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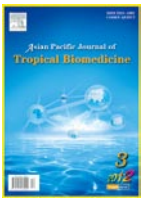

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Sub-chronic toxicity and heavy metal toxicity study on *Kappaphycus alvarezii* in albino rats

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ABSTRACT

Objective: This study aimed to evaluate the toxicity of *Kappaphycus alvarezii* methanolic extracts in albino rats. **Methods:** Sub-chronic toxicity was tested with a single dose of intraperitonal administration of the extract as per the OECD guidelines in the experimental group rats and the control group rats was fed with standard diet and water *ad libitum*. Mortality, behaviour changes, clinical signs and symptoms, food intake, body weight and any abnormalities of the visceral organs were observed. **Results:** The results revealed that the algal extract resulted in neither mortality nor any abnormalities. The Most of the serum biochemical parameters and hematological values were similar in control and experimental groups, histopathological examination of the vital organs like liver, kidney, spleen, brain and heart revealed no obvious abnormality in the control group and *Kappaphycus alvarezii* treated group. **Conclusion:** It may be concluded that *Kappaphycus alvarezii* rich in nutrient and nutraceutial potentials and also safety food for human consumption.

1. Introduction

Kappaphycus alvarezii (*Eucheuma cottonii*) are marine macroalgae belonging to Rhodophyceae. *Kappaphycus alvarezii* are grown in abundance as dominant communities in the shores of Kanyakumari and Ramanthapuram districts of Tamilnadu State, India. The coastal region of Tamilnadu, South India, supports a rich vegetation of marine algae. It has been shown that a great diversity exists in the macroalgal community of the marine algal vegetation of the region; more over 2500 species were identified. Kappaphycus are abundantly present in the intertidal and sub tidal zone regions. The species so cultured yield relatively large amounts of good quality seaweed by labour-intensive means at low cost in the less-developed countries [1].

For the past four decades, this species has been globally introduced to many maritime countries for experimental and commercial cultivation as a sustainable alternate livelihood for coastal villagers [2]. Accompanying the introduction is an increasing concern over the specific effects on the biodiversity of endemic ecosystems; they suggested the utility of *K. alvarezii* as a health food and nutraceutical supplement. *K. alvarezii* could be exploited for their multifunctional properties in the form of food, energy, medicine and as biotechnological tools [3]. Recently we reported the nutrient and nutraceutical potentials of Ulva lactuca and *Kappaphycus alvarezii* [4]. Though *K. alvarezii* are consumed by coastal people in many regions of the world, systematic studies on sub chronic toxicity of these seaweeds are not available in India. Moreover, seaweeds tend to accumulate metal ions and hence data on metal toxicity are also scarce. So the purpose of toxicity studies is the detection of valid biological evidence for any toxic substance being investigated. This study provides information on possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post weaning maturation and growth well into adulthood. Hence the present investigation tests (1) the hypothesis that *K.alvarezii* does not cause toxicity in terms of haematology, biochemical and histopathology parameters in albino rats and (2) to create predictions that it is safe to use the macroalgae for human consumption as novel source of macro and micro nutrients. To test these predictions, we conducted an investigation to test the subchronic toxicity and metal toxicity in animals and thereby recommend for use in Protein Energy Malnutrition (PEM) and chronic

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degenerative diseases.

2. Materials and methods

2.1 Preparation of seaweed extract

Kappaphycus alvarezii were collected from cultivation sites of Mandapam region in Ramanathapuram district, TamilNadu (Palk Bay coast) India. They were washed thoroughly in seawater and then in tap water and the extract was prepared. The techinque involved in the preparation of extracts of *K.alvarezii* is given below. Initially, the seaweeds were shade dried for 48-72 hours. The dried seaweeds were coarsely powdered and 250g of this seaweed powder was packed in soxhlet extractor of one litre capacity. The solvent methanol was added into the flask and heated. The temperature was maintained at 600C throughout the extraction. The soluble active constituents of the extract remained in the flask and the process was repeated until the compounds were completely extracted. Then the mixture was concentrated by vaccum drier and kept in dessicator Ganesan et al. [5]. This extract attained a solid viscous residue with a total weight 4 to 5g. The crude extract was kept in dry place and it was used for toxicity study and heavy metal analysis.

