

## Safety Evaluation of Amaranth Extract by Acute, Sub-Chronic and Chronic Exposure in Rats

Praveen Thaggikuppe Krishnamurthy<sup>1</sup>, Merina Benny<sup>2\*</sup>, Benny Antony<sup>2</sup>, Binu T. Kuruvilla<sup>2</sup> and Nishant Kumar Gupta<sup>2</sup>

<sup>1</sup>Department of Pharmacology, JSS College of Pharmacy, JSS Academy of Higher Education and Research, Ooty, Tamil Nadu, India.

<sup>2</sup>Development Laboratory, Arjuna Natural Private Ltd., Erumathala PO, Aluva, Kerala, India.

\*Corresponding Author E-mail: research@arjunanatural.com

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Amaranth is one of the popularly grown leafy vegetables in tropical regions globally and contains a large amount of nitrate. The present study's objective was evaluation of acute and repeated dose toxicity of amaranth extract as per the OECD guidelines. The acute oral toxicity was conducted in 6 female rats (150-170 g; 8-10 Weeks old) as per OECD 423 guidelines. The amaranth extract had no adverse/toxic effects and no mortality was noted at the dose of 2000mg/kg. The oral LD<sub>50</sub>, therefore, was considered greater than 2000mg/kg. The sub-chronic (28-day repeated dose) toxicity was studied in 40 rats (150-170 g; 8-10 Weeks old) as per OECD 407 guidelines whereas chronic (365-days repeated dose) toxicity study was conducted in 200 rats (150-170 g; 8-10 Weeks old) as per OECD 452 guidelines. Sub-chronic study confirmed the safety of amaranth extract at the highest dose of 1000 mg/kg/day. The 1000 mg/kg in rats was considered as NOEL (No Observed Adverse Effect Level). The chronic toxicity study established a NOEL of 180 mg/kg in rats. In the repeated dose toxicity studies, body weight, food consumption, blood profile, biochemistry parameters and histopathology of major organs were similar in test and control groups. The current study results indicated that amaranth extract was safe upon acute, sub-chronic and chronic administration in rats, under testing conditions and at dose levels employed.

**Keywords:** Chronic toxicity; OECD guidelines; Red spinach; Safety profile; Toxicity study.

Vegetables and fruits in diet are considered essential for long life and human health maintenance. These are considered rich in several vitamins, minerals and other potentially metabolically active compounds, e.g. polyphenols<sup>1</sup>. Apart from these nutritious compounds, nitrate (NO<sub>3</sub><sup>-</sup>) present in vegetables has unique role in vasodilation<sup>2,3</sup>. The facultative anaerobic bacteria present in the oral cavity, facilitates the conversion of NO<sub>3</sub><sup>-</sup> ions into nitrite (NO<sub>2</sub><sup>-</sup>)

ions<sup>4</sup>. Once NO<sub>2</sub><sup>-</sup> is formed, it further converts to NO (nitric oxide) via various pathways<sup>5</sup>. During low oxygen saturation (also called hypoxia) in blood, the conversion of NO<sub>2</sub><sup>-</sup> to NO takes place at faster rate<sup>6</sup>. Endothelial nitric oxide synthase (eNOS or NOS3) is the primary source of NO in the vascular endothelium. Under a hypoxia state, the expression of eNOS decreases, resulting in low NO production. In such state, formation of NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup> and then conversion to NO works

as an alternate system for NO production inside the body<sup>7</sup>. NO is considered as one of the primes signaling molecule with multiple roles in humans. These include maintenance of muscles contraction, flow of blood in arteries and veins, homeostasis of certain molecules like calcium and glucose etc<sup>8,9</sup>.

It has been scientifically proved that NO<sub>2</sub> level can be improved in significant manner by ingesting NO<sub>3</sub> in diet. This helps in lowering the blood pressure by dilating the blood vessels<sup>10-12</sup>. Consumption of NO<sub>3</sub> in diet also increases endurance to exercise as a response to physiological benefits<sup>13</sup>. In a study by Stokes *et al.*, C-reactive protein level was decreased after NO<sub>3</sub> and NO<sub>2</sub> intake in high cholesterol fed mice. It also decreased the vascular inflammation and significantly reversed the endothelial dysfunctions<sup>14</sup>. It has been documented that during old age, the supply of amino acid L-arginine (an important NOS-substrate) and tetrahydrobiopterin (one of the cofactors) reduces<sup>15</sup>. This along with poor level of NO<sub>2</sub>, makes the regular nitric oxide pathway less efficient during ageing<sup>16</sup>. Apart from this, overproduction of O<sub>2</sub><sup>-</sup> (free radical superoxide) in old age, decreases the bioavailability of NO by formation of peroxy-nitrites<sup>17</sup>. This reduced availability of NO may increase the chances of endothelial dysfunctions during ageing process<sup>18</sup> and may cause arterial hyper-tension<sup>19</sup> of old age. Thus, increase of NO<sub>3</sub> consumption in the diet of old age peoples may be beneficial for overall vascular health and adequate supply of NO in bioavailable form.

The vegetables like Spinach, Cabbage and underground edible parts like beetroot are known to contain high percentage of NO<sub>3</sub>. Amaranth (also known as red spinach) is one of the popular vegetables rich in various nutrients along with significantly higher amount of NO<sub>3</sub> present in leaves<sup>20</sup>. It is cultivated as gluten free pseudo-cereal primarily in Asia, Mexico, South America and all tropical places of the world<sup>21</sup>. Amaranth is a fast-growing plant and easy to maintain and consume as leafy vegetables throughout the year. The leaves as well as seeds of amaranth are considered highly nutritious<sup>22,23</sup>. Both leaves and seeds are rich sources of various proteins. Quantitatively, leaves contain about 15-30% protein whereas seeds contain 15-45% of fresh matter. The leaves also contain Vitamin C (one of the widely known

antioxidant), dietary fibers and traces of essential minerals<sup>24,25</sup>. The composition of amino acid present in amaranth proteins is considered well balanced, highly bioavailable and good functional characteristics<sup>26</sup>. Apart from these nutrients, amaranth leaves are also rich in secondary plant metabolites, which may provide potential health benefits<sup>27</sup>. Recent research has indicated that leaves and other aerial parts of amaranth are important sources of phenolic compounds<sup>28-30</sup>. Among these, hydroxycinnamic acids, benzoic acids, flavonols and their glycosides have been reported in amaranth leaves and flowers<sup>31</sup>. Other phytochemicals present in amaranth with antioxidant activity are betalains, especially betacyanins<sup>32</sup>. The contents of these pigments vary among amaranth species and genotypes<sup>33</sup>.

