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Antiproliferative Effect of Carrageenans on Epidermoid Carcinoma (A431NS) Cells Through Inhibition of DNA Synthesis and Translationally-Controlled Tumour Protein (TPT1) Gene Expression

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Carrageenans are polysaccharide constituents of red seaweed cell walls used in food and medicine as well as thickening agents and excipients in cosmetics and skincare products. Carrageenans have antioxidants, anti-inflammatory and pro-apoptotic properties that could potentially cause antiproliferative effects against cancer cells. The primary aim of this study was to assess the antiproliferative effect of iota (ι) and kappa (κ)-carrageenan as well as their combination with α-tocopherol on epidermoid carcinoma (A431NS) cells concerning DNA synthesis and translationally-controlled tumour protein (TPT1) gene expression. Carrageenans exhibited cytotoxic effects against A431NS cells with CD_{50}<100ug/ml and an antiproliferative effect in a concentration-dependent manner after 24, 48 and 72 hours of treatment by inhibiting DNA synthesis in these cells. The expression of the TPT1 gene increased significantly ($p<0.05$) in untreated A431NS cells. However, the expression was either lowered or completely suppressed in groups treated with carrageenans and/or α-tocopherol compared to the untreated cells. This study indicates that carrageenans can exhibit an antiproliferative effect via inhibition of DNA synthesis and downregulation of TPT1 gene expression in A431NS cells. The anticancer activity of carrageenans deserves further studies to explore their potential applications as an ingredient in cosmetics or skin care products for the prevention of skin cancer.

Keywords: carrageenans; epidermoid carcinoma cells (A431NS); antiproliferation; DNA synthesis; translationally-controlled tumour protein (TPT1) gene

I. INTRODUCTION

Skin cancer is a locally destructive cancerous growth of the skin and can be divided into non-melanoma skin cancers (NMSC) and melanoma. NMSC are associated with excessive exposure to ultraviolet radiation (UVR) from sunlight (Melnikova & Ananthaswamy, 2005) and usually develops in the epidermis, often named after the type of skin cells from which they developed. Melanoma is a cancer of the pigment-producing cell, the melanocytes (NHS, 2017).

The two types of NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC starts in cells lining the bottom of the epidermis and accounts for about 75% of skin cancers and causes high morbidity (So, 2008). SCC accounts for about 20% of skin cancers and starts in cells lining the top of the epidermis or from a precancerous lesion such as actinic keratosis that grows on sun-damaged skin (Hawrot *et al.,* 2003). It is highly invasive and may progress to lethal metastases (Waterman *et al.,* 2007).

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Skin cancer progresses through active DNA synthesis that supports cancer cell proliferation as well as when the apoptotic mechanism is disrupted due to mutations of related gene or pathway that allows for uncontrollable cell growth (Yukio, 2013). One such gene involved in skin cancer is the Translationally-Controlled Tumour Protein 1 (TPT1) gene, however, it has not been extensively studied. TPT1 is a gene coding for the translationally controlled tumour protein (TCTP) (Li *et. al.,* 2001; Andree *et al.,* 2006). The TCTP is an anti-apoptotic protein, which is highly expressed in several human cancer types, including colon, prostate and liver cancer (Wu *et. al.,* 2012; Chan *et al.,* 2012).

TCTP has been implicated in important cellular processes, such as cell growth, cell cycle progression, malignant transformation, calcium binding, microtubule stabilisation and in the protection of cells against various stress conditions. Silencing of TCTP has been shown to enhance apoptotic cell death in cancer cells (Gnanasekar *et. al.,* 2009; Rho *et al.,* 2011). Hence, downregulating the expression of this gene would significantly induce apoptosis and inhibit DNA synthesis in skin cancer cells, thus, preventing cell proliferation.

Seaweeds are a potential source of bioactive compounds comprised of proteins, peptides and carbohydrates. The major polysaccharides of seaweeds include alginates, fucans, laminarans, agaran, agaropectin and carrageenans (Alvaro *et al.,* 2010). Carrageenan is the generic name for the family of natural water-soluble, gel-forming, viscosifying sulphated galactans that are present in the extracellular matrices of red algae from the genera *Chondrus, Eucheuma, Gigartina* and *Iridae*. There are three major isomers of carrageenans, namely kappa (κ), iota (ι) and lambda (λ) carrageenans (Liners *et al.,* 2005).

Photoprotective, anti-inflammatory, free radical scavenging (Thevanayagam *et al.,* 2014) and antioxidative (Sokolova *et. al.,* 2011; Sun *et al.,* 2010) activities of carrageenans have been reported. Carrageenans have also been shown to exhibit anti-tumour (Haijin *et. al.,* 2003; Zhou *et. al.,* 2004, 2006; Luo *et al.,* 2015), anti-proliferative (Yuan *et al.,* 2006), anti-viral (Kalitnik *et al.,* 2013), antibacterial (Seol *et al.,* 2009) and immunomodulatory (Zhou *et al.,* 2004) activities.

A study by Coombe *et al.* (1987) suggested that the anticancer mechanism is due to sulphated polysaccharides interfering with the passing of tumour cells across the capillary walls. In addition, sulphated polysaccharides display anti-metastatic activity by blocking the interactions between cancer cells and the basement membrane (Rocha *et al.,* 2005), inhibiting tumour cell proliferation and tumour cell adhesion to various substrates (Yamamoto *et al.,* 1986). Based on these promising anticancer effects of sulphated polysaccharides, we hypothesise that carrageenans exhibit cytotoxic and antiproliferative effects against A431NS cells while inducing apoptotic cell death through the halt of DNA synthesis and TPT1 downregulation.