2.2 Selection of experimental animals

Young healthy Wistar strain albino rats (Rattus norvegicus) of three to four months old, weighing 130-140g were chosen for the study. The rats were obtained from the stock colony of animal laboratory; the healthy animals which were not subjected to previous experimental procedures were used for this study. All the animals were acclimatized to the laboratory conditions for a period of one week. Each group consisted of six rats and were housed in individual stainless steel cages in institutional animal care and house facility at room temperature 28±2℃. A 12hours light and dark cycle was maintained as per the animal ethical guidelines. The standard rat pellet feed supplied by Pranav Auro Industries Ltd, Sangli, India was used as animal feed for both the groups. Ethical Committee Clearance was obtained from the Institutional Animal Ethics Committee (IAEC).

2.3 Sub-chronic toxicity study:

Rats were randomized into two groups each containing three males and three females respectively. The amount of extract supplemented to the rats was taken in accordance to its weight. As per OECD guidelines, 2000mg of extract/kg of body weight may be used as supplement to the experimental group. Single dose was given throughout the study period. Except for the treatment with the test substance, animals in the control group were handled in an identical manner to those in the test group. Control group was fed with standard diet and water ad libitium. The body weight of each rat was assessed by using a weighing balance, once before commencement of dosing, first day, once weekly, during the period of dosing and on the day of sacrifice. During the 90 day period, all the animals were observed daily for clinical signs like mortality pattern, abnormalities or distress once before dosing, immediately after dosing and up to four hours after dosing. Any additional findings were recorded as observed. The blood samples were collected by retro orbital plexus technique for both control and experimental group just before sacrificing. Blood samples for clinical chemistry tests were collected in plain tubes without any anticoagulant, whereas blood samples for haematological tests and heavy metal analysis were collected in heparinized tubes.

2.3.1. Haematological parameters

Hemoglobin, RBC count, WBC count, Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) were assessed by standard procedures [6].

2.3.2. Clinical Chemistry

Glucose - Hexokinase method, Total protein-Biuret method, Albumin-BCG Dye binding method, Calcium -Arenazo III method, Inorganic phosphorus-Phosphomolybdate method, Electrolytes- Sodium, Potassium, Chloride - ISE method, Serum cholesterol -CHOD PAP method [7].

2.3.3. Liver function tests

ALT (SGOT) - Modified IFCC/UV kinetic method, AST (SGPT) - modified IFCC/UV kinetic method, Alkaline phosphatase -PNPP-AMP kinetic method [7].

2.3.4. Renal function test

Blood urea nitrogen - Urease GLDH/UV kinetic method, Creatnine - Jaffe/kinetic method [7].

2.3.5. Histopathology study

On the 90 day of dosing period, all the animals were euthanized and sacrificed. Vital organs like liver, kidney, spleen, heart and brain were carefully dissected out and weighed immediately without drying. Then the organs were preserved in 10 per cent formalin. Full histopathology was carried out on the preserved organs and histological changes were observed and recorded which was compared with the corresponding controls.

2.6. Assessment of heavy metals in plasma of rats

At the end of the experimental study period the blood samples were collected in heparanized tubes. Then it was centrifuged (1000× g) at 40c for 30 minutes to separate RBC′s from plasma and stored at -800C until heavy metal analysis was done by Atomic Absorption Spectroscopy [8].

2.7. Assessment of heavy metals in organ samples

Liver, kidney and brain samples from each animal was separated immediately, rinsed in saline and weighed. All the samples were kept in dry ice and stored until heavy metal analysis was carried out by AAS. Each organ sample was digested in 2 ml of an acid mixture containing 5:1:1 concentrated nitric, sulfuric, and perchloric acids, respectively. The tissue samples were digested cooled and the digested samples were diluted to 10 ml, as per the procedure Makos et al [9].

2.8. Statistical analysis

The data thus obtained were processed and the results were interpreted. The haematological and biochemical parameters of the control and experimental group were subjected to student's 't' test to determine the significant difference between the groups using sigma stat software. Data were accepted as statistically significant at $P<0.05$.

3. Results

3.1. Effect of seaweed extract on body weight of rats

The initial body weights of the albino rats ranged from 130- 140g and the mean weights were similar among the two groups of albino rats. At the end of the study period, it was noticed that the weight gain was higher in experimental group. The mean weight gain in the control group was 243.77g and experimental group it was 293.19g. *K.alvarezii* supplemented rats showed higher weight gain which may be due to the higher protein content and enhanced protein digestibility and biological value and further studies need to be explored. The body weight values of rats on the 90th day that was fed *K.alvarezii* are presented in Table 1.

3.2. Effect of seaweed extract on haematological parameters

The haematological values of rats on the 90th day that was fed *Kappaphycus alvarezii* were presented in Table 2. It was observed that there was no significant difference in haematological parameters like Red Blood Corpuscles (RBC), White Blood Corpuscles(WBC), Packed Cell Volume(PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin(MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) values between the *Kappaphycus alvarezii* treated groups and the control group. However there was a significant difference between the control and treated group.

