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CYTOTOXIC ACTIVITY OF *EUCHEUMA COTTONII* ON MCF-7 HUMAN BREAST CANCER

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ABSTRACT Studies of seaweeds are essential as these marine macro algae are the only source that produces agar, alginate and carrageenan. *Eucheuma cottonii* belongs to the genus Eucheuma, in the division of Rhodophyta. It is one of the important genera of carrageenophytes. It was reported to possess anticancer effects for the prevention and management of cancers. This study investigates the cytotoxic effect of crude extracts from *E. cottonii* on breast cancer cell line (MCF-7) in vitro. *E. cottonii* collected from Sabah was used for preparation of crude extracts using absolute methanol. The cytotoxic effect of the extract was determined using MTT assay whereas cell apoptosis was detected using GeneTex Enhanced Apoptotic DNA Ladder Detection Kit. Result of MTT assay shows that the growth of MCF-7 cancer cells was suppressed by the crude extracts of *E. cottonii* in a dose-dependent manner of IC50 at 3.5mg/mL (24h) and 0.85mg/mL (48h). The presence of fragmented DNA of cells treated with the crude extract indicated cytotoxic effect is through apoptosis. In conclusion, *E. cottonii* extract that exerts cytotoxic effect on the MCF-7 cancer cell line is dose dependent and via apoptosis induction. It can be a potential source of anticancer drugs from nature.

ABSTRAK Kajian rumpai laut adalah penting sebab makro alga laut ini adalah satu-satunya sumber yang menghasilkan agar, alginat dan carrageenan. *Eucheuma cottonii* yang tergolong dalam genus Eucheuma adalah di bahagian Rhodophyta dan ia adalah salah satu genus penting carrageenophytes. Ia dilaporkan mempunyai kesan anti kanser. Oleh itu, ia boleh digunakan untuk pencegahan dan pengurusan kanser. Kajian ini menyiasat kesan sitotoksik ekstrak mentah *E. cottonii* terhadap sel kanser payudara manusia (MCF-7) secara in vitro. *E. cottonii* yang dikumpul dari Sabah digunakan untuk penyediaan ekstrak mentah dengan menggunakan metanol pekat. Kesan sitotoksik ekstrak ini ditentukan dengan menggunakan ujian MTT, manakala apoptosis sel dikesan menggunakan GeneTex Enhanced Apoptotic DNA Ladder Detection Kit. Keputusan yang diperoleh dalam ujian MTT menunjukkan bahawa pertumbuhan sel-sel kanser MCF-7 itu telah dihalang oleh ekstrak mentah *E. cottonii* dengan cara yang bergantung kepada dos, dengan nilai kepekatan perencatan 50% pertumbuhan (IC50) di 3.5mg/mL (24 jam) dan 0.85mg/mL (48 jam). Kewujudan fragmen DNA daripada sel-sel yang dirawati dengan ekstrak mentah E. cottonii menunjukkan bahawa sitotoksik adalah melalui apoptosis. Kesimpulannya, ekstrak *E. cottonii* mengenakan sitotoksik pada sel kanser payudara manusia (MCF-7) adalah bergantung kepada dos dan melalui induksi apoptosis. Ia boleh menjadi satu sumber ubat anti kanser yang berpotensi dari alam semulajadi.

(**Keywords**: surface bacteria, mobile phones, different form factors, *Staphylococcus aureus*)

INTRODUCTION

Seaweeds are marine macro algae that can be found attached to the bottom of shallow coastal waters which contain characteristics of non-flowering plant such as no flowers, true shoot, root, stem and leaf system [1]. Their polysaccharides, phospholipid and glycolipid fractions were proven to have antitumor activity to mice even though the host immunological functions were yet to be clarified [2, 3]. The compounds that are proposed to be found in red seaweed are Chlorophyll *d*, Pycobiliproteins, Allophycocyanin, C-phycocyanin, R-phycocanin, B-phycoerythrin, β-carotene, α-carotene, sterols (C27 sterols, cholesterol, desmosterol, 22-dehydrocholestrol), unsaturated C20 fatty acid (20:4 ω6, 20:5 ω5), Vitamin B12 and Taurine [4]. The sample of this study, *Eucheuma cottonii* Weber-van Bosse, is a type of edible red seaweeds that mainly harvested in the Phillipines and Indonesia. This kind of red seaweeds has a high commercial value as it is one of the main sources of kappa carrageenan [5]. Carrageenans, a sulfate linear polysaccharides extracted from red seaweeds have been found of consisting several potential pharmaceutical uses such as antitumor, antiviral, anticoagulant and immunomodulation functions [6, 7, 8].