The epidermoid carcinoma cell line A431NS is a model that can be used to assess tests related to NMSC since it is derived from epidermoid carcinoma of the skin or epidermis. A431 cell line was derived from an 85-year-old female having epidermoid carcinoma of the skin or epidermis. A431NS derived from the A431 cell line (ATCC CRL-1555) in 1997 by repeated subculturing to select the cells that detached from the substrate easily without affecting other properties (ATTC, 2022). A431NS cells are used in studies evaluating NMSC as well as the anticancer and anti-proliferative effects of selected compounds (Baechler *et. al.,* 2009; Khamar *et al.,* 2012). The choice of using the A431NS cell line to test for skin cancer and anticancer effect in this study was based on these factors.

The primary aim of this study was to assess the antiproliferative effect of carrageenans on A431NS cells and its downregulation effect on the TPT1 gene. Although studies have been conducted on the antiproliferative effects of carrageenan on other cancer cell lines (Liu *et al.,* 2019), no studies concentrated on skin cancer (A431Ns) cells. Being one of the widely used excipients in cosmetics and skin care products, carrageenan is important to be evaluated for its potential effect as an anticancer agent for skin cancers. No studies have been conducted on the use of carrageenan to downregulate the TPT1 gene in skin cancer, even though the TPT1 gene was found to be an important gene related to the apoptotic process (Gnanasekar *et al.,* 2009). The findings of this study will be a novel contribution towards the development of a new natural-based anticancer agent for preventing or treating skin cancer from a different approach.

II. MATERIALS AND METHOD

A. Cell Culture

The A431NS cell line used in this study was a generous gift from Dr Charlton Keith from the University of Aberdeen, Scotland. The cells were cultured and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO Life Technologies, USA), 1% penicillin-streptomycin (10,000 units of penicillin (base), 10,000 µg of streptomycin (base) ml^{-1}(GIBCO Life Technologies), stored at 37°C in an atmosphere of 5 % CO_2 and 99 % humidity incubator.

B. Carrageenans

Two isomers of purified food grade carrageenans, namely ι from *Eucheuma denticulatum*, and κ-carrageenan from *Eucheuma cottonii* (PT. Wahyu Putra Bimasakti, Indonesia) were used. Vitamin E (α-tocopherol) of 96% purity (Sigma Aldrich, USA) was used as a positive control. Stock solutions of ι, κ- carrageenan and α-tocopherol were prepared by dissolving the chemicals in dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, USA). For the experiments, compounds were taken from the stock solutions and diluted to the desired concentrations using complete DMEM.

C. Cell Treatment with Carrageenans And A-Tocopherol

For all the tests, the cells were treated with ι-, κ- carrageenan, α-tocopherol combined with ι, α-tocopherol, and κ with α-tocopherol at final concentrations, ranging from 3.125 to 200 µg ml^{-1} via serial dilutions. For the combination, an equal volume and a concentration of the individual solution were added to a final concentration of 3.125-200 µg ml^{-1}. The treatment solution was added in triplicates for each concentration into microplate wells containing $2x10^4$ cells per ml unless stated otherwise. After the treatment, the samples were incubated for another 24, 48 or 72 hours before the analysis of the various parameters. All experiments were carried out in triplicates unless stated otherwise. The control consisted of cells in complete DMEM without the addition of carrageenans and/or α-tocopherol diluted to the same concentration as the test group.

D. Cytotoxic Effect of Carrageenans and α-tocopherol

A431NS cells were harvested and seeded in a 96-well microplate and incubated for 24 hours before proceeding with the treatment as described in section C. After the treatment, the standard MTT assay was conducted (van Meerloo *et al.,* 2011). The percentage of cell viability was obtained based on the comparison between the treated and control groups.

$$Cell\ Viability\ (\%) = \frac{ODtreated}{ODuntreated} cells\ x\ 100$$

E. Cell Antiproliferation Assay

The antiproliferative effect of carrageenans on the A431NS cells was assessed using CyQUANT® Direct Cell Proliferation Assay Kit (Invitrogen, California, USA). The cells were prepared for further analysis as per the manufacturer's protocols after being subjected to the treatment described in section C. The absorbance obtained was directly related to the number of live cells since only healthy cells would be stained. This allows a direct quantification of cell proliferation, which is expressed in the percentage of cell viability, determined using the formula below:

$$Cell\ Viability\ (\%) = \frac{ODtreated}{ODuntreated} cells\ x\ 100$$

F. Quantification of DNA Synthesis

DNA synthesis in A431NS cells was quantified using the Click-iT EdU Microplate Assay (Invitrogen, California, USA). A431NS cells were harvested, seeded and treated with carrageenan and/or α-tocopherol at selected concentrations of 12.5, 25, 50 and 200 µg ml^{-1} and incubated for another 24 or 48 hours. The test was then performed according to the manufacturer's protocols. The absorbance was read using a microplate reader to quantify the DNA synthesis in the cells, which was converted to percentage according to the formula below:

$$DNA\ Synthesis\ (\%) = \frac{ODtreated}{ODuntreated} cells\ x\ 100$$

G. Assessment of Apoptosis and Necrosis Cell Death

Annexin V FITC Assay Kit (Cayman, USA) was used for this study. The cells were harvested using Accutase (PAA laboratories, Austria) and cultured in a 96-well black microplate (Falcon, USA). The cells were seeded at a density of $1x10^6$ cells per ml and incubated for 24 hours before proceeding with the treatment described in section C. After the desired treatment time, further tests were conducted following the manufacturer's instructions. The percentage of apoptotic and necrotic cell death compared to the control was based on the OD readings, calculated according to the formula below:

$$Cell\ death\ (\%) = \frac{ODtreated - ODuntreated}{ODuntreated} cells\ x\ 100$$