3.3 Effect of seaweed extract on biochemical parameters

Table 3 reveals the effect of seaweed extract like *K.alvarezii* on different biochemical parameters in rats. It is clear that most of the serum biochemistry values of rats were similar among both the groups. There was no significant difference in the serum levels of glucose, cholesterol, total protein, albumin, SGOT, SGPT, sodium, calcium and phosphorus.

3.4. Effect of seaweed extract on organ weight of rats

The mean weight of all the vital organs like brain, kidney, liver, spleen, heart were similar among both control and experimental groups. There was no significant difference in the organ weight in both the groups. The mean weights of other organs were found to be similar (Table 4).

3.5. Histopathology examination and heavy metal toxicity

Histopathological examinations of all the vital organs like liver, kidney, spleen, heart and brain were noted. The heavy metal contents like mercury, cadmium, lead and arsenic levels in plasma are shown in table 5.

It was revealed that in brain, mercury content was not detected in both the groups. In the experimental group, mercury was not detected in kidney and brain and 0.012ppm was detected in liver, which is shown in Table. 6.

Table-1

BODY WEIGHT OF RATS OF THE TWO GROUPS ON 90TH DAY

Groups	Control group	Experimental group
1st week	137.46±5.95	139.76±4.79
2nd week	136.21±6.27	148.57±4.12
3rd week	139.82±5.66	160.2±4.92
4th week	142.5±5.17	172.4±4.13
5th week	148.5±4.12	195.64±16.1
6th week	151.5±2.30	204.92±13.24
7th Week	169.3±10.20	221.11±9.11
8th week	180.4±15.04	245.7±4.96
9thweek	199.0±15.04	260.35±5.33
10th week	218.70±14.61	272.05±4.64
11th week	232±13.09	280.92±7.05
12th week	243.33±13.76	293.19±7.51

Values are mean ±SD of n = 10 animals in each group.

Table 2

HAEMATOLOGICAL VALUES OF RATS WITH TREATED GROUP ON 90TH DAY

Parameters	Controlgroup	Experimental group	Treatment effect
Haemoglobin(g/dl)	16.34±0.34	16.92±0.35	*
RBC(×106cells/mm^3)	8.67±0.32	8.34±0.73	NS
WBC(×10^{3}cells/mm^3)	8.12±0.34	8.21±0.89	NS
PCV(%)	45.4±0.12	44.32±0.77	NS
MCV (cumm)	52.36±0.008	54.23±0.67	NS
MCH (pg)	23.78±0.89	23.98±0.98	NS
MCHC(%)	42.78±0.12	42.89±0.23	NS

*$P<0.05$, **$P<0.01$ as compared to control group, by students 't' test and Values are mean ±SD of n = 10 animals in each group.

NS- Not significant

Table 3

BIOCHEMICAL VALUES OF RATS OF THE TWO GROUPS ON 90TH DAY

Parameters	Control group	Experimental group	Treatment effect
Glucose (mg/dl)	109.74±0.81	87.31±3.55	NS
Cholesterol (mg/dl)	60.408±9.3	61.67±1.80	NS
Blood urea nitrogen(mg/dl)	15.86±2.76	16.77±0.72	NS
Total protein (g/dl)	6.08±1.56	7.60±0.90	NS
Albumin (g/dl)	4.32±0.34	4.21±0.12	NS
SGOT(AST) (U/L)	84.79±4.59	89.61±1.06	NS
SGPT(ALT) (U/L)	39.03±5.60	43.83±5.33	NS
Sodium (mEq/L)	145.35±2.47	141.13±1.51	NS
Potassium (mEq/L)	6.39±0.43	7.12±0.80	**
Calcium (mg/dl)	10.92±0.64	11.28±1.17	NS
Phosphorus (mg/dl)	7.20±0.45	7.34±0.23	NS
Alkaline Phosphatase (U/L)	120.72±17.0	101.29±0.66	*

*P<0.05, **P<0.01 as compared to control group, by students 't' test and Values are mean ±SD of n = 10 animals in each group.

NS– Not significant

Table 4

MEAN WEIGHT OF VITAL ORGANS OF THE TWO GROUPS

Organs	Control group	Experimental group	Treatment effect
Liver	3.34±0.07	3.91±0.003	NS
Spleen	0.34±0.003	0.37±0.007	NS
Heart	0.4±0.160	0.37±0.012	NS
Kidney	1.06±0.140	1.14±0.012	NS
Brain	0.48±0.010	0.59±0.003	NS

*P<0.05, **P<0.01 as compared to control group, by students 't' test and Values are mean ±SD of n = 10 animals in each group.

