

Evaluation on Semi-chronic Toxicity of Carrageenan extracting from *Kappaphycus alvarezii* Grown in Vietnam



Bui Huy Chich¹, Vu Ngoc Boi^{2,*}, Do Van Ninh³, Nguyen Xuan Hoan⁴, Nguyen Thi My Trang², Duong Hong Quan⁴, Dinh Huu Dong⁴, Nguyen Thi Phuong⁴, Hoang Thai Ha⁴, Pham Van Thinh⁴, Dang Xuan Cuong⁵

Department of Science and Technology, Vung Tau, Vietnam¹

Nha Trang University, Khanh Hoa, Vietnam²

Pacific Oceanography University, Khanh Hoa, Vietnam³

Ho Chi Minh University of Food Industry, Ho Chi Minh, Vietnam⁴

Nha Trang Institute of Technology Application and Research, VAST, Nha Trang, Vietnam⁵

Abstract— *Kappaphycus alvarezii* is commonly cultured in Vietnam and mainly contains carrageenan that is useful in food, beverages, functional food, and pharmaceuticals. The study focused on the evaluation for the semi-chronic toxicity of carrageenan on guinea pig (*Cavia porcellus*) for 42 days, inside, 21 first days (experiment), and 21 next days (recovery). Carrageenan extracting from *Kappaphycus alvarezii* by the enzyme-assisted method and the purification of carrageenan by using 96 % ethanol. Mice were observed on clinical, weighed, evaluated on the chemical composition of urine and blood, and sliced cells (liver, kidney, and spleen). The results showed that carrageenan extracting by the enzyme-assisted method and purifying by 96 % ethanol was non-toxicity. Carrageenan did not impact the liver, kidney, and spleen of experimental mice, exhibited on the microscopy figure. The fresh weight of the liver, kidney, and spleen increased according to adulthood time, suitable for the weighing increase of mice. The chemical composition of urine and blood was a non-significant difference between mice groups drinking carrageenan and the control group, except for mononuclear leukocyte (M) (MPV) ($p < 0.05$).

Keywords— Red algae; carrageenan; enzyme; *Kappaphycus alvarezii*; semi-chronic toxicity

1. Introduction

Red algae species *Kappaphycus alvarezii* is cultured and naturally developing in the central coast of Vietnam. This species mainly contain carrageenan using for food and beverages, for example, jelly-jelly, juice, candy, and cheese. [1]

Nowadays, carrageenan is known as a safety additive using popularly in the food processing industry. The consumption yield of carrageenan is estimated per all the world and supported mainly from aquaculture. Nearly, carrageenan exhibited a potential of application into the pharmaceutical with the adjuvant role and the treatment of stomach ulcers in functional food because of inertness to the human body. [2, 3] Carrageenan is also known as anti-cancer, immunomodulation, and anticoagulant activity. [4] Unlike when used as a food additive, in the pharmaceutical and functional food industries, the requirements for purity and content of active ingredients are more demanding. [5] Nowadays, the enzyme-assisted extraction of carrageenan orient towards the environment-friendly and possible to create high purity carrageenan products that meet the quality requirements when applying this product in the functional food and pharmaceutical. [6]

The structure characteristics, [7] some methods of extraction and purification, [8] and acute toxicity for carrageenan from *Kappaphycus alvarezii* grown in Vietnam were found, [9] except for semi-chronic toxicity.

According to international and Vietnamese regulations, researching and developing a new pharmaceutical product such as functional food or pharmaceutical for human under any form is required evaluating the toxicity and activity on animals. According to international and Vietnamese regulations, researching and developing a new pharmaceutical product such as functional food or pharmaceutical for human under any form is required evaluating the toxicity and activity on animals. Results of research on toxicity and product activity on laboratory animals, also known as preclinical studies, are a necessary component of the product profile (IB) used to register with the authority.

Therefore, the paper focused on the semi-chronic toxicity evaluation of carrageenan extracting from *Kappaphycus alvarezii* grown in Vietnam based on the application into functional food and pharmaceutical. In the current study, carrageenan was isolated by using the enzyme-assisted method of extraction and purified by ethanol.

