

The Pharmacological Effects of Kappa Carrageenan on Different Human Cell Lines and Genomic DNA: An *in vitro* study

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Abstract

Carrageenan extract is a compound of sulfated polyglycan that is taken out from red seaweeds. Being hydrocolloid in nature, carrageenan has gelling, emulsifying and thickening properties allowing it to be commonly used in the oral healthcare products and cosmetics. Due to its bioactive compounds, carrageenan has been shown to have antimicrobial, antiviral, and antitumor properties. The purpose of this work is to study the probable use of carrageenan on the diseases that are related to oral cavity and on the genomic DNA in *in vitro* experimental model. In this study, the effects of κ -carrageenan on four different cell lines related to the cancer and normal cells which cultured on selective media were done. Moreover, the effect of κ -carrageenan on the DNA molecule using an *in vitro* model was investigated in order to explain the antiproliferative effect of carrageenan. Kappa-carrageenan inhibited the cancer cell growth and fibroblast cell lines growth (*in vitro*) experimental model. In addition, κ -carrageenan solution completely and significantly damaged the DNA molecule by the evidence that the mean \pm SD absorbance of the mixture of κ -carrageenan and DNA solution is 0.0 ± 0.0 . This study shows that the κ -carrageenan pharmaceutical preparations exert biological activities as anticancer in *in vitro* studies.

Keywords: Carrageenan, Cell line, DNA, Antitumor, *In vitro*.

التأثيرات الدوائية لمادة كابتا كراجينان على خطوط الخلايا البشرية و د.ن.أ الجينومي : دراسة داخل الأنبوب

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الخلاصة

كاراجينان هو مركب يحتوي على البولي غلايكان الكبريتي المتعدد وهو مستخلص من الطحلب الاحمر. الكراجين بطبيعته الغروية المائية يمتلك خواص هلامية، مثخنة ومستحلبة تسمح باستخدامه بكثرة في مستحضرات الصحة الفموية والكماليات. وبسبب مركباته الحياتية الفعالة فقد اظهر الكراجينان امتلاكه خواص مضادة للخلايا السرطانية. لقد هدف هذا البحث الى دراسة التطبيق الممكن للكراجينان في خطوط الخلايا السرطانية والخلايا الليفية وتأثيره في الحمض النووي الجيني البشري في نموذج مختبري خارج الزجاج (في الأنبوب) تم دراسة تأثير كراجينان في أربعة انواع من الخلايا السرطانية والطبيعية والتي تم زرعها في وسط انتقائي لمعرفة مدى تأثير كراجينان في اثباط نمو الخلايا المزروعة، كما امتدت الدراسة لتشمل تأثير كراجينان في الحمض النووي في أنموذج تجريبي خارج الزجاج. وقد بينت هذه الدراسة ان كابتا كراجينان يمنع خطوط الخلايا السرطانية والخلايا الليفية في التجارب الاختبارية بالاضافة الى اذى تام في جزيئة الحمض النووي الجيني البشري. يستدل من ذلك ان الكراجينان الذي استعمل في هذه الدراسة على شكل محلول لديه نشاط مضاد للسرطان في الدراسات المختبرية .

الكلمات المفتاحية: كراجينان، خطوط الخلايا البشرية المختلفة، الدنا، ضد الاورام، في الزجاج .

Introduction

The carrageenan extract is a (sulfated – polyglycan) that is taken out from red seaweeds from the genera (*Gigartina*, *Chondrus*, *Eucheuma* and *Iridaea*)^(1,2). Carrageenans are mainly utilized as a diet improver because of its emulsifying, thickening and gelling activities making it a vegetarian substitute for the gelatin⁽²⁾. In addition to their use in food, carrageenans are generally used as excipients in a personal lubricant, toothpaste, many cosmetics besides many of the pharmaceutical products⁽³⁾. Commercially, three forms are there of

carrageenan that are presented (kappa, lambda and iota) that are differing in degree and composition of sulfation in the polymeric structures. (Figure 1).

These actions of carrageenan have been considered, particularly on the animal model, where it had been reported that carrageenan extract has anti-microbial^(4,5) and anti-tumor criteria^(6,7).

The tissue of the oral cavity is mainly formed by oral-keratinocytes, which are stratified squamous epithelium that form a main barrier to physical, chemical and microbial against agents that can cause localized cellular injury⁽⁸⁾.

