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Safety Assessment of AYUSH SC-3 through Acute and 90 Days Repeated Dose Oral Toxicity Study

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Abstract

The present study is focused on establishing the safety of the formulation through acute and 90 days of repeated oral dose toxicity as per the Organization for Economic Cooperation and Development (OECD) guidelines. During the acute toxicity test, the drug was orally administered at a limited test dose of 2000 mg/kg. Clinical signs, feed and body weight were recorded. At the end of 14 days, the animals were euthanized and subjected to a detailed post-mortem examination (necropsy). As per OECD, 408 the 90 days repeated dose oral toxicity study was carried out with three different doses of test drug i.e., 1500, 1000 and 500 mg/kg. Cage side observations, body weight and feed intake were recorded. Upon termination of the study, urine analysis, haematology and clinical biochemical examinations were performed. Finally, the rats were subjected to euthanasia, a gross necropsy was conducted and vital organs were weighed and made prone for histopathological evaluation. Cage-side observation of AYUSH-SC-3 treated animals showed no signs of toxicity, and the mortality or moribund state was observed in both of the studies. No significant change in body weight and feed intake was seen in AYUSH-SC-3 treated animals. Gross morphology and necropsy findings of the animals revealed no treatment-related. Haematological and biochemical parameters of rats treated with AYUSH-SC-3 were found to be non-significant when compared to the control group. Necropsy findings and relative organ weights did not change significantly. Further, the histopathological analysis of major organs showed no major lesions and treatment-related changes. The LD_{50} of AYUSH SC-3 is greater than 2000 mg/kg and NOAEL is up to 1500 mg/kg.

Keywords: Acute Toxicity, AYUSH SC-3, LD_{50}, NOAEL, OECD, 90 Days Repeated Dose Oral Toxicity

1. Introduction

The roots of experimentation for ensuring the safety of food and drugs have been in vogue since ancient times. There are indications that Ayurveda is accomplished with the testing on animals i.e., Vishanna/Virudhanna Pareeksha organized on animals. Sushruta mentioned the animal experiments like testing the food/drug by giving it to animals like birds (pigeons, peacocks), and animals (rabbits, monkeys) in order to establish safety and toxicity[1]. Charak Samhita explains the significance of the safety of Ayurvedic medicine by demarcating that medicine is safe and effective if it attenuates the disease and does not produce any consequent adverse effects[2]. In

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Ayurveda, plants, animal products and minerals-based medicines are used for healthcare and treatment of various disease conditions[3]. Nonclinical assessment of drug toxicity typically consists of definitive animal toxicology investigations. Animal toxicity studies are required to be carried out to evaluate the potential unwanted pharmacodynamic effects of drugs on anatomic functions in relation to susceptibility within the therapeutic limit[4].

The safety of the Ayurvedic formulation was evaluated as per OECD (Organization for Economic Co-operation and Development) Guidelines for the Testing of Chemicals (OECD TG). These are a set of globally approved specifications for the testing of chemicals decided on by the Organization for Economic Co-operation and Development (OECD). The Organization for Economic Co-operation and Development (OECD) Guidelines for the testing of chemicals or drugs are a distinctive tool for analysis of the prospective effects of chemicals on public health and nature. OECD Guidelines provide analytically designed experiments to comprehend the toxic effect of drug administration through various routes and durations. OECD 423 (the acute toxic class method) provides a stepwise procedure with the use of 6 animals (3 control + 3 test) of a single sex per step. Overall, OECD423 confirms NOEL (No Observed Effect Level) and NOAEL (No Observable Adverse Effect Level) by estimating the fatality as well as noticeable signs of toxicity[5]. Choosing pre-established doses regardless of the test substance, with a category explicitly tied to the number of animals distinguished in different states enhances the chances for laboratory-to-laboratory reporting, steadiness and replicability. The limit test is first and foremost used in circumstances where the researcher has information to specify that the trial compound is likely to be safe, i.e., having toxicity only more than regulatory limit doses. In usual practice, the test drug at a single limit dose of 2000 mg/kg may be given to a set of 3 female animals (three per step).

Most of the Ayurvedic preparations are usually intended to be used for multiple dosing regimens. This makes it necessary to screen Ayurvedic drugs for long-term oral toxicity testing. OECD guideline 408 explains the procedure for testing drugs for their 90-day repeated dose oral toxicity. In general, repeated dose toxicity studies are intended to reveal and determine any physiological, anatomical or pathological changes by the repeated administration of the test drug and to identify the dose-response relationships[6]. Unlike single-dose oral toxicity testing, 90 days of repeated dose oral toxicity involves an intense understanding of organ levels' toxic effects as evident from biochemical and histological data[7]. The test is designed in such a way as to cover the post-weaning maturation and adulthood of the test animals. The clinical biochemical parameters determined during the 90-day repeated dose oral toxicity focus on exploring the toxicity of the test drug on specific tissues/organs by measuring the possible markers of general tissue damage.

It is essential to assess the toxic potential/hazard-causing properties of any drug before it can be recommended for its desired properties. Therefore, the test drug has to be tested to assess its safety profile by performing repeated dose oral toxicity studies in laboratory rats.

2. Materials and Methods

2.1 Procurement and Storage of Test Drug

The test-coded drug AYUSH SC-3 was received from the CCRAS (Central Council for Research in Ayurvedic Sciences), New Delhi in the form of tablets of 500 mg each and stored in a plastic bottle at room temperature in racks designed for storing chemicals and drugs.

2.2 Animals

Principles of laboratory animal care guidelines were followed and prior permission was sought from the Institute Animal Ethics Committee (IAEC) for conducting the experiments (Approval No. IAEC/NARIP/2021-22/02).

Female and male *Sprague-Dawley* rats were procured from a small animal breeding station, Veterinary College, Mannuthy, Thrissur, Kerala. (CPCSEA reg. No. 28/PO/C/01/CPCSEA) and Animals were maintained as per CPCSEA (Committee for the Purpose of Control And Supervision of Experiments on Animals) guidelines.

2.2.1 Chemicals and Kits

Normal saline, Formaldehyde, Sodium citrate, Phosphate buffer solution, Urine strips, Thromborel, Vaccutainers, Pipette tips, Centrifuge tubes, Test tubes and Plastic bottles for collection of organs, ERBA (TRANSASIA, India) fully auto analyzer kits, Rat Thyroxine (T4) BT LAB (Zhejiang, China) (Cat.No.EA0027 Ra, DOE:16/02/2023), urine strips (DEKA PHAN LAURA, TRANSASIA, India).

2.2.2 Instruments

Hematology analyzer (Lab X, Trivandrum, India), semi-automated clinical chemistry analyzer (Robonic, Thane, India), electrolyte analyser (Krishlyte, Krish biomedicals, Delhi, India).

2.3 Statistical Analysis

Results were analyzed by using student's t-test with Graph pad prism 8.4.2 software. Data were expressed as mean ± standard error of the mean. $P<0.05$ was considered statistically significant.

3. Acute Oral Toxicity Study

3.1 Animals

Female *Sprague Dawley* Rats are one of the recommended species for oral toxicity studies as per OECD guidelines.

3.2 Husbandry and Environment

Animals were maintained at the experimental animal's house facility at a temperature of 25 ± 2 °C, relative humidity of 55 ± 5 % and light and dark cycles of 12 h. Animals were housed in cages designed for rats and provided with a standard pellet diet (Champaka feeds, Bengaluru), water *ad libitum*.

