Acute Oral Toxicity of Test Substance AMRITH NONI CANCI-CARE in Wistar Rats (OECD-423)

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ABSTRACT

To access the safety or toxicity of our novel formulation against Cancer. The study was conducted in rats using OECD-423 guidelines. In the Acute Oral Toxicity study rats were administered with our novel formulation AMRITH NONI CANCI-CARE orally and then observed individually for the first four hours, then over a period of 24 hours and once daily for 4 days. General behaviour, adverse effects and mortality were observed throughout the experimental period. All the animals were necropsied and pathologically examined macroscopically. No abnormalities were detected for the animals necropsied at terminal sacrifice. The limit doses of 2000mg/kg did not cause any mortality or signs of toxicity in rats during the study. As per OECD-423 guidelines if there is no lethality > 2000mg/kg body weight, then the dose falls under Category 5 and considered safe to use or administer.

Keywords: limit dose, mortality, morbidity, pathological examination, necropsied, Dennett's test.

1. INTRODUCTION

Cancer is a major cause of morbidity and mortality worldwide. According to the statistical report presented by Kulothungan et al., 2022 which is based on the National Cancer Registry Programme, the Cancer burden in India for 2021 is 26.7 million and is expected to increase to 29.8 million in 2025[3].

Generally for Cancer chemotherapy with radiation and surgery methods are used to treat the patients. Although chemo and radiation are designed to kill Cancer cells while sparing healthy tissue, these treatments sometimes damage or destroy normal cells. The main side effects were neutropenia, lymphedema, hair loss, nausea, vomiting, deep vein thrombosis, tiredness, difficulties in eating, depression, anaemia (low red blood cell counts), appetite changes, constipation, diarrhoea, sores, peripheral neuropathy or other nerve problems, such as numbness, tingling, and pain, skin and nail changes such as dry skin and colour change, urine, bladder and kidney problems, weight loss, chemo-brain (which can affect concentration and focus), mood swings, fertility problems and etc. [1].

From ancient times, to treat various ailments we have used Ayurvedic treatment, which doesn't have/less, side effects with a higher percentage of efficacies towards the betterment of the healthy life of the patients. Ayurveda is a science of life with historical roots in the Indian subcontinent. In India for Cancer, various plants extract were used to treat Cancer however there is no reliable/ lacking scientific evidence to support that these extracts can cure and improve the lives of Cancer patients.

Even World Health Organization (WHO) reported that approximately 80% of the global population relies on traditional medicines, plant extracts, or plant-based substances for primary healthcare [5].

For Cancer treatment, 25% of the 247 newly approved Anti-Cancer drugs were derived from natural products [4]. Therefore, medical plant research is very much needed to promote the appropriate use of herbal medicine and to assess its potential use for treatment.

Researchers have found that Ayurvedic treatment can help in relieving Cancer symptoms. It can also improve the quality of life. Meditation can reduce anxiety, lower blood pressure, boost general well-being and reduce the growth of Cancer with animal models, whereas, there is no evidence that Ayurvedic medicine can prevent and cure Cancer in humans and even we won't know until we carry out large randomized clinical trials.

We introduce our novel formulation **AMRITH NONI CANCI-CARE** which is made from 23 plants. The main plants source used were Noni (fruit extract), Lakshman Phala/ Soursop (fruit and leaf extract) other anti-Cancerous and anti-tumor herbs. The molecules present in these herbs aid in suppressing Cancer by performing functions like apoptosis (natural tumor cell death), anti-proliferative (suppressing Cancer cell growth), by decreasing glucose supply to Cancer cells (suppressing the ATP) and free radical scavenging thus aiding in better living.

Most of the herbs in this combination act as the best anti-oxidant and anti-inflammatory to help in the management of all kinds of Cancers. The combination is designed in such a way as to ensure one's overall well-being and to reduce any unwanted extra growth in the body. It also acts as an adjuvant in Cancer therapy by helping to reduce the side effects of chemotherapy and radiation treatment.