To get clinical benefits, heavy intake of vegetables rich in NO<sub>3</sub> is practically very difficult in routine day-to-day life. Along with nitrates, a large amount of oxalic acid (an anti-nutrient) also gets inside the body and may result in kidney damage on prolonged use. Moreover, it has been reported that food rich in NO<sub>3</sub> didn't increase the nitrate levels in blood whereas consumption of NO<sub>3</sub> in the form of dietary supplement increased the same in old age peoples<sup>34</sup>. In another published study, the absorption of NO<sub>3</sub> from extract of amaranth leaves (2 g dose) was studied where single dose of extract, significantly increased ( $p < 0.001$ ) the plasma NO<sub>3</sub> levels in healthy adults as compared to the subject's consumed placebo<sup>35</sup>. The authors concluded that one dose of extract from red spinach leaves can enhance the NO<sub>3</sub> levels in plasma for more than eight hours. The higher levels of NO<sub>3</sub> may be beneficial in various sports activities and routine exercises.

Apart from multiple benefits of dietary NO<sub>3</sub>, a few studies have reported some adverse effects/toxicity of synthetic potassium and sodium nitrate in animals<sup>36</sup>. These were mainly due to development of methemoglobinemia. At the same time, toxic effects of potassium nitrate on some biochemical parameters of rats were completely ameliorated by simultaneous feeding of ascorbic acid<sup>37</sup>. The rationale of current study is to establish safety of extract from amaranth leaves with high dietary nitrates and to show its high safety profile compared to toxicity associated with synthetic NO<sub>3</sub> and NO<sub>2</sub>.

## MATERIALS AND METHODS

### Test sample and animals

Commercial batch of Oxystorm<sup>®</sup> from Arjuna Natural Private Ltd., Kochi, Kerala, India was used as test sample of Amaranth extract. It is standardized to contain approximately 9% dietary nitrate. Male and female Wistar albino rats weighing 150-170 g were kept at animal house conditions (Temperature 24±2°C; Relative humidity 55-70%; 12/12 h light/dark cycle). Filtered potable water and extruded rodent diet supplied by M/s. Amruth labs, Bangalore, India was used *ad libitum*. Acute and sub-chronic toxicity studies were approved by IAEC of JSS College of Pharmacy, Ooty, Tamilnadu, India (approval no. JSSCP/IAEC/CADRAT/2014-15). SD rats of 160-180 gram were used for the long-term chronic toxicity and study was approved by IAEC of Arjuna Natural Private Ltd., Kochi, Kerala, India (approval no. ANEL/IAEC/2016-I/1607015). SD rats were chosen for long term study due to longer life span of SD rats as compared to Wistar rats. The animal house conditions for long term study were same as mentioned for acute and sub-chronic toxicity study.

### Acute toxicity study

OECD 423 guidelines were followed to conduct this study in stepwise manner<sup>38</sup>. Three rats were used in the each step to determine adequate classification of the test material (amaranth extract) by acute toxicity test. Six Wistar albino rats (female only) weighing 150-170 g (Age, 8-10 Weeks) were used in this study and acclimatized for 7 days before each step's commencement. The amaranth extract was dissolved in distilled water and administered to rats by oral route at 2000 mg/kg body weight. A metal canula fitted to a syringe was used for this purpose. Based on the results, the next set of animals were administered with a 2000 mg/kg dose of the test item. The rats were carefully observed by an experienced veterinarian for 14 days. Special attention was paid during initial 4 hours on day 1 of the study. Body weight of all the rats was noted at baseline, day 7 and day 14. On the last day of study, necropsy was conducted by a veterinarian to see any gross lesions or hemorrhage in major organs.

### Sub-chronic (28 days repeated dose) toxicity study

OECD 407 guidelines were followed to

conduct sub-chronic toxicity study in rats<sup>39</sup>. Forty rats (M/F: 1/1; 150-170 g; 8-9 weeks old) were included in this study and randomly distributed into four groups. Ten rats comprising of 5 males and 5 females were kept in each group. Acclimatization period was one week in standard animal house conditions. Amaranth extract was dissolved in water and administered at 100, 500 and 1000 mg/kg to the rats of group 1, 2 and 3 as low, medium and high dose, respectively. Required quantity of extract was daily weighed and freshly dissolved in water before administration to the rats. Extract feeding was continued for 28 days using metal cannula attached with syringe. The fourth group of ten rats (M/F: 1/1) was fed with distill water alone for the same duration (28 days) and was considered as the control group.

Daily cage side observations were done by a qualified veterinarian for any abnormal behaviour or symptom. At the end of the study, sensory reactivity towards different stimuli (e.g., various reflexes, visual, auditory and proprioceptive stimuli), measurement of grip strength and motor coordination assessment were performed as per the standards published procedures<sup>40-43</sup>. In brief, flexion reflex (tests spinal cord) was assessed by pinching the toes of rat with forceps, the response was to move the foot away. Grasping reflex (tests cerebral cortex) was assessed by picking up the rat and palm was touched with a wire; the response was to grip the wire. Righting reflex was tested by putting the rat on its back and it turns over immediately. Auditory startle was assessed by putting the rat on a level surface in quiet environment and then a loud hand clap was given. The rat flexed forelimbs, extended hind-limbs and arched the body. For assessment of grip strength, the animal was kept on the top of the wire-bottomed cage. The tail was clenched at the base and the animal was pulled along the surface to measure its capacity to hold on to the wired surface. The motor coordination was evaluated by Rotarod apparatus. At the end of the experiment, animals were fasted for 16 h and blood samples were collected from retro orbital plexus of all the animals. The blood samples were used as such (with K3EDTA as anti-coagulant) for hematology whereas serum was separated by centrifugation at 3000 rpm for 15 min and used for the biochemical estimations. Routine urine analysis was also conducted as per the standard

procedures. Body weight of rats was recorded weekly and on the last day of study, all the rats were sacrificed and major tissues were preserved in 10% buffered formalin for histopathology using rotary microtome.

#### Chronic toxicity study

OECD 452 guidelines were followed to conduct chronic toxicity study<sup>44</sup>. Two-hundred rats (100 male/100 female) were used to conduct this study. The rats were divided into four main groups of 40 rats in each group. Ratio of male/female rats was kept 1:1 for all the groups. An acclimatization period of seven days was followed before start of dosing of extract. First three group of rats were fed at 45, 90 and 180 mg/kg of amaranth extract as low medium and high dose groups. Distill water was fed to the fourth group of rats and designated as control group. Dosing was continued once daily for one year duration.