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These cells are also actively involved in the pro-inflammatory route through the making of some cytokines^(9,10). Some of the toothpastes and oral gels contained carrageenan, and if exposure to an extended period will cause contact with oral cells besides the oral microorganisms. As stated before, carrageenan possessed anti-microbial activity⁽¹¹⁾. In earlier work, κ -carrageenan oligo-saccharides from *Kapaphycus stratum*; in vitro and in vivo were proved to have immuno-modulation action on S180 bearing mice^(12,13).

Chemical adjustment of carrageenan oligosaccharides can increase their antioxidant activities⁽¹⁴⁾. The aim of the study is to evaluate the effect of κ -carrageenan on the human cancer and fibroblast cell lines and on the human genomic DNA in *in vitro* experimental study.

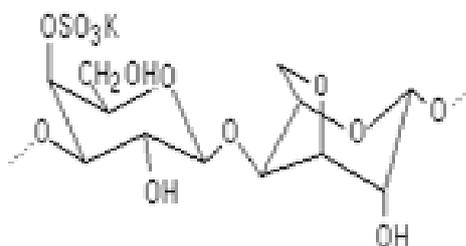


Figure 1 Chemical structure of κ -carrageenan

Materials and Method

Materials

κ -carrageenan purchased from Sigma-Aldrich (USA)⁽¹⁵⁾

Cell line:

- Ahmed Majeed 2003(AM3) Transplantable mammary adenocarcinoma line.
- HeLa established by (George Gey) at the Johns Hopkins medical school.
- Primary Rat Embryo Fibroblast (REF) provided and established from the Iraqi Centre for Cancer & Medical Genetics Research, Baghdad, Iraq (ICCMGR).
- Rhabdomyosarcoma (RD) cell line (got from the pelvic rhabdomyosarcoma of a seven-years-old Caucasian girl)^(16,17).

Methods

Effect of κ -carrageenan on cancer cell line and fibroblast cells line

In this study, four types of the cell lines are used. These are HeLa cell, Rhabdomyosarcoma, Mammary cell carcinoma, and fibroblast cells.

Media and solutions preparing for the (*in vitro* cell culture) experiment:

1. Rosswell-Park-Memorial/ Institute RPMI/1640 Media

The procedure of the media is made as following:

- a. The (RPMI/1640) powder with the (HEPES.) buffer, with L-glutamine (8.2g)

was dissolved in a (400) ml of the distilled water, and afterwards other constituents were added.

- b. The sodium-bicarbonate powder is equal to 1.1 gm Streptomycin (0.25 ml) of (1gm) vial that is dissolved within a (5 ml) of distilled water.
- c. Ampicillin (0.5)ml of a (500) mg vial that is dissolved in (5 ml) of distilled water.
- d. Fetal calf serum/FCS (100 ml) afterward this volume is accomplished for up to (500) ml with distilled water and this media was sterilized with Nalgen filter by a 0.2 μ m filtering unit^(16,17).

2. Phosphate buffer saline/PBS solution.

This solution is made up using the following procedure:

- a. Firstly dissolving an amount of 5 gm (PBS) powder in a (400) ml of distilled water, and then the other constituents were added.
- b. Streptomycin (0.25) ml of (1gm) vial was dissolved in a 5 ml of distilled water.
- c. Ampicillin 0.5 ml of (500 mg) vial was dissolved in (5 ml) of distilled water.

Afterwards the volume is completed up to 500 ml by adding distilled water and this media was sterilized by Nalgen filter with a (0.2 μ m) filter unit.

3. Trypsine/Versen s olutions

The solution is made as the following:

- a. Dissolving amount of (5.05gm)Trypsine/Versene powder in a (400 ml) of distilled water, and after that adding other constituents.
- b. Adding sodium-bicarbonate powder amount of 1 gm.
- c. Streptomycin (0.25) ml of (1gm) vial is dissolved in a 5 ml of distilled water.
- d. Ampicillin amount of (0.5 ml) of a 500 mg vial which is dissolved in a 5 ml of distilled water).Then the volume is finished to (500ml) with distilled water and then media is sterilized by Nalgen filter with (0.2) μ m filtering units^(16,17).