Hence, need for Study of Acute oral Toxicity was needed to determine the dosage and safety of the novel formulation. The study was conducted in rats using OECD-423 guidelines. In the Acute Oral Toxicity study rats were administered with our novel formulation AMRITH NONI CANCI-CARE orally and then observed individually for the first four hours, then over a period of 24 hours and once daily for 4 days. General behaviour, adverse effects and mortality were observed throughout the experimental period. Body weights were recorded on the test day 0 (prior to administration), day

3, day 7 and day 14. All the animals were necropsied and pathologically examined macroscopically.

2. OBJECTIVE

The objective of the study is to assess the toxicity/safety of our novel formulation "AMRITH NONI CANCI-CARE" by acute oral administration in adult healthy female wistar albino rats.

3. GUIDELINES

OECD guidelines for testing of chemicals, Acute Oral Toxicity – Acute Toxic Class Method 423, 2001: 1-14.

4. AMENDMENT AND DEVIATION PROCEDURES

This study was conducted as per OECD guidelines and we have not made any amendments or deviated from the procedure.

5. ANIMAL HUSBANDRY

5.1. Animal Welfare

Animal experiment was conducted in accordance with the guidelines of the committee for the purpose of control and supervision of experiments on animals. (CPCSEA Registration Number – 1803/PO/RcBi/S/2015/CPCSEA).

5.2. Animal Housing Condition

Animals were housed under temperature 22 ± 3°C, relative humidity 30-70%, 12 hour light and 12 hour dark cycle. Animals were housed in a standard polypropylene cage with stainless steel top grill having facilities for food and water. Sterile corncob (Source: Hylasco Biotechnology, Hyderabad) was used as bedding material and changed every day.

5.3. Feed and Water

Normal chow diet (Purina lab diet 5L79 Rat and Mouse 18%) (PMI nutrition International) was provided to all the animals throughout the experiment. Fresh water was provided *ad libitum.* Animals were provided access to fresh, potable, uncontaminated drinking water. Periodic monitoring of microbial contamination of water was done. Drinking water bottles and their tubes were examined routinely to ensure their proper operation.

5.4. Randomization

Each animal was marked by picric acid and numbering was given individually for each animal. Each cage was numbered separately to identify the groups. All animals were randomized based on their body weight. Randomization was done using Microsoft Excel Worksheet.

5.5. Animal Care

All procedures involving animals were conducted humanely and were performed by or under the direction of trained or experienced personnel. The study was commenced after the protocol reviewed and approved by Institutional Animal Ethical Committee (IAEC) of Radiant Research Services Pvt. Ltd.

5.6. Pain or Distress Category

In the event, adverse reactions indicate pain or distress, the study director and veterinarian was conducted promptly. The animals were treated or euthanized according to the professional judgement of the veterinarian, in consultation with the study director when possible. Treatment or euthanasia was based upon the circumstances and in reference to the CPCSEA pain and distress protocol guidance.

5.7. Justification of selection of vehicle

Demineralized water is universally accepted and routinely used vehicle in oral route for animal studies. The test substance forms a uniform suspension in Demineralized water as evidenced by in house suspend ability test. Hence, Demineralized water was used as a vehicle for test formulation.

5.8. Rationale of Doses

The dose and the frequency were selected based on the guidelines of OECD 423. Hence, the selected dose level for this study was 2000mg/kg body weight of animal.

5.9. Justification of Dose, Route, Number and Species

The rat species are accepted by regulatory authorities for this type of studies. The number of animals used in this study is the minimum needed for this type of acute oral toxicity study. The number of animals used in the study is also appropriate statistical analysis (ANNOVA, Dennett's test, etc.) of the data generated from the study. The dose levels were selected based on our recommendation. The oral route is intended clinical route of administration. Dosing was conducted via oral gavage using disposable polypropylene syringes with sterilized stainless steel gavage tubes.

5.10. Safety Precaution

Routine hygiene procedure: Protective gloves and face mask, aprons and goggles were used to ensure the health and safety of the personnel.