3.3 Grouping and Randomization

Animals were randomized as per body weight and divided into 2 groups (control and test) containing each of 3 animals. Females were nulliparous and non-pregnant. The vehicle control group was treated with distilled water, while the test group was treated with a 2000 mg/kg dose of AYUSH SC-3.

3.4 Study Design

The study was conducted as per the OECD guideline 423 (Acute Toxic Class Method). The dose selected for the present study is a limited test dose as mentioned in the guidelines[8]. AYUSH SC-3 was suspended in distilled water as a vehicle and administered once in a dose volume of 1.0 ml/100g of BW (body weight).

3.5 In-Life Phase Observations

Cage side observations included changes in fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behavior pattern, morbidity and mortality daily for 14 days. Change in the bodyweight and feed intake was recorded weekly.

3.6 Pathology - Necropsy

Animals were sacrificed with CO_2 asphyxiation method on the 15th day of the study and subjected to a detailed post-mortem examination.

4. 90 Days Repeated Dose Oral Toxicity Study

90 days toxicity (sub-chronic toxicity) of the test drug was performed as per the OECD guideline 408 to establish the NOAEL.

4.1 Animals

Male and female SD rats were used in the present study which is the one of recommended species as per OECD 408 guidelines.

4.2 Husbandry

Animals were acclimatized for 7 days before initiation of the study. The health examination was performed during the acclimatization period. Animals were maintained at the Experimental animal house facility, NARIP (National Ayurveda Research Institute for Panchakarma), Cheruthuruthy under standard housing conditions of temperature 25 ± 2 °C, relative humidity 55 ± 5 % and light and dark cycles of 12 h. They were housed in polypropylene cages (43×27×15 cm) group of three animals/cage with lids and rice husk bedding. Animals were provided with a standard pellet diet (Champaka feeds, Bengaluru) during the study period. The purified filtered water was provided *ad libitum* using plastic nozzle bottles.

4.3 Grouping, Randomization and Identification

Animals were randomized based on the body weight and divided into 5 groups (Groups I, II, III, IV and V). Group I serves as vehicle control group which was treated with distilled water. While the Groups II, III, and IV serve as test drug treatment groups that received the AYUSH SC-3 at 1500 mg/kg (high dose), 1000 mg/kg (average dose)

and 500 mg/kg (low dose) respectively, Group V serves as a satellite group and was treated with a high dose of AYUSH SC-3 to check the reversibility of toxicity if any. Vehicle, test drug groups consist of 20 rats (10 males and 10 females) and the satellite group consists of 10 rats (5 males and 5 females). The female animals used in the present study were nulliparous and non-pregnant. The grouping and randomization of the study are shown in Table 1.

4.4 Methodology and Study Design

The vehicle and AYUSH SC-3 were administered orally for 90 days to individual animals of control and test groups, respectively in a dose volume of 1.0 ml/100g BW. The body weight and feed consumption were recorded weekly. Mortality, signs and symptoms of toxicity were observed daily for the entire study period. At the end of the study blood samples were collected for hematology and serum biochemistry. Animals were euthanized and a detailed post-mortem was carried out. Internal organs were weighed and tissue samples were preserved in 10% formalin for histopathology[9]. The organs subjected for weighing and histopathology shown in Table 2. Histopathology was carried out at Regional Ayurveda Institute for Fundamental Research, Pune.

5. Results

5.1 Acute Oral Toxicity Study

The AYUSH SC-3 treated animals had shown no abnormality in terms of clinical signs i.e., Changes in fur, Eyes, Mucous membrane, Respiratory, Circulatory, CNS, Somatomotor and behavioural pattern. No mortality and the moribund stage were observed. No significant

Table 1. Grouping and randomisation during 90 days repeated dose oral toxicity

Name	Animals	
	Male	Female
Vehicle Control	NCM (10 rats)	NCF (10 rats)
High Dose 1500 mg/kg	HDM (10 rats)	HDF (10 rats)
Medium Dose 1000 mg/kg	ADM (10 rats)	ADF (10 rats)
Low Dose 500 mg/kg	LDM (10 rats)	LDF (10 rats)
Satellite (1500 mg/kg dose)	SDM (05 rats)	SDF (05 rats)

Table 2. List of organs collected during 90 days repeated dose oral toxicity

Organ/Tissue	Collection and Preservation	Histopathology	Weighing
Skin	✓	✓	X
Adrenals (2)	✓	✓	✓
Brain	✓	✓	✓
Heart	✓	✓	✓
Kidneys (2)	✓	✓	✓
Lungs	✓	✓	✓
Liver	✓	✓	✓
Sciatic nerve	✓	✓	X
Spleen	✓	✓	✓
Thymus	✓	✓	✓
Testes (2)/ Ovaries	✓	✓	✓
Intestine	✓	✓	X

change in the weekly body weight of the AYUSH SC-3 treated animals when compared to the control ($p>0.05$) (Figure 1). The feed intake of test-drug-treated animals did not differ significantly when compared to the control animals ($p>0.05$) (Figure 2). The gross necropsy findings were normal and the organ weights were not significantly changed as compared to the control ($p>0.05$) (Figure 3).

5.2 90 Days Repeated Dose Oral Toxicity Study

The AYUSH SC-3 treated animals had shown no abnormality in terms of clinical signs i.e., Changes in fur, Eyes, Mucous membrane, Respiratory, Circulatory, CNS, Somatomotor and behavioural pattern. No mortality

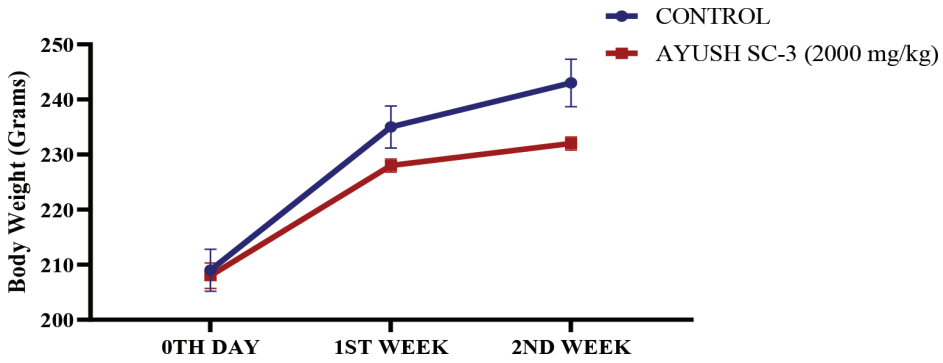


Figure 1. Body weight.

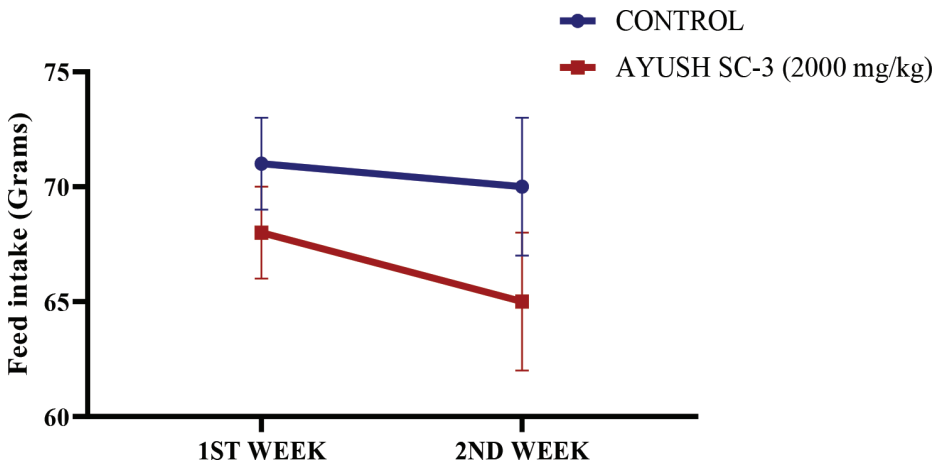


Figure 2. Feed intake.

