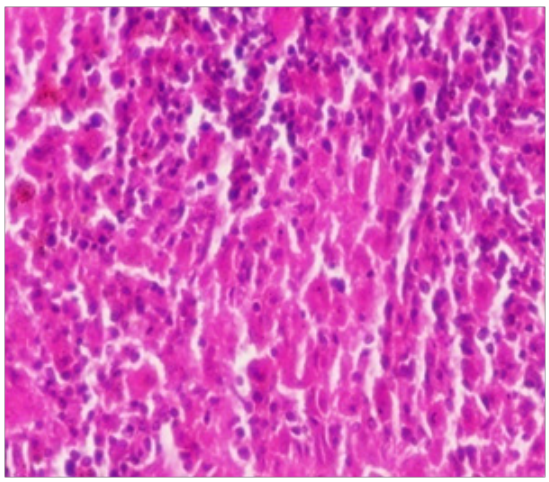


(c) Group-III- 400 mg/Kg

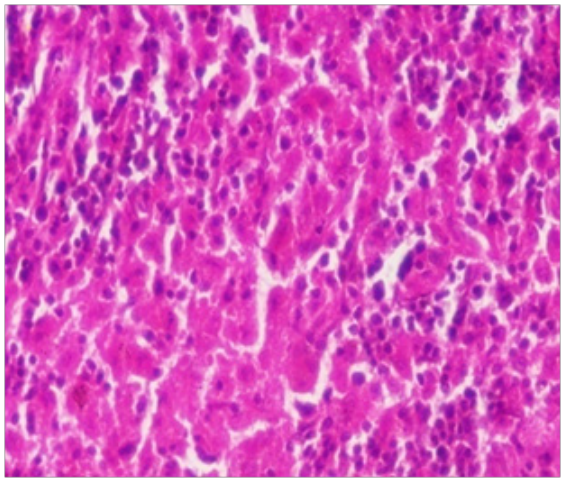


(d) Group-IV-800mg/Kg

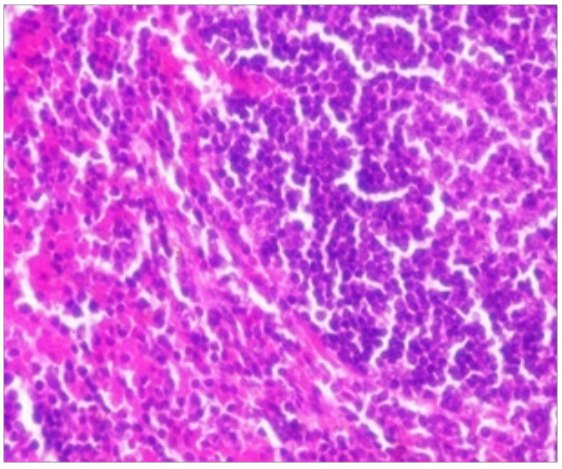
Figure 3. Liver. **(a-d).** Histopathological changes of the liver in Group I - normal control, Group II - 200mg/kg, Group III - 400mg/kg and Group IV - 800mg/kg, respectively.



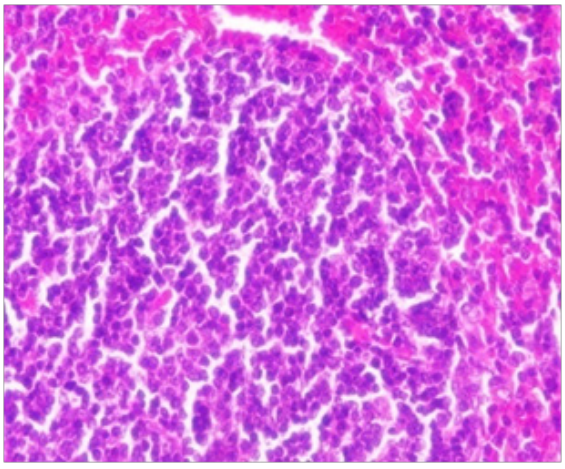
(a) Group-I Normal Control



(b) Group-II- 200 mg/Kg



(c) Group-III- 400 mg/Kg



(d) Group-IV-800mg

Figure 4. Spleen. **(a-d).** Histopathological changes of the spleen in Group I - normal control, Group II - 200mg/kg, Group III - 400mg/kg and Group IV - 800mg/kg, respectively.

4. Discussion

Siddha medicine is a traditional medical system practiced in the southern regions of India, that flourished due to the dedicated efforts of individuals known as *Siddhars*, who embraced obscurity and austerity. Those who attained these remarkable abilities were referred to as *Siddhars*[13]. According to WHO traditional medicine is defined as the "sum aggregate of knowledge, skill and practices rooted in the theories, beliefs, and experiences unique to various cultures, whether or not they can be scientifically explained, used to maintain health, and to prevent, diagnose, treat, or improve both physical and mental illnesses."[14]. A significant role has been played by traditional medicine in addressing the primary healthcare needs of many developing countries, and the use of traditional medicine has increased in numerous developed countries[15]. In a series of three global surveys conducted by WHO between 2005 and 2018, 99 out of 113 responding member states (88 per cent) identified that more technical guidance is required for evaluating the safety, quality, and efficacy of traditional systems of medicines[16]. The lack of adequate evidence regarding the safety and efficacy of Traditional Chinese Medicine led to criticism of its inclusion in the 11th revision of the WHO International Classification of Diseases (WHO-ICD) in 2019[17].