Apart from four main study groups, two groups of 20 animals in each (10M/10F) were fed with amaranth extract 180 mg/kg and distill water, respectively and designated as 'Recovery groups'. These groups were also fed for the duration of one year but after the end of one year period, these recovery group animals were observed for one more month (without feeding of extract) for any reversible effect or delayed toxicity.

Daily observations on any abnormal behaviour or toxic symptom were conducted by a qualified veterinarian. Blood samples were collected at the end of study period and serum was separated by centrifugation to conduct biochemistry. Hematology was performed with as such whole blood (EDTA was used as an anti-coagulant). Terminally, the rats were sacrificed and

all the major organs were collected, preserved in formalin and studied for histopathological changes.

#### Statistical analysis

Bartlett's test was conducted to analyze the homogeneous nature of data by GraphPad Prism Software. The data was further analyzed by ANOVA and if 'F' was found significant, an individual comparison of means of control and treated groups was done using Dunnett's test. P value <0.05 was considered significant.

## RESULTS

#### Acute toxicity study

The body weight gain of all the rats was similar and in normal range over the study duration of 2 weeks (Table 1). All the animals were healthy and no animal died after feeding of amaranth extract at 2000 mg/kg dose. The behavior of all the rats was normal as observed by veterinarian. The animals didn't show any symptom of toxicity or abnormality throughout the study period. The cavities and orifices were normal when observed on the day of sacrifice. There was no change in skin or eye color and mucous membrane was also normal. The gross necropsy revealed that animals were healthy and all the internal organs were normal. The LD<sub>50</sub> of amaranth extract was calculated as >2000 mg/kg in rats. As per the OECD 423 guidelines, the extract falls in the category 5 of Globally Harmonized System.

#### Sub-chronic toxicity study

There was no mortality in extract treated or control rats. All the rats were clinically fit and their behavior was normal throughout the study period. Gain in body weight was almost similar in

**Table 1.** Body weight (g) and mortality data in acute toxicity study of amaranth extract

Dose in mg/kg	Rat	M/F	Initial	Day 8	Body weight (g)		No. dead / No. tested
					Weight change (day 8 – Initial)	Weight change (day 15 – Initial)	
2000	1	Female	158	165	7	169	0/6
	2	Female	161	170	9	176	
	3	Female	157	163	6	174	
	4	Female	149	152	3	163	
	5	Female	150	155	5	172	
	6	Female	149	154	5	171	

**Table 2.** Hematology profile for male rats in 28 days sub-chronic toxicity study of amaranth extract

Group & Dose	Clotting time (sec.)	WBC (cells/ $\mu$ l) $\times 10^3$	RBC (cells/ $\mu$ l) $\times 10^6$	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT (cells/ $\mu$ l) $\times 10^3$	PCT (%)	MPV (%)	Differential count (%)			
												N	L	E	M
Control (0 mg/kg)	123.4 $\pm$ 18.1	12.5 $\pm$ 1.6	6.4 $\pm$ 1.1	13.7 $\pm$ 1.1	53.9 $\pm$ 9.2	85.7 $\pm$ 18.5	21.9 $\pm$ 4.6	26.1 $\pm$ 5.3	557.8 $\pm$ 112.0	0.6 $\pm$ 0.0	10.4 $\pm$ 2.4	22.2 $\pm$ 2.4	57.2 $\pm$ 6.6	2.0 $\pm$ 1.0	18.6 $\pm$ 6.0
Low dose (100 mg/kg)	109.6 $\pm$ 25.3	13.2 $\pm$ 1.3	7.1 $\pm$ 1.2	14.1 $\pm$ 1.1	48.0 $\pm$ 7.1	69.0 $\pm$ 15.7	20.3 $\pm$ 4.2	29.7 $\pm$ 3.6	547.4 $\pm$ 103.5	0.6 $\pm$ 0.1	10.4 $\pm$ 1.9	20.6 $\pm$ 2.1	58.6 $\pm$ 5.9	2.2 $\pm$ 1.8	18.6 $\pm$ 7.6
Medium dose (500 mg/kg)	109.6 $\pm$ 24.9	11.1 $\pm$ 2.2	6.6 $\pm$ 0.9	14.1 $\pm$ 1.3	56.7 $\pm$ 7.7	86.8 $\pm$ 17.2	21.8 $\pm$ 5.2	25.5 $\pm$ 4.9	575.4 $\pm$ 76.9	0.6 $\pm$ 0.1	10.4 $\pm$ 0.8	20.6 $\pm$ 1.1	53.2 $\pm$ 4.5	2.2 $\pm$ 2.0	24.0 $\pm$ 6.9
High dose (1000 mg/kg)	117.4 $\pm$ 19.3	11.7 $\pm$ 2.9	6.9 $\pm$ 1.2	15.0 $\pm$ 1.4	51.2 $\pm$ 10.8	76.0 $\pm$ 20.0	22.4 $\pm$ 4.9	30.4 $\pm$ 7.9	490.0 $\pm$ 26.2	0.6 $\pm$ 0.1	11.3 $\pm$ 1.9	22.2 $\pm$ 1.9	52.2 $\pm$ 6.4	1.8 $\pm$ 1.3	23.8 $\pm$ 7.5

Data presented as Mean $\pm$ SD, n=5. ANOVA, p>0.05 as compared to control in each case. There was no significant difference between treated and control groups. HCT=Haematocrit; Hb=Hemoglobin; MCH= Mean Corpuscular Hemoglobin; MCHC= Mean Corpuscular Concentration; MCV= Mean Corpuscular Volume; PCT=Plateletcrit; MPV=Mean platelet volume; PLT=Platelets count; E=Eosinophils; L=Lymphocytes; N=Neutrophils; M=Monocytes