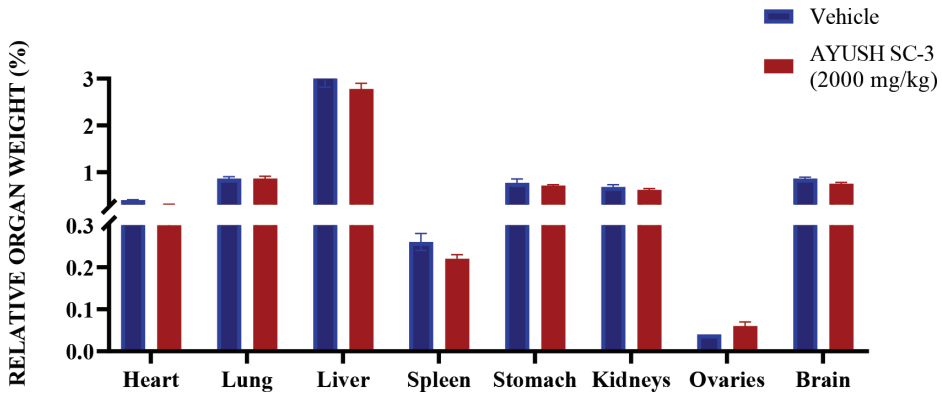


Figure 3. Relative organ weight.

and the moribund stage were observed throughout the treatment period. Weekly body weights of the drug-treated animals didn't change significantly throughout the study period as compared to the vehicle-treated animals ($p>0.05$) (Figures 4, 5). Feed intake was found to be normal. The results of feed consumption didn't show any significant changes as compared to the control ($p>0.05$) (Figures 6, 7).

The haematological parameters such as RBC (Red Blood Cells), WBC (White Blood Cells), Platelet counts as well and other indices were found to be within the range. The haematological data of the AYUSH SC-3 treatment didn't show any significant change as compared to the vehicle-treated rats ($p>0.05$) (Figures 8, 9). Similarly, the biochemical analysis of serum has shown no significant change in blood glucose, total cholesterol, HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), VLDL (Very Low Density Lipoprotein, triglycerides, AST, ALT, ALP, total bilirubin, thyroxine and creatinine levels when compared to the vehicle-treated animals ($p>0.05$) (Figures 10, 11). The electrolyte analysis of serum had shown no significant effect on calcium, chloride, sodium and potassium levels as compared to the vehicle-treated animals ($p>0.05$) (Figures 12, 13).

Urine analysis data of the AYUSH SC-3 treated animals did not show any noticeable changes in the colour and specific gravity of the urine. The pH of the urine was found to be in the range of 6.0-9.0. AYUSH SC-3 treated animals had shown the absence of Glucose, Bilirubin, Ketones, Proteins, Nitrites, leukocytes, and Haemoglobin similar to the control animals. The gross necropsy of the AYUSH SC-3 treated animals was found to be similar to that of control animals. The relative organ weight data of the test drug-treated animals didn't show any significant difference when compared to the control animals (Figures 14, 15).

Specified organs/tissues as in the study protocol from the control group and test dose group animals

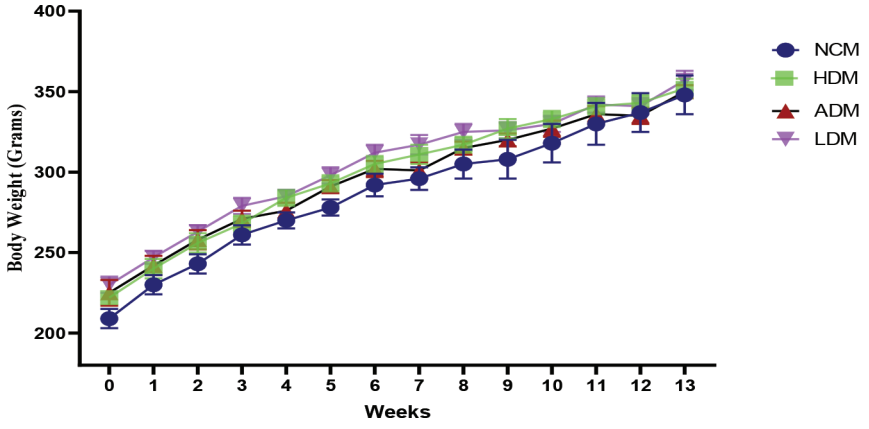


Figure 4. Body weight-males.

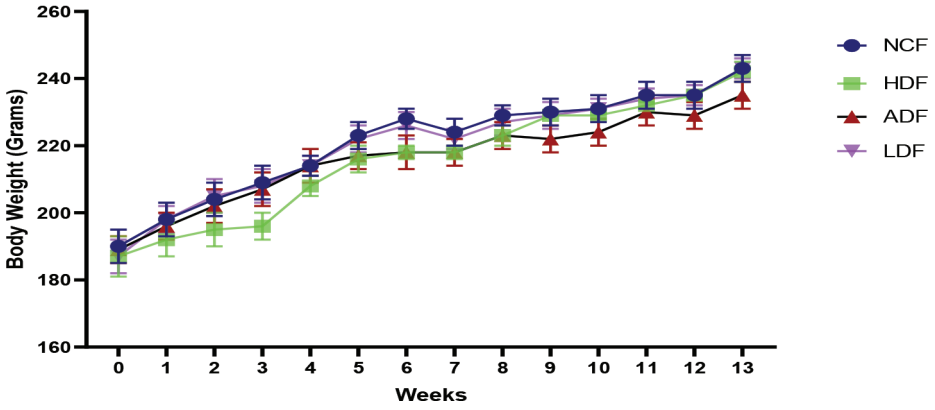


Figure 5. Body weight-females.

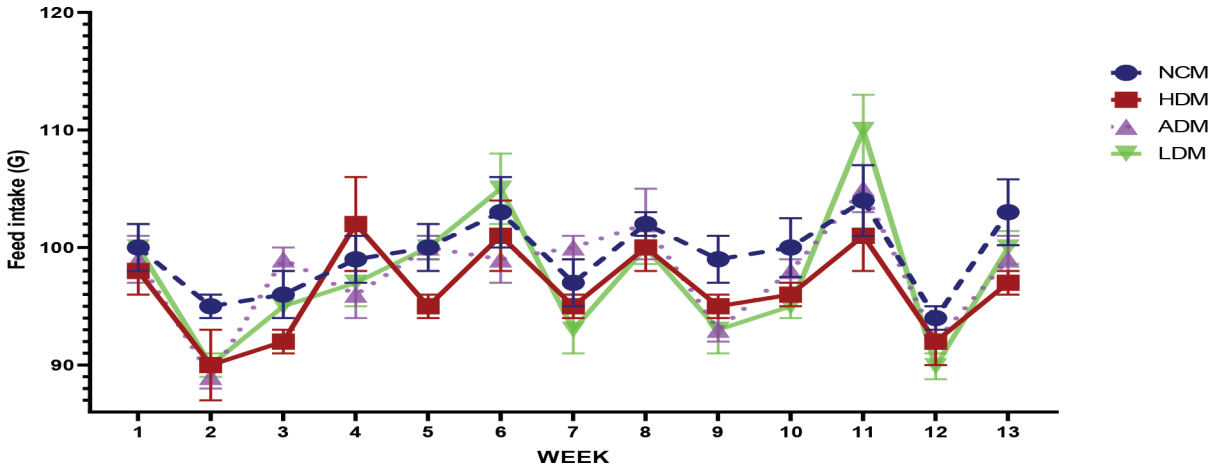


Figure 6. Feed intake- males.

