Philippine Journal of Science
129 (2): 85-91, December 2000
ISSN 0031-7683

Anti-Blood Coagulant Activity and Hypocholesterolemic Property of Philippine Carrageenan

A.V. Briones*, W.O. Ambal, R.R. Estrella, E.A. Lanto
F. Sison and M.A. Villanueva

Industrial Technology Development Institute
DOST Compd.,General Santos Avenue, Bicutan
Taguig, Metro Manila, Philippines

The anti-coagulant and hypocholesterolemic properties of Philippine carrageenan were studied. *Kappa* and *iota* type carrageenan were recovered from *Eucheuma* species while *lambda* type carrageenan was extracted from *Halymenia durvillaei,* Bory de Sainte Vincent. The three types of carrageenan used in this experiment conformed with the specifications set by USP XXII (1990). The acute oral toxicity test (LD_{50}) for *kappa* and *iota* carrageenan is 10.6610 ± 0.1514 g/kg. The *lambda* carrageenan administered orally to male Swiss mice, at a high dose of 15 g/kg, did not cause death in the test animals. *Lambda* carrageenan effected significant higher anti-blood coagulant activity (*in vitro*) than *kappa* and *iota* type.

The influence of the administration of carrageenan by different modes in rats were determined against the coagulation time of blood. By intravenous route, its effect was instantaneous while intra-peritoneal route recorded a time of 4.339 minutes. Subcutaneous administration recorded a time of 2.73 minutes.

No traces of deactivated carrageenan were detected in the blood of rats 30 minutes after injection.

A 3.0% concentration of iota carrageenan added to the specially prepared diet showed an 11.68% decrease in cholesterol level in rats after feeding them for 4 to 6 weeks. *Lambda* carrageenan elicited 1.95% decrease in cholesterol level after 2 to 6 weeks feeding. An increase in weight by 14.73% to 20.17% was observed in rats fed with the three different types of carrageenan.

Key words: *Anti-blood coagulant, hypocholesterolemic, kappa carrageenan, iota carrageenan, lambda carrageenan*

A great number of deaths today is attributed to vascular diseases of which the most prevalent form is atherosclerosic heart disease. Within recent years cholesterol has been believed to play an important role in the occurrence of atherosclerosis in man. Atherosclerosis may be defined as degenerative changes in the intima of medium and large arteries (Gennaro, 1990). The degeneration includes the accumulation of lipids, complex carbohydrates, blood and blood products and is accompanied by the formation of fibrous tissue and calcium deposition in the intima of the blood vessels. These deposits or "placques" decrease the lumen of the artery, reduce its elasticity, and may create foci for thrombic and subsequent occlusion of the blood vessel.

*Corresponding author: bel@pacific.net.ph

A lot of drugs were developed and studied to ascertain their efficacy in the long-term treatment of this disease (Gennaro, 1990). Yet, the results showed no evidence to indicate that any of these drugs could be recommended for long-term therapy. In 1969, Tsuchiya studied the hypocholesterolemic activities of marine algae. The findings confirmed the hypocholesterolemic property of carrageenan, agar and alginic acid of which carrageenan came out to be the excellent one. Among the algal hydrocolloids carrageenan by far has the widest application in the food, pharmaceutical, textile and cosmetic industries (Whistler 1973). Considering the fact that, in the well-fed part of the world, more people die from circulatory disturbance, than from any other disease group, these are good reasons for a substantial increase in pharmaceutical research on substances derived from seaweeds and for increase production of anti-atherosclerotic drugs. This reason alone, the lowering of blood cholesterol level by carrageenan is sufficient to warrant an increase in human consumption of seaweed.

