Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 899-908
Received: 04-05-2019
Accepted: 06-06-2019

Suganya V
PG & Research Department of Biochemistry, Mohamed Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, India

Anuradha V
PG & Research Department of Biochemistry, Mohamed Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, India

Correspondence
Anuradha V
PG & Research Department of Biochemistry, Mohamed Sathak College of Arts & Science, Sholinganallur, Chennai, Tamil Nadu, India

Toxicity evaluation of encapsulated and non-encapsulated Astaxanthin in Swiss albinos mice

Suganya V and Anuradha V

Abstract
Astaxanthin is a keto carotenoid found in many crustaceans and microorganism especially in *Haematococcus pluvialis.* Astaxanthin was encapsulated with liposomal substances. The objective of the study was to investigate the acute and sub-acute toxicity of encapsulated and non-encapsulated astaxanthin. The test samples in the range of 250, 500 and 1000 mg/g bw was taken. Each group consists of 5 animals. No deaths occurred and no treatment-related changes were observed in the detailed clinical observations, manipulative tests, hematology, blood chemistry, organ weight, but some changes like grip strength, motor activity, body weights, food consumption and histopathology was founded. Body color changes were also found in higher dose of both encapsulated and non-encapsulated astaxanthin. Based on this results, it was concluded that the 250 mg/kg bw has no side effect when compared to 500 and 1000 mg/kg bw in acute toxicity study. Further, in sub-acute toxicity all the dose like 20, 40 and 80 mg/kg bw shows no adverse effect. Hence, the dosage for anticancer study can be fixed at 20 mg/kg bw respectively.

Keywords: Acute toxicity, sub-acute toxicity, Astaxanthin, *Haematococcus pluvialis*, mice

Introduction
Astaxanthin (Ax) is a fat-soluble compound classified into xanthophyll's that are oxygenated derivatives of carotenoids. In nature, Ax is a naturally-occurring red pigment and is widely found in microorganisms (e.g., bacteria, microalgae, yeast), crustaceans (e.g., lobster, krill, shrimp), fish (e.g., salmon, trout) and some birds (e.g., flamingo, quail) [1]. The ultimate producers of oxy carotenoids however are photosynthetic or microbial species that can be found in the aquatic environment (e.g., algae) or as terrestrial plants and microorganisms. Although we recognize the presence of astaxanthin by its red or orange color, its cellular biochemical role is thought to be based on the powerful antioxidant characteristics of this molecule [2].

Astaxanthin can exist as several stereoisomers since the molecule has two chiral centres at carbons 3 and 3'. Both chiral centres can exist in either the R or S form and there are four possible stereo (or optical) isomers: SS, RS, SR, or RR. Astaxanthin can also be present in cells in a free form or esterified to certain fatty acids [3]. Astaxanthin produced by the algae *Heamatococcus pluvialis* is esterified and in the 3S, 3'S form, whereas that produced by the yeast *Phaffia rhodozyma* is non-esterified. Synthetic astaxanthin is used extensively worldwide as a colouring agent in salmon aquaculture and it is a racemic mixture of all optical isomers and in the non-esterified form [4].

Chemically synthesized astaxanthin has been mainly used as color additives for fish and shrimp due to the benefit of the large scale and low coat productions. Therefore, astaxanthin produced by chemical synthesis approach as an aquaculture use for decades [5]. Several studies in animals and humans have shown that astaxanthin possesses antioxidant properties [6] and a wide variety of biological activities, including anti-inflammatory [7], anticancer [8] and ocular and heart hypertension protective effects [9]. Thus, the objective of the present study is to investigate the acute and sub-acute toxicity of non-encapsulated astaxanthin and liposomal encapsulated astaxanthin.

Materials and Methods
Experimental Animals
Swiss Albino mice (20-25 g) were used for acute and subacute toxicity study. These animals were kept in polypropylene cages and were maintained at a temperature of 22°C ± 2°C with a relative humidity of 60% ± 10%. They were fed with standard animal diet and water *ad libitum*. Animals were acclimatized to laboratory conditions before the test. Each cage contained 5 mice of the same sex with a bedding of husk, and 12-hour light/dark cycles were

provided. Experiments were designed and conducted in accordance with ethical norms approved by Institutional animal ethical committee IAEC No: 05/321/PO/Re/S/01/ CPCSEA Dated 12/10/2018.

Test samples used for toxicity study

A. Non-encapsulated astaxanthin: Astaxanthin was purchased from Rudra Bio ventures Pvt Ltd, Bangalore.

B. Preparation of liposome encapsulated astaxanthin

The method for production of liposome-encapsulated astaxanthin was done as follows: L- phosphatidylcholine (0.04 g/ml of DMSO) and cholesterol (0.01 g/ml of DMSO) were liquefied in 5 mL of mixed solvent of chloroform/methanol (2:1 v/v). To the above mixture, an appropriate amount of astaxanthin was added. Lastly, concentration of astaxanthin was made to 1.0 mM. This solution was ultra-sonicated and concentrated under decreased pressure to totally drive off the organic solvents until a membranous product formed on the inner wall of the concentrator. The desiccation process was done for another 2 hours in vacuum drier. The product membrane was dissolved in 15% of ethanol/double distilled water. The solution was sonicated for 30 min with a probe Sonicator. The dispersal was filtered through a 0.2-µm membrane. The filtrate was lyophilized to obtain Liposome- encapsulated Astaxanthin [10].

Acute oral toxicity studies

Acute oral toxicity study was performed as per the guidelines of Organization for Economic Co-operation and Development (OECD, 425) [11]. Healthy young mice were selected and divided into seven groups; each group consists of five animals. Totally thirty five animals were used for acute oral toxicity studies. The both non-encapsulated and encapsulated astaxanthin was given separately in various doses (250, 500, 1000 mg/kg) by oral route.

Group I (control): animals were administered orally with vehicle (normal saline).
Group II: administered 250 mg/kg body weight of non-encapsulated astaxanthin
Group III: administered 500 mg/kg body weight of non-encapsulated astaxanthin
Group IV: administered 1000 mg/kg body weight of non-encapsulated astaxanthin
Group V: administered 250 mg/kg body weight of encapsulated astaxanthin
Group VI: administered 500 mg/kg body weight of encapsulated astaxanthin
Group VII: administered 1000 mg/kg body weight of encapsulated astaxanthin

After administration of the test samples, the animals were observed continuously for the first 30 min, then for the 24 hours, with special attention during the first 4 hour and daily thereafter for a total of 14 days to detect changes in skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems and for behavioural pattern. On 15th day animals were anaesthetized, blood was collected from the mice for haematological and biochemical analysis.

Subacute toxicity studies

The sub-acute oral toxicity study was conducted according to the guidelines of the Organization for Economic Co-operation and Development (OECD, 407) [12]. Thirty five animals were used for subacute toxicity studies. The mice were divided into seven groups; each group consists of five animals.

Group I (control): animals were administered orally with vehicle (normal saline).
Group II: administered 20 mg/kg body weight of non-encapsulated astaxanthin
Group III: administered 40 mg/kg body weight of non-encapsulated astaxanthin
Group IV: administered 80 mg/kg body weight of non-encapsulated astaxanthin
Group V: administered 20 mg/kg body weight of encapsulated astaxanthin
Group VI: administered 40 mg/kg body weight of encapsulated astaxanthin
Group VII: administered 80 mg/kg body weight of encapsulated astaxanthin

The test samples based on above dosage were given orally for 28 days. Animals were observed for signs and symptoms, behavior alteration. During the treatment period food consumption and water intake for all the groups were observed from day 1 to 28 days.