**Table 3.** Hematology profile for female rats in 28 days sub-chronic toxicity study of amaranth extract

Group & Dose	Clotting time (sec.)	WBC (cells/ $\mu$ l) $\times 10^3$	RBC (cells/ $\mu$ l) $\times 10^6$	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT (cells/ $\mu$ l) $\times 10^3$	PCT (%)	MPV (%)	Differential count (%)			
												N	L	E	M
Control (0 mg/kg)	99.4 $\pm$ 11.9	12.7 $\pm$ 1	6.5 $\pm$ 0.8	13.1 $\pm$ 0.9	53.3 $\pm$ 11.5	83.1 $\pm$ 23.5	20.3 $\pm$ 3.3	25.8 $\pm$ 8	515.4 $\pm$ 100	0.5 $\pm$ 0.1	10.8 $\pm$ 2.6	20.8 $\pm$ 1.6	53.6 $\pm$ 6.7	1.6 $\pm$ 1.1	24 $\pm$ 6.9
Low dose (100 mg/kg)	103.8 $\pm$ 22.5	11.1 $\pm$ 2.4	5.9 $\pm$ 0.7	14.7 $\pm$ 1.4	50.1 $\pm$ 13.5	87.3 $\pm$ 30.4	25.3 $\pm$ 4.6	31 $\pm$ 8.4	500.6 $\pm$ 65	0.6 $\pm$ 0.1	11.8 $\pm$ 1.8	20.8 $\pm$ 2.5	53 $\pm$ 6.9	2.6 $\pm$ 1.3	23.6 $\pm$ 8.3
Medium dose (500 mg/kg)	121.8 $\pm$ 4.8	11.5 $\pm$ 1.6	7.0 $\pm$ 1.0	13.7 $\pm$ 2	51.3 $\pm$ 12.9	75.9 $\pm$ 26.7	20 $\pm$ 3.9	28.6 $\pm$ 9.7	519.8 $\pm$ 61.5	0.6 $\pm$ 0.0	11.8 $\pm$ 1.5	21.4 $\pm$ 1.9	54.6 $\pm$ 7.3	2.8 $\pm$ 1.3	21.2 $\pm$ 7.6
High dose (1000 mg/kg)	111.8 $\pm$ 18	10.9 $\pm$ 2.2	6.9 $\pm$ 1.1	14.2 $\pm$ 1	56 $\pm$ 10.2	84.1 $\pm$ 25.2	21.2 $\pm$ 5	26.2 $\pm$ 5.5	606.2 $\pm$ 55.3	0.5 $\pm$ 0.1	8.6 $\pm$ 0.7	22.4 $\pm$ 2.2	54.8 $\pm$ 4.7	3 $\pm$ 1	19.8 $\pm$ 6.1

Data presented as Mean $\pm$ SD, n=5. ANOVA, p>0.05 as compared to control in each case. There was no significant difference between treated and control groups. HCT=Haematocrit; Hb=Hemoglobin; MCH= Mean Corpuscular Hemoglobin; MCHC= Mean Corpuscular Concentration; MCV= Mean Corpuscular Volume; PCT=Plateletcrit; MPV=Mean platelet volume; PLT=Platelets count; E=Eosinophils; L=Lymphocytes; N=Neutrophils; M=Monocytes

**Table 4.** Clinical chemistry data for male rats in 28 days sub-chronic toxicity study of amaranth extract

Group & Dose	FBS (mg/dl)	ALKP (U/L)	CHO (mg/dl)	BUN (mg/dl)	Cre (mg/dl)	T.Pro (gm/dl)	Alb (gm/dl)	SGOT (U/L)	SGPT (U/L)
Control (0 mg/kg)	85.2±5.9	144.3±8.1	118.7±8.5	44.8±14.1	0.9±0.0	11.9±1.9	7.3±0.6	119±12.9	74±11.2
Low dose (100 mg/kg)	84.8±6.2	146.6±12.3	112.1±7.6	46.9±14.4	0.9±0.0	12.6±2.0	7.7±1.0	130.5±12.7	69.4±10.5
Medium dose (500 mg/kg)	87.0±4.6	151.5±8.8	108.1±6.9	51.3±7.1	0.9±0.1	11.1±1.6	7.7±1.0	126.4±20.8	68.8±7.0
High dose (1000 mg/kg)	85.5±4.2	149.3±10.6	113.0±8.1	42.8±9.0	0.8±0.0	12.6±2.4	7.5±0.7	125.9±5.4	74.5±3.6

Data presented as Mean±SD; n=5. ANOVA, p>0.05 as compared to control in each case. There was no significant difference between treated and control groups.

FBS=Fasting Blood Sugar; ALKP=Alkaline phosphatase; CHO=Cholesterol; Cre=Creatinine; BUN=Blood urea nitrogen; SGOT=Serum glutamic oxaloacetic transaminase; SGPT=Serum glutamic pyruvic transaminase; T.Pro=Total Protein; Alb=Albumin.

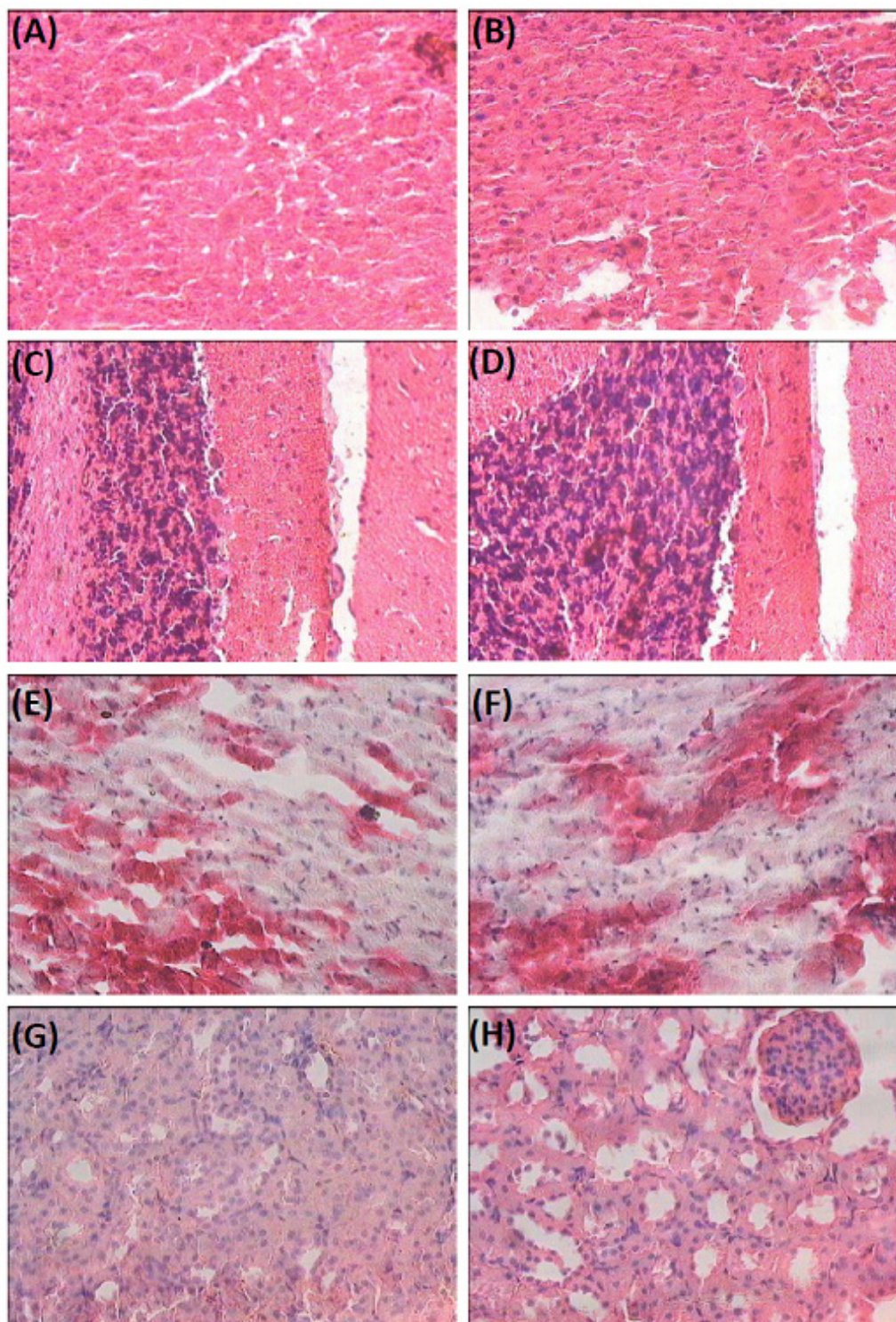
**Table 5.** Clinical chemistry data for female rats in 28 days sub-chronic toxicity study of amaranth extract