6. MATERIALS

6.1. Chemicals

Sl. No	Chemicals	Make	Code/Lot No.
1.	Demineralized Water	MRCL, India	B245/212/2022
2.	Picric Acid	Himedia, India	0000175497
3.	Isoflurane USP	Raman & Weil Pvt. Ltd., India	ISI-22017
4.	Ethanol	CS Reagents, India	AQ-MRN0000ST15

6.2. Test Sample Details

Test sample	Amrith Noni Canci-Care
Sample Number	RR220044
Batch Number	NA
Mfg. Date	NA
Storage Conditions	Room Temperature

6.3. Test System Details

Test Species	Rat
Strain	Albino Wistar
Sex	Female
Age	8-10 weeks
Body Weight	180-200gms
No. of Animals	09 Rats
Source	In house breed

7. METHODOLOGY

7.1. Procedure

Female wistar rats with normal diet were selected and acclimatized for seven days. Animals were fasted prior to dosing (with food but not water overnight). Followed the period of fasting, the animals were weighed and test substance at a starting dose level at 2000mg/kg body weight was administered (three animals) in a single dose by orally. After administration of test substance, food was withheld for 3-4 hours in rats. After administration, animals were observed for first 4 hours then periodically for 24 hours and daily thereafter, for a total of 14 days.

7.2. Limit Test

Animals at a starting dose of 2000mg/kg body weight didn't show any mortality, then a limit test was conducted at one dose level of 2000mg/kg body weight with six animals.

7.3. Formulation

Required quantity of test sample was taken in a mortar and pestle, and it was triturated and adequate quantity of vehicle was added, mixed well and transferred to a volumetric flask. Additional quantity of vehicle was added to the beaker, rinsed and transferred to the volumetric flask. Required volume was made up by adding sufficient quantity of vehicle to the volumetric flask, mixed well and transferred to labelled beakers with magnetic beads. Homogeneity of the test sample formulation was maintained by continuous stirring using a magnetic stirrer. The amount of test item and volume of the formulation was varied depending on the requirement or body weight of the animals. The exact amount of the test item\, volume of the formulation prepared and volume of formulation administered were recorded in the raw data.

7.4. Dose Administration

The substance formulation was administered only once by oral route at the dose level of 2000mg/kg body weight of the animal. The dose volume administered to each animal was 10ml/kg/day. The dose volume was calculated for individual animal on the day of treatment based on body weight.

8. OBSERVATIONS

8.1. Mortality and Morbidity

The cage side examinations were conducted to detect moribund or dead animals and abnormal behaviours and/or appearance in animal's at least twice a day through the study.

8.2. Clinical Signs

Cage sided evaluation was conducted for visible clinical signs once daily throughput the study period. Detailed clinical examinations were conducted prior to the treatment (once during acclimatization and once during randomization). Detailed clinical examinations include changes in skin, fur, eyes and mucous membranes and also respiratory, circulatory and behavioural pattern. Attention was also given to tremors, convulsions, salivation, diarrhea, lethargy and coma.

8.3. Body Weight

Individual body weights were recorded at receipt, on the day of randomization, on the first day of treatment before dosing (day 0), day 3, day 7 and day 14. The change in the body weight for animals were calculated and reported along with the body weight data.

8.4. Necropsy and Gross Pathology

At the end of the treatment period (day 15), all the animals were sacrificed using Isoflurane and subjected to gross pathological examination.

8.5. Statistical Analysis

All data including body weight and clinical symptoms were statistically analysed using Graph-Pad Prism Software, version 5.01. All the values were expressed as Mean ±SD. The significant difference between the treated and the control group was estimated using one-way ANNOVA with Dennett's test. All the results of the statistical analysis were summarized in separate tables. In any case the values were considered statistically significant at $P<0.05$.

9. RESULTS

9.1. Mortality and Morbidity

Mortality and Morbidity was not observed in the test substance treated animals throughout the experimental period (Refer Table 1).

9.2. Clinical Signs

All animals were observed to be normal throughout the experimental period (Refer Table 2a and 2b).

9.3. Body Weight and Body Weight changes

During the study period the test substance treated animals body weights were significantly increased when compared to day 0 body weight of animals (Refer Table 3 and Figure 1 and 2).

9.4. Gross Pathology

The Gross pathological examination of test substance treated animals was found to be normal (Refer Table 4 and figure 3).