Serum Isolation and Hematology

On 29th day, the overnight fasted rats were anaesthetized with diethyl ether inhalation in a jar containing cotton soaked with diethyl ether. Then blood samples were withdrawn from retro-orbital sinus and the collected blood samples were evaluated for hematological parameters [13] viz. red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), platelet count, packed cell volume (PCV), differential count, Mean Platelet Volume (MPV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and non-heparinized tube from which serum was isolated by centrifugation at 3000 rpm for 10 minutes and used for biochemical estimations [14-20] viz. Cholesterol, triglycerides, VLDL levels SGOT, SGPT, ALP, total bilirubin, total protein, albumin, globulin, urea, uric acid and creatinine. Biochemical investigations were carried out in an auto analyzer.

Organ Weight and Histopathology

Animals were sacrificed under mild ether anesthesia. After sacrifice, organ weights (liver, kidney, spleen) were recorded. Vital organs like Liver, kidneys and spleen were excised from the anesthetized animal, rinsed in 0.9% saline. Tissue pieces were fixed in 10% paraformaldehyde for paraffin histology and processed in paraffin embedding as per the standard protocol. Sections of each tissue were stained with hematoxylin and eosin, and observed for possible histopathological damages.

Statistical analysis

The values were expressed as mean ± SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. *P* values < 0.05 were considered as significant.

Results

Acute toxicity studies

When certain tests were conducted in mice with different dose of 250, 500 and 1000 mg/kg bw of both non-encapsulated and encapsulated astaxanthin, no mortality was observed. Test animals were observed with special attention for first 30 min to 4 hours. Observations were recorded at regular time intervals throughout the study period i.e. 14 days. Results are as follows:

Table 1: Behavioral signs of acute oral toxicity (Encapsulated astaxanthin)

Group	Control	250mg/kg	500 mg/kg	1000 mg/kg
Body weight	Normal	Normal	Reduced	Reduced
Assessments of posture	Normal	Normal	Normal	Normal
Signs of Convulsion Limb paralysis	Normal	Absence of sign (-)	Absence of sign (-)	Absence of sign (-)
Body tone	Normal	Normal	Reduced	Reduced
Lacrimation	Normal	Normal	Normal	Normal
Salivation	Normal	Absence	Absence	Absence
Change in skin color	NS color change	NS color change	Change in skin color	Change in skin color
Piloerection	Normal	Normal	Erect	Erect
Defecation	Normal	Normal	Normal	Normal
Sensitivity response	Normal	Normal	Normal	Normal
Locomotion	Normal	Normal	Slow	Slow
Muscle gripness	Normal	Normal	Normal	Normal
Rearing	Mild	Normal	Slow	Slow
Urination	Normal	Normal	Normal	Normal

Table 2: Behavioral signs of acute oral toxicity (Non- Encapsulated astaxanthin)

Group	Control	250mg/kg	500 mg/kg	1000 mg/kg
Body weight	Normal	Normal	Reduced	Reduced
Assessments of posture	Normal	Normal	Normal	Normal
Signs of Convulsion Limb paralysis	Normal	Absence of sign (-)	Absence of sign (-)	Absence of sign (-)
Body tone	Normal	Normal	Reduced	Reduced
Lacrimation	Normal	Normal	Normal	Normal
Salivation	Normal	Absence	Absence	Absence
Change in skin color	NS color change	NS color change	Change in skin color	Change in skin color
Piloerection	Normal	Normal	Erect	Erect
Defecation	Normal	Normal	Normal	Normal
Sensitivity response	Normal	Normal	Normal	Normal
Locomotion	Normal	Normal	Slow	Slow
Muscle gripness	Normal	Normal	Normal	Normal
Rearing	Mild	Normal	Slow	Slow
Urination	Normal	Normal	Normal	Normal

The body weight and body tone of test animals of both control and 250 mg/kg of encapsulated and non-encapsulated astaxanthin were found to be normal whereas other treated group at 500, 1000 mg/kg bw concentration were decreased progressively throughout the study period as shown in Table 1 and 2. Behavioral observation of the test animals after dosing showed that Assessment of posture, lacrimation, and defecation, sensitivity of response, muscle gripness and urination was to be normal for both test and control group. Salvation was normal in control but absence in test group of both encapsulated and non-encapsulated astaxanthin. Signs of convulsion was normal in control where as it is absence in test group of both the drug. A change in skin color was not seen in control and 250 mg/kg bw of test sample however changes occur in 500 and 1000 mg/kg bw of test samples.

Table 3: Changes found during acute toxicity studies: Encapsulated astaxanthin

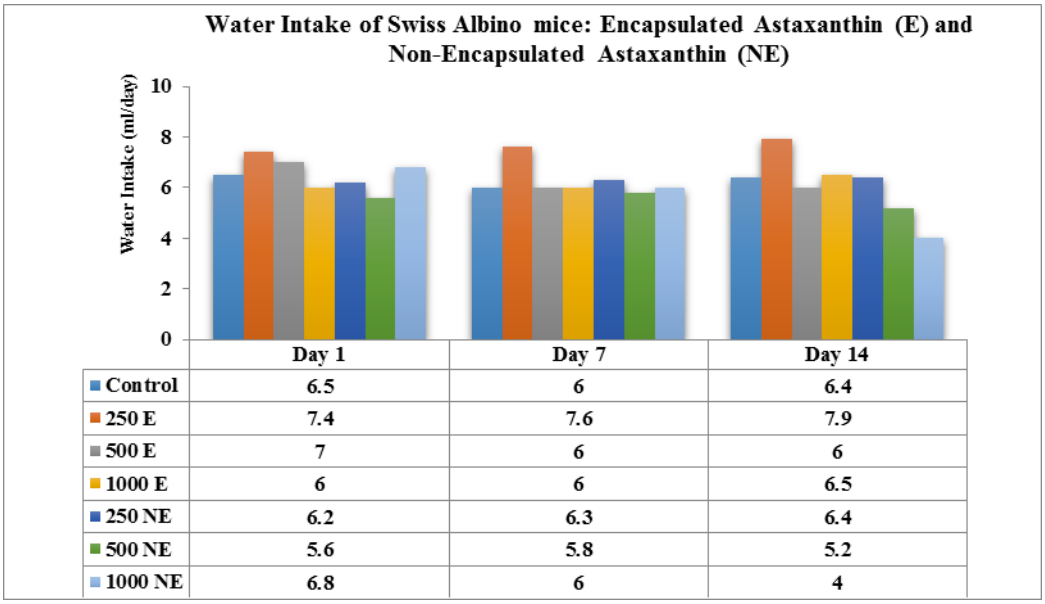
Dose	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Control	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
250 mg/kg	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
500 mg/kg	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1000 mg/kg	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4: Changes found during acute toxicity studies: Non- encapsulated astaxanthin

Dose	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Control	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
250mg/kg	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
500mg/kg	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1000mg/kg	-	-	-	+	-	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-