4. Fetal calf serum(FCS)

This serum is thermally deactivated, set for uninterrupted use for the tissue culture media and it was sterilized^(16,17).

5. Preserving Serum Free Media / SFM.

The media was made by the same process that is described for RPMI./ media except that the FCS was not added.

Solution of methyl-thiazolyl tetrazolium /MTT.

3-Dimethylthiazol-2-yl-2,5-Diphenyl-tetrazoliumbromide (0.2 gm), which was dissolved in a 100 ml of the PBS in order to get (2 mg /ml) concentration of the dye. This solution was filtered by using a 0.2µm Millipore filter to get rid of any blue color formazan material, and then kept in sterile, dark, glass container at a (4°C). The solution must be used within 2weeks of its preparation^(16,17).

Types of the Cell Lines used in this study**1. HeLa cell:**

This cervical cancer cell line of human which was firstly recognized by "George Gey" at the Medicine school of Johns Hopkins in the year 1951 obtained from a mother 31-year-old that had four children, her name is "*Henrietta Lacks*". The HeLa cell were different from another cervical cancers explant in which they raised horribly in culture, could be too violently. Passage/13-14 that was used in the study, and the RPMI-1640 media with a (5%) FCS was used in preserving these cells.

2. Rhabdomyosarcoma (R.D. Cell Line):

This human cell line (RD) derived from a biopsy sample got from the pelvic rhabdomyosarcoma of a (7 years old) Caucasian girl. The Passage of (20-21) used in this study, and the RPMI-1640 media with 5% /FCS was used in preserving these cells.

3. Ahmed/Mohammed/Nahi-2003(AMN-3)**Cell Line:**

This is a murine mammary-a type of adenocarcinoma- cell line is a derivative of the initially (*in-vivo*) passage for the spontaneous mammary adenocarcinomas of a female BALB-c mouse. Passage/182-183 was utilized in this work, and the RPMI/1640 media with 5% FCS utilized in preserving these cells.

4. The primary rats embryo fibroblasts/R.E.F

The R.E.F cell line is recognized and delivered by the help of Dr. Ahmed M. Al- Shammery from the (ICCMGR). These cells of this standard rats cell lines were a combination of epithelial and fibroblastic cell with ordinary chromosomal image. Tumorigenicity trial of the cell lines has shown no tumor or growth development in the rats that are injected during 3 months of observing. Passage of (77/78) was utilized in this study, and the RPMI/1640 media with 5%/ FCS was used in preserving these cells.

Four cell lines are subjected to carrageenan. Those cells are (HeLa, fibroblasts mammary-AMN3, and rhabdomyosarcoma. These cells are stored in a deep freezing as stock cells at the National Centre of Cancer. Such cells are rebooted before the testing. Many trials of the cell revival are made so that to get a single layer cell in a precise falcon volume of 25 ml which can be accustomed under the microscope to search for the presence of monolayer cell. Cell cultivation was carried on according to the instructions of The National Center of Cancer in Baghdad, using the following procedures:

1st step: During the first day, when the development of a single layer cells that have been formed in the cell falcons which is a precise falcon of 25 ml of volume, the last growth media got rid of and washed with the phosphate buffer saline (PBS) buffer only once, then add amount of 0.5-1.0 ml Trypsin-versin solution, shaking the mixture and allowed to set down for 1 min. Then, at that moment, a gentle shake by hand is followed for a minute, after that add 10 ml of sterile growth media in amount of 5% of fetal calf serum to the cells in the falcons, then start to pipette in order to diffuse the cells within the newly made media. Then the isolated cells moved into a germ-free microplate. A whole volume of (200) µl cells suspension or cell suspension-culture media (which is considered as control) is moved to a number of wells of a microplate. The microtitre plate is protected with a (plate-cover) to avoid the opportunity of impurity and then incubated at a temperature (37°C) for 24 -hours so that to maintain a single layer cells progress.

2nd step: During the second day meaning after 24-hour of cell culturing sin microplates, when the monolayer cell growth is formed in the microtitre plate, culture media was thrown away and the subsequent measurements of culture media and then carrageenan was added in a number of wells

- 200 µl of cell cultures media
- 200µl of the cultured media were added into the wells with the monolayer cells growing
- 175 µl of the culture media having no cells in addition to 25µl of 0.5% w/v freshly made κ-carrageenan solution.
- The prepared κ-carrageenan solution poured into the wells with monolayer cells growth.