Carrageenan is a high molecular weight sulfated galactan. This is obtained by aqueous or alkali extraction of some carrageenan-bearing red seaweeds. The term carrageenan comes from the name of the small coastal town *carrageenan*, in Ireland where commercial harvests of *Chondrus crispus* (Irish moss) were made in the late 19th century (Whistler 1973). The backbone of the carrageenan polymer consists of α-1,3 and β-1,4 linked D- galactoypranose units which vary in the degree and locations of sulphate esterification (Percival 1979 and Rees 1972). *Kappa* carrageenan consist mostly of alternating polymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose. *Iota* carrageenan is similar, except that 3,6-anhydrogalactose is sulfated at carbon 2. Between *kappa* carrageenan and *iota* carrageenan is a continuum of galactan with intermediate compositions differing in degree of sulfation at carbon 2. In *lambda*-carrageenan, the alternating monomeric units are mostly D-galactose-2-sulfate (1,3-linked) and D-galactose-2,6-disulfate (1,4-linked). The ester sulfate content of carrageenan ranges from 18% to 40%. In addition, it contains inorganic salts that originate from the seaweed and the process of recovery from the extract. Several types of carrageenan have been identified due to differences in their chemical structure (i.e., differences in altering sugar unit components) of which *kappa, lambda* and *iota* are the industrially important ones.

Another interesting application of polysaccharides from seaweeds is a discovery by Elsner, Broser and Bunger in 1937, which may yet prove to be important from the medical point of view. A water-soluble extract can be obtained from carrageenan which, even in very dilute concentration, acts as an anti-blood coagulant compound. Anti-coagulants are substances or drugs which delay blood coagulation (Gennaro 1990). They are of three general types namely : calcium sequestrating agent, heparin and heparin substitute; prothromboponic anticoagulant (Oral Anticoagulant). Agar may also have a use in this respect, Iridoglycan, the carbohydrate- sulphate esters from *Iridophycus flaccidum* (Chapman 1980) has a similar property. In solution the substance occurs principally as the sodium salt . The beautiful red seaweed *Delesseria sanguinea* also possesses a strong anti-coagulating action which is as good or even better than heparin. The effect of this extract can be stopped immediately by the injection of thionin. Attention has been directed in recent years to this preparation of synthetic anti-blood coagulant as substitute for heparin. The sulfated derivative of gum and locust bean gums, agar, laminarins, cellulose, starch, glycogen, dextran, polyvinyl alcohol, chondroitin sulfuric acid, xylan and synthetic glucose have been investigated. Some of these derivatives show activity *in-vivo* and though none approached the activity of heparin, certain sulfuric acid derivatives such as the sulfated 2-amine ethyl ether of laminarin showed about half the activity of heparin. Alginic acid sulfate is no more toxic than heparin and its effect lasts twice as long. Extracts of *Chondrus crispus* have about 40 per cent of the activity of heparin while fucoidin, a fucan sulfate is inactive (Smith 1959).

A study conducted by Dumelod, 1999 showed that *lambda* carrageenan incorporated into arroz caldo has a good hypoglycemic effect on humans. Results of the short trem *in vivo* study proved the importance of *lambda* carrageenan in the prevention and management of metabolic conditions such as diabetes in man.

The utilization of carrageenan as substitute for existing anticoagulants and antihyperlipidemic agents is an innovative technology that will help boost the industry since this is an indigenous material. It may also reduce the bulk of importation of heparin and other anti-coagulants including quite a number of antihyperlipidemic drugs.

Materials and Methods

Kappa and *iota* carrageenan were provided by Marcel Trading Corporation. *Lambda* type carrageenan was extracted from *Halymenia durvillaei* Bory De Sainte Vincent. All chemicals and reagents used were of analytical grade.

Analysis for the physico-chemical properties of carrageenan was based on the procedures found in USP XXII, 1990.

Heavy metals such as Hg, Cd and Pb were determined according to the Official Methods of Analysis for Heavy Metals, AOAC, 1990.

Acute Oral Toxicity (LD_{50}) Test

Kappa and *Iota* Samples:

Preliminary dosing was done to determine the expected dose that will cause 50% death of the experimental animals. Three increasing log doses of the test substance were given orally in serial to the animals in four groups of ten including the control. The number of death and other adverse/abnormal signs and manifestations were closely observed and noted for the first two hours after administration of test sample. This was continued in the next twenty-four to forty-eight hours, daily up to fourteen days. The median lethal dose (LD_{50}) was computed using the Probit Analysis Method by Fisher and Yates, 1952.