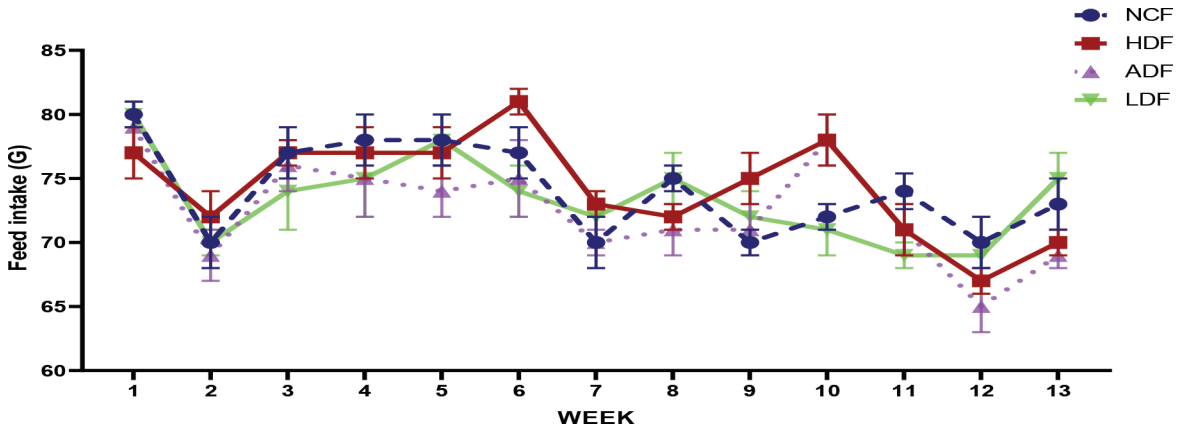


Figure 7. Feed intake-females.

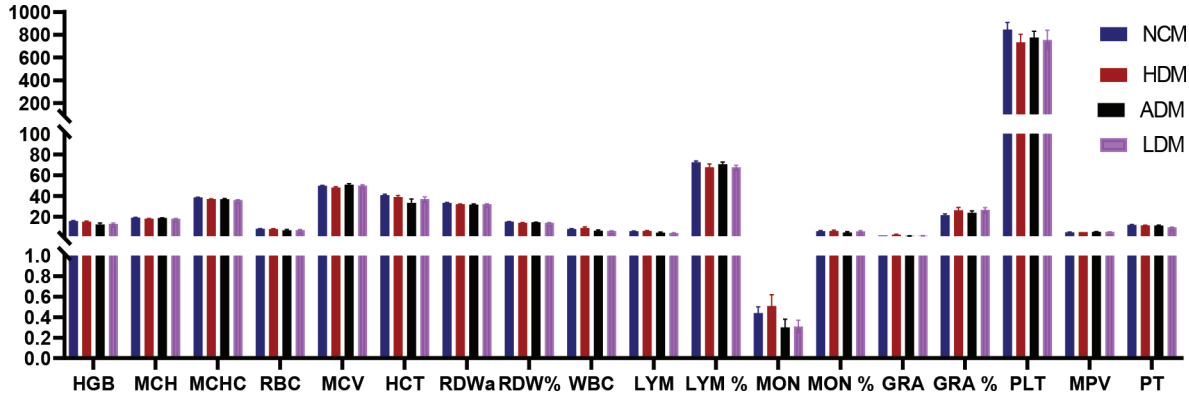


Figure 8. Haematology-males.

were trimmed, processed, embedded in paraffin blocks, sectioned and stained with routine Haematoxylin and Eosin (H and E) and evaluated for histopathological changes at Regional Ayurveda Research Institute, Pune.

Histopathology evaluation of various organs/tissues of the control group and test dose group did not reveal any treatment-related effect. Lungs of a few of the animals of the control group and test dose group showed mild to moderate focal as well as diffused lymphocytic infiltration surrounding airways and perivascular lymphocytic infiltration. The changes observed in lungs are commonly seen in animals housed in non-

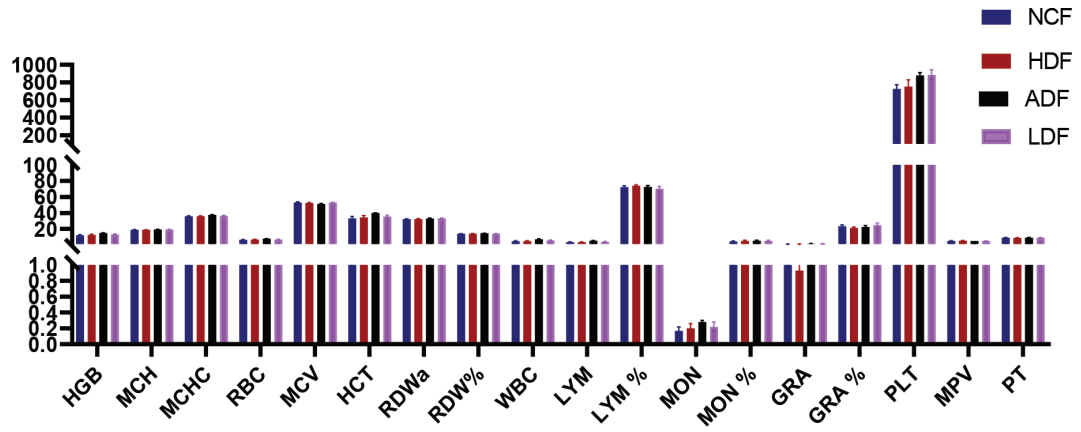


Figure 9. Hematology-females.

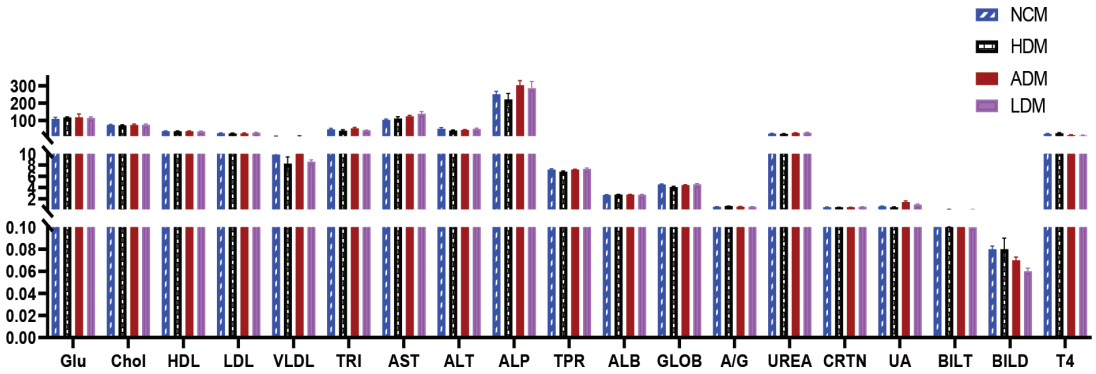


Figure 10. Serum biochemistry-males.