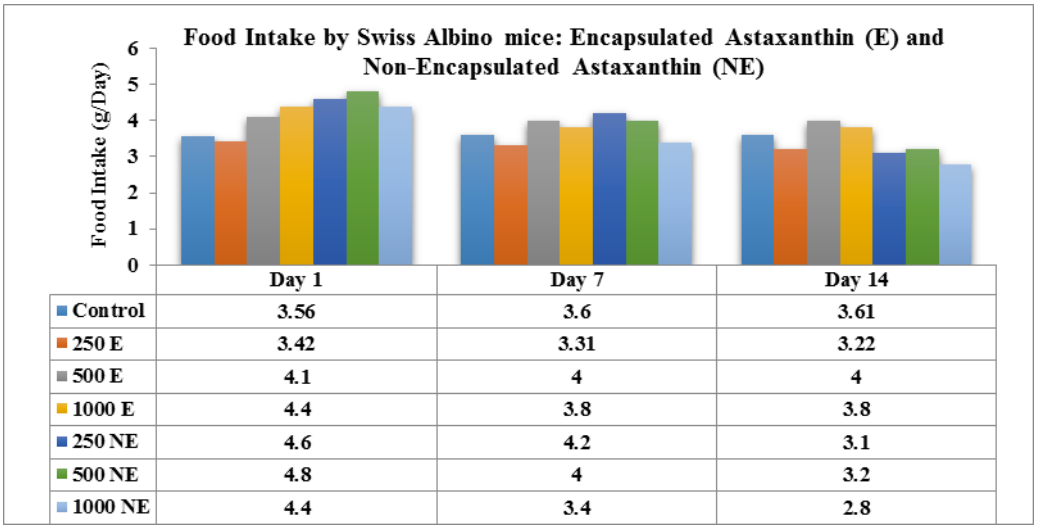
1. Alertness 2. Aggressiveness 3. Pile erection 4. Grooming 5. Gripping 6. Touch Response 7. Decreased Motor Activity 8. Tremors 9. Convulsions 10. Muscle Spasm 11. Catatonia 12. Muscle relaxant 13. Hypnosis 14. Analgesia 15. Lacrimation 16. Exophthalmos 17. Diarrhea 18. Writhing 19. Respiration 20. Mortality

Based on Table 3 and 4, Behavioral changes of the test animal after dosing showed alertness for control and 250 mg/kg bw whereas it was negative for 500 and 1000 mg/kg bw of test samples. Aggressiveness, Gripping, Tremors, Muscle spasm, Muscle relaxant, Hypnosis, Analgesia, Lacrimation, Exophthalmos, Diarrhea and Respiration was normal during studies. At 1000 mg/kg bw of encapsulated astaxanthin decreased motor activity was found to be presence. Similarly, at 1000 mg/kg bw of non-encapsulated astaxanthin Convulsion, Catatonia, Writhing was seen.



	Day 1	Day 7	Day 14
Control	6.5	6	6.4
250 E	7.4	7.6	7.9
500 E	7	6	6
1000 E	6	6	6.5
250 NE	6.2	6.3	6.4
500 NE	5.6	5.8	5.2
1000 NE	6.8	6	4

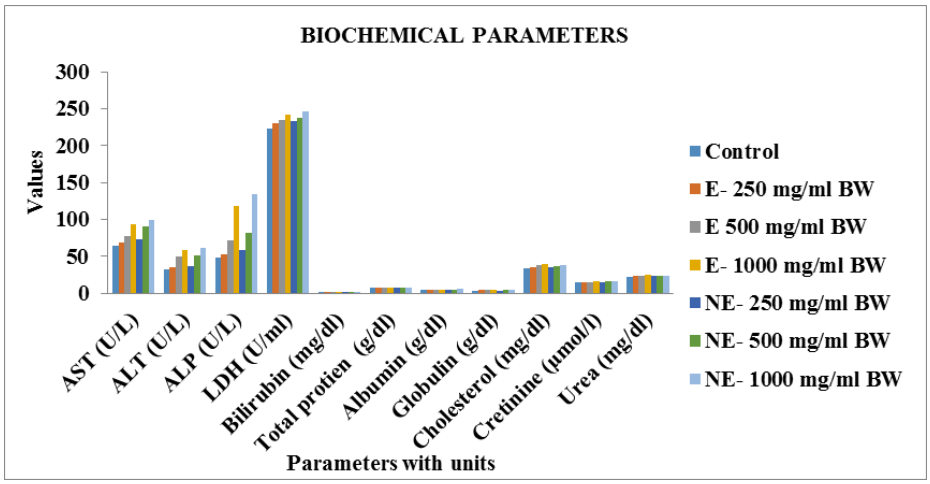
Graph 1: Water intake measured during acute studies



	Day 1	Day 7	Day 14
Control	3.56	3.6	3.61
250 E	3.42	3.31	3.22
500 E	4.1	4	4
1000 E	4.4	3.8	3.8
250 NE	4.6	4.2	3.1
500 NE	4.8	4	3.2
1000 NE	4.4	3.4	2.8

Graph 2: Food intake measured during acute studies

Water and food consumption of mice were continuously monitored, where there is no significant change in consumption was observed for both the control and drug treated groups (Graph 1 and 2).



Graph 3: Biochemical Parameters in blood of Swiss albino mice group exposed to Encapsulated and Non-encapsulated astaxanthin

Biochemical parameters in blood of tested mice show similar activity at 250 & 500 mg/kg bw concentration when compared to the normal group but at 1000 mg/kg bw both the test samples shows higher amount of biochemical compounds. The results were interpreted in Graph 3. No significant changes was observed in AST, ALT & ALP of tested animals

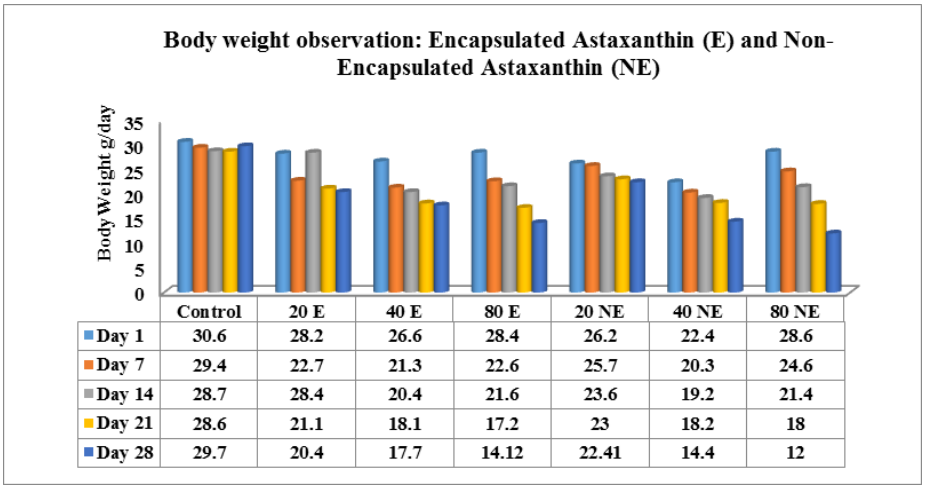
when compared with the normal control group. Also no significant alteration observed in creatinine, urea and uric acid levels of treated group animals when compared with control group animals.