Lambda Sample:

Four preliminary increasing doses were done to determine the expected dose that will cause 50% death of the experimental animals. No death was observed even at a high dose of 15 g/kg given orally in serial parts/aliquot to the animals in two groups of ten including the control. The adverse/abnormal signs and manifestations were closely observed and noted for the first two hours after dosing. This was continued in the next 24-48 hours and daily up to fourteen days.

Anti-blood Coagulation Tests

Preparation of Sample:

About 25 mg of carrageenan was dissolved in sufficient saline TS to give a concentration of 1 mg per ml.

Preparation of Plasma:

Porcine blood samples were collected directly into a vessel containing 8% sodium citrate solution in the proportion of one volume to each 19 volumes of blood. The mixture was mixed immediately by gentle agitation and inversion of the vessel. The blood was centrifuged and the separated plasma was collected and pooled.

Anticoagulant Activity:

One milliliter of the carrageenan solution was added to 1 ml of porcine plasma and the coagulation time was measured. The method is based on the studies conducted by Kimura, 1941 on the anti-blood coagulation test for *Laminaria japonica* extract.

Hypocholesterolemic Test

Preparation of Basic Diet:

The basic diet consisted of 63.26% of sucrose, 22% of casein, 5% of celluflour, 4% of a salt mixture, 0.24% of Choline - HCl, 0.5% of a vitamin mixture (casein, p-aminobenzoic acid, inositol, tocopherol, ascorbic acid, B1, Ca-pantothenate, niacin, B12, B6, A, D, folic acid, menadion, and biotin), and 5% of cotton seed oil.

Procedure:

Swiss Female Rats were used in the experiment. The animals were grouped into seven with 5 members per group. Group I was fed with the basic diet. Group II were fed with the prepared basic diet added with 1% cholesterol and bile salts. Group III - V were fed with the prepared basic diet added with 1% cholesterol and bile salts and mixed with 3% *kappa*, *iota* and *lambda* carrageenan. Group VI was fed with the prepared basic diet added with 0.034% Lipostat (commercial anti-hyperlipidaemic agent). Group VII was fed with commercial feeds as control group. Feeding was done for about 10 weeks.

Cholesterol Analysis:

Blood samples were collected from the rats every after 2 weeks and were submitted to the Department of Health for analysis of cholesterol. Standard methods for determination of cholesterol were used in the experiment.

Results and Discussion

Kappa, *iota* and *lambda* carrageenan were analyzed for their physicochemical properties. The results are shown in Table1. All samples conform with the specifications set by USP XXII, 1990.

Acute oral toxicity test LD_{50} was determined for all samples. The median lethal dose (LD_{50}) of the *kappa* and *iota* samples administered orally in male Swiss mice is 10.6610 $\pm$0.1514 g/kg. Toxidrome ranged from decreased motor activity, increased respiratory rate, hyperemia, passivity, loss of righting reflex and grip strength, tremors, convulsion and death of mice. Details of acute oral toxicity test is shown in Table 2. The sample, carrageenan (*lambda*), administered orally to male Swiss mice, at a high dose of 15 g/kg, did not cause death in the test animals. Further increase of dosing will require larger amount of sample to be administered to the test animals which will exceed the maximum limit a mouse can normally take. Since the determination of the lethal dose (LD_{50}) is dependent upon the number of death occurrence during the period of experiment, the results will be erroneous and doubtful, for the cause of death of animals will be attributed either due to bloating or to the toxic effect of the sample/test drug, Toxidrome ranged from decreased motor activity and respiratory rate, and excretion of sample after twenty-four hours. No death occurred within fourteen days observation. Details of acute oral toxicity test is shown in Table 3.

Table 1. Physico-chemical properties of carrageenan.