2. Materials and methods

2.1. Materials

Red algae species *Kappaphycus alvarezii* was collected in Ninh Thuan coast, Vietnam, cleaned, dried under the infrared-freeze drying, and stored in the nylon bag at the lower temperature of 15°C for the further studies.

2.2. Sample preparation

2.2.1. Extraction of carrageenan from *Kappaphycus alvarezii*

K. alvarezii maceration was at 42 °C for 60 minutes in the buffer-enzyme (1.45 %) solution (pH 5.1) according to the solution and algae ratio of 20/1 (v/w). The residue was then soaked in aqueous with the aqueous to residue ratio of 50/1 (v/w) for 80 minutes at 90 °C for selecting the supernatant. Using 80 % ethanol for precipitating carrageenan that dried by using the freeze-drying method for the further studies. [9]

2.2.2. Carrageenan purification using ethanol Removing protein and impurities in carrageenan was by the solution preparation composed of 5 % of carrageenan and 25 % ethanol at 70 °C for 15 minutes and then the centrifugation at 10.000 rpm for 15 minutes. The supernatant was mixed with 60 % ethanol for 40 minutes and filtered for selecting carrageenan. Carrageenan was continuously cleaned twice in 96 % ethanol and dried at 45±2 °C by the freeze drying method with the velocity ratio of 2 m/s for selecting purifying carrageenan powder. [9]

2.3. Experimental design

Semi-chronic toxicity assay of carrageenan extracting from *Kappaphycus alvarezii* in Vietnam was on guinea pig (*Cavia porcellus*) via the oral using water for injection for dissolving carrageenan with the control of carrageenan-non water for injection. Guinea pigs division were into three groups of the high, the medium, and the low dosage of carrageenan, corresponding to 1.5%, 1.0%, and 0.5% carrageenan in drinking solution, respectively. Each group of guinea pigs had continuously two groups (four mice for each group) for evaluating the chemical composition of urine and blood, weighing, and clinical observation on day 21st(Table 1) and day 42nd(Table 2). Mice number to the first male and late females were from 101 to

120, 201 to 220, 301 to 320, and 701 to 720 corresponding to a group A, B, C, and control (salt solution), respectively (Table 5).

The experiments animal were approved by the Vietnam Institute of Vaccines and Medical Biologicals (300-500 g bodyweight) and tested according to the Ethics Committee on Animal Experimentation of the Vietnam Institute of Vaccines and Medical Biologicals basing on the guidelines of the Vietnam Ministry of Health on Animal Experimentation. Mice cultivation was in the closed and controlled environment at the temperature (22-25 °C) with the relative humidity (53–67 %), and light-dark cycle (12 h x 12 h).

Table 1. Experiment design for evaluating semi-chronic toxicity of carrageenan

Study	Dosage/mouse	Mice/group	Drink days	Observation days	Anatomy and Testing	
					Days	Sample number
Main study	High dosage (1.5 % w/v)	4	20	21	21	2
	Average dosage (1.0 % w/v)	4	20	21	21	2
	Low dosage (0.5 % w/v)	4	20	21	21	2
	Placebo	4	20	21	21	2
Recovery study	High dosage (1.5 % w/v)	4	20	42	42	2
	Average dosage (1.0 % w/v)	4	20	42	42	2
	Low dosage (0.5 % w/v)	4	20	42	42	2
	Placebo	4	20	42	42	2
Total		56				28

Table 2. Evaluation standard semi-chronic toxicity of carrageenan on guinea pig

Criteria	Evaluation standard
Clinical manifestations	The observation and the record of the number of living rats and death, and the disease signs or any other clinical manifestations of rats
Weight increase	Weighing the rats once times per week. Comparing and finding the difference in the weight between the mice group
Urine analysis criteria	pH, Density (SG), Protein (PRO), Glucose (GLU), Red Blood Cell (BLO), White Blood Cell (LEU), Bilirubin (BIL), Urobilinogen