Table 5: Hematological parameters of Swiss albino mice group exposed to encapsulated and Non-encapsulated astaxanthin

Parameters with Units	Control group	Encapsulated			Non-Encapsulated		
		250 mg/kg bw	500 mg/kg bw	1000 mg/kg bw	250 mg/kg bw	500 mg/kg bw	1000 mg/kg bw
Hb (g/dl)	11.2 ± 0.257	11.5 ± 0.643	12.0 ± 0.301	12.8 ± 0.312	11.2 ± 0.203	11.4 ± 0.647	11.9 ± 0.492
Total RBC ($x10^6/mm^{3)}$	7.25 ± 0.065	7.31 ± 0.163	7.45 ± 0.601	8.10 ± 0.843	7.28 ± 0.031	7.35 ± 0.261	7.89 ± 0.521
MCV (Fl)	42.3 ± 0.106	44.1 ± 0.031	45 ± 0.217	45.21 ± 0.654	42.4 ± 0.006	43.1 ± 0.301	43.8 ± 0.322
MCHC (g/dl)	33 ± 0.751	34.2 ± 0.937	34.8 ± 0.632	35.3 ± 0.523	33.1 ± 0.246	33.5 ± 0.341	34.1 ± 0.352
Platelet Count ($x10^3/ mm^3$)	4.31 ± 0.642	4.78 ± 0.526	5.01 ± 0.561	5.23 ± 0.024	4.34 ± 0.135	4.41 ± 0.413	4.89 ± 0.649
WBC count ($x10^6/ mm^3$)	6.78 ± 0.033	7.01 ± 0.962	7.21 ± 0.753	7.89 ± 0.453	6.89 ± 0.049	7.12 ± 0.511	7.25 ± 0.931
Neutrophils (%)	13 ± 0.897	14 ± 0.453	15 ± 0.153	15 ± 0.023	14 ± 0.065	15 ± 0.611	15 ± 0.923
Lymphocytes (%)	83 ± 0.231	83 ± 0.025	83 ± 0.756	84 ± 0.257	82 ± 0.642	83 ± 0.357	83 ± 0.616
Monocytes (%)	2 ± 0.454	2 ± 0.951	1 ± 0.425	1 ± 0.152	2 ± 0.157	1 ± 0.642	1 ± 0.535
Eosinophils (%)	2 ± 0.245	1 ± 0.520	1 ± 0.152	1 ± 0.953	2 ± 0.315	1 ± 0.044	1 ± 0.133
MCH (Pg)	15.8 ± 0.325	16 ± 0.920	16.2 ± 0.502	17.0 ± 0.025	16.1 ± 0.064	16.8 ± 0.666	17.5 ± 0.543
HCT (%)	33.1 ± 0.064	33.8 ± 0.620	34.3 ± 0.530	35.1 ± 0.423	34.1 ± 0.032	34.9 ± 0.946	35.3 ± 0.455

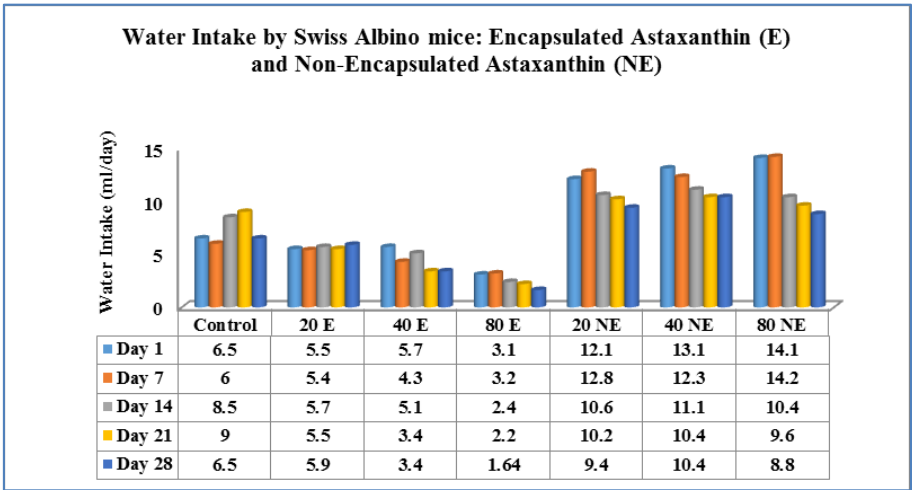
The results of hematological investigations (Table 5) conducted on day 15th day for acute toxicity study revealed no substantial changes was found in the values of RBC, WBC, Hb, platelet count, PCV, differential count, MPV, MCV, MCH, MCHC of treated groups when compared with the control mice.

Sub-Acute Toxicity

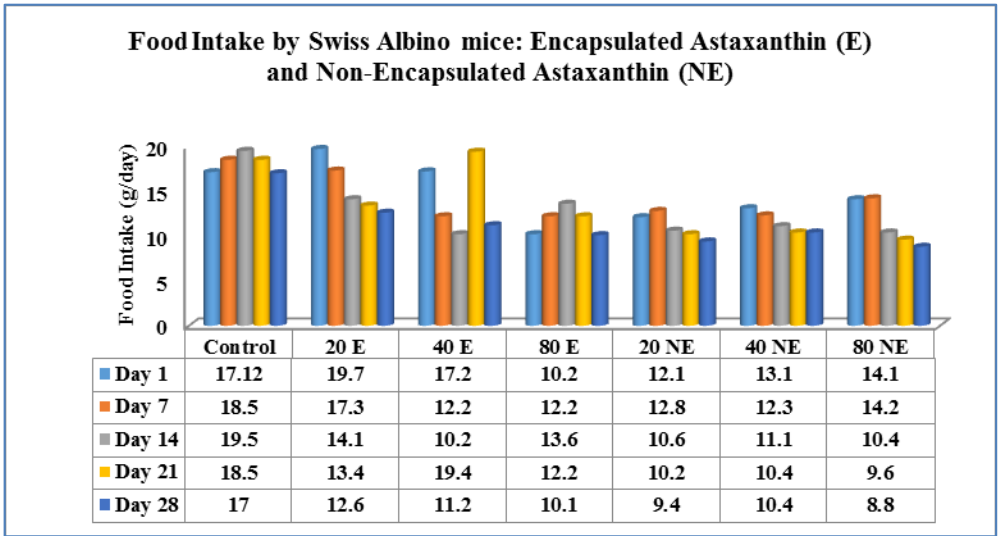


Graph 4: Body weight observations during Sub-acute studies

Body weight of test mice were observed after the dosage given at different concentration of drug samples (20, 40 and 80 mg/kg bw). Loss in body weight is frequently the first indicator of the onset of an adverse effect. A dose, which causes 10% or more reduction in the body weight, is considered to be a toxic dose. From Graph 4, it is concluded that except control group, the body weight of the test animals tends to decreases slowly from day 1 to day 28 which may be due to lower intake of food and water.