The solutions of carrageenan were sterilized by filtering using a 0.2µm Millipore/filter before the adding step. The microtitre plate was covered by a plate cover to inhibit the possibility of impurity and then was incubated at a 37°C for 24 hours so that to get a mono-layer cell growth.

3rd step: After 24 hours, an amount of 30µl M.T.T dye was added into all of the wells in dark room (to prevent oxidation of the dye), covering the microtiter plate by a foil and is kept for incubation for about two hours at a 37.5°C in the incubator. Then the wells containing solution discarded from the plate and, 100µl of dimethyl/sulfoxide added to each well and then the plates were shaken for 15 minutes using horizontal shaker. Then the absorbance of all wells were recorded at 540nm by the ELISA-reader

Effect of κ-carrageenan on the genomic DNA of human

The genomic DNA of human has been generously gotten from Prof. Dr. Adil Al Huseiny, at the Medicine Department, College of Medicine/ Diyala University. Briefly genomic DNA of human is extracted from the anti-coagulated blood by EDTA by using the (proteinase K) solutions and

genomic purification kit/ Geneaid, Taiwan, and this process of extraction is followed like the instruction of the manufacturer company of the kit. Absorbance of the extracted DNA, which is dissolved in the phosphate buffer solutions, recorded wavelength of 260nm and 280nm using a UV-Visible spectrophotometer. A ratio of absorbance at λ 260nm to λ 280nm approximately equal to 1.8-2.0, indicating the genomic DNA contaminated with RNA.

A total number of six genomic DNA obtained from six participants were tested with 0.5% (w/v) κ -carrageenan; where, 5 μ l κ -carrageenan was added to 5 μ l genomic DNA in a quartz cuvette then completed the volume to the 4ml by distilled water. The absorbance of genomic DNA was recorded at 260nm wavelength using a UV-Visible spectrophotometer. A decrease in the absorbance of genomic DNA compared with the absorbance before adding κ -carrageenan indicated DNA damage (a term known as hypochromasia). While an increase of the genomic DNA absorbance after adding κ -carrageenan, indicating separation of the DNA strands (a term known as hyperchromasia).

Statistical analysis:

Descriptive and inferential statistics were done by the use of the Microsoft Excel /2007 program. All results were stated as numbers (%), and every time possible as mean and standard deviation (SD). Data were analyzed by the utilization of unpaired two/tailed Student's t-test by taking ($p \leq 0.05$) as the lowest limit of the significance.

Results

Effect of κ -carrageenan on the cancer cell line

κ -carrageenan inhibits the development of cancer cell and fibroblasts cell lines *in-vitro* experimental model. It significantly ($p \leq 0.05$) stops the growth of HeLa cell/line by 81.2% (the mean absorbance value at λ 540 nm is reduced from 0.706 to 0.133 (Figure2).

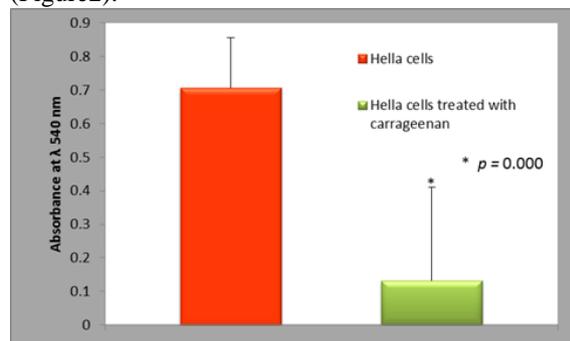


Figure 2 The effect of κ -carrageenan on HeLa cell growth *in vitro* experimental model. The results expressed as mean \pm SD (n=8).

κ -carrageenan completely and significantly suppressed the growth of mammary cell carcinoma i.e. the inhibitory percent is 100% (the mean absorbance value at λ 540 nm is reduced from 0.108 to 0.0 (Figure 3). κ -carrageenan failed to suppress the growth of rhabdomyosarcoma, in fact it improves the growth of these cells by the evidence that the mean absorbance value is increased from 0.463 to 0.524, i.e. 13% increment (Figure 4).

Figure 5 shows that κ -carrageenan **completely and significantly** suppressed the growth of fibroblast cells i.e. the inhibitory percent is 100% (the mean absorbance value at λ 540 nm is reduced from 0.196 to 0.0).