10. DISCUSSION

The study was conducted as per OECD guideline 423, revealed that the test substance **"AMRITH NONI CANCI-CARE"** did not produce any mortality throughout the study period of 14 days even when the limit was maintained at 2000mg/kg body weight of the animals. All the animals appeared normal throughout the experimental period (Refer Table 2a and 2b). Behavioural changes were observed carefully after the dose administration. There was no abnormal signs observed throughout the study in all the animals. All the surviving animals had gained body weight by 3rd, 7th and 14th day as compared to day 0 (Refer Table 3, Figure 1 and 2). Increased body weight in animals was observed in all the animals and it is a normal pattern with healthy animals.

All surviving animals were sacrificed at the end of the experiment and discarded after the gross/macroscopic pathological changes were observed and recorded (Refer Table 4 and Figure 3).

The test group at single oral dose of 2000mg/kg body weight did not cause any death or clinical symptoms in rats observed over a period of 14 days. The median lethal dose of test substance is more than 2000mg/kg body weight.

11. CONCLUSION

Based on clinical signs, behavriol and gross pathological observations it can be concluded that the novel formulation/test substance **"AMRITH NONI CANCI-CARE"** was safe acute oral administration to wistar rats at dose of 2000mg/kg body weight. **The lethal dose value of novel formulation /test substance in female rats after single oral administration of above 2000mg/kg body weight was found to be non-toxic and is classified as category 5 and safe to use.**

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Table 1: Summary of Mortality and Morbidity Data of all groups

Animal Id.	Mortality & Morbidity Data													
RA01	1	2	3	4	5	6	7	8	9	10	11	12	13	14
RA02	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RA03	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RA04	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RA05	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RA06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RA07	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RA08	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RA09	0	0	0	0	0	0	0	0	0	0	0	0	0	0
* 0 indicates No mortality and +1 indicates Moribund														

Table 2a: Clinical and Behavioural Signs and Symptoms

CODES	OBSERVATIONS	SIGNS/SYMPTOMS
1	No Abnormal Diseases	02-40 observations are not seen
2	Accidental death	.
3	Partial Cannibalism	An animal of a species consuming part of another animal of same species
4	Total Cannibalism	An animal of a species consuming major organs of another animal of same species
5	Dead	Irreversible cessation of all body function, manifested by absence of spontaneous breathing and total loss of cardio-vascular and cerebral functions
6	Moribund Condition	Approaching death animal will not be available for examination next day
7	Weakness	A weak bodily state as expressed by difficulty in rising, shuffling, disinclination to move, eating slowly and a drooping posture
8	Lethargy	A level of consciousness characterized by decreased interaction with objects in the environment, sluggishness, abnormal drowsiness
9	Salivation	Flow of saliva, drooling
10	Lacrimation	Flow of tears
11	Discharge	Abnormal discharge
12	Snuffling (Unusual Respiratory Pattern)	A bubbling sound from the nasal cavity
13	Bronchial rales	Abnormal respiratory sound in auscultation of lungs
14	Cough	A forceful release of air from lungs
15	Dyspnoea	Shortness of breath
16	Corneal opacity	Opaque with white spots on cornea
17	Cataract	Opacity of the crystalline lens of the eye
18	Diarrhea	Diarrhea is the frequent passage of loose, watery, soft stools
19	Haematuria	Presence of blood in urine
20	Piloerection	Erection of hair
21	Response to Handling	Normal response to approach
22	Convulsions	Violent involuntary contraction of muscle or muscles
23	Repetitive circling	Continuous circling
24	Head tilted on one side	Head facing towards some other direction other than straight
25	Ataxia	Inability to control voluntary muscle movement
26	Dermatitis	Inflammation of the skin
27	Blister	A local swelling of the skin that contains watery fluid
28	Utricaria	An itchy skin eruption characterized by weal's with pale interiors and well defined red margins
29	Necrosis	Death of a portion of tissue differently affected by local injury
30	Erythema	redness of the skin
31	Oedema	A swelling from the effusion of watery fluid in the cellular tissue beneath the skin or mucous membrane
32	Cynosis	Bluish decolouration of the skin and mucous membrane
33	Paralysis	Loss of sensation over a region of body
34	Edema	An excessive accumulation of serous fluid in the tissue spaces or body cavity
35	Crepitation	A dry, Crackling sound or sensation
36	Dehydration	Loss of water and salts. The skin turns pale and cold, the mucous membranes lining lose their natural moisture
37	Dull	Lacking responsiveness or alertness
38	Posture	Position of the body or body parts
39	Epistaxis	Bleeding from the nose
40	Urine dribbling	Leaking of Urine