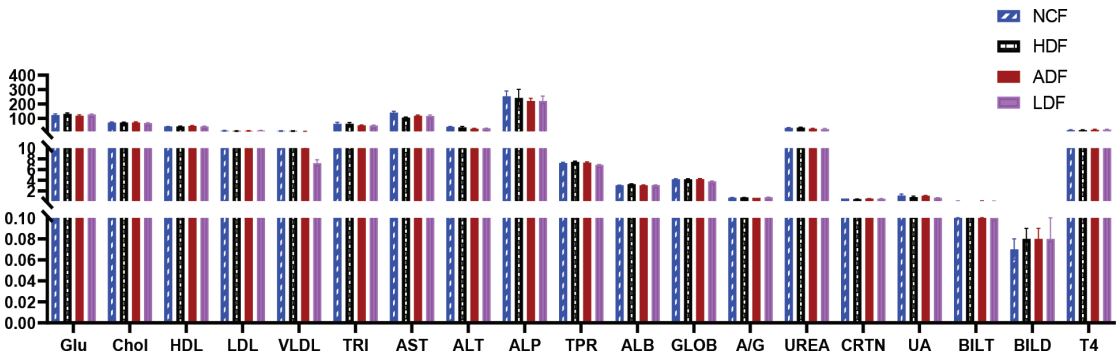


Figure 11. Serum biochemistry-females.

barrier animal housing facilities as in the present case. Incidental changes like MNC infiltration in lamina propria of the small intestine, stomach and focal MNC (Mono Nuclear Cells) infiltration in the liver were also observed in the animals from control as well as test groups. (Figures 16-31).

Histopathology evaluations of various organs/tissues from males and females of the control and test dose group did not show any lesion or histopathological observation which was treatment-related / dose-dependent of AYUSH SC-3 administration by oral route for 90 consecutive days in *Sprague Dawley* rats.

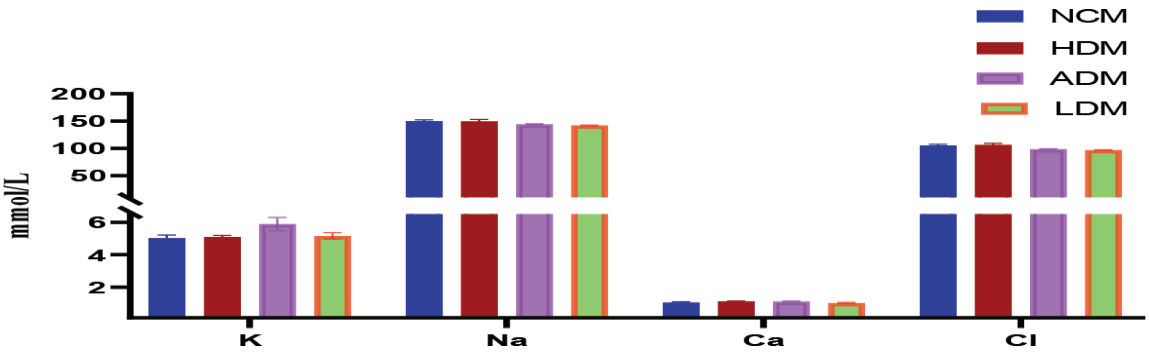


Figure 12. Serum electrolytes-males.

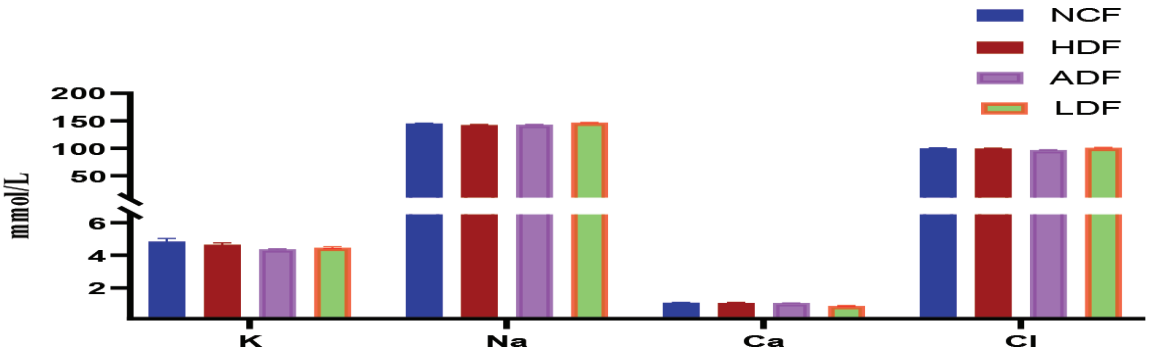


Figure 13. Serum electrolytes-females.

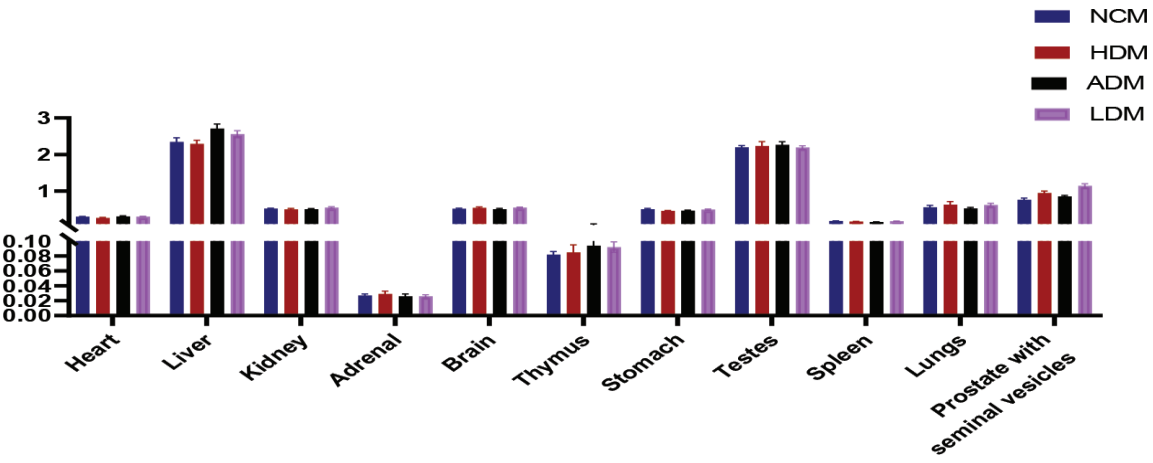


Figure 14. Relative organ weight – males.

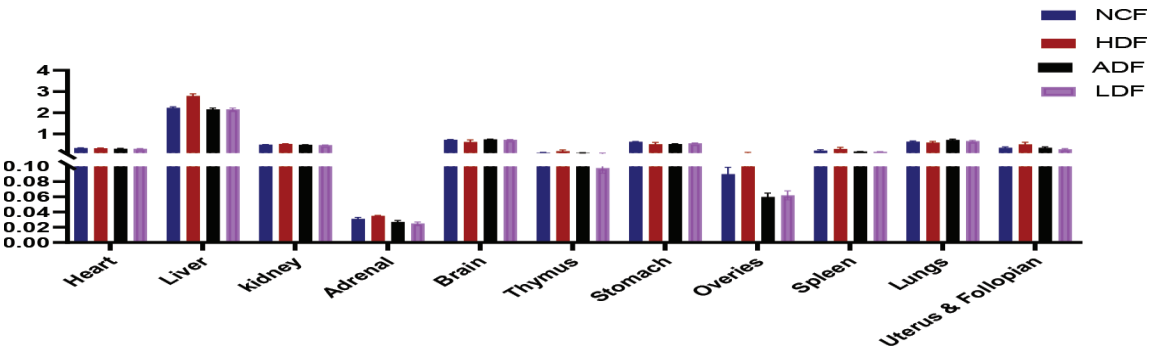


Figure 15. Relative organ weight – females.