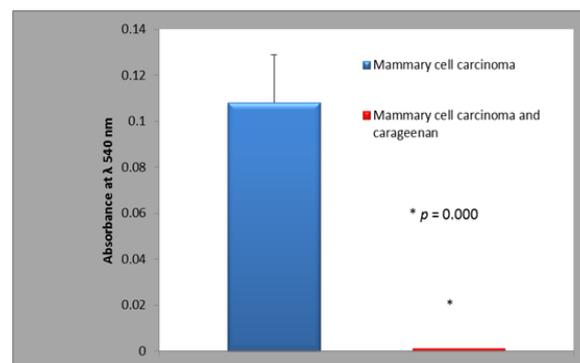


Figure 3 The effect of κ -carrageenan on mammary cell growth *in vitro* experimental model. The results expressed as mean \pm SD (n=8).

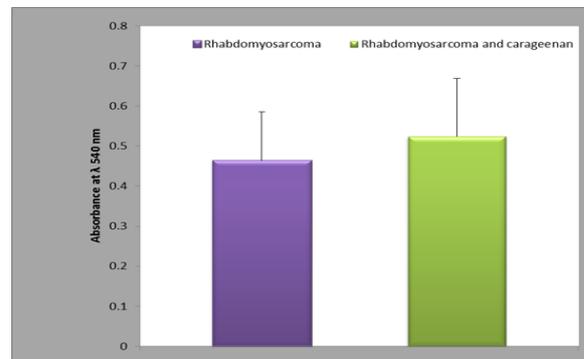


Figure 4 Effect of κ -carrageenan on the rhabdomyosarcom growth *in vitro* experimental model. Results expressed as mean \pm SD(n=8).

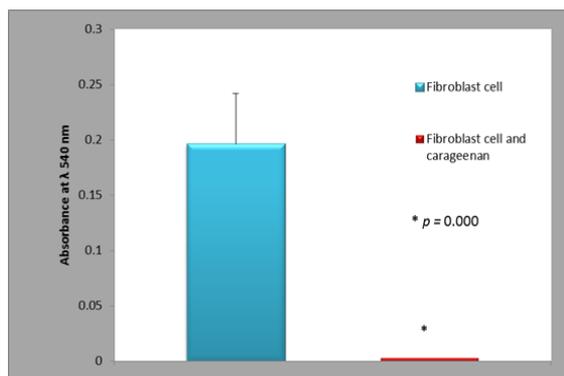


Figure 5 Effect of κ -carrageenan on the fibroblast cell growth *in vitro* experimental model. Results expressed as mean \pm SD(n=8)

Effect of κ -carrageenan on the human genomic DNA

The mean \pm SD concentration of human genomic DNA that isolated from 6 subjects was 1.783 ± 0.397 $\mu\text{g/ml}$ which corresponding to the absorbance of 0.0178 ± 0.0039 at wavelength of 260 nm. Kappa- (κ -) carrageenan solution completely and significantly ($p < 0.001$) damaged the DNA molecule by the evidence that the mean \pm SD absorbance of the mixture of κ -carrageenan and DNA solution is 0.0 ± 0.0 (Figure 6)

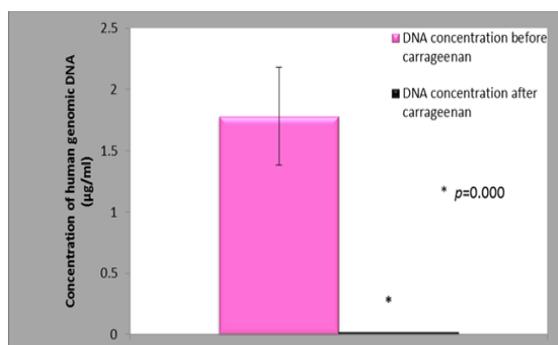


Figure 6 Effect of κ -carrageenan solution (0.5% w/v) on the human genomic DNA *in vitro* experimental model. Results are expressed as mean \pm SD of six subjects.