	(URO), Nitric (NIT), Keton (KET)
Blood analysis criteria	WBC [$\times 10^9/L$]: Number of WBCs; N [%]: neutrophils; L [%]: lymphocytes; E [%]: leukocytes; B [%]: white blood cells; M [%]: mononucleosis; RCB [$\times 10^{12}/L$]: Number of red blood cells; Hb [g/dL]: Hemoglobin; HCT (Hematocrit) [%]: Ratio of red blood cells to the volume of whole blood; MCV [fL]: Mean volume of erythrocytes; MCH [pg]: Average amount of hemoglobin in 1 red blood cell; MCHC [g/dL]: Mean hemoglobin concentration in 1 red blood cell; PLT [$\times 10^9/L$]: Number of platelets; MPV [fL]: mean platelet volume;
Blood biochemistry	ALAT (Alanine aminotransferase) (SGPT [U/L]) ASAT(Aspartate aminotransferase) (SGOT [U/L])
Anatomy	- Observe and describe the general and detail of the macroscopic organs (colour, shape) of the liver, kidney, and spleen organs of the mouse. - Determine the fresh weight of the liver, spleen, and kidney (2 sides).
Histological examination results	Histological examination (microscopic) of organs: liver, kidney, and stomach

2.4. Data analysis

All the experiments were analyzed and in triplication. The results was statistically analysed using Microsoft Excel 2010 and expressed as mean values \pm standard deviation (SD). Differences were analysed on ANOVA with significant level ($p \leq 0.05$).

3. Results and discussion

3.1. Clinical observations

Clinical observations showed that all experimental rats were alive, healthy, eating well and gaining weight after 21 days and 42 days of the experiment. The mouse responded well to external effects, for example, fluffy hair, dry eyes, nose, mouth, and anus; did not find in the following abnormalities: Struggling / stimulating / walking on tiptoes; Lethargy, poor reflexes with the outside; Body sweating; Ruffled feathers; Shivering; Skinny; Not eat; Difficulty breathing or wheezing; Exudate (for example, watery eyes, runny nose or saliva); Red eyes are often flustered; Bloating, vomiting (vomiting) or diarrhoea.

3.2. Mice weighing

The rats increased their weight continuously for experimental weeks and the difference in the rats weight between the control group and the carrageenan-drinking group was not significant ($p > 0.05$). The difference was similar to the group drinking the other dosages ($p > 0.05$) (Table 3).

Table 3. Mice weighing drinking carrageenan and non-carrageenan (control)

Group	Mice weighing (g)						
	1 st week	2 nd week	3 rd week	4 th week	5 th week	6 th week	7 th week
High dosage	362.5 \pm 15.811	382.5 \pm 15.119	419.75 \pm 19.869	455.625 \pm 18.792	485 \pm 23.805	502.5 \pm 15	523.75 \pm 17.017

Average dosage	370 ± 19.272	387.5 ± 19.64	418.75 ± 20.659	459.375 ± 23.366	468.75 ± 8.539	495 ± 22.73	500 ± 28.284
Low dosage	348.75 ± 12.464	366.875 ± 15.57	400 ± 15.811	433.125 ± 14.377	453.75 ± 18.875	471.25 ± 24.622	480 ± 27.08
Control	350.625 ± 10.155	391.25 ± 14.577	426.25 ± 15.98	465.625 ± 20.948	490 ± 16.833	502.5 ± 9.574	525 ± 12.91

3.3. Urine composition

Urine analysis were performed in all groups on day 21 and day 42 (Table 4).

Table 4. Biochemistry indicators in urine of guinea pigs.

Group	Code	Results									
		LEU	NIT	URO	PRO	pH	BLO	SG	KET	BIL	GLU
Carra (21 days)	101	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	102	20	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	103	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	104	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
Placebo (21 days)	701	20	(+)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	702	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	703	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	704	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
Carra (42 days)	105	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	106	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	107	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	108	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
Placebo (42 days)	705	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	706	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	707	15	(-)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)
	708	15	(+)	0.2 (3.5)	30 (0.3)	9	(-)	1000	(-)	4 (70)	(-)

* abbreviation words see in table 2

Indexes of red blood cells, nitrite, ketin, and urinary tract were mostly negative, except for one to two rats had mild positive (+), but non-significance on the clinical. The urobilin, protein, pH, density, and bilirubin indices were the same in all rats. The leukocyte count ranged slightly from negative to 20, evenly distributed in all groups. Urine test results showed a non-significant difference in the biochemical indices of rats taking carrageenan or distilled water, and no difference between high, medium or low doses (Table 3).