H. Assessment on TPT1 Gene Regulation by Carrageenans and α-tocopherol

A431NS cells were harvested and seeded in a six-well plate and treated with selected concentrations of 12.5, 25 and 50μg ml^{-1}. After 24 or 48 hours, the cells were harvested for RNA extraction (RNeasy Kit, Qiagen, California, USA). The purity of the RNA obtained was assessed at A260/280nm. The RNA was then converted to cDNA using Qiagen RT² First Strand Kit real-time PCR (RT² qPCR Primer Assay, Qiagen, California, USA). A specific primer (PPH18268A; QIAGEN) was used for the amplification of the TPT1 gene. Phosphoglycerate Kinase 1(PGK1) was used as the housekeeping gene (HKG) (PPH02049A; QIAGEN). Immortalised normal human keratinocyte (HaCaT) cells were used as the control and all groups were compared to the delta C_T values from HaCaT. The PCR test was conducted using a real-time cycler (BIO-RAD CFX96, Hercules, California, USA). The delta delta C_T method was used for data analysis according to the Qiagen RT² First Strand Kit manufacturer's instructions. The C_q values of the gene of interest (GOI) and HKG were obtained for each sample and the difference between the values was calculated as shown below:

delta C_T (Control): C_q(GOI) - C_q(HKG) = **X**

delta C_T (Experimental): C_q(GOI) - C_q(HKG) = **Y**

For each pair-wise set of samples compared, the difference in delta C_T values for each GOI and HKG was calculated.

delta delta C_T = Y – X = **Z**

The fold change in gene-expression was then calculated using the $2^{(-Z)}$ equation.

III. RESULTS AND DISCUSSION

A. Cytotoxicity of Carrageenans and α-tocopherol on A431NS Cells

The percentage of cell viability decreased in a dose-dependent manner concerning the concentrations of carrageenans and α-tocopherol tested (Figure 1). After 24 and 48 hours of treatment, the lowest cell viability was in cells treated with ι-carrageenan followed by κ-carrageenan and those treated with the combination of carrageenans and α-tocopherol. α-tocopherol was not effective in promoting cell death at low concentrations (3.125-12.5 μg ml^{-1}) but promoted cell proliferation instead. The cell viability was reduced only after treatment with high concentrations (25 to 200 μg ml^{-1}) of α-tocopherol. The CD_{50} was 60-80 μg ml^{-1} after 24 hours and 62-70 μg ml^{-1} after 48 hours for all treated groups. The percentage of cell death was >50% at 100μg ml^{-1} (Figure 1 (a) and (b)).

After 24 hours of ι-carrageenan treatment, 0.06 to 0.9-fold greater cytotoxicity was observed and 0.04 to 0.9-fold in the κ-carrageenan group. However, a 0.003- 0.2-fold increase in cell viability was observed after 3.125-12.5 μg ml^{-1} α-tocopherol treatment. Only at concentrations 25-200 μg ml^{-1}, 0.07-0.6-fold greater cytotoxicity activity was observed. For the ι and α-tocopherol combination group, 0.007 to 0.6-fold greater cytotoxicity activity was observed and 0.03 to 0.8-fold for κ and α-tocopherol treatment for concentrations between 3.125 to 200 μg ml^{-1}, respectively.

After 48 hours of ι-carrageenan treatment, 0.09 to 1-fold greater cytotoxicity was observed and 0.1 to 1-fold in the κ-carrageenan group. 0.1- 0.8-fold greater cytotoxicity after α-tocopherol treatment. For the ι and α-tocopherol combination group, 0.1 to 0.9-fold greater cytotoxicity activity was observed and 0.1 to 0.9-fold for κ and α-tocopherol treatment.

The CD_{50} ranged between 13-33 μg ml^{-1} after 72 hours of treatment. The percentage of cell death was >50% at 50 μg ml^{-1}. 0.3 to 1-fold greater cytotoxicity was observed after ι-carrageenan treatment and 0.4 to 0.9-fold in the

κ-carrageenan group. After α-tocopherol treatment, 0.2-0.6-fold greater cytotoxicity was seen. For the ι and α-tocopherol combination group 0.3 to 0.7-fold greater cytotoxicity activity was observed and 0.3 to 0.7-fold for κ and α-tocopherol treatment (Figure 1 (c)).

It is important to determine the toxicity profile for carrageenan and α-tocopherol. This allows a selection of appropriate concentrations for evaluating the effects. A range of concentration was selected since there was no standard concentration for carrageenan in cosmetic preparation or cancer treatment to date. Since there were findings that recommended a ratio of 0.1-1.5% of carrageenan based on its isomer in a skincare formulation (Aria, 2021; Fransiska *et al.,* 2021), it is crucial to test for its toxicity and effect from a low to higher concentration range. Moreover, some studies have shown that a minimal concentration of carrageenan is sufficient to exhibit antiproliferative effects against cancer cell lines (Namvar *et. al.,* 2012; Murad *et al.,* 2015) while others were tested for at a much higher concentration (Luo *et. al.,* 2015; Prasedya *et al.,* 2016). In this study, we considered the concentrations based on the cytotoxicity profile of carrageenan on normal keratinocyte, HaCaT cells (data not shown) from our previous studies (Thevanayagam, 2013; 2017). Therefore, a concentration suitable for both normal and cancerous cells can be determined from this finding. This is important to ensure carrageenan used is not toxic to normal skin cells, at the same time, able to exhibit antiproliferation or toxic effects on cancer cells.