	Kappa Carrageenan	*Iota* Carrageenan	*Lambda* Carrageenan	Standard Values/USP XXII Specifications
Moisture (%)	11.7	11.4	11.0	<12.5
Acid-Insoluble Ash (%)	0.8	1.0	1.5	<2.0
Ash (%)	25.5	27	23	<35.0
Sulfate (%)	18.4	24.7	38.0	15 - 40
Viscosity, 1.5% solution (cp)	82	85	110	>5.0
Heavy metals (ppm)				
Hg	<0.10	<0.10	0.15	–
Pb	5	5.0	3.9	max. 10
Cd	0.3	0.7	1.5	–

Table 2. Acute Oral Toxicity Test (LD_{50}) of *Kappa* and *Iota* Carrageenan in mice.

$$\text{Mortality Ratio} = \frac{\text{Number of mice with positive sign (death)}}{\text{Total number of animals tested}}$$

Dose (g/kg)	n	Observation
0	0	No effect
10	10	Five (5) minutes after dosing, the mice manifested decreased motor activity, increased respiratory rate, hyperemia, tremors and convulsion. One (1) mouse died after dosing and another moused died after seventeen (17) minutes. The eight (8) remaining mice recovered after twenty four (24) hours.
11.0675	10	Immediately after the last dosing, the mice manifested decreased motor activity, increased respiratory rate, passivity (++), loss of grip strength (+) and righting reflex (+), hyperemia, tremors and convulsion. Two (2) mice died afte dosing, and three (3) mice died within three (3) hours. One (1) of the remaining five (5) mice died after twenty-four (24) hours. Four (4) of the remaining mice recovered after twenty-four (24) hours.
12.2490	10	Immediately after the last dosing, the mice manifested decreased motor activity, increased respiratory rate, hyperemia, loss of grip strength (++) ang righting reflex (++) passivity (++++), tremors and convulsion. Five (5) mice died immediately after the last dosing and one (1) mouse after three (3) hours. Four (4) of the remaining mice died after twenty-four (24) hours.

Autopsy findings: Animals which died twenty-four (24) hours and those sacrificed after fourteen (14) days had grossly normal findings.

Table 3. Results of acute oral toxicity test (LD_{50}) in mice for lambda carrageenan.

$$\text{Mortality Ratio} = \frac{\text{Number of mice with positive sign (death)}}{\text{Total number of animals tested}}$$

Group Number	Dose (g/kg)	n	Number of Deaths				
			Day 1	Day 2	Day 3	Day 7	Day 14
I	0	10	0/10	0/10	0/10	0/10	0/10
II	8	2	0/2	0/2	0/2	0/2	0/2
III	10	2	0/2	0/2	0/2	0/2	0/2
IV	12	2	0/2	0/2	0/2	0/2	0/2
V	15	10	0/10	0/10	0/10	0/10	0/10

Dose (g/kg)	n	Observation
0	0	No effect
8	2	Thirth (30) minutes after dosing, the mice manifested decreased motor activity and respiratory rate lasting for twenty-five (25) minutes. No other adverse/abnormal signs or death occurred within the fourteen (14) days observation.
10	2	
12	2	
15	10	Twenty nine (29) minutes after the last serial dosing, the mice manifested decreased motor activity and respiratory rate. Excretion of the test substance was observed after twenty-four (24) hours. All mice were normal within twenty-four (24) hours. No death occurred within fourteen (14) days observation.

Autopsy findings: Animals which died within twenty-four (24) hours and those sacrificed after fourteen (14) days had grossly normal findings.

Table 4. Effect of varying concentrations of carrageenan (*kappa, iota, lambda*) on anti-blood coagulation activity.

Concentration (%)	Coagulation Time (Mins.)					
	Kappa	Iota	Lambda	Control	Commercial Anti-Coagulant (Heparin), 1000 IU/ML	Concentration IU
0.1	177.5 ± 14.5	395 ± 11.18	605 ± 11.18	6 ± 0.71	1215 ± 11.18	100
0.5	232.5 ± 21.65	535 ± 11.18	7695 ± 12.27	9.5 ± 1.11	1500 ± 14.79	500
1	282.5 ± 9.68	720 ± 7.07	1440 ± 21.21	5.5 ± 0.5	1800 ± 14.79	1,000

Table 5. Effect of carrageenan on blood cholesterol level in rats.