Considering the above-mentioned facts one among the 32 *Agamarunthukal* mentioned under *Vinnavarurai*[18], *Navuppu Mezhugu* indicated for various ailments on approval of the Institutional Animal Ethical Committee, of K.M. College of Pharmacy, Madurai, Tamil Nadu, India (IAEC approval number IAEC/RADHAS/TNMGRMU/M.D(S)/3355/KMCP/122/2021) was selected for preclinical profiling.

The ingredients of the investigational drug include *karasaaram* (mineral salts) like *Indhuppu, Kariuppu, vazhayaluppu, potiluppu, Amuriuppu, Moongiluppu, Kaluppu, Paaraiuppu, Pooneeru* and toxic substance like *Veeram* (salt of mercury), *Karpooram.* These raw materials have common indications such as gastritis, indigestion, dysmenorrhea, rheumatism, pricking pain of joints (*santhu vaatha soolai*), joint stiffness and other vaatha-related diseases[3]. Also, it has pepper, long pepper, dried ginger, croton seeds, root of *thuthi* (*Abutilon indicum*), cow's milk, juice of *thummati* (*Citrullus colocynthis*). The pharmacological profile of pepper, long pepper, dry ginger, *thuthi, thumati* and croton seeds reveal their analgesic, antiarthritic, anti-inflammatory, anticancerous, antidiabetic, antihyperlipidemic, anti-HIV and immune modulatory effect[19-25]. In addition, *Abutilon indicum*, dry ginger, pepper and long pepper possess hepatoprotective activity[26-29]. Moreover piperine the active principle in pepper and long pepper is known for its antidote and antivenomous properties which could protect the liver from toxicity if any[30]. Piperine is also well known for its bioavailability-enhancing action that could aid in the absorption of drugs[31,32].

In the acute oral toxicity study, no mortality was observed among the test animals, indicating its non-toxic nature. The animals were monitored daily for any clinical signs for 14 consecutive days following administration. No fatalities occurred during the study period. Data on recorded home cage activities and handheld activities were found normal in both groups indicating that there were no significant toxicity-related behavior changes in the animals.

In a subacute repeated oral toxicity study, of the four groups, none of the groups exhibited changes in behaviour or food. Normal growth pattern was noted in both groups and weight gain ($p<0.05$) was specifically noted in test group animals. The results are presented in (Table 1).

Changes in liver enzyme levels, serum levels of cholesterol and proteins are regulated by their synthesis in the liver and variation in their serum concentrations implies liver toxicity. The results of administering *Navauppu Mezhugu*, clearly indicate that the investigational drug, at all concentrations, had not produced any liver damage (Table 2).

The study demonstrated a notable decrease ($p<0.05$) in plasma glucose levels in treated rats, particularly Group III rats, compared to Group I rats. A decrease ($p<0.05$) in other lipid profile parameters was observed, along with a notable increase ($p<0.05$) in HDL-cholesterol levels in all animals of the test group compared to the control group. Liver enzymes were found normal in all the animals treated with *Navauppu Mezhugu* indicating no severe toxicity associated with the administration of a high dosage of *Navauppu Mezhugu* (Tables 3 and 4).

When translating animal data to human data, changes in the haematological system are regarded as

having a higher predictive value for assessing toxicity[33]. The blood-forming organs are sensitive to toxic compounds, also they reflect the physiological and pathological status in man and animals[34]. In this study, an increase in hemoglobin ($p<0.01$) and erythrocyte count was noted at a dosage level of 800mg/kg, compared to the control group suggesting that one of the constituents of *Navauppu Mezhugu* may have enhanced iron absorption (Table 4).

Serum calcium levels remained unchanged in all treated animals and the control group (Table 4). Overall, the results indicate that *Navauppu Mezhugu* is non-toxic to the haematopoietic and leucopoietic systems, suggesting it is non-hepatotoxic. Histopathological examinations of major organs, including the liver, heart, kidneys, and spleen, revealed no remarkable changes across all three dosing levels.

5. Conclusion

The non-toxic nature of the *Siddha* formulation *Navauppu Mezhugu* is evident from the acute oral toxicity study conducted according to OECD guidelines, with no mortality observed throughout the study period. The normal behaviour of the test animals over 14 days further supports the non-toxic nature of the investigational drug, indicating it may be safe at doses up to 2000mg/kg body weight. No toxic symptoms were noted following the repeated oral administration of *Navauppu Mezhugu* for 28 days. Thus, it can be concluded that the dosage recommended in the literature is safe and suitable for long-term use in clinical practice.

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7. Author Contribution

S. Radha: Contributed in framing the study design and implemented the study; A. Mariappan: Analysed the statistical data, prepared graphical abstract; V. A. Mekala- Collected the data from literatures; K. Namasivayam- Collected the data from literatures; N. J. Muthukumar- Contributed in forming the unstructured data into article. All authors have read and agreed to the published version of the manuscript.

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