When evaluating cytotoxicity, inhibitory concentration (IC_{50}) values of less than 100 µg/ml indicate a potentially cytotoxic compound. IC_{50} values in the range of 100-1000 µg/ml were considered to correspond with moderate cytotoxic effects, whereas, compounds with IC_{50} values greater than 1000 µg/ml were considered non-toxic to the cells (Zainal Ariffin *et al.,* 2014). Based on this, it was rational to include a range of low to high concentrations for initial screening and narrow it down to specific concentrations based on our data (not shown) on the cytotoxicity and photoprotective profile of carrageenan and tocopherol on normal skin cells, which was used against the A431NS cells in selected parts of the test.

A431NS cells treated with carrageenans and/or α-tocopherol showed a cytotoxic effect compared to the cells without treatment. Antitumor agents that can affect the cell cycle could be a good target for antitumor drugs. Carrageenans have been found to have the potential to arrest the cell cycle at either G2 or S phase (Prasedya *et al.,* 2016). Their study showed that κ- and λ-carrageenan exhibited a cytotoxic effect on human cervical carcinoma cells (HeLa) based on the MTT assay, where cell cycle arrest was also seen to take place. Cells treated with κ-carrageenan had longer cell phase periods while the division of cells treated with λ-carrageenan was inhibited. The suppression of cell growth and division following the treatment with λ-carrageenan suggested that carrageenan has a strong antiproliferative effect.

Carrageenans have also been shown to significantly inhibit HeLa growth not only by arresting the cell cycle in specific phases but also delayed the cell cycle progress, resulting in a longer cell cycle (Prasedya *et al.,* 2016). Additionally, different types of carrageenans have different effects on cell cycle progression and this effect of carrageenans towards cancer cells is worth exploring for developing the bioactive compound into a tumour-cell-specific anticancer agent (Prasedya *et al.*, 2016).

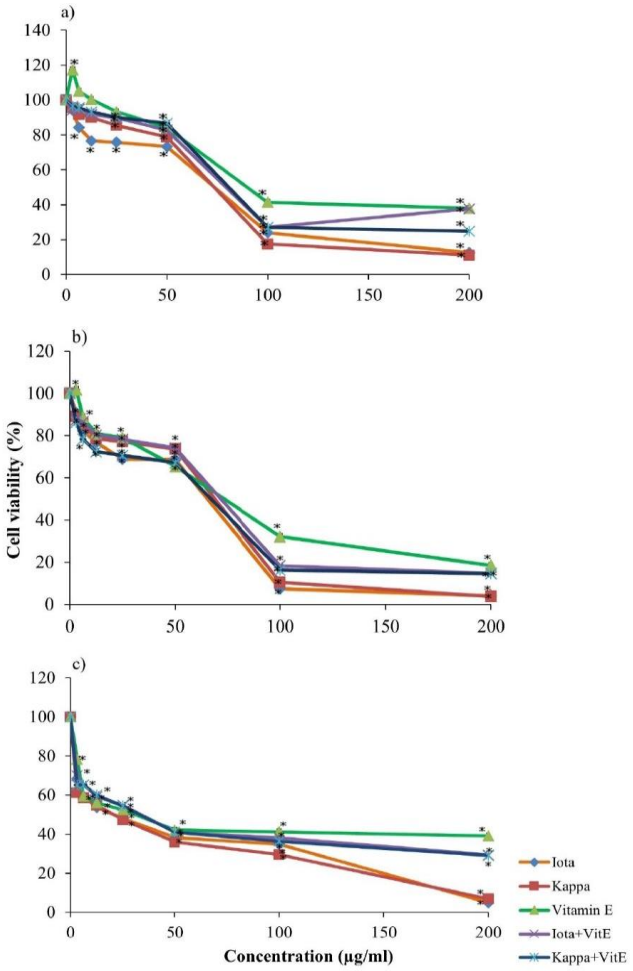

Figure 1. Percentage of A431NS cell viability after (a) 24 hours, (b) 8 hours, and (c) 72 hours treatment with carrageenans and/or vitamin E. The percentage of viability was determined based on OD_{570} of the treated cells compared to the control (taken as 100%), following MTT assay. The data are presented as mean of triplicates of each dose ± SD (n=3). SD=0.00-0.28 * indicates significant difference from the control (non-treated cells) value with ANOVA Bonferroni *post hoc* test, $p<0.05$, compared with treated cells for all concentrations.

B. Antiproliferative Effect

The results showed that carrageenans and/or α-tocopherol exhibited antiproliferative effects on A431NS cells in a dose-dependent manner since the cell proliferation was significantly decreased (Figure 2). After 24 and 48 hours of treatment, the combination of carrageenans and α-tocopherol showed more pronounced antiproliferative effects than carrageenans alone. No significant difference ($p > 0.05$) was observed between the isomers in terms of antiproliferative effects. α-tocopherol exhibited a lower cell proliferation only at high concentrations (50-200 µg ml^{-1}). Overall, the percentage of decrease in cell proliferation after 24 hours of treatment was 1.93 - 66.79% and 16.32 - 65.81% after 48 hours of treatment compared to the untreated cells (Figures 2 (a) and (b)). After 72 hours of treatment, no significant difference was observed between carrageenans alone and their combination with α-tocopherol since all groups showed a similar percentage of cell proliferation. Overall, the percentage of cell proliferation after 72 hours of treatment decreased to 10.88 - 50.40% (Figure 2 (c)).