	Feeding	Number in Experimental Group	Weight Gain (g/rat)				Whole Blood Cholesterol (mmol/liter)			
			0 Week	2nd Week	3rd Week	4th Week	0 Week	2nd Week	4th Week	6th Week
I.	Basic Diet	5	395.334	387.418	427.818	475.104	3.02 ± 1.02	2.52 ± 0.147	1.736 ± 0.21	1.982 ± 0.62
II	I + cholesterol (1%) and bile salts	5	385.384	310.38	388.28	446.315	1.96 ± 0.62	3.7 ± 0.6	5.58 ± 1.13	5.47 ± 1.38
III.	II + 3% κ-carrageenan	5	349.862	328.046	380.666	438.246	2.28 ± 0.36	3.8 ± 1.35	5.192 ± 1.54	5.776 ± 1.07
IV.	II + 3% ι-carrageenan	5	333.13	319.248	360.256	405.976	2.56 ± 0.25	3.66 ± 0.66	5.136 ± 0.56	4.536 ± 1.46
V.	II + 3% λ-carrageenan	5	346.174	332.22	366.716	405.966	1.70 ± 0.23	2.82 ± 0.12	2.804 ± 0.61	2.765 ± 0.74
VI.	II + 3% Liposat (commercial antihyperlipidaemic agent	5	371.81	324.975	364.585	427.58	2.34 ± 0.59	9.025 ± 0.64	4.995 ± 0.83	5.975 ± 0.85
VII.	Commercial feeds	5	342.796	344.375	346.302	362.132	1.70 ± 0.41	2.32 ± 0.73	2.886 ± 0.93	2.754 ± 1.19

The effect of varying concentrations of carrageenan (*kappa, iota, lambda*) on anti-blood coagulation activity is shown in Table 4. *Lambda* carrageenan extracted from *Halymenia durvillaei* showed significant activity than *kappa* and *iota* carrageenan. Probably this is due to the difference in chemical structure. *Kappa* type carrageenan has a D-galactose group containing 6-sulphate ester groups and some of the 3,6 - anhydro-D-galactose contains 2-sulphate ester groups. *Iota* carrageenan is characterized by having 4-sulphate ester groups on all D-galactose residues and 2-sulphate ester groups on all 3,6 - anhydro-D-galactose residues. *Lambda* carrageenan differs from *kappa* and *iota* carrageenan by having a disulphated β (1→4) - D- galactose residue and no 4-sulphate in the α (1→3) - D-

galactose residue. Instead of 4-sulphate ester groups *lambda* carrageenan contains variable amounts of 2-sulphate ester groups.

Heparin the most common anti-blood coagulant is an acidic carbohydrate with a positive optical rotation capable of forming salts with metals and has uronic acid, glucosamine, and sulfate components (Jorpes 1939 and Jacques 1966). Experimental evidence indicates that N-sulfate groups may be related to anticoagulant activity whereas O-sulfate groups may determine clearing factor activity. More recent studies however, suggest that anticoagulant activity is not produced by individual sulfate groups, but rather by a combination of such groups with carboxyl functions in the molecule. According to Lindahl, 1977 from the studies of the biosynthesis of heparin, showed that single sugars in the chain can vary; for example, both sulfamino or acetylamino glucosamine may occur, and iduronic acid and glucuronic acid may occur in both unsulfated or sulfate forms. The inhibitory activity with respect to the blood coagulation system is possibly dependent on the presence of such variant sugars and that the binding site for AT-III involves a dodesaccharide sequence with a variant sugar.

The degree of sulfation and variations in chemical structure of heparinoids showed importance on its biological activity (Jacques 1978). Since *lambda* carrageenan contains higher amount of sulfate this could probably contribute to its significant inhibitory activity of blood coagulation as compared to *kappa* and *iota* type.

Preliminary experiment on the effect of the different modes of administration of *lambda* carrageenan in rats against coagulation time of blood samples was conducted. The dose prepared was 1 mg/kg. Results showed that intravenous route was lethal, while intraperetonial route recorded a time of 4.339 mins. Subcutaneous administration recorded 2.73 mins.

Lambda carrageenan was deactivated by the addition of acetic acid. A 1 mg/kg dose of deactivated carrageenan was injected to the rats and after 30 minutes the blood samples were collected and analyzed for the presence of carrageenan. The result of analysis showed no traces of carrageenan in blood as compared to heparin which has the ability to be activated *in vivo* (Jacques 1973).