Group & Dose	FBS (mg/dl)	ALKP (U/L)	CHO (mg/dl)	BUN (mg/dl)	Cre (mg/dl)	T.Pro (gm/dl)	Alb (gm/dl)	SGOT (U/L)	SGPT (U/L)
Control (0 mg/kg)	88.7±6.2	145.0±3.7	105.4±4.2	43.6±10.0	0.9±0.1	13.8±2.1	7.3±0.9	118.4±14.3	68.1±6.3
Low dose (100 mg/kg)	85.7±5.4	137.5±4.2	112.0±12.0	46.4±13.0	0.9±0.1	11.6±2.0	7.5±0.8	124.0±16.0	71.9±6.5
Medium dose (500 mg/kg)	83.2±6.2	150.3±13.4	111.8±9.6	42.7±6.3	0.9±0.0	13.2±2.5	7.4±1.0	123.7±21.7	69.0±8.1
High dose (1000 mg/kg)	87.3±2.3	141.7±14.0	110.2±12.7	45.9±11.5	0.9±0.1	11.6±1.8	7.5±0.4	125.4±13.9	71.6±5.5

Data presented as Mean±SD; n=5. ANOVA, p>0.05 as compared to control in each case. There was no significant difference between treated and control groups.

FBS=Fasting Blood Sugar; ALKP=Alkaline phosphatase; CHO=Cholesterol; Cre=Creatinine; BUN=Blood urea nitrogen; SGOT=Serum glutamic oxaloacetic transaminase; SGPT=Serum glutamic pyruvic transaminase; T.Pro=Total Protein; Alb=Albumin.





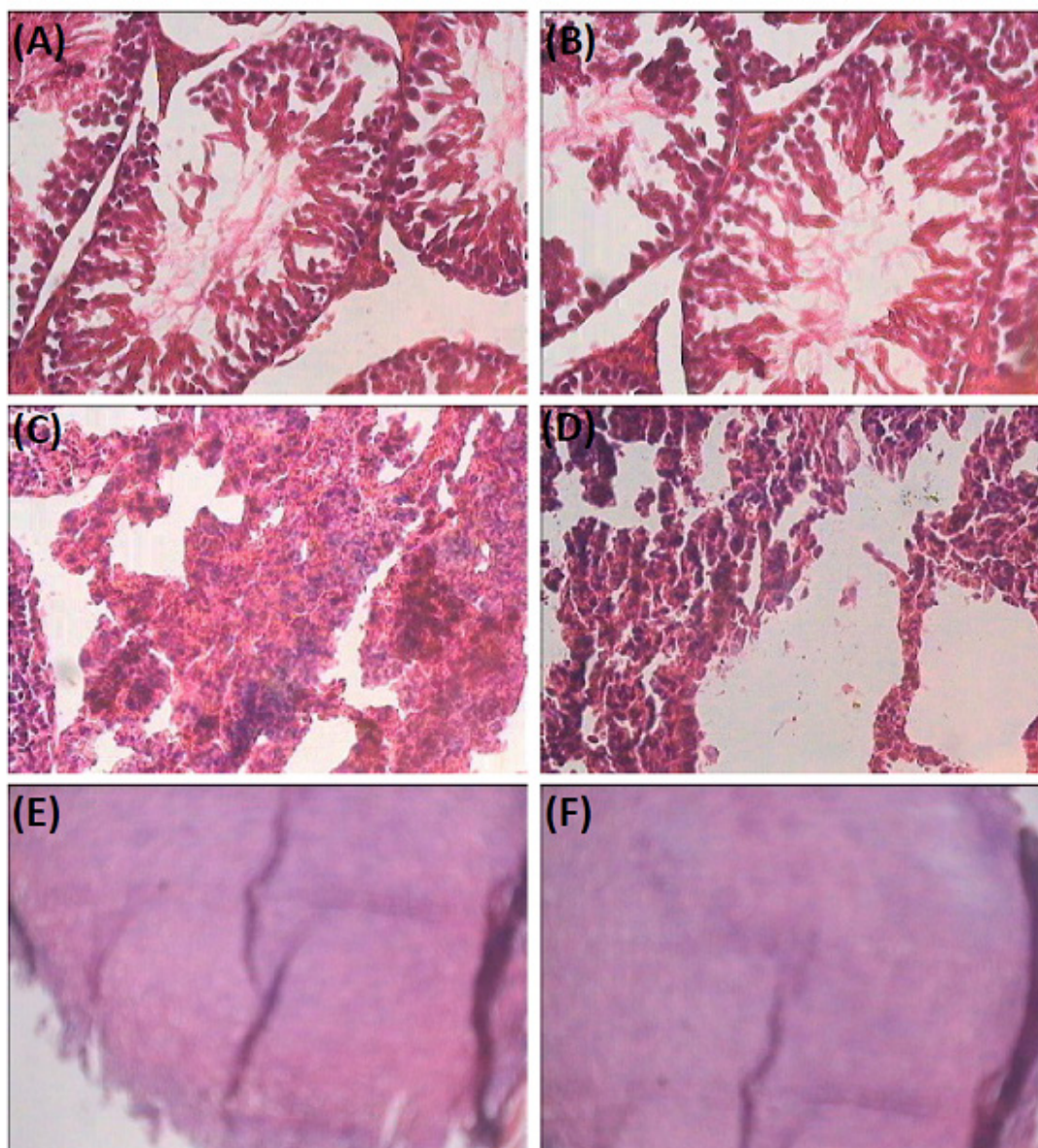
**Fig. 1.** Microscopic sections of major organs in 28 days sub-chronic toxicity (X100). (A) Control liver (B) High dose liver (C) Control brain (D) High dose brain (E) Control heart (F) High dose heart (G) Control kidney (H) High dose kidney