Table 2b: Clinical Signs and Behavioural observations during the study in Rats

OBSERVATION														
Animal Id	Before Treatment	Days of post treatment examination												
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
RA01	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RA 02	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RA 03	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RA 04	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RA 05	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RA 06	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RA 07	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RA 08	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RA 09	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01

***Observation for first four hours after treatment: 01 – NAD (02-40 codes observations are not seen)**

Table 3: Body weights of rats during the study

Animal ID	Dose	Treatment			
		Before	After		
		Day 0	Day 3	Day 7	Day 14
RA 01 RA 02 RA 03	2000 mg/kg	186.84±0.59	193.03±0.49	200.21±0.95	213.61±1.38
RA 04 RA 05 RA 06 RA 07 RA 08 RA 09	Limit test (2000 mg/kg)	186.09±0.35	192.50±1.10	201.29±1.19	214.66±1.06

Values were expressed as Mean ± SD

Table 4: Results of Gross pathological examination

Animal ID. No	Dose	Macroscopic lesions
RA 01	2000 mg/kg	No macroscopic alteration occurred
RA 02		No macroscopic alteration occurred
RA 03		No macroscopic alteration occurred
RA 04	Limit test (2000 mg/kg)	No macroscopic alteration occurred
RA 05		No macroscopic alteration occurred
RA 06		No macroscopic alteration occurred
RA 04		No macroscopic alteration occurred
RA 05		No macroscopic alteration occurred
RA 06		No macroscopic alteration occurred

13. LIST OF FIGURES

Figure 1: Body weight of animals at 2000mg/kg dose from day 0 to day 14.