The antiproliferative role of λ-carrageenan has been previously revealed in mice mammary carcinoma (4T1) cell lines (Luo *et. al.,* 2015; Mi *et al.,* 2008) and degraded κ-carrageenan has shown moderate cytotoxic effects to both intestine Caco-2 and liver HepG2 human cancer cell lines (Zainal Ariffim *et al.,* 2014). In another study, the antiproliferative ability of algal polysaccharides against HeLa cells was positively correlated with the sulphate content (Costa *et al.,* 2010). Similarly, the antiproliferative effects against A431NS cells observed in the present study could be due to the sulphate contents of carrageenans. However, further studies are required to validate this surmise. Our previous study also demonstrated the potential antioxidant effects of carrageenans, which could also be attributed to the sulphate moieties of the compound (Thevanayagam *et al.,* 2014). It would be worthwhile to investigate how the anticancer and antioxidative effects of carrageenans are related.

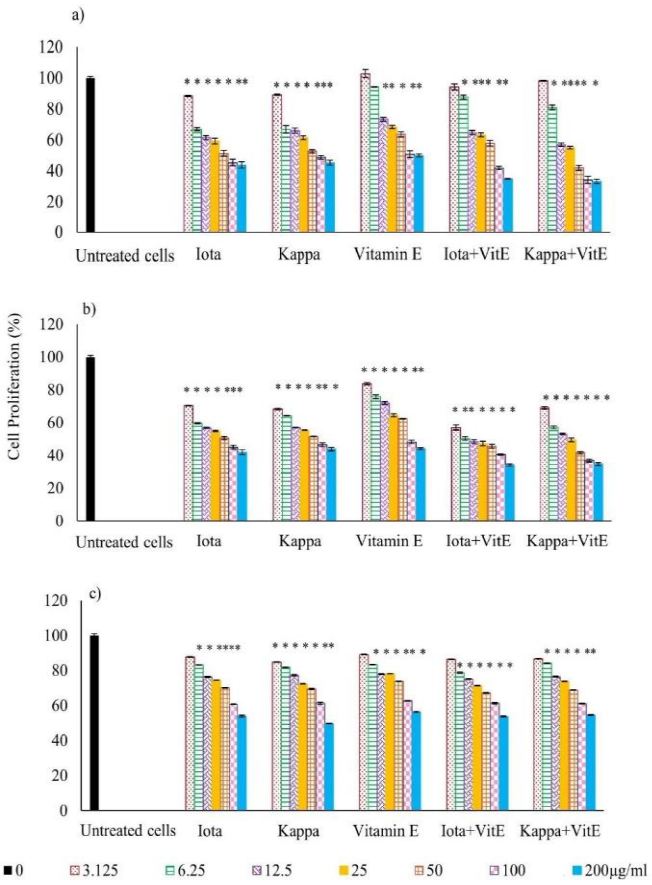

Figure 2. Percentage of A431NS cell proliferation after (a) 24 hours, (b) 48 hours, and (c) 72 hours treatment with carrageenans and/or vitamin E. The percentages were determined based on $OD_{480/535}$ following direct cell proliferation assay and calculated by comparing to the control (cells without treatment). The data are presented as mean ± SD (n=3). SD=0.12-2.55 * indicates significant difference from the control (non-treated cells) value with ANOVA Bonferroni *post hoc* test, $p<0.05$, compared with treated cells for all concentrations.

C. DNA Synthesis

A decline in active DNA synthesis was concentration dependent. After 24-hour treatment, DNA synthesis in the treated cells was reduced compared to the control. The decrease in DNA synthesis by various treatments is as follows: ι-carrageenan, 12.21 - 30.00%; κ-carrageenan, 9.40 - 30.71%; α-tocopherol, 4.42 - 28.32%; ι-carrageenan + α-tocopherol, 17.05 - 35.50%, and κ-carrageenan + α-tocopherol, 16.14 - 38.63% (Figure 3 (a)). After 48 hours, the percentage of DNA synthesis reduced further compared to the 24-hours treatment (Figure 3 (b)). In general, the treatment with carrageenans and/or α-tocopherol reduced the DNA synthesis of A431NS cells, whereas, the combination of carrageenans and α-tocopherol displayed the most profound inhibitory effect.

The present study showed the impaired function of DNA synthesis in both ι and κ treated cells compared to the untreated cells. The limitation of DNA synthesis was expected to cause antiproliferation in those cells. Impaired DNA synthesis could have occurred due to DNA fragmentation as observed via confocal microscopy (image not enclosed) (Thevanayagam, 2017). Thus, the proliferation of A431NS cells was limited after the treatment with carrageenans and/or α-tocopherol.