Graph 5: Water intake measured during sub-acute toxicity



	Control	20 E	40 E	80 E	20 NE	40 NE	80 NE
Day 1	17.12	19.7	17.2	10.2	12.1	13.1	14.1
Day 7	18.5	17.3	12.2	12.2	12.8	12.3	14.2
Day 14	19.5	14.1	10.2	13.6	10.6	11.1	10.4
Day 21	18.5	13.4	19.4	12.2	10.2	10.4	9.6
Day 28	17	12.6	11.2	10.1	9.4	10.4	8.8

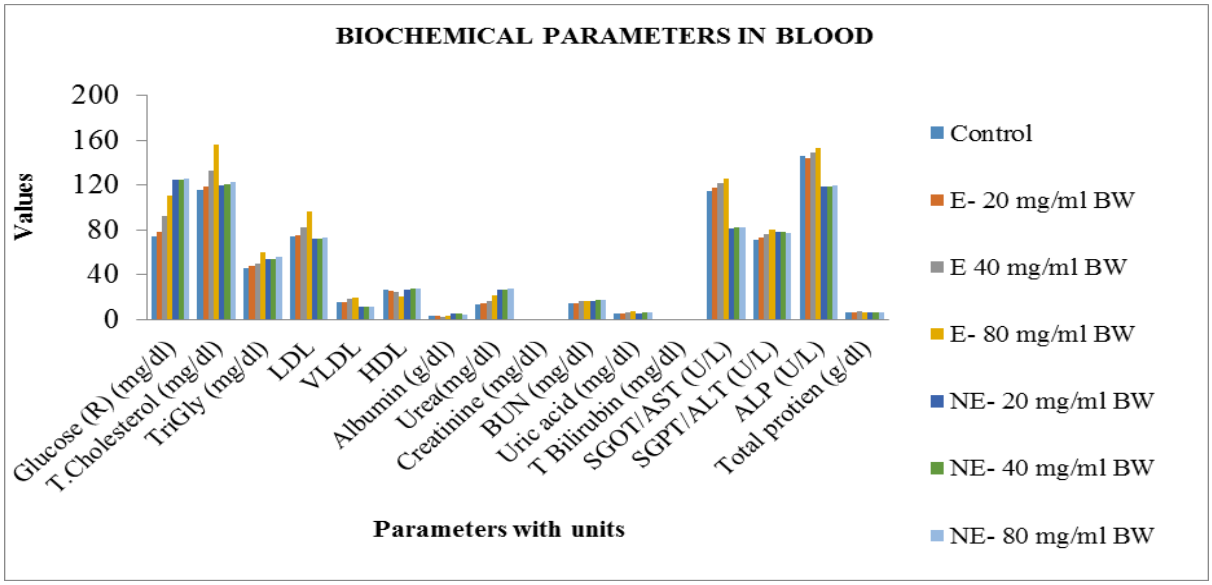
Graph 6: Food intake measured during Sub-acute toxicity

Measured quantities of water and feed were supplied daily for each animal. The consumption of water and food was estimated from the amount of water remaining in feeding bottles and from the amount of feed remaining in the feed hopper. The data for food and water consumption is given in Graph 6. There was no significant change in food and water intake of the test animals at all dose levels.

Table 6: Hematological parameters of Swiss albino mice group exposed to Encapsulated and Non-encapsulated astaxanthin

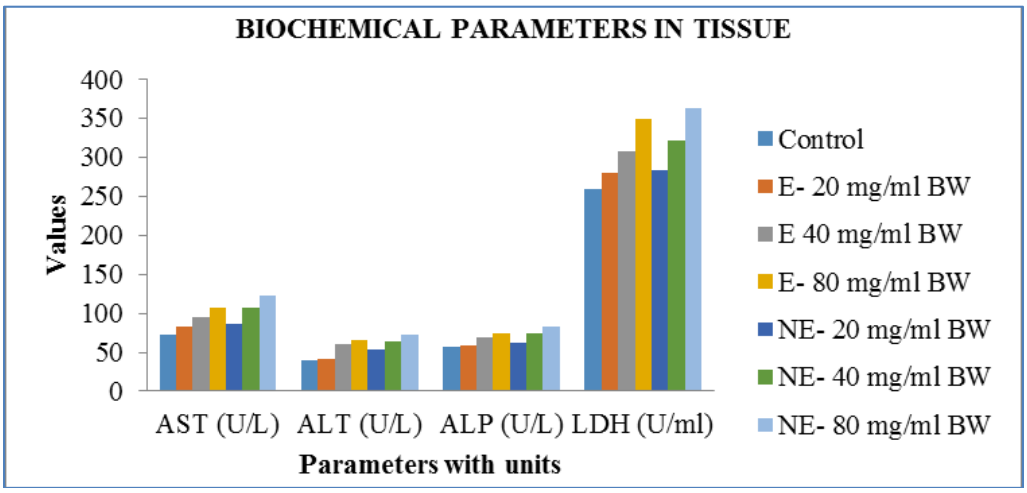
Test name	Control	Encapsulated Astaxanthin			Non Encapsulated Astaxanthin		
		20 mg/kg body weight	40 mg/kg body weight	80 mg/kg body weight	20 mg/kg body weight	40 mg/kg body weight	80 mg/kg body weight
Haemoglobin(g/dl)	13.8±0.88	13.90±1.16	11.14±0.66	9.28±1.16	16.60±0.24	16.5±0.23	16.82±0.16
Total WBC ($\times 10^3$ l)	11.91±0.59	11.85±1.23	10.08±1.21	8.110±2.27	10.64±0.21	10.54±0.42	9.60±1.12
Neutrophils(%)	33.65±0.06	33.3±1.24	32.11±2.16	30.20±1.10	31.02±0.12	32.11±1.22	33.02±6.21
lymphocyte (%)	70.24±1.48	70.02±1.12	69.20±1.16	66.10±1.26	72.12±1.32	73.10±2.34	73.20±2.44
Monocyte (%)	0.86±0.07	0.85±0.19	0.72±0.13	0.71±0.60	0.9±0.01	0.9±0.04	0.9±0.03
Eosinohil(%)	0.54±0.09	0.54±0.12	0.62±0.16	0.72±0.04	0.5±0.04	0.5±0.05	0.5±0.08
Platelets cells10^3/µl	687.17±8.76	678.71±9.16	623.18±2.20	627.16±3.74	682.41±4.12	682.13±2.02	684.10±2.34
Total RBC 10^6/µl	7.99±0.12	7.79±1.57	7.62±0.19	7.05±0.12	8.46±0.53	8.49±0.44	8.74±0.46
PCV%	37.79±0.6	37.35±1.23	32.98±1.18	28.82±2.14	42.62±1.02	43±1.20	44.40±2.10
MCHC g/dL	33.6±2.23	33.29±1.19	30.18±1.12	34.03±1.14	34.2±1.10	34.8±1.70	34.33±1.30
MCV fL(µm^3)	49.07±3.64	47.28±8.12	45.20±1.24	4.22±1.94	59.2±1.10	58.9±1.40	58.8±1.20

The results of hematological investigations (Table 6) conducted on 28th day for sub-acute toxicity study revealed some significant changes in the values of RBC, WBC, Hb, platelet count, PCV, differential count, MCV and MCHC of treated groups when compared with the respective control mice respectively.



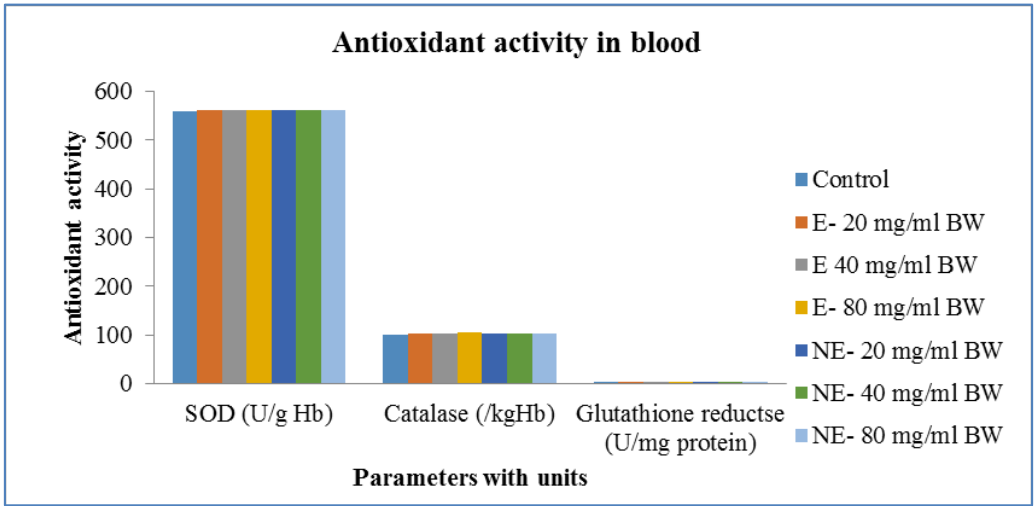
NS- Not Significant, ** ($p > 0.01$), * ($p > 0.05$), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Graph 7: Biochemical Parameters in blood of Swiss albino mice group exposed to Encapsulated and Non-encapsulated astaxanthin