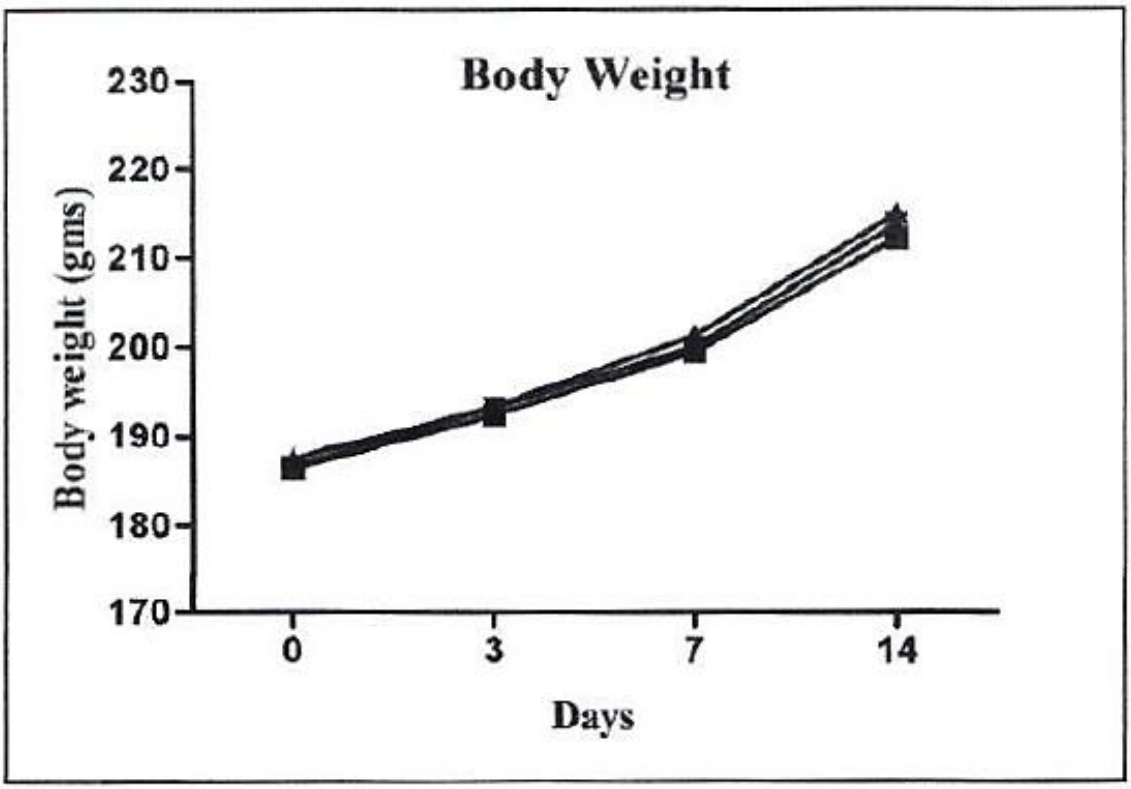

Figure 2: Body weight of animals at limit test dose (2000mg/kg) from day 0 to day 14