all the groups. Similarly, there was no significant difference in the food intake pattern of all the groups. In this study, no significant changes in the haematology (Table 2-3) and bio-chemistry profile of control and treated rats were observed after 28 days. These parameters for treated groups were comparable to respective control group of rats (Table 4-5).

The histological observations of all the vital organs in control and treated groups were same. There was no treatment related abnormality

(Figure 1-2). Some haemorrhage and alveolar oedema was observed in the lungs of extract treated (high dose) as well as control rats. In few rats hydronephrosis and cysts in kidney was also noted but it was similar for control and high dose extract treated rats. Liver of a few rats in both the groups have shown vacuolation and cellular swelling at some places. These findings were considered normal/incidental by the histopathologist and concluded as non-toxic nature of the test extract.



**Fig. 2.** Microscopic sections of major organs in 28 days sub-chronic toxicity (X100). (A) Control testes (B) High dose testes (C) Control lung (D) High dose lung (E) Control adrenal (F) High dose adrenal



**Table 6.** Hematology profile for male rats in chronic toxicity study of amaranth extract

Treatment	WBC (X 10 <sup>3</sup> )	RBC (X 10 <sup>6</sup> )	Hb (gm/dl)	N%	E%	L%	PCV (%)	MCV (fl)	MCH (pg)	MCHC (%)	Platelet count (X 10 <sup>5</sup> )	RDW- CV (%)
Control (0 mg/kg)	8.3±1.0	7.0±0.8	14.6±0.5	43.6±3.4	2.2±0.8	54.2±2.8	50.4±3.6	57.2±2.3	18.1±1.2	31.3±2.8	6.4±0.7	17.4±0.4
Low dose (45 mg/kg)	8.0±0.8	7.4±0.9	14.3±0.6	42.0±2.7	2.4±0.9	55.6±3.0	48.3±6.1	55.5±3.3	18.8±1.2	29.7±2.4	6.6±0.8	17.7±1.0
Medium dose (90 mg/kg)	8.4±1.1	6.6±0.8	14.5±0.5	41.4±3.8	2.4±1.1	56.2±3.5	51.0±3.2	57.4±1.9	19.6±1.9	28.1±2.3	5.8±0.8	18.6±1.4
High dose (180 mg/kg)	8.8±0.8	6.4±1.0	13.8±0.4	46.8±4.4	2.2±0.8	51.0±3.8	53.6±3.0	53.5±2.1	17.1±1.5	29.8±3.1	6.8±1.0	17.7±2.6
Control recovery (0 mg/kg)	8.2±1.1	6.5±1.1	15.3±0.7	42.6±4.0	2.4±0.5	55.0±3.9	49.6±4.4	59.3±3.4	19.9±3.2	28.4±3.4	7.0±0.7	19.3±2.1
High dose recovery (180 mg/kg)	8.9±0.6	6.0±1.1	14.4±1.1	48.6±4.6	2.6±0.5	48.8±4.4	51.0±3.8	55.8±7.8	18.3±3.2	29.6±3.5	6.0±0.7	18.5±2.3

Data presented as Mean±SD; ANOVA, p>0.05 as compared to control in each case. There was no significant difference between treated and control groups.

MCH= Mean Corpuscular Hemoglobin; MCHC= Mean Corpuscular Hemoglobin Concentration; Hb=Hemoglobin; MCV= Mean Corpuscular Volume; E=Eosinophils; L=Lymphocytes; N=Neutrophils; RDW= Red Blood Cell Distribution Width

**Table 7.** Hematology profile for female rats in chronic toxicity study of amaranth extract

Treatment	WBC (X 10 <sup>3</sup> )	RBC (X 10 <sup>6</sup> )	Hb (gm/dl)	N%	E%	L%	PCV (%)	MCV (fl)	MCH (pg)	MCHC (%)	Platelet count (X 10 <sup>5</sup> )	RDW- CV (%)
Control (0 mg/kg)	8.1±1.2	6.6±1.1	13.4±0.4	43.4±2.9	2.2±0.8	54.4±3.2	49.2±5.1	56.5±4.6	18.3±1.3	30.4±4.5	6.0±0.7	17.2±0.5
Low dose (45 mg/kg)	8.0±0.9	7.0±0.4	13.7±0.5	42.4±3.8	2.0±1.0	55.6±4.0	46.8±4.9	56.0±3.5	18.7±1.1	29.2±2.3	6.2±0.9	17.7±0.9
Medium dose (90 mg/kg)	8.3±1.3	6.4±0.6	13.6±0.5	40.8±5.1	2.2±0.8	57.0±5.1	45.3±3.5	54.8±3.3	19.0±2.8	30.0±3.7	6.2±0.5	19.2±1.9
High dose (180 mg/kg)	7.9±0.7	6.7±1.1	13.0±0.6	46.6±5.5	2.4±0.5	51.0±5.2	46.2±5.9	54.3±2.8	16.9±1.3	28.6±2.0	6.5±1.3	17.9±3.5
Control recovery (0 mg/kg)	8.1±0.6	6.6±1.2	13.6±0.4	41.2±3.3	2.4±0.5	56.4±3.6	47.5±5.6	57.0±3.4	18.5±3.3	27.7±3.4	6.8±0.9	18.8±2.9
High dose recovery (180 mg/kg)	8.9±0.6	6.5±1.1	13.9±0.2	47.6±2.3	2.2±0.8	50.2±2.2	51.3±3.3	55.2±6.7	18.8±1.1	26.8±3.4	6.0±0.5	18.5±1.1

Data presented as Mean±SD; ANOVA, p>0.05 as compared to control in each case. There was no significant difference between treated and control groups.