Discussion

The results of this study showed that κ -carrageenan significantly prevent growth of fibroblasts, HeLa cell, and mammary cells; while, its effect against rhabdomyosarcoma cell is negligible, indicating its anti-growth effect is specific. These observations agreed with a previous study that showed the specificity of carrageenan as anti-cancer; furthermore, it is imperative to declare that the anti-cancer effect of carrageenan is strictly associated with the molecular weight, carbohydrates structure and the contents and the linking site of sulfur group⁽¹⁸⁾.

In previous results of the *in vivo* and *in vitro* experimental study, it has been found that degraded

iota (ι)-carrageenan inhibits the osteosarcoma growth in established xenograft tumor models in mice and enhanced survival rate of animals (*in-vivo* study) as well as it inhibits the growth human osteosarcoma cell line. The authors suggested that carrageenan induced apoptosis (not necrosis), and arrest the cell cycle at G1 phase⁽¹⁹⁾.

In another study, the small molecular-weight, highly-sulfated lambda λ -carrageenan oligosaccharide inhibits the angiogenesis (a process that is involved in initiation and also promotion of cancer), as well as inhibits the cellular invasion, migration and proliferation⁽²⁰⁾. Mi *et al* 2008 linked those effects to the upregulation of apoptotic genes like TNF-alpha, p-53, caspase 8, caspase 3 and increase level of active caspase 3⁽²¹⁾. Tobacman and Walters (2001) used transmission electron microscopy to illustrate the interaction between mammary myoepithelial cells of human and lambda-carrageenan and found that carrageenan entered the cells by membrane-associated endocytic vesicles and accumulate in endosomes and lysosomes⁽²²⁾. As a result of the release of proteolytic enzymes from the distorted lysosome, the mammary myoepithelial cells are destroyed. On the other hand, Tobacman *et al* (2001) verified that increasing intake of numerous gums with carrageenan associates positively with high frequency of breast carcinoma⁽²³⁾.

Authors found that carrageenan oligosaccharides can inhibit the growth of HeLa cell as well as the endothelial cell via a mechanism related to inhibition of the heparanase enzyme activity; moreover, this finding pointed out that carrageenan inhibited the cell growth at the molecular level⁽²⁰⁾. The effects of κ -carrageenans on fibroblast indicate that this substance is not free from harmful effect on the normal human cells. This observation is of great importance because carrageenan is pharmaceutically formulated as salt cast film in dressings for drug delivery to wound and to allow active adherence to and protection for the wound⁽²⁴⁾. According to results obtained from the current study, it is expected that healing of the wound is either delayed or even not occurred.

The results of this study demonstrated that κ -carrageenan induced DNA (human genomic) damage in *in vitro* model. These results are in agreement with other studies that used carrageenan as inflammatory inducing agents. Cuzzocrea *et al* (2001) demonstrated that intra-pleural injection of carrageenan into rats caused DNA damage in lung tissue that accompanied with generation of reactive nitrogen species (RNS)⁽²⁵⁾; moreover, this important finding should be considered in the oral medicine-practice because, the soluble carrageenan is used as a pharmaceutical formula of the bio-adhesive buccal mucosa in which the carried medication is released and dissolved in saliva within 40 minutes⁽²⁶⁾. Therefore, using of such formula may induce DNA

damage or oral mucosa. Bhattacharyya *et al* (2014) demonstrated an increase in m-RNA expression in a mouse colonic epithelium and the colonic epithelial cells of human⁽²⁷⁾. There is no evidence in human that dietary carrageenan induced expression of mRNA in the cells of large intestine. Also Bhattacharyya *et al* (2008) found that carrageenan induced cell necrosis rather than apoptosis in the colonic epithelial cell lines of human (NCM460) and in the primary colonic epithelial cells of humans; moreover, there is evidence that carrageenan can induce DNA damage and arrest the cycle of the cell⁽²⁸⁾. On the other hand, Weiner (2014) concluded that, using dietary carrageenan is safe and there is no evidence that it has undesirable effects on the normal cell growth or on the reproductive system⁽²⁹⁾.

Conclusions

The results showed that the effects of carrageenan against cancer cell line prohibit the claim that the dietary carrageenan plays a role in carcinogenesis; whereas, its effect against fibroblast pointed to limit its uses in tissue injuries as it may limit the healing process. The damaging effect of carrageenan on the human genomic DNA in *in vitro* experimental study should be supported by further studies including *in vivo* experimental animal models to demonstrate the safety of using carrageenan.

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