Lambda carrageenan has been shown to inhibit the proliferation of MDA-MB-231 breast cancer cells by up-regulating the pro-apoptotic genes caspase-8, caspase-9 and caspase-3 (Jazzara *et al.,* 2016). The cells treated with λ-carrageenan compared to the untreated one have shown visible shrinkage. The DAPI/PI stain demonstrated DNA condensation in the treated cells, which increased in a dose-dependent manner. Other observations, such as DNA fragmentation, cell shrinkage and DNA condensation indicated that the cells underwent programmed cell death (Jazzara *et al.,* 2016). In another study, ι-carrageenan exhibited cytotoxic activity against breast cancer cells by inhibiting cell proliferation and induced cell death through nuclear condensation and DNA fragmentation. It was revealed that induction of apoptosis occurred via the activation of the extrinsic apoptotic caspase-8 gene, which suggests that carrageenans are a potential therapeutic agent to target breast cancer via prompting apoptosis (Murad *et al.,* 2015).

In a previous study, mice fed with high calcium diet inhibited the DNA replication of colorectal cancer cells. DNA replication, cell division, and regulation of transcription were significantly downregulated by calcium (Aggarwal *et al.,* 2017). Carrageenans being high in calcium content could be playing a similar role by affecting DNA replication, hence, limiting the proliferation of the cancer cells. Based on the findings from the studies mentioned, carrageenans seemed to exhibit antiproliferative activities by promoting caspases-dependent apoptosis, cell cycle regulation and nuclear fragmentation. Hence, the present study postulates that the antiproliferative effects of carrageenans could be due to

their ability to induce apoptosis and inhibit DNA synthesis besides their antioxidative properties.

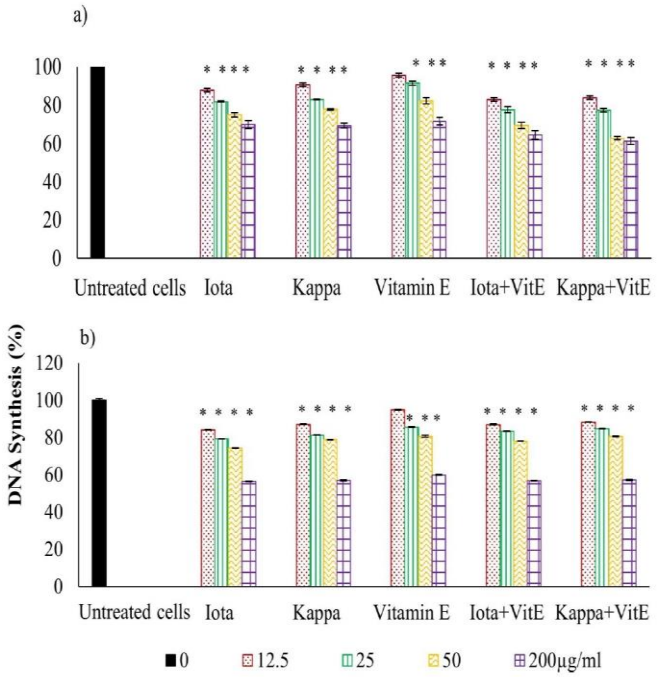

Figure 3. Percentage of DNA synthesis of A431NS cell death after (a) 24 hours, and (b) 48 hours treatment with carrageenans and/or vitamin E. The percentages were determined based on $OD_{568,585}$ following active DNA synthesis quantification assay and calculated by comparing with the control (cells without treatment). The data are presented as mean of triplicates of each dose ± SD (n=3). SD=0.09-2.24 * indicates significant difference from the control (non-treated cells) value with ANOVA Bonferroni *post hoc* test, $p<0.05$, compared with treated cells for all concentrations.

D. Apoptotic and Necrotic Cell Death

The results showed that carrageenans favoured apoptotic cell death rather than necrosis in A341NS cells after 24-, 48- and 72-hour treatments, which was concentration-dependent. After the 24-hour treatment, ι- carrageenan caused the highest apoptotic cell death (18.5 - 43.46%), followed by κ- (16.42 - 33.45%), then, the combination of ι- and α-tocopherol (15.11 - 21.18%) as well as κ- and α-tocopherol (2.88 - 18.9%). Necrotic cell death was very low (0.04 - 2.11%) in cells treated with carrageenans. The combination of carrageenans and α-tocopherol did not induce necrotic cell death. As for α-tocopherol, at the lowest concentration (3.125µg ml^{-1}), neither apoptotic nor necrotic cell death was observed but apoptotic cell death took place at higher concentrations (Figure 4(a)).

After the 48-hour treatment, the percentage of cell death increased in all groups, predominantly apoptosis. Cells treated with ι-carrageenan showed the highest apoptotic cell death (29.55 - 60.59%), followed by κ- (29.89 - 57.47%), as well as the combination of κ- and α-tocopherol (30.32 - 35.56%) and ι- and α-tocopherol (19.56 - 30.72%). Necrotic cell death was only 0.52 - 3.62% in cells treated with carrageenans. α-tocopherol treatment did not induce cell death at the low concentration tested but apoptotic cell death was observed at higher concentrations, which was 18.34 - 25.42% and necrotic cell death was 0.56 - 7.57% (Figure 4 (b)).

At the 72-hour treatment, both apoptotic and necrotic cell deaths increased in all the treated groups. In cells treated with ι-carrageenan, apoptotic cell death was between 35.89 - 46.31%, while cells treated with κ-carrageenan and α-tocopherol were 35.62 - 46.69% and 32.83 - 45.40% respectively. For the combination of carrageenans and α-tocopherol, apoptotic cell death was 34.71 - 45.40%. The percentages of necrotic cell death in all these groups ranged between 20.95 - 34.42% (Figure 4 (c)). Carrageenans and/or α-tocopherol treated cells could induce apoptotic cell death and the percentage was higher compared to necrotic death.