MCH= Mean Corpuscular Hemoglobin; MCHC= Mean Corpuscular Hemoglobin Concentration; Hb=Hemoglobin; MCV= Mean Corpuscular Volume; E=Eosinophils; L=Lymphocytes; N=Neutrophils; RDW= Red Blood Cell Distribution Width

**Table 8.** Clinical chemistry data for male rats in chronic toxicity study of amaranth extract

Treatment	CHO (mg/dl)	TG (mg/dl)	ALKP (U/l)	T.Bil (mg/dl)	T.Pro (g/dl)	Alb (g/dl)	Glo (g/dl)	SGOT (U/l)	SGPT (U/l)	Urea (mg/dl)	Cre (mg/dl)	FBS (mg/dl)
Control (0 mg/kg)	83.2±6.3	58.6±9.6	143.4±8.9	0.5±0.1	7.9±0.5	4.3±0.5	3.6±0.5	99.6±11.7	65.4±7.5	41.8±4.7	0.7±0.2	84.0±6.2
Low dose (45 mg/kg)	79.6±9.4	57.2±8.3	136.4±8.2	0.6±0.1	7.8±0.8	4.0±0.3	3.8±0.5	91.6±14.8	64.6±6.1	41.2±8.8	0.7±0.2	85.0±9.0
Medium dose (90 mg/kg)	82.4±8.4	60.0±13.1	141.8±4.8	0.5±0.1	7.9±0.6	4.3±0.3	3.5±0.4	96.2±9.6	66.6±7.6	41.2±5.1	0.7±0.1	83.4±4.9
High dose (180 mg/kg)	84.4±10.5	54.2±6.5	143.8±6.9	0.6±0.1	7.6±1.1	3.9±0.6	3.7±0.7	104.6±8.7	66.0±3.5	42.4±4.4	0.7±0.1	83.0±8.1
Control recovery (0 mg/kg)	84.2±7.9	56.4±16.6	140.2±10.7	0.5±0.1	8.1±1.1	4.2±0.5	3.8±0.6	91.4±8.7	67.2±5.1	43.2±4.5	0.6±0.2	81.8±7.8
High dose recovery (180 mg/kg)	90.2±4.8	57.6±7.9	137.8±11.1	0.7±0.1	7.5±1.0	3.9±0.6	3.6±0.5	91.8±7.0	69.6±6.0	41.2±5.6	0.7±0.1	81.6±10.6

Data presented as Mean±SD; ANOVA,  $p > 0.05$  as compared to control in each case. There was no significant difference between treated and control groups.

FBS=Fasting Blood Sugar; ALKP=Alkaline phosphatase; CHO=Cholesterol; Cre=Creatinine; SGOT=Serum glutamic oxaloacetic transaminase; SGPT=Serum glutamic pyruvic transaminase; T.Pro=Total Protein; Alb=Albumin; Glo=Globulin; TG=Triglycerides; T.Bil=Total Bilirubin.

**Table 9.** Clinical chemistry data for female rats in chronic toxicity study of amaranth extract

Treatment	CHO (mg/dl)	TG (mg/dl)	ALKP (U/l)	T.Bil (mg/dl)	T.Pro (g/dl)	Alb (g/dl)	Glo (g/dl)	SGOT (U/l)	SGPT (U/l)	Urea (mg/dl)	Cre (mg/dl)	FBS (mg/dl)
Control (0 mg/kg)	80.4±5.8	62.0±8.4	143.6±10.4	0.6±0.1	7.8±0.5	4.0±0.4	3.8±0.6	96.2±9.8	66.2±8.1	41.2±4.0	0.6±0.2	82.0±8.3
Low dose (45 mg/kg)	78.4±6.9	57.6±10.2	140.0±10.3	0.7±0.2	7.9±0.7	3.9±0.4	4.0±0.3	92.6±14.9	64.6±8.5	39.4±6.8	0.7±0.1	88.2±4.4
Medium dose (90 mg/kg)	80.8±4.4	59.6±11.8	144.8±7.7	0.7±0.2	8.2±0.6	4.3±0.5	3.9±0.3	96.0±8.9	65.2±7.9	41.4±4.9	0.6±0.1	83.0±8.5
High dose (180 mg/kg)	82.0±9.1	57.8±6.8	145.8±7.0	0.6±0.2	8.2±0.8	4.3±0.6	3.9±0.4	99.4±5.2	66.6±3.8	42.8±6.3	0.7±0.2	82.4±9.0
Control recovery (0 mg/kg)	79.8±5.5	57.6±13.0	143.6±14.2	0.6±0.2	8.1±1.0	4.3±0.5	3.8±0.5	88.4±11.1	67.6±7.5	44.8±4.8	0.5±0.1	83.0±6.3
High dose recovery (180 mg/kg)	88.2±11.0	59.2±12.2	147.8±11.0	0.7±0.2	7.6±0.8	4.2±0.4	3.4±0.6	96.2±3.0	70.8±5.2	49.2±9.4	0.7±0.2	82.6±10.1

Data presented as Mean±SD; ANOVA,  $p > 0.05$  as compared to control in each case. There was no significant difference between treated and control groups.

FBS=Fasting Blood Sugar; ALKP=Alkaline phosphatase; CHO=Cholesterol; Cre=Creatinine; SGOT=Serum glutamic oxaloacetic transaminase; SGPT=Serum glutamic pyruvic transaminase; T.Pro=Total Protein; Alb=Albumin; Glo=Globulin; TG=Triglycerides; T.Bil=Total Bilirubin.

In the treated as well as control group of rats, body weight and organ weight on 28<sup>th</sup> day was in normal range. The organ weight to body weight ratios were comparable to respective control rats for males as well as females. In the medium dose (500 mg/kg) males, there was significant reduction in fasting body weight and a significant enhancement in weights of gonads and heart. In addition, medium dose females showed increase in absolute gonads weight. However, the same was not found with high dose rats (treated at 1000 mg/kg) and there was no dose correlation was observed, therefore, considered incidental.

In the 28-days repeated dose study with amaranth extract, the maximum dose of 1000 mg/kg in rats didn't produce any toxic effect and all the findings were comparable to respective control counterparts. The NOEL (No Observed Adverse Effect Level) in this study was found as 1000 mg/kg in rats.

#### **Chronic toxicity study**

The chronic toxicity study was conducted at slightly low dosages to observe the accumulated effect over a period of one year. There was no death of rats at any dose level of extract treated or control group of animals. The body weight and food intake of all the rats was in normal range. When compared to the control rats, there was no abnormality noted with extract treated rats as far as visible clinical symptoms are concerned.