NS– Not significant

Table 5

Heavy Metals in Plasma of Two Groups of Rats (ppm)

Heavy Metals (ppm)	Control group	Experimental group
Cadmium	0.027±0.12	0.029±0.47
Arsenic	0.0036±0.10	0.057±0.18
Lead	nd	0.073±0.24
Mercury	nd	nd

Values are mean ±SD of n = 6 animals in each group.

nd– Not detected

Table 6

Heavy Metals in Organs of Two Groups of Rats (ppm)

Organs	Control group				Experimental group			
	Hg	As	Cd	Pb	Hg	As	Cd	Pb
Kidney	nd	0.07±0.02	0.31±0.02	nd	nd	0.42±0.06	0.46±0.04	0.86±0.12
Liver	nd	0.012±0.02	0.44±0.04	0.038±0.02	0.012±0.16	0.012±0.05	0.38±0.04	0.32±0.08
Brain	nd	0.02±0.04	0.028±0.02	nd	nd	nd	0.18±0.05	nd

Values are mean ±SD of n = 6 animals in each group.

4. Discussion

K.alvarezii supplemented rats showed higher weight gain which may be due to the higher protein content and enhanced protein digestibility and biological value and further studies need to be explored on these lines [10].

During the 90 days treatment period with the selected seaweeds, no mortality was observed among the rats. Initially, the experimental group showed hyperactivity and sneezing and no adverse clinical manifestations like diarrhoea, haemeturia and diurea was observed in the experimental group rats during the dosage period. There was no changes in the nature of stool, urine and eye ball colour of all the animals, Hence all the rats in the experimental group tolerated the dose level of seaweed.

It was observed that there was a significant difference in the alkaline phosphatase level in control group and also significant increase in the potassium content of the seaweed treated groups. All seaweeds offer an extraordinary level of potassium that is very similar to our natural plasma level [11].

However, the histopathological observation of experimental group rats showed no obvious abnormalities and the results are similar to the control group. According to Banu and Mageswari [12] the red seaweed Acanthophora spicifera were safe at dosing levels 2000mg/kg body weight, which was exactly correlated to the present study as the similar tolerable limit for the red seaweed *Kappaphycus alvarezii*. The kidney of experimental group rats showed undamaged renal glomeruli and tubules. Thus Kappaphycus did not reveal any toxicological lesions and proved to be non toxic and compared to the observations seen in the control group. It is clear that experimental group had high amounts of heavy metals when compared to the control group. This may be due to the accumulation of metals from the seaweeds. In general, the plasma heavy metals concentration was found to be low in the range of 0.003 to 1.003ppm in experimental groups of rats.

In general, the heavy metal contents were found to be low and the contribution of these heavy metals from seaweeds, when compared to the permissible tolerable weekly intake is negligible as suggested by WHO [13].

The results indicated that seaweeds originate from marine source; they tend to accumulate heavy metals which may cause toxicity. Hence, sub-chronic toxicity of the extract *Kappaphycus alvarezii* was done in albino rats for a period of 90 days. The haemotalogical and biochemical picture did not show significant changes in comparison to the control group. *Kappaphycus alvarezii* did not show any abnormalities. Heavy metal toxicity studies showed that in both blood samples and organs of the rats supplemented

with seaweed extracts, the heavy metal contents were found to be within the tolerant value prescribed as safe levels. Likewise, results from haematological and biochemical levels were in the standard range for rats. The normal levels of BUN, creatinine AST and ALT indicated that *K.alvarezii* doesn't altered kidney or liver function. Hence *K.alvarezii* can be used safely by humans. Further in-depth studies on nutraceutical properties and incorporation of seaweeds in common dietaries are recommended. Nutrition education to the local population in coastal areas of South India could be used for consumption and for enhancing monitory benefits through marketing of K. alverzii in fresh and dried forms. Continued use of this seaweed in the dietaries would serve as a sustainable strategy to combat macro and micronutrient deficiencies in India thereby helping in ecological balance.

Conflict of interest statement

We declare that we have no conflict of interest.

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[13]WHO Tolerable Weekly Intakes for other inorganic elements: CD (cadmium) 7 μg/kg adult weight; Hg (mercury) 5μg/kg adult weight; Pb (lead) 50 μg/kg adult http://www.seaveg.com/shop/index.php?main_page=page&id=21&chapter=1