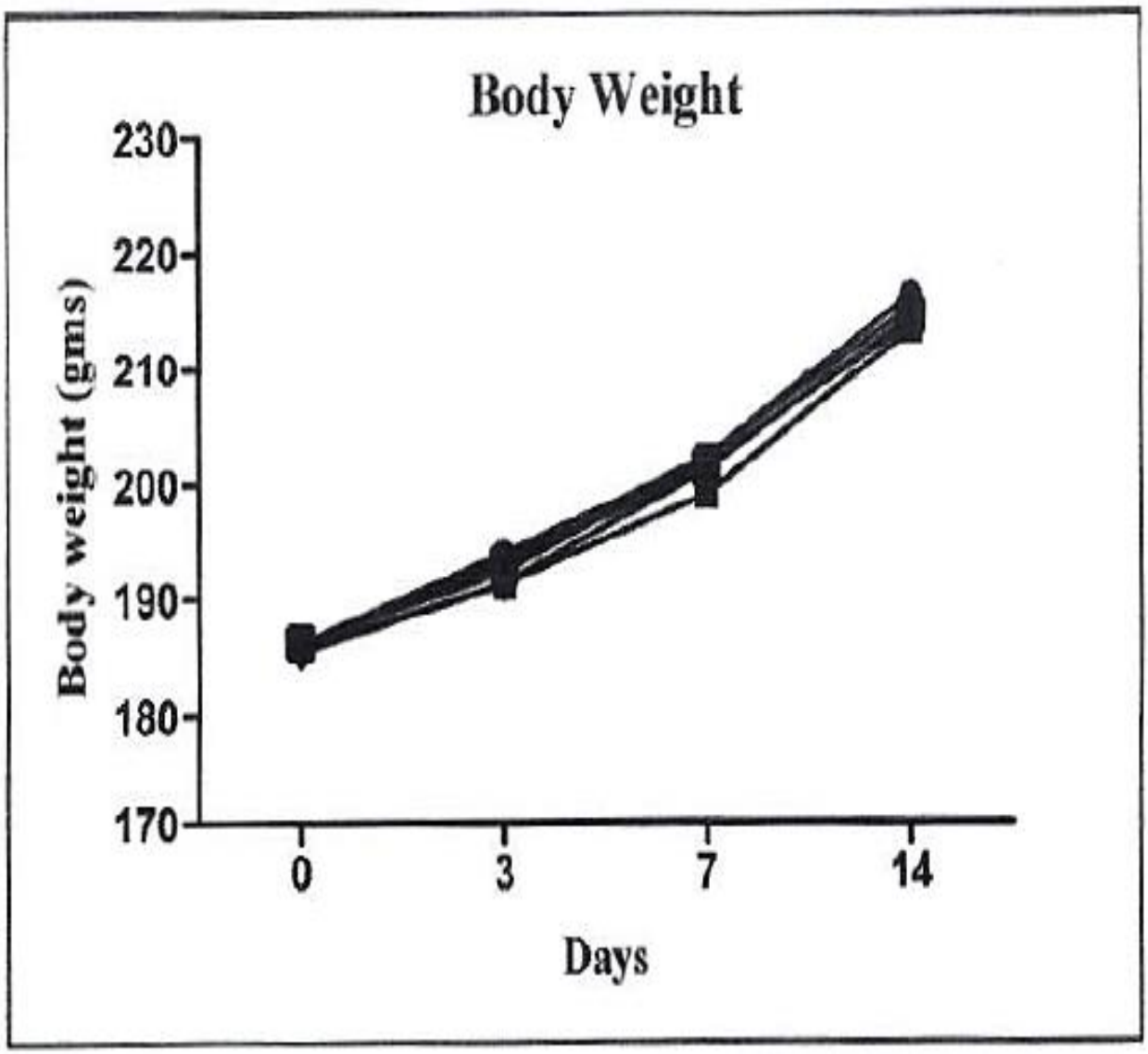

Figure 3: Gross Pathological examination of treated animals (Macroscopic Observation)

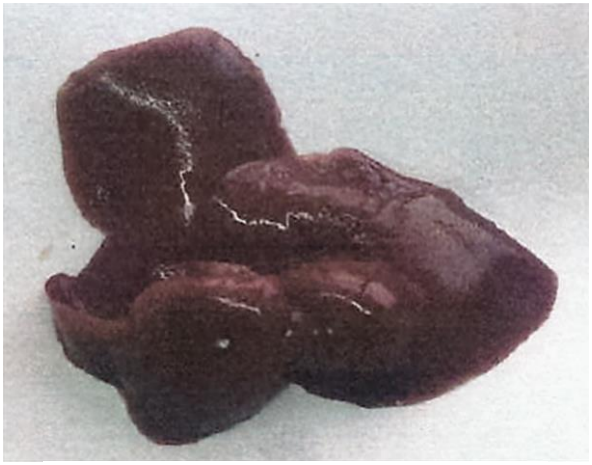

Figure 3a: Liver

Figure 3b: Kidney

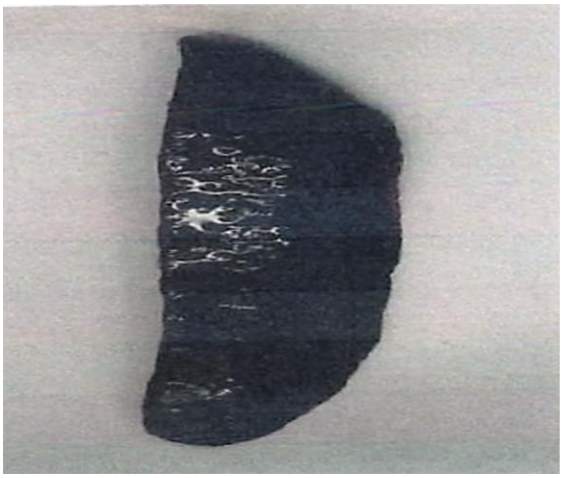

Figure 3c: Spleen

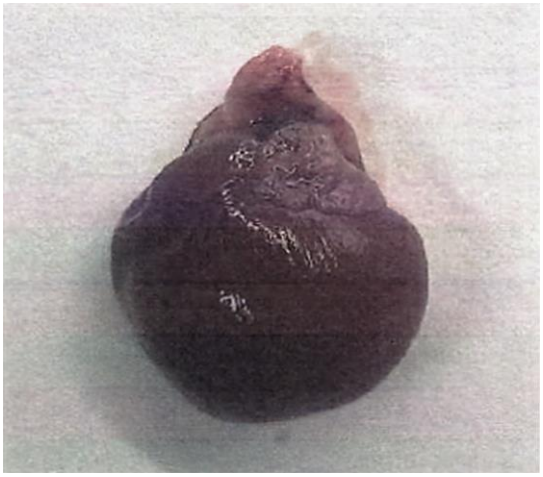

Figure 3d: Heart

14. REFERENCE

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