The level of RBC, WBC, Hemoglobin etc was in normal range in extract treated as well as control group of rats. Full hematological profile is presented as Table 6-7 for the rats of both the sexes. Furthermore, the liver functions, kidney functions and lipid profile of all the rats was normal after one year of extract treatment. The whole biochemistry data of control and amaranth extract fed rats were similar when side by side comparison was conducted (Table 8-9). The gross pathology and histopathology of all the collected organs was normal in all the control and treated rats.

### **DISCUSSION**

The traditional Japanese and Mediterranean food habits are considered most healthy all over the world. These are found to increase the life span and less occurrence of heart related disorders<sup>45</sup>. Both of these diets contain

plenty of fresh vegetables and fruits. Japanese diet is also rich in fish whereas olive oil is one of the ingredients in Mediterranean diet. These diets do not contain red meat except a few occasional dishes. One of the common things is the presence of high dietary nitrate content in both of these diets. In Mediterranean diet, vegetables (leafy) are used as such. These may include red-spinach, lettuce and rocket salad etc. Whereas Japanese diet contains ta cai, spinach, garland chrisantemum, etc<sup>46</sup>. Food rich in vegetables is normally considered to bring down the blood pressure and less occurrence of fatal coronary heart disease, nonfatal myocardial infarction or acute stroke<sup>47</sup>.

The ADI (Acceptable Daily Intake) for nitrate is 3.7 mg per kg of body weight as set by the European Food Safety Authority. This equals to 0.06 mmol/kg body weight in human. For an average built human of roughly 70 kg body weight, it will be equal to 260 mg daily<sup>48</sup>. This ADI was set on the basis of toxicity and safety studies conducted with synthetic nitrate. Since the natural nitrate supplements like amaranth or red spinach extract contains a lot of anti-oxidants and other phyto-nutrients, these extracts are safer than synthetic nitrates. Moreover, the recommended dose of amaranth extract is 1000 to 2000 mg/day for an adult. This equates to around 90 to 180 mg of nitrate per day which is well within the set ADI limits.

There are a number of side effects are known due to ingestion of synthetic nitrates. These include toxicity to reproductive organs, methylation of hemoglobin (methemoglobinemia) and other endovrine or metabolic disorders. In one study, male Wistar rats were treated with sodium nitrate at 19 mg/kg, 66 mg/kg and 150 mg/kg once daily by oral route for a period of 10 days<sup>49</sup>. Methemoglobinemia was observed in all the rats and nitrate got accumulated in the liver of high dose rats. The functions of liver were also got impaired as evidenced by increased levels of transferases, lactates, triglycerides, and glucose in medium (66 mg/kg) as well as high dose (150 mg/kg) groups. In their investigations, histopathology further confirmed the inflammation of liver cells, necrosis, steatosis etc in high dose group. In contrast to the study by González *et al.*, in present study 28 days repeated feeding of amaranth extract at highest dose of 1000 mg/kg daily (equivalent

to 90 mg nitrate/kg per day) to rats did not induce any observable toxic effect or liver injury. The polyphenols and other anti-oxidants present in the amaranth extract might have a protective effect on liver and other organs by avoiding production of nitrosamines.

Acute toxicity in rats and mice is considered first step in toxicity evaluation. In the present study, non-toxic nature of amaranth extract at 2000 mg/kg in rats confirmed the safety of the test material as LD50 was computed as >2000 mg/kg in rats. The 28 days study by daily feeding to rats at maximum dose of 1000 mg/kg in rats is another evidence of safety of amaranth extract. Therefore, the NOEL of amaranth extract in rats was found as 1000 mg/kg. This corresponds to 11.2 g for a 70 Kg human<sup>50</sup>.

The sub-chronic toxicity had certain limitations. A few histopathological findings in lungs, kidney and liver of control as well as amaranth extract treated group were noted. These were considered to be safe as seen in such studies, and were considered as incidental. Similarly, the male rats of 500mg/kg dose had slight decrease in fasting weight and an enhancement in weights of gonads and heart. The females of 500 mg/kg group showed a remarkable enhancement in the weight of gonads. However, same was not found with 1000 mg/kg daily dose group and there was no correlation with dose was observed, therefore, considered incidental.

Since food supplements are supposed to be taken for long term, the chronic toxicity study was also conducted by feeding amaranth extract to rats for one year duration. There was no toxic sign or symptoms observed in chronic toxicity study at low, medium or high dose of amaranth extract. The biochemical, hematological and histopathological observations further confirmed the safety of amaranth extract in rats. In this study, the three dosages 45, 90 and 180 mg/kg in rats were chosen to represent the human equivalent dose of 500, 1000 and 2000 mg. In this study, the gross observation of behavior, appearance and toxicological findings like changes in pupil size, color of skin and unusual respiratory pattern was normal, thus detailed ophthalmic examination using ophthalmoscope was not done. In sub-chronic toxicity study, the neurological and functional

examinations (proprioceptive stimuli) was normal in all rats examined, thus detail examination on these parameters was not conducted in the chronic toxicity study. These parameters are optional as stated in the OECD 452 guidelines.

Several human clinical studies have also been reported with amaranth extract (red spinach extract). In one such study, acute effect of amaranth extract (1000 mg dose) was determined on vascular reactivity in peripheral conduit and resistance arteries<sup>51</sup>. In another study on healthy human subjects, amaranth extract (1000 mg dose which equals to 90 mg nitrate) was orally given as a single dose to study the exercise performance and endurance. The authors found that amaranth extract delayed the ventilatory threshold and response was ergogenic<sup>52</sup>. Interestingly, there were no adverse effects reported in the above human studies which further confirm the safety of amaranth extract in human. In chronic toxicity study 180mg/kg was the maximum dose in rats. While converting to human dose, it will be approximately 2 g per day for a 70 Kg human. Overall, the results of these studies confirm the safety of amaranth extract in rats at the tested dosages.

## CONCLUSION

The amaranth extract tested in the present study has shown non-toxicity of the product at 2000 mg/kg in acute toxicity test. There were no adverse effects at 1000 mg/kg daily dosing for 28-days in sub-chronic toxicity study and this dose was considered as NOEL. In the chronic toxicity study also, the extract was non-toxic at 180 mg/kg daily in rats. Thus, it can be concluded that amaranth extract is safe at the doses tested in this study as per the guidelines laid by OECD.

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## Conflict of Interest

The authors declare that there is no conflict of interest.

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