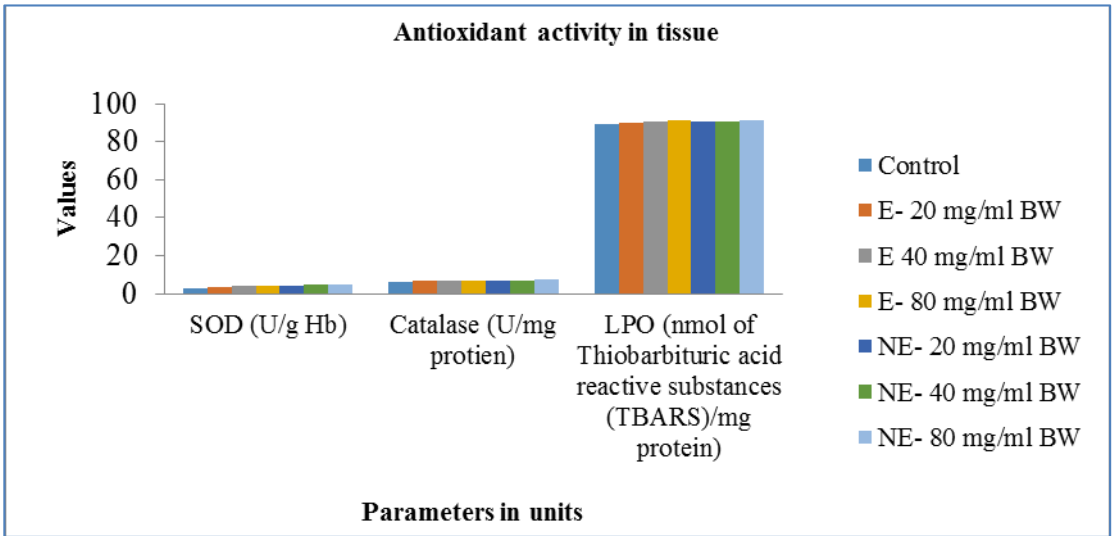


Graph 8: Biochemical Parameters in tissue of Swiss albino mice group exposed to Encapsulated and Non-encapsulated astaxanthin

Biochemical investigations were performed in order to review any toxic effects produced after administration of encapsulated and on-encapsulated astaxanthin on liver and kidney. There was no significant alteration in cholesterol, triglycerides and VLDL levels in control and encapsulated astaxanthin treated groups of sub-acute toxicity study when compared with no-encapsulated astaxanthin group of mice (Graph 7 & 8). No significant change observed in (serum glutamic oxalo acetic transaminase) SGOT, (Serum glutamic pyruvic transaminase) SGPT, (alkaline phosphatase) ALP and total bilirubin content of encapsulated astaxanthin treated group animals when compared with control group animals and non-encapsulated astaxanthin. There was no significant alteration observed in creatinine, urea and uric acid levels of treated group.

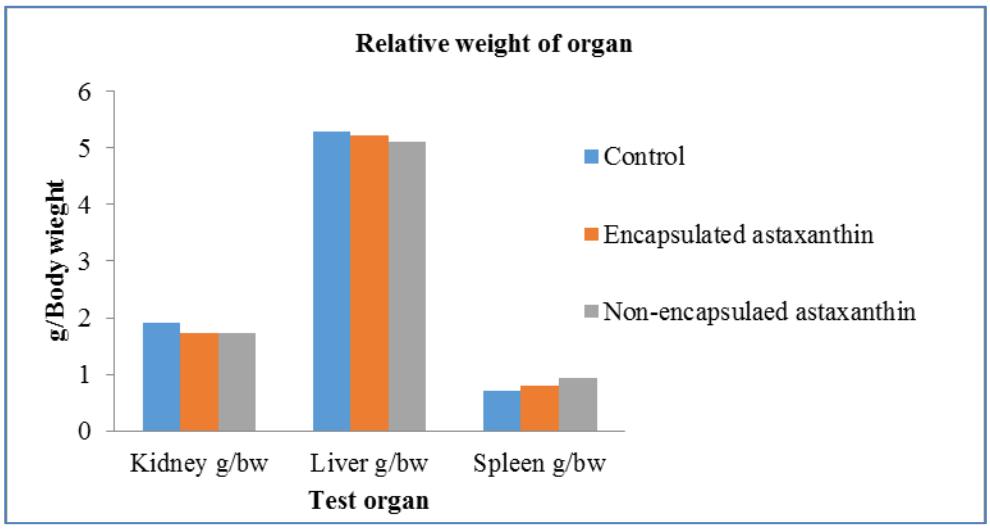


Graph 9: Antioxidant activity of Swiss albino mice group exposed to encapsulated and Non-encapsulated astaxanthin in blood



Graph 10: Antioxidant activity of Swiss albino mice group exposed to encapsulated and Non-encapsulated astaxanthin in tissue

Antioxidant activity such as SOD, Catalase, and Glutathione reductase in mice exposed to encapsulated and non-encapsulated astaxanthin in blood shows no significant changes at different concentration along with control group (Graph 9). Similarly, the antioxidant activity like SOD, Catalase, LPO, Glutathione peroxidase, Glutathione reductase and Glutathione-S-transferase were performed in tissue sample of drug treated mice and found that all the groups exhibit better activity (Graph 10).



Graph 11: Relative weight of organ after administration of test dose (Encapsulated and Non-encapsulated astaxanthin)

No abnormal change in the relative weight of internal organs of mice was observed when compared to control group. Similarly, no lesion was found on examination of isolated vital organs such as kidney, liver and spleen from testing animals. Organ to body weight index was calculated and summarized in Graph 11 which shows that there was no significant variation present among the groups.

Figure 1 to 3, represent the histopathology image of control group along with encapsulated astaxanthin and non-encapsulated astaxanthin with high dose of 80 mg/kg bw of mice. Major differences were not founded between the test and control group.