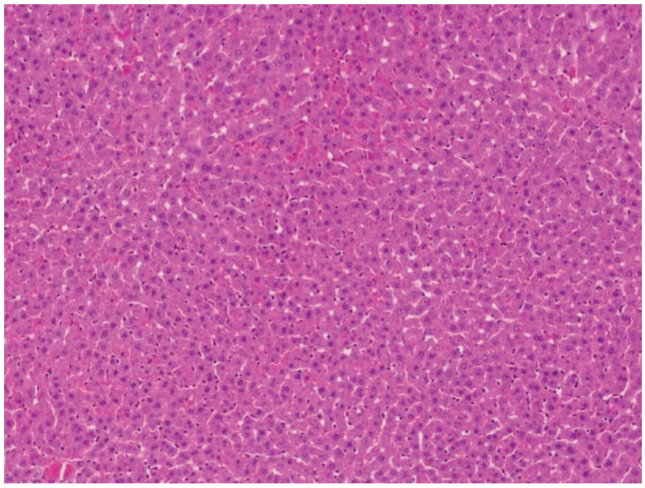


Figure 16. Liver high dose: NAD (10 x 10x).

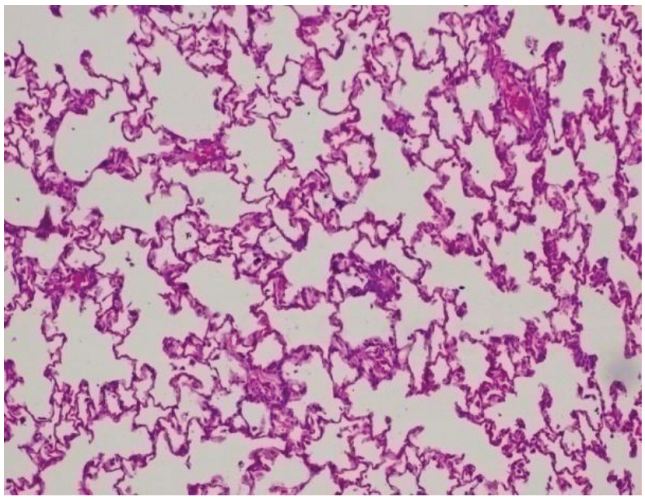


Figure 17. Lung high dose- NAD (10x 10X).

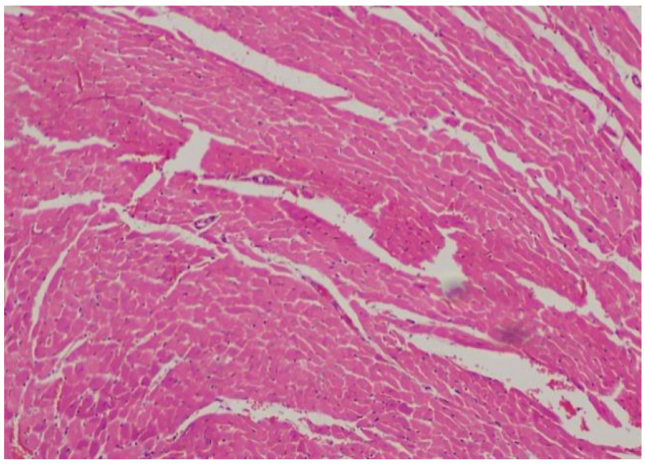


Figure 18. Heart high dose - NAD (10x 10X).

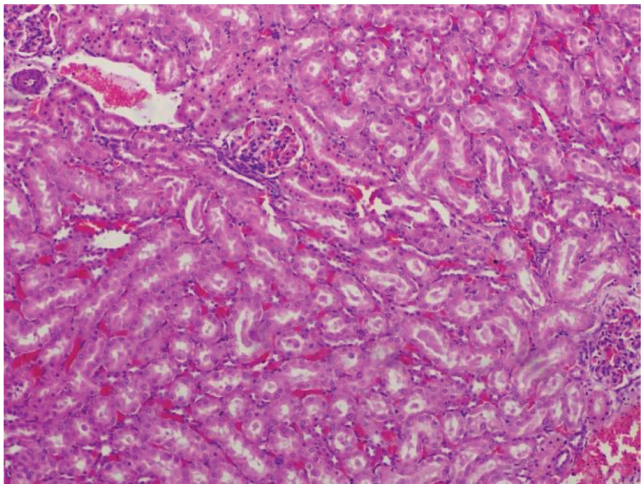


Figure 19. Kidney high dose - NAD (10x 10X)

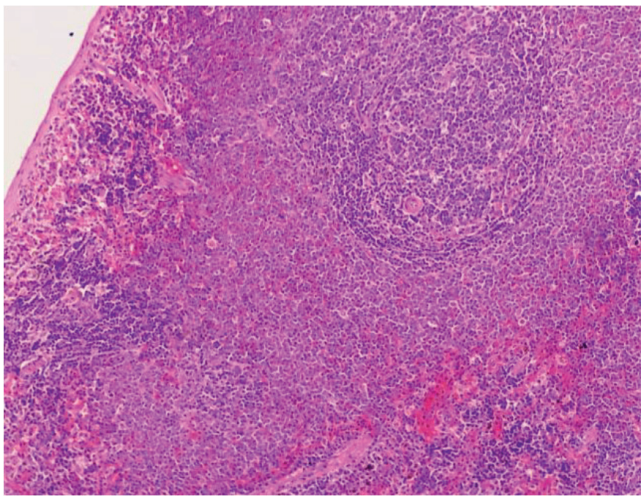


Figure 20. Spleen high dose - NAD (10x 10X).

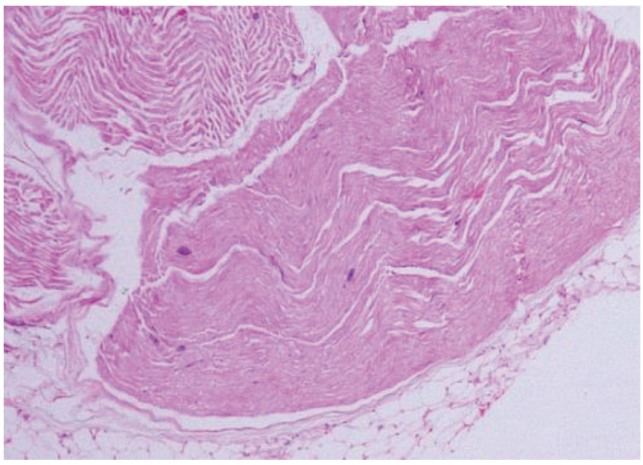


Figure 21. Sciatic nerve high dose - NAD (10x 10X)

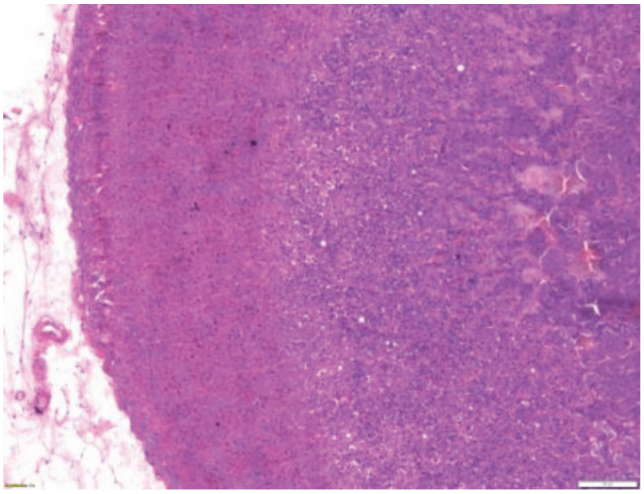


Figure 22. Adrenal gland high dose - NAD (10x 10X).