3.4. Blood haematology and biochemistry

Table 5. The indicators of the haematology - blood biochemistry in guinea pigs after 21 and 42 days.

Code	WBC	N	L	M	E	B	RBC	Hb	Hc	MCV	MCH	MCH _C	PLT	MPV	SGP _T	SG _{OT}
	[×10 ⁹ /L]	[%]	[%]	[%]	[%]	[%]	[×10 ¹² /L]	[g/dL]	[%]	[fL]	[pG]	[g/D]	[×10 ⁹]	[fL]	[U/L]	[U/L]
The indicators of the haematology - blood biochemistry in guinea pigs on day 21 st																
101	6.2	32	61	6	1	1	5.53	13	39.1	70.7	23.9	33.8	484	7.1	50.7	39.7

102	5.68	39	51	7	1	2	5.52	14	40.2	72.8	24.6	33.8	498	7.7	98.9	39.3
103	2.06	43	45	5	6	1	4.37	12	35.4	81	27.5	33.9	412	6.9	238	38.1
104	4.42	31	59	5	5	0	4.83	13	38.1	78.9	26.3	33.3	416	7.3	48.1	30.9
201	8.26	44	49	4	3	0	5.39	14	39.4	73.1	25.8	35.3	451	7	78	47.9
202	5.11	28	65	5	1	1	5.4	15	43.1	79.8	27.4	34.3	396	6.7	54	41
203	6.07	53	40	5	1	1	5.03	13	37.3	74.2	25.4	34.3	514	7.3	62.6	42.3
204	2.95	32	58	4	5	1	5.33	14	41.9	76.7	25.7	33.4	478	6.6	53.1	40.6
301	6.89	38	55	5	2	1	5.34	14	39.5	74.7	25.7	34.7	471	6.9	67.2	48.2
302	4.79	41	46	3	9	0	5.33	13	37.6	70.5	24.2	34.3	407	7.1	76.8	40.8
303	4.54	33	55	8	3	1	4.66	12	34.6	74.2	24.7	33.2	517	7.4	154	50.6
304	Blood clotting does not take a haematology test.													226	47.8	
701	4.46	45.3	46.9	5.81	1.8	0.2	5.26	13.4	39.4	74.9	25.5	34.0	544	6.5	53.0	43.0
702	5.08	44.7	49.6	4.11	1.2	0.4	4.77	12.2	36.1	75.7	25.6	33.8	491	6.2	42.9	36.5
703	3.36	41.3	50.7	5.02	2	0.8	4.82	12.5	36.6	75.9	25.9	34.2	424	6.4	152.2	42.9
704	2.98	24.5	69.5	5.40	3	0.3	4.61	12.5	36.1	78.3	27.1	34.6	370	6.7	91.0	57.4
p	0.51	0.82	0.999	0.29	0.40	0.63	0.33	0.17	0.29	0.39	0.53	0.37	0.99	0.03	0.77	0.08

The indicators of the haematology - blood biochemistry in guinea pigs onday 42nd

105	5.68	39	51	7	1	2	5.52	14	40.2	72.8	24.6	33.8	498	7.7	98.9	39.3
106	5.37	34.7	57.2	5.2	2.2	0.7	4.80	12.7	36.30	75.6	26.50	35.0	440	6.90	52.0	48.7
107	5.91	19.7	68.2	4.9	5.2	2.0	5.05	13.5	39.70	78.6	26.70	34.0	465	7.10	48.5	30.3
108	6.18	24.5	70.6	2.3	2.4	0.2	4.95	13.4	38.80	78.4	27.10	34.5	504	6.80	59.7	29.7
205	6.07	53	40	5	1	1	5.03	13	37.3	74.2	25.4	34.3	514	7.3	62.6	42.3
206	4.50	31.6	60.0	6.0	2.2	0.2	5.37	13.6	39.50	73.6	25.30	34.4	412	7.00	95.2	70.8
207	9.04	35.4	54.6	6.7	2.7	0.6	5.78	14.9	43.70	75.6	25.80	34.1	400	6.80	97.9	57.6
208	3.71	34.5	51.2	5.7	7.8	0.8	5.13	13.1	36.90	71.9	25.50	35.5	498	7.40	69.5	45.3
305	6.01	35.4	52.2	7.8	3.8	0.8	5.58	14.0	40.30	72.2	25.10	34.7	384	7.50	107.9	56.5
306	5.58	35.9	48.7	7.5	6.5	1.4	5.63	14.4	42.20	75.0	25.60	34.1	445	6.70	59.3	39.8