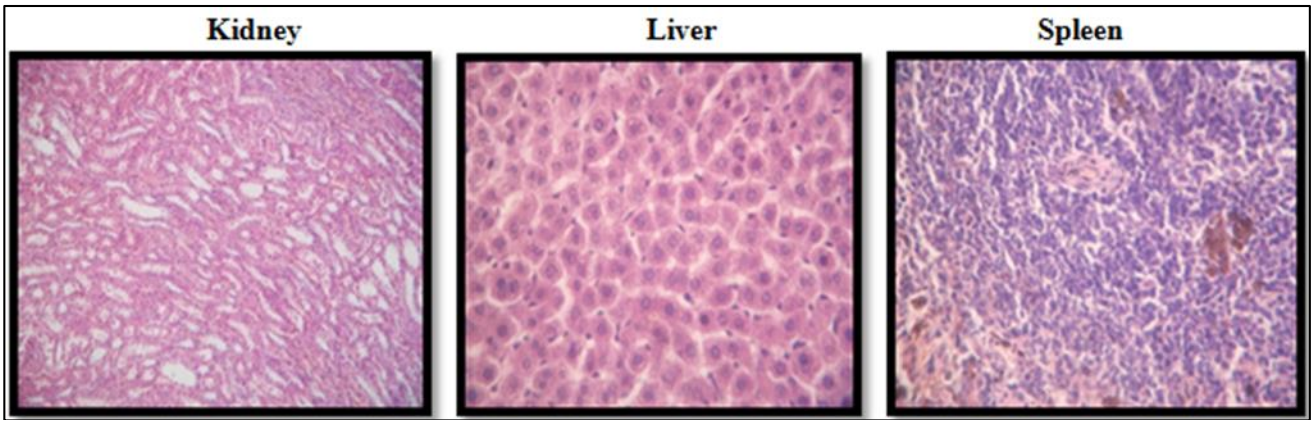


Fig 1: Histopathology image of Control Group

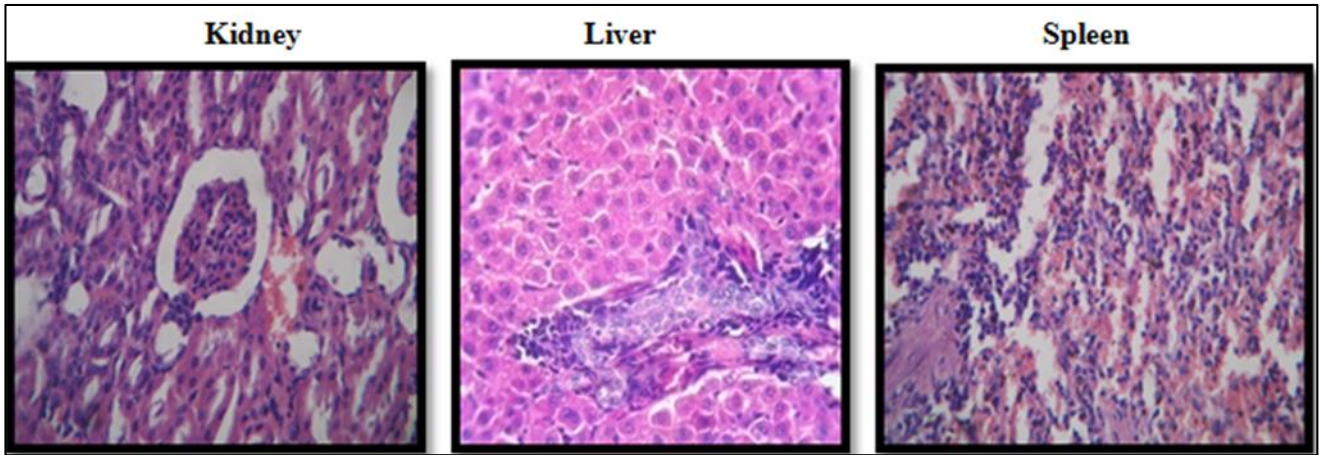


Fig 2: Histopathology image Test Group (High Dose): Encapsulated Astaxanthin

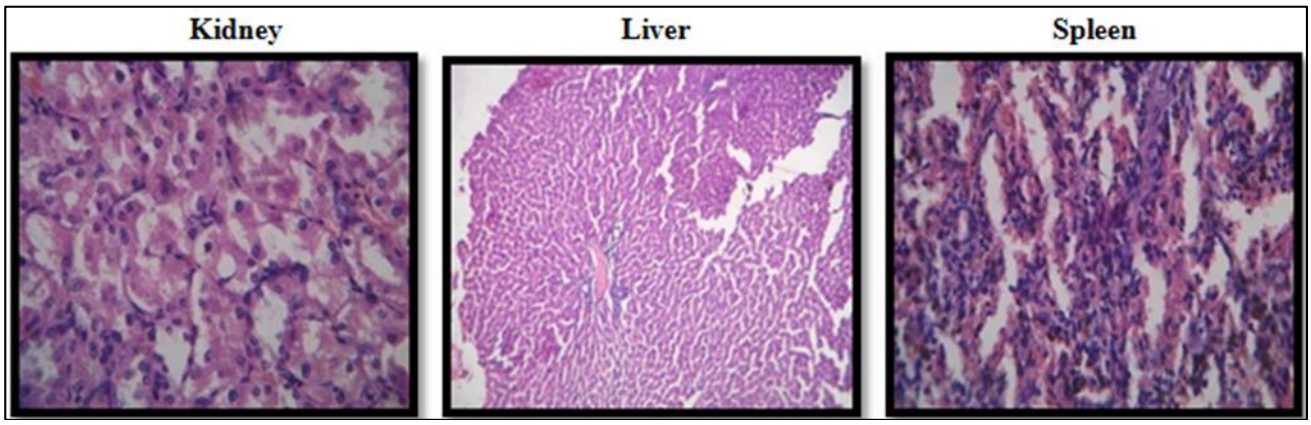


Fig 3: Histopathology image Test Group (High Dose): Non- Encapsulated Astaxanthin

Liver

Normal lobular architecture and normal portal tract was observed in histopathological section of liver in control group mice in 10 x and 40 x magnifications respectively. Drug treated mice at a dose of 80 mg/kg showed abnormal lobular architecture, central veins and sinusoids but no toxic signs like inflammation, fatty change or fibrosis were observed.

Spleen

Histological spleen section showed red pulp congestion in control group mice and in drug treated mice (80 mg/kg). Variation in white pulp, pencillar artery and the red pulp was slightly observed.

Kidney

Normal cortex, medulla and normal glomeruli were observed from the histological sections of kidney from control and drug treated mice (80 mg/kg). Section also showed abnormal interstitial at 80 mg/kg for both encapsulated and non-encapsulated astaxanthin when compared to control group.

Discussion

Astaxanthin has received much attention in recent years as a cosmetic and functional food ingredient and presentation of safety information for astaxanthin to consumers are an important responsibility of manufactures. Specifically, the dietary exposure to Wistar rat of pure astaxanthin at a concentration of 0.04% (equivalent to approximately 20 mg/kg/day) for 41 days did not cause toxicological effects [21]. In agreement, another study proved that dietary exposure to F344 rats of *P. rhodozyma* product at concentrations of 5% (equivalent to 0.06% astaxanthin; approximately 30 mg/kg/day) for 90 days did not cause adverse or toxic effects [22]. The administration of 1000 mg/kg astaxanthin-pre (equivalent to 50 mg/kg astaxanthin) allowed a 500 fold safety margin based on the expected daily human consumption of 6 mg astaxanthin [23].

Several safety studies of *H. pluvialis* astaxanthin have been published. Among these, the NOAEL of astaxanthin rich extracts (50mg astaxanthin) from *H. pluvialis* in sub-chronic administration to rats was found to be 9259 mg/kg [24]. The safety of astaxanthin rich extract from *H. pluvialis* using rats as model organism was also evaluated and reported that the NOAEL was approximately 500 mg/kg astaxanthin [25].