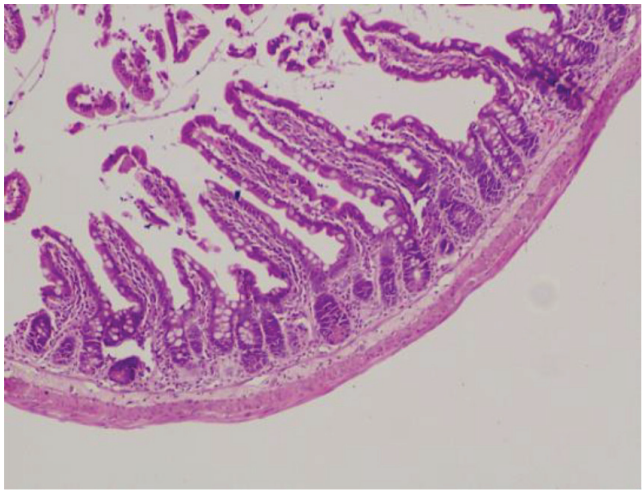


Figure 25. Small intestine high dose - NAD (10x 10X).

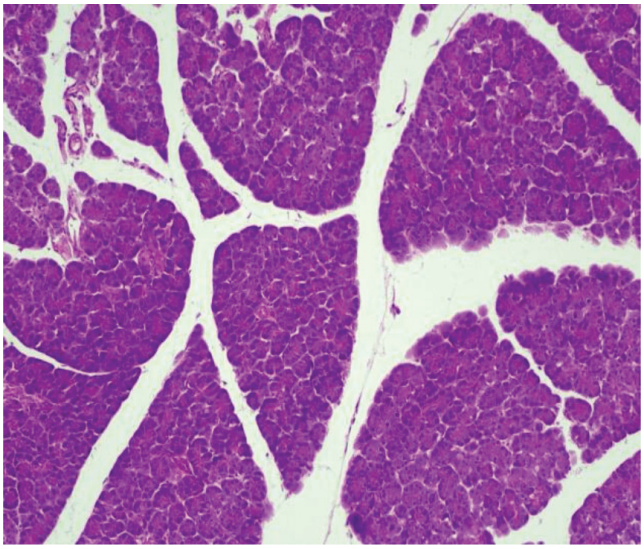


Figure 23. Pancreas high dose - NAD (10x 10X).

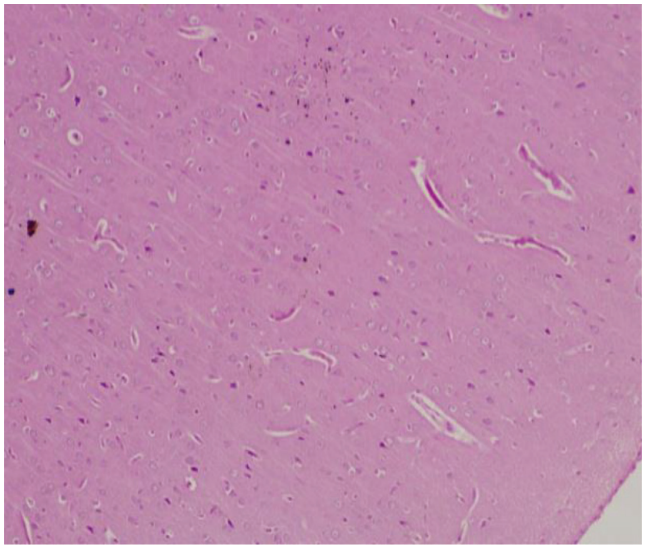


Figure 26. Brain high dose - NAD (10x 10X).

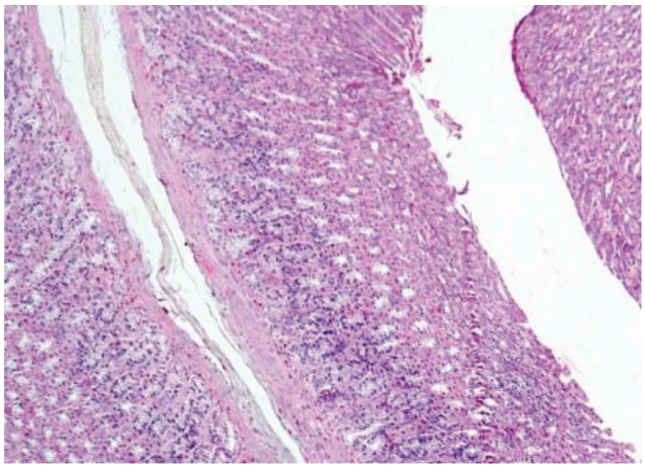


Figure 24. Stomach high dose - NAD (10x 10X).

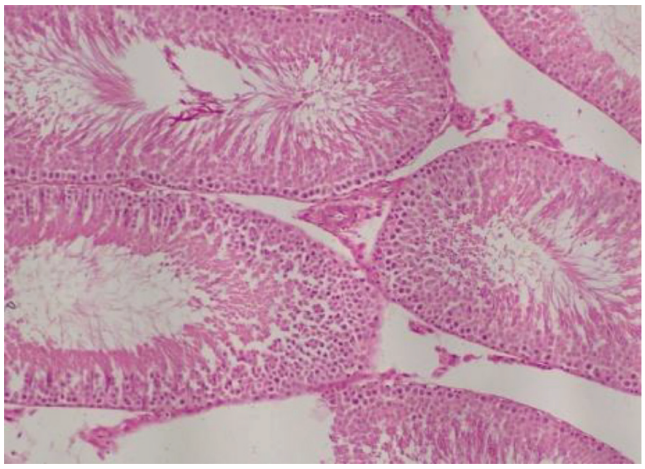


Figure 27. Testis high dose - NAD (10x 10X).

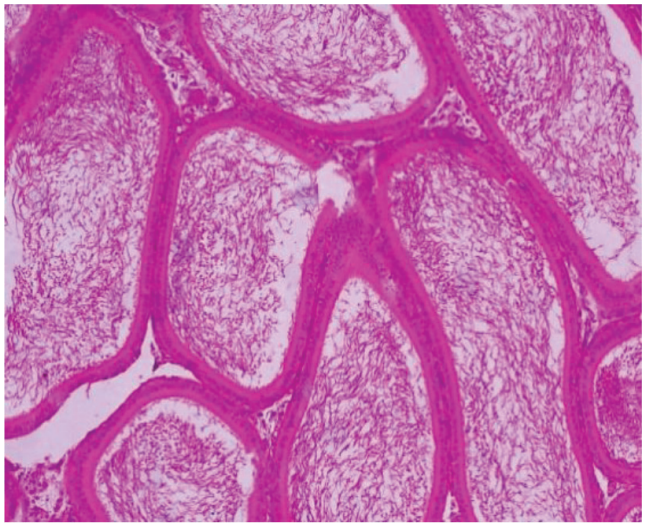


Figure 28. Epididymis high dose - NAD (10x 10X).