30 7	9.04	8.3	80.4	6.4	1.8	3.1	4.88	13.3	38.3	78.4	27.3	34.7	334	7.00	63.0	35. 8
30 8	2.27	38.7	48.5	8.8	3.1	0.9	5.03	13.3	38.7	76.9	26.4	34.4	495	7.00	77.3	33. 8
70 5	8.42	23.3	68.9	5.6	1.6	0.5	5.1	12.4	40.2	75.7	26.5	35.6	310	7.1	85.6	39. 7
70 6	3.33	34.9	57.6	4.0	2.4	1.5	5.2	14.5	41.3	77.7	27.4	34.9	453	7.0	44.5	36. 7
70 7	5.4	39.1	45.5	5.2	2.7	0.7	4.8	12.4	36.5	76.1	26.3	32.3	470	7.4	121	44. 4
70 8	6.53	49.2	43.4	6.5	3.2	1.2	4.5	13.3	34.6	83.2	27.4	30.4	362	7.3	62.6	36. 2
p	0.284	0.22 8	0.35 0	0.01 5	0.09 9	0.12 3	0.10 2	0.14	0.209	0.17 7	0.236	0.52 0	0.658	0.05 0	0.24 7	0.1 24

* abbreviation words see in table 2

Almost indexes of rats in other group were non-significant difference after 21 days, except for the platelet volume index ($p > 0.05$). Mononuclear leukocyte (M) (MPV) was different between other rat groups ($p < 0.05$), and other indexes of rats were not similar to the thing (Table 5).

3.5. Pathology

No abnormal signs of shape, colour, and properties of liver, spleen, kidney, intestine organs; stomach, bladder, genitals and no lymph nodes, lumps, signs of haemorrhage, or abnormal fluid stagnation in the abdominal or thoracic cavities were detected. Microscopic observation of liver, spleen and kidney organs (both sides) showed no abnormality and no difference, compared to the control group (Figure 1).