Increasing the levels of apoptosis in cancer cells may be an effective method of chemopreventative and chemotherapeutic intervention in numerous types of cancer (Kim & Karagozlu, 2011). The present study demonstrated that carrageenans and/or α-tocopherol treated cells could induce apoptotic cell death and the percentage was higher compared to necrotic death. Necrotic cells that were observed may be due to late-stage apoptosis, at a point where the cells finally enter necrosis after a long period of treatment (72 hours) and at the highest concentration (200µg ml^{-1}).

Apoptotic effects of carrageenans against other cancer cells have been reported. For instance, λ-carrageenan was shown to induce apoptosis in MDA-MB-231 cells. The Annexin-V analysis has demonstrated that 8% and 32% of the cells experienced apoptotic cell death after treatment with 12.5 and 25 µM of λ-carrageenan for 24 hours, respectively, while apoptotic cells did not exceed 3% of the population of the

non-treated cells. It has been reported that λ-carrageenan altered the apoptotic gene's activity in MDA MB 231 cells and could trigger the caspases-3 activity by 17 to 20% compared to untreated cells, which resulted in defective DNA repair and apoptotic cell death (Jazzara *et al.,* 2016). The domination of apoptosis over necrosis in all pre-treated cell populations in the present study is a good indicator that carrageenans contributed to the regulation of cell death pathways. This observation suggested that carrageenans toxicity via apoptotic rather than necrotic cell death in cancer cells could avoid inflammatory reactions and further adds value as a potential anticancer agent.

The present study showed that low concentrations of carrageenans (3.25 - 12.5 μg ml^{-1}) induced progressive flipping of the membrane phospholipids (revealed by the increase of Annexin V FITC) without the loss of membrane integrity (revealed by the PI negativity) of the cells. Intermediate concentrations (25-50 μg ml^{-1}) induced a prolonged roundup phase suggesting a delayed induction of the late apoptotic phases (Annexin V positive). In contrast, high concentrations (100 and 200 μg ml^{-1}) induced necrosis as revealed by the positive Annexin V FITC/PI test.

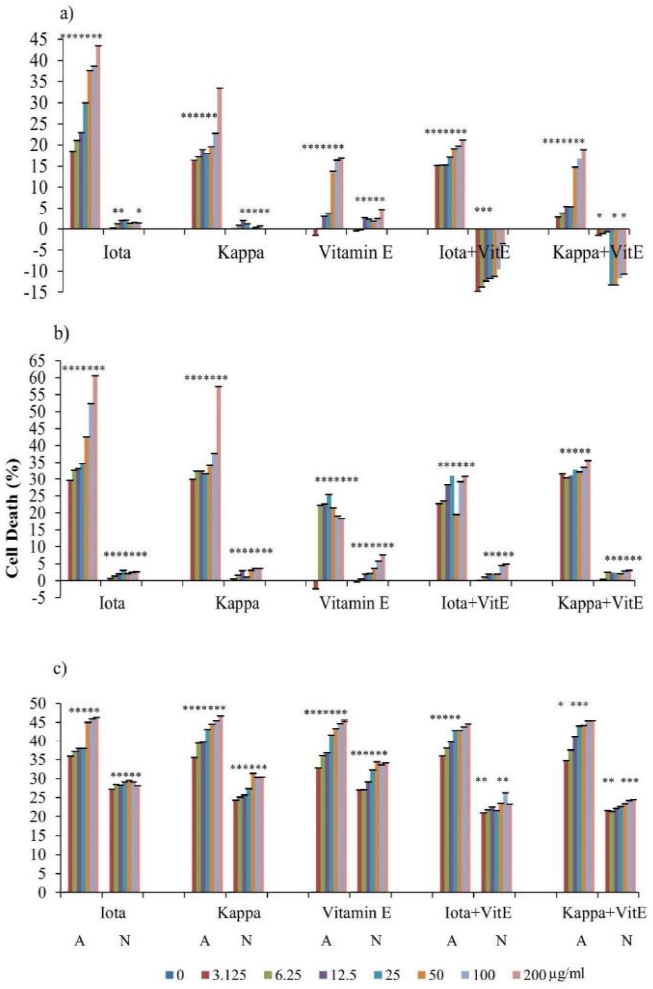

Figure 4. Percentage of apoptotic (A) and necrotic (N) cell death of A431NS cells after (a) 24 hours, (b) 48 hours, and (c) 72 hours treatment with carrageenans and/or vitamin E. The percentages were determined based on $OD_{485,560}$ following Annexin V FITC/PI assay and calculated by comparing to the control (cells without treatment). The control (non-treated cells) is taken as 0% cell death. The data are presented as mean of triplicates of each dose ± SD (n=3). SD=0.00-3.12 * indicates significant difference from the control (non-treated cells) value with ANOVA Bonferroni *post hoc* test, $p<0.05$, compared with treated cells for all concentrations.

E. TPT1 Gene Analysis

The expression of the TPT1 gene in cells treated with carrageenans or α-tocopherol was either suppressed or not expressed at all (value < 1.0), in contrast to the control (Figure 5). The gene expression in the untreated A431NS cells after 24 hours of seeding increased by 28.84-fold, indicating the up-regulation of the TPT1 gene. Furthermore, cells treated with α-tocopherol at 12.5 and 25μg ml^{-1} expressed this gene after 24 hours of treatment. The upregulation of this gene was also observed in cells treated

with the combination of ι- carrageenan and α-tocopherol at 12.5 μg ml^{-1}, and ι-carrageenan and κ-carrageenan at the highest concentration (50 μg ml^{-1}). Other treatment groups did not express the TPT1 gene, indicating a downregulation of the gene.