From subchronic study of the 13-week repeated dose oral administration of a natural astaxanthin-rich extract (ARE) from *P carotinifaciens* to rats at dose levels of 250, 500 and 1000 mg/kg/day showed nil death and without any significant toxic changes. They reported the no observed adverse effect level (NOAEL) was at least 1000 mg/kg/day for both males and females [4]. There were no treatment-related changes in body weight gain in any of the groups and the growth curves of the male and female animals during the study were consistent with historical background data from this test facility. The food consumption data also tracked historical norms from this facility, but statistical analysis revealed that higher food consumption was observed in the high dose (1000 mg/kg) male group on day 84 and in the high dose (1000 mg/kg) female group on day 77. However, this was due to excretion of the coloured test material into faces and was not suggestive of toxicity. These changes were judged to be incidental fluctuations since they were observed at only one time point during the study. Likewise a low food consumption recorded on day 14 of administration in females in the 500 mg/kg group was also judged to be incidental since it was a single point and not dose-related.

An investigation reported no abnormality was apparent when adult subjects ingested Ax-containing *H. pluvialis* oil product, Puresta®, up to 20 mg (equivalent to Ax dialcohol) once daily for 4 weeks [1]. The safety of Puresta® has been investigated previously in a clinical study where 17 subjects were administered Puresta® amounts equivalent to 8 mg of Ax dialcohol twice daily for 12 weeks. Thus their findings support the safety of this product at a dose of 20 mg/day, with no negative effects on blood chemistry or haematology results over time, and no subject reports of adverse experiences during treatment. Similarly, our findings also matches with above work which shows good activity at 250 mg/kg bw for acute toxicity and 20 mg/kg bw for sub-acute toxicity. Only treatment-related changes were observed in all mice of treated groups throughout the administration period which may be due to fluctuations in food consumptions, water intake etc. Hence, the dosage of 20 mg/kg bw of encapsulated and non-encapsulated astaxanthin was fixed for further studies.

Conclusion

From the overall results, when compared between two types of drugs such as encapsulated and non-encapsulated astaxanthin, the toxicity was found to be lower with the drug astaxanthin encapsulated with liposomal compound than the free astaxanthin. Further, the encapsulated drugs also reach the site and release the astaxanthin with fewer side effects which can be further confirmed by bioavailability studies. Hence, encapsulated astaxanthin at fixed dosage is explored for its protective role against hepatocellular carcinoma in mice.

Conflict of Interest
The authors declare that there are no conflicts of interest.

Acknowledgements
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References
1. Satoh A, Tsuji S, Okada Y, Murakami N, Urami M, Nakagawa K *et al.* Preliminary clinical evaluation of toxicity and efficiency of a new astaxanthin rich *Haematococcus pluvialis* extract. Journal of Clinical Biochemistry and Nutrition. 2009; 44:280-284.
2. Naguib YMA. Antioxidant activities of astaxanthin and related carotenoids. Journal of Agricultural and Food Chemistry. 2000; 48:1150-1154.
3. Renstrom B, Liaaen-Jensen S. Fatty acids of some esterified carotenoids. Comparative Biochemistry and Physiology. 1998; 69:625-627.
4. Katsumata T, Ishibashi T, Kyle D. A sub-chronic toxicity evaluation of a natural astaxanthin-rich carotenoid extract of *Paracoccus carotinifaciens* in rats. Toxicology Reports. 2014; 1:582-588.
5. Surmatis JD, Thommen R. A total synthesis of astaxanthin dimethyl ether. Journal of Organic Chemistry. 1967; 32:180-184.
6. Nakagawa K, Kiko T, Miyazawa T, Carpentero BG, Kimura F, Satoh A *et al*. Antioxidant effects of astaxanthin on phospholipid peroxidation in human erythrocytes. British Journal of Nutrition. 2011; 105:1563-1571.
7. Andersen LP, Holck S, Kupcinskas L, Kuidwlis G, Jonaitis L, Janciauskas *et al*. Gastric inflammatory markers and interleukins in patients with functional dyspepsia treated with astaxanthin. FEMS Immunology and Medical Microbiology. 2007; 50:244-248.
8. Tanaka T, Maita H, Ohnishi M, Mori H, Satoh K, Hara A. Chemoprevention of rat oral carcinogenesis by natural occurring xanthophyll's, astaxanthin and canthaxanthin. Cancer Research. 1995; 55:4059-4064.
9. Fassett RG, Coombes JS. Astaxanthin: a potential therapeutic agent in cardiovascular diease. Marine Drugs. 2011; 9:447-465.
10. Chiu CH, Chang CC, Lin ST, Chyau CC, Peng RY. Improved Hepatoprotectivity effect of Liposome-encapsulated astaxanthin in lipopolysaccharide-induced acute hepatotoxicity. International Journal of Molecular sciences. 2016; 17:1-17.
11. OECD. Test Guideline 425. Acute Oral Toxicity – Up-and-Down Procedure, 2001.
12. OECD (testing guideline, 407), Repeat dose 28 days oral toxicity study in rodents. In-Guidance document for the development of OECD guideline for testing of chemicals. Environmental monogr. 1995, 76.
13. Docie JV. Practical haemotology (J&A Churchill Ltd., London, 1958, 38-42p.
14. Hugget ASG, Nixon DA, The Lancet 270 (6991), 368 1957. http://dx.doi.org/10.1016/S0140-6736 (57)92595-3.
15. Wybenga DR, Pileggi VJ, Dirstine PH, Giorgio JD. Giorgio, Manual and automated methods for urea nitrogen measurement in whole serum. Journal of Clinical Chemistry. 1970; 16:891-895.
16. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. American Journal of Clinical Pathology. 1957; 28:56-63.
17. Mallay HT, Evelyn KA. Ameliorated Activity Of *Murraya koenigii* Leaves Chloroform Extract (Mkce) Against Lead Induced Hepatic Dysfunctions In Mice. Journal of Biology and Chemistry. 2017; 8:2619-2623.
18. Natelson S. Micro techniques of clinical chemistry for the routine laboratory (Thomas Springfield, Dilliners, 1957, 381.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of Biology and Chemistry. 1951; 193:265-275.
20. Henry RJ. Clinical chemistry principles and techniques, 2nd ed. (Harper and Row Publication, New York, 1974, 225p.
21. Nishikawa Y, Minenaka Y, Ichimura M. Physiological and biochemical effects of carotenoid (beta carotene and astaxanthin) on rats, Bulletin Koshien University A. 1997; 25:19-58.
22. Onodera H, Motsumori K, Yasuhara K, Takegawa K, Takahashi M. 13 week subchronic oral toxicity study of *phaffia* colour in F344 rats, Kokuritsu Iyakuhin Shokuhin Eisei Ken Hokoku. 1997; 115:99-106.
23. Tago Y, Fujii T, Wada J, Kato M, Wei M, Wanibuchi H, *et al*. Genotoxicity and subacute toxicity studies of a new astaxanthin containing *Phaffia rhodozyma* extract, Journal of Toxicology Science. 2014; 39:373-382.
24. Takahasi J, Tsukahara H, Minota H. Toxicological studies of astaxanthin from *Haematococus pluvialis*-ames test, oral single doe and 90 days sub-chronic toxicity studies in rats, J. Clin. Therap. Med. 2004; 20:867-881.
25. Stewart JS, Lignell A, Pettersson A, Elfving E, Soni MG. Safety assessment of astaxanthin-rich microalgae biomass: Acute and subchronic toxicity studies in rats. Food and Chemical Toxicology. 2008; 46:3030-3036.