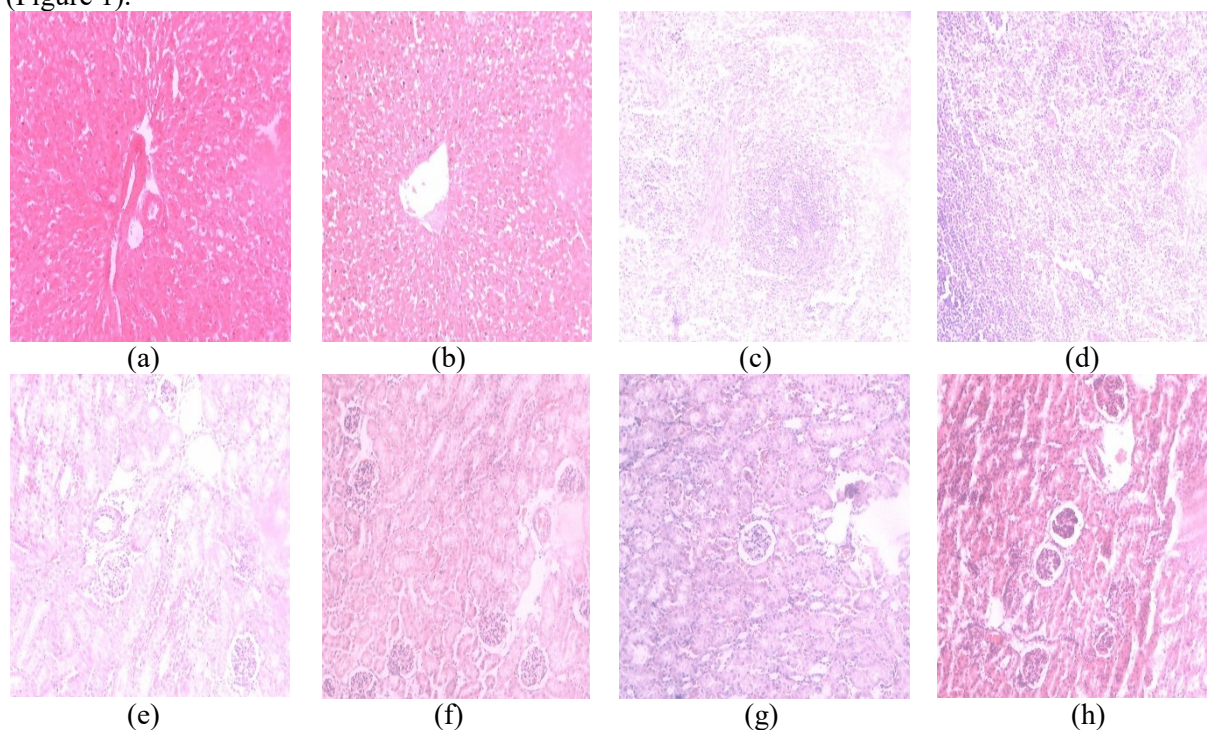


Figure 1. Microscopic observation on liver, spleen and kidney organs of mice. (a) Liver of mice using Carragennan; (b) Liver of mice using placebo; (c) Spleen of mice using Carragennan; (d) Spleen of mice using placebo; (e) Kidney of mice using Carragennan; (f) Kidney of mice using placebo; (g) Kidney of mice using Carragennan; (h) Kidney of mice using placebo

Table 6. Average fresh weight of liver, spleen, and kidney organs (both sides) of the first surgery

Average fresh weight of liver, spleen, and kidney organs (both sides) of the first surgery			
Liver	Spleen	Left Kidney	Right Kidney
On day 21st			
17.688 ± 2.117	0.499 ± 0.119	1.469 ± 0.119	1.499 ± 0.189
On day 42nd			
20.144 ± 2.117	0.697 ± 0.119	1.586 ± 0.119	1.592 ± 0.189

Carrageenan is a sulfated polysaccharide deriving from red algae and useful in conventional medicine, for example, a decrease of pain and swelling, and used in the acidic form as a bulk laxative and the treatment for peptic ulcers. Carrageenan also has a role as a thickening and firming agent in healthcare products, for example, toothpaste, and fermented milk products. [10] However, some side effects of carrageenan were noticed as using them in intravenous, for example, liver cancer, ulcerative colitis, fetal toxicity, and immune suppression. [11] Human bodies are not absorbent or metabolite carrageenan, so they are excreted in the feces, similar to other fibers, and rarely cause acute toxicity that depended on the molecular weight of carrageenan. Carrageenan helped the decrease of abnormality in pancreatic tissues, but not impact the pancreatic tissue and the amylase level in the blood. [12]

The toxicity of carrageenan depends on numerous different factors, for example, algae species, the structure characteristics, and the content of carrageenan. In the current study, the results showed that carrageenan is non-toxicity on mice, because the difference in the clinical observation, weighing, urine composition, blood hematology and biochemistry, and pathology occurred between different mice groups. Carrageenan extracting from red algae species *Kappaphycus alverazii* grown in Vietnam fully applies well into functional food and pharmaceutical. The enzyme-assisted method of carrageenan extraction and the purification of carrageenan by using ethanol is environmentally friendly, suitable to Chich et al. The results will contribute to open the research and application direction on carrageenan extracting from *Kappaphycus alverazii* grown in Vietnam.

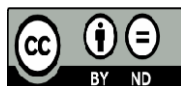
3. Conclusion

Carrageenan of red algae species *Kappaphycus alverazii* commonly grown in Vietnam is non-toxicity on experimental mice. The clinical observation on mice did not detect any abnormalities. The mice weighing between the mice group drinking carrageenan and the placebo group was non-significantly different. There was also no abnormality in the blood biochemical composition of mice at the assay times. The chemical composition in the urine of the mice did not change after culture. The fresh weighing of spleen, liver, and kidney increased according to adulthood time and the non-significant difference between other mice groups. It was not finding any abnormalities in the cell of the spleen, liver, and kidneys as an observation by microscopic. Carrageenan extracting from seaweed grown in Vietnam can be useful in functional foods and pharmaceuticals.

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