After 48 hours, the TPT1 gene was expressed in almost all the groups with 4.08-12.82-fold change in cells treated with carrageenans and α-tocopherol, compared to the untreated cells, which had the highest gene expression (101.83-fold change). Cells that did not express the gene were those treated with ι- carrageenan (0.46-fold change) and κ- carrageenan (0.16-fold change) at 12.5μg ml^{-1}. Overall, the treatment with carrageenans and/or α-tocopherol suppressed the expression of the TPT1 gene compared to the untreated cells. However, α-tocopherol by itself showed a lower suppression compared to the other treatment groups, including those treated with the combination of carrageenans and α-tocopherol (Figure 5). α-tocopherol showed stronger suppression of TPT1 gene expression only at high concentrations (25 and 50 μg ml^{-1}).

The present study showed a downregulation or complete suppression of the TPT1 expression in cells treated with carrageenans and/or α-tocopherol compared to the untreated cells. The ability of carrageenans to suppress TPT1 gene expression suggests the potential use of the bioactive compound as an anti-cancer agent against skin cancer. The TPT1 gene was significantly downregulated in revertant tumour cells, suggesting that inhibition of the gene expression could revert cancer cells into normal phenotypes (Tuynder *et al.,* 2002). The expression of TPT1 upregulation confers protection against cell death by enhancing the antiapoptotic response and suppressing proapoptotic activities (Liu *et. al.,* 2005; Lucibello *et al.,* 2011). Similar to the reported observation, the present study also showed that after 48 hours of incubation, the untreated group's TPT1 expression was higher than in the 24 hours period, indicating the role of TPT1 in inhibiting apoptosis while promoting cancer cell proliferation. Furthermore, Wu *et al.* demonstrated that TPT1 prevented apoptosis by destabilising p53, whereas, the overexpression of TPT1 promoted the degradation of p53. They also demonstrated that TCTP was overexpressed in cutaneous SCC cell line A431 compared to normal skin keratinocyte cell line, HaCaT, which significantly increased with the malignancy grade. Using a small interfering RNA (siRNA) gene silencing approach, the authors also showed that downregulation of TCTP expression was associated with decreased cell proliferation and increased apoptosis in A431 cells (Wu *et al.,* 2012).

The anti-cancer effect following the knockdown of TPT1 gene expression has been demonstrated in other cancer cell lines as well as in animal models. For instance, in a cultured colon cancer cell line, knockdown of TPT1 was shown to inhibit cell proliferation, migration, and invasion. In an in vivo study, the knockdown of TPT1 reduced tumour growth and liver metastasis when the colon cancer cells were injected into nude mice (Ma *et al.,* 2009). Similarly, siRNA-mediated downregulation of TPT1 in prostate cancer cells inhibited cell growth and enhanced apoptosis through the activation of caspase-8 and caspase-3 (Gnanasekar *et al.,* 2009). The regulation of TPT1 gene expression is proven to be important in cancer therapy, therefore, the ability to downregulate the expression of this gene could be one of the key anticancer mechanisms of carrageenans.

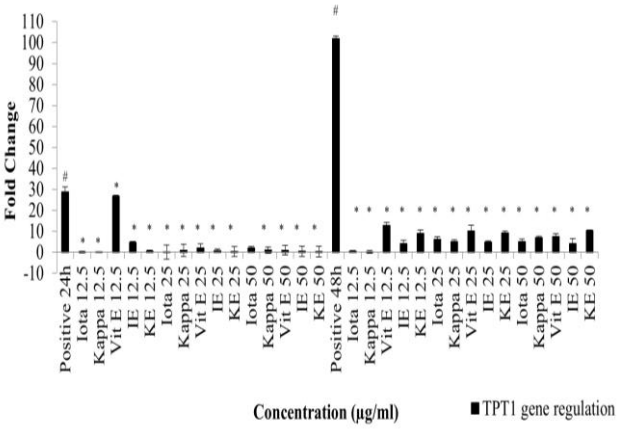

Figure 5. TPT1 gene expression in A431NS cells after 24- and 48-hours treatment with carrageenans and/or vitamin E. The gene expression was determined by fold change values using real-time PCR assay with the reference to the control delta C_T values of HaCaT cells and housekeeping gene (PGK1). The data are presented as mean of triplicates of each dose ± SD (n=3). SD=0.15-5.12 * indicates significant difference from the positive control (non-treated cells) value with ANOVA Bonferroni *post hoc* test, $p<0.05$, compared with treated cells for all concentrations. # indicates significant difference from the control (HaCaT cells) value with ANOVA Bonferroni *post hoc* test, $p<0.05$ compared with positive cells.IE is iota and vitamin E, KE indicates kappa and vitamin E.

IV. CONCLUSION

Carrageenans were found to exhibit cytotoxic and antiproliferative effects against A431NS cells, thus, showing the potential of the seaweed polysaccharide as an anticancer agent. Furthermore, the present study showed that carrageenans could inhibit DNA synthesis in cancer cells while exhibiting apoptotic action and downregulating the TPT1 gene expression.

The antiproliferative effect of carrageenans could be due to the DNA synthesis arrest and the enhancement of apoptotic cell death. The downregulation of the TPT1 gene expression observed in the present study warrants further work to explore the potential for gene-targeted anticancer drugs or treatment for skin cancer based on carrageenans.

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VI. CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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