The hypocholesterolemic activity of carrageenan in rats was determined. Table 5 showed the results of the study. Among the three types of carrageenan used in the experiment *lambda* carrageenan showed a decrease in cholesterol level after 6 weeks of feeding. *Kappa* carrageenan has no significant effect on the lowering of cholesterol level while *iota* type showed an 11.68% decreased in cholesterol level from the 4th week of feeding to the 6th week of feeding. Group II had 60% mortality while Group VI had a 20% mortality. The Groups fed with carrageenan had no mortality including the control Group and Group I. Groups I-VI gained more weight than the control group which is group VII.

The hypocholesterolemic activity of carrageenan maybe possibly due to the binding of cholesterol with carrageenan. Thus, in effect lowers the concentration of cholesterol.

Summary and Conclusion

The three types of carrageenan used in the experiment conform with the specifications set by USP XXII, 1990. Acute oral toxicity test LD_{50} for *kappa* and *iota* carrageenan is 10.6610 ± 0.1514 g/kg. The *lambda* carrageenan administered orally to male Swiss mice, at a high dose of 15 g/kg, did not cause death in the test animals.

Lambda carrageenan showed significant anti-blood coagulant activity than *kappa* and *iota* type.

Administration of carrageenan by intravenous route is lethal while intra-peritoneal route recorded a time of 4.339 minutes for coagulation time of blood. Subcutaneous administration recorded a time of 2.73 minutes.

Deactivated carrageenan when injected in rats did not show any traces in the blood when investigated.

At 3.0% concentration of *Iota* carrageenan which have been added to the diet showed an 11.68% decrease of cholesterol level in rats after feeding them from 4th week to 6th week. *Lambda* carrageenan showed 1.95% decrease of cholesterol level from 2nd week feeding to 6th week feeding.

An increase in weight by 14.73% to 20.17% was observed for rats fed with three different types of carrageenan.

Acknowledgement

We wish to thank the Department of Health for the analysis of cholesterol levels and Marcel Corporation for providing the samples.

References

Chapman VJ & Chapman DG. 1980. Seaweeds and their uses. Chapman and Hall, New York.

Dumelod, BD, Ramirez, RP, Tiangson, CL., Barrios, EB, & Panlasigui LN. 1999. Carbohydrate availability of arroz caldo with *lambda* carrageenan, Journal of Food and Science and Nutrition, July 50 (4):283-289.

Elsner H. *et al.* 1937. Z. Physiol. Chem. 246, 244.

Fisher RA & Yates F. 1952. Statistical Tables. Oliver and Boyd Ltd. Edinburgh.

Gennaro A R. 1990. Remington's pharmaceutical sciences. 15th edition Mack Publishing Company, Easton, Pennsylvania, 18040.

Horwitz W. 1990. Official methods of analysis, 15th edition AOAC., Inc. V.A. USA.

Jacques LB. 1966. Proceedings of the third international pharmacology meeting. Pergamon Press. New York. 6: 25.

Jacques LB. 1978. Heparin: An old drug with a new paradigm. Science 206:528-533.

Jorpes E. 1939. *Heparin*: Its chemistry, physiology and applications in medicine. Oxford Univ. Press, London.

Kimura, J. 1941. Studies on the substances of anti-blood coagulation activity in marine algae, IV. In *Laminaria japonica*. Hokkaido Igaku Zasshi (Acta Medica Hokkaideonesia). 19:427-436.

Lindahl U. 1977. Heparin, structure, cellular functions, and clinical applications. Uppsala J. Med. Sci. 82:167.

Percival E. 1979. The polysaccharides of green, red and brown seaweeds. Their basic structure, biosynthesis and function. British Phycol. J. 14:103-117.

Rees DA. 1972. Shapely polysaccharides. Biochem. J. 126:257-73.

Smith F. & Montgomery R. 1959. The chemistry of plant gums and mucilages. Reinhold Publishing Corporation, New York.

Tsuchiya Y. 1969. Comparative hypocholesterolemic activities of marine algae. Proceedings of the 6th international seaweed symposium (ed. by Margreef, R.) Sub-secretaria de la Marina Mercante, Madrid Spain. pp. 747-757.

Whistler RL. 1973. Industrial gums, polysaccharides and their derivatives. 2nd Edition Academic Press. London/New York.