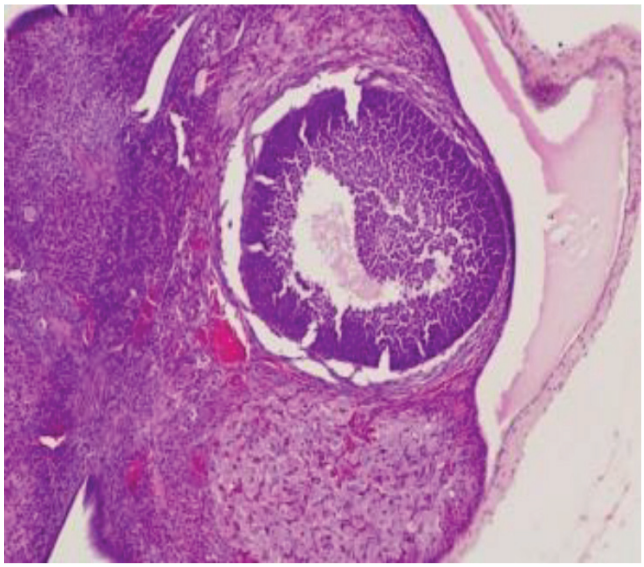


Figure 29. Ovary high dose - NAD (10x 10X).

6. Discussion

Traditional Ayurvedic herbal medicines are regularly prescribed for the treatment of different types of diseases which possess fewer side effects[10]. However, continued use of plant-based medicines without validly reporting on their toxic profile may negatively affect human health[11]. Hence it is necessary to carry out a comprehensive safety analysis as per the regulatory guidelines[12]. To validate the safety of AYUSH SC-3, the current study was undertaken to assess the toxicity when orally administered for a period of 90 days.

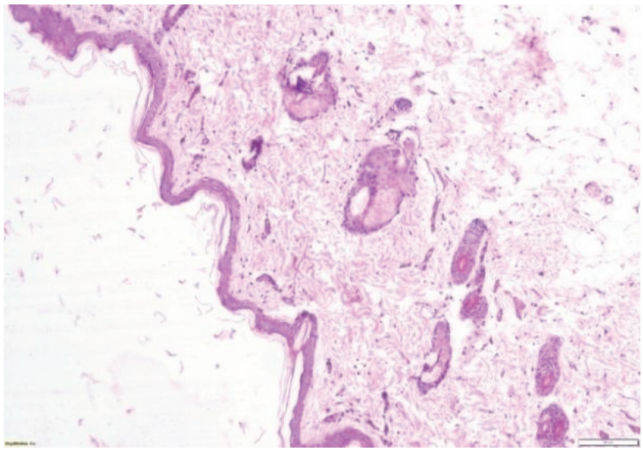


Figure 30. Skin high dose - NAD (10x10X).

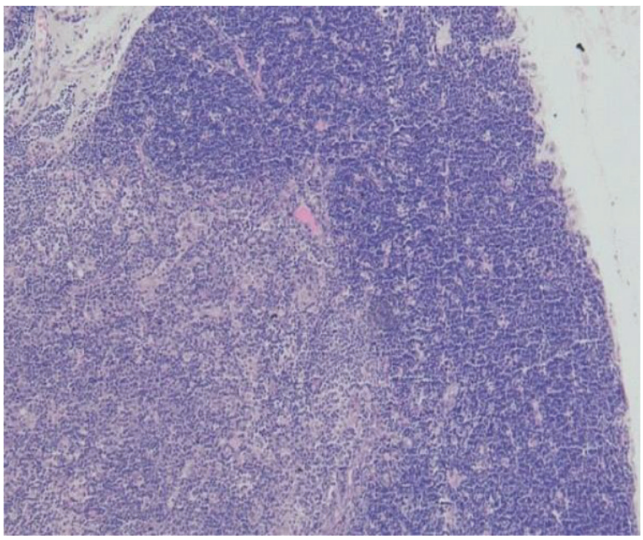


Figure 31. Thymus high dose – NAD.
NAD: No Abnormality Detected

Parameters of general clinical signs, mortality, body weight and feed intake are the main indices to evaluate the first signs of toxicity[13,14]. Body weight loss is more generally pronounced at the end of the study and it is often used to derive an endpoint for long-term risk associated with the test compound[13]. There were no observable signs of toxicity or mortality in AYUSH SC-3 treated rats which demonstrates its safety. There were no significant changes in body weight and feed intake when compared to the vehicle-treated animals for the entire study period.

Haematological and biochemistry parameters indicate the toxic effects of drug substances[15]. Changes

in the hematopoietic system are one of the important targets of toxic compounds and it is also a critical index of physiological and pathological status like anemia[16,17]. In the present study administration of AYUSH SC-3 for a period of 90 days produced no significant changes in blood parameters.

Determination of kidney and liver function is important for estimating the toxicity of plant products for the survival of organisms. Blood creatinine, Urea and Uric Acid are the important parameters for diagnosis of renal function. An increase in the level of these parameters is associated with kidney failure[14,18]. In this study no difference was observed in above said parameters when compared to the control group. These findings were supported by a histopathological examination of kidneys showing normal architecture.

Although considered to be safer to use, herbal medicines are also reported to have potential hepatotoxicity[19]. Herbal hepatotoxicity is often attributed to the presence of Pyrrolizidine alkaloids symptomized as Hepatic Sinusoidal Obstruction Syndrome (HSOS), a potentially life-threatening condition[20]. The abnormal increase in AST and ALP is associated with liver damage. Hence it is meaningful to evaluate the safety of the liver by measuring these enzyme levels in blood[21]. In the present study, the liver enzyme levels of AYUSH SC-3 treated rats were within the range which was further supported by histology of the liver indicating the absence of lesions.

Determination of Glucose, Total Cholesterol, HDL, LDL, and Tri-glyceride indicates the status of hyperglycaemic and hyperlipidaemic conditions associated with toxic drugs. In the present study, the test drug AYUSH SC-3 didn't produce any significant changes in these parameters, establishing the safety of the test compound on general physiological status.

Thyroid targets are also incorporated in repeat-dose toxicity studies. The OECD requires measurements of thyroid hormone levels in the 90-day rat study[9]. Accordingly, the T4 levels were measured and it was found to be within the range, no significant variation was seen as compared to the control animals. This signifies the safety of the test drug on the thyroid gland.

Calcium metabolism markedly altered during uraemia[22]. Increased sodium concentration is associated with water loss. The elevation of chloride is associated with certain kidney diseases[23]. Serum Potassium is the major determinant of the normal functioning of all cells[24]. Hence it is important to measure the serum electrolyte to assess the toxicity. In the present study, Potassium, Sodium, Calcium and Chloride levels were found to be normal.

Exogenous chemicals might result in toxicity to vital organs[25]. Therefore the assessment of histopathological alterations is considered as a basic test of safety assessment of tested material[18]. In this study, AYUSH SC-3 at a dose of 1500 mg/kg showed no change in the histopathological structure of the Liver, Kidney, Adrenals, Heart, Stomach, Pancreas, Lungs, Brain and Spleen etc. The general architecture of vital organs is normal as that of a control animal. There were no toxic changes confirming the safety of AYUSH SC-3 on vital organs. The results of the present study confirm that no adverse events were due to the administration of AYUSH SC-3 in rats.

7. Conclusion

The AYUSH SC-3 was found safe on acute oral administration. The LD_{50} of AYUSH SC-3 was more than 2000 mg/kg furthermore test drug was proved to be safe during 90 days repeated-dose-oral-toxicity study and NOAEL was established as 1500 mg/kg.

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9. References

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