It has previously reported that polyphenol-rich *E. cottonii* extract can suppress breast tumour via hormone modulation and apoptosis induction [9]. As in other countries, breast cancer is described as the most common cancer that happens among women in Malaysia [10]. Previously, there is evidence showing that *E. cottonii* has good potential in treating cancer which can be explained by the properties of this seaweed that comprised of high amount of dietary fibres, minerals, vitamins, antioxidants, polyphenols, phytochemicals, proteins and polyunsaturated fatty acids [11]. It is concluded that this seaweed can be utilized in the future for the purpose of prevention and management of cancers, especially on breast cancer as *E. cottonii* polyphenol-rich extract (ECME) was found to have the ability to suppress breast tumour through hormonal modulation, apoptosis induction, and oxidative status modulation [9].

The objectives of this study are to determine the cytotoxic activity of breast cancer cell line (MCF-7) using the *E. cottonii* crude extracts and to determine the effective concentration of this crude extract that able to induce apoptosis on this cell line. In this study, cell viability assay and DNA ladder kit assay have been used to investigate the cytotoxic effect of crude extracts from *E. cottonii* on breast cancer cell line (MCF-7). The viability of cells was determined using MTT assay whereas GeneTex Enhanced Apoptotic DNA Ladder Detection Kit was used to detect cell apoptosis.

MATERIALS AND METHODS

Preparation of crude extracts from Eucheuma cottonii

E. cottonii seaweed samples used were from Sabah, East Malaysia. Seaweed samples were washed and grinded in liquid nitrogen [12]. A total of 10g of grinded samples were weighed and transferred into a 250mL conical flask. Next, 150mL of methanol was added into the flask and the samples were incubated overnight at room temperature with stirring at 200rpm. After 24h, the solvent extracts were filtered through a filter paper and the filtrate was concentrated by rotary evaporation at 45-50°C. Once the evaporation process was done, the resulting crude extracts were dissolved in DMSO and kept in 4°C.

Cell culture

MCF-7 (Human Breast Adenocarcinoma cell line) was cultured with EMEM medium supplemented with 10% of FBS. The T-25 flask was incubated in laminar flow. The spent medium was discarded and 2mL of 1x PBS was added into the flask to rinse the cell monolayer. The PBS was then discarded followed by adding 1mL of trypsin solution. Next, the T-25 flask was slightly rotated to cover the entire cell monolayer with trypsin. The flask was then incubated for 5-10 min or until the monolayer can be seen detaching from the culture surface. Then, the cells were examined under an inverted microscope to ensure the cells were detached and floating [13]. A total volume of 2mL EMEM medium was added into the flask and the cells were dispersed by repeat pipetting. Subculture was carried out in a ratio of 1:4 (cell suspension: culture medium) by adding 1mL of cell suspension with 4mL of EMEM medium to a new T-25 flask. The T-25 flask was then incubated in CO_2 incubator at 37°C and the cells were checked routinely using inverted microscope to monitor the cell growth.

Cell viability test with MTT assay

A total of 14 sets of triplicate test including blank, positive control, negative control, solvent control and a range of concentration of *E. cottonii* crude extracts: 0.1mg/mL, 0.3mg/mL, 0.5mg/mL, 0.7mg/mL, 0.9mg/mL, 1.0mg/mL, 3.0mg/mL, 5.0mg/mL, 10.0mg/mL and 15.0mg/mL were carried out in a 96 well plate. The positive control was 50% DMSO, prepared by adding 100µL of absolute DMSO with 100µL of EMEM, and the negative control used in this study was 10,000 cells with a total volume of 200µL EMEM. MCF-7 cells were counted with hemocytometer by trypan blue exclusion assay followed by plating 10,000 cells in each well with a total volume of 200µL EMEM with the exception of the blank set. The 96 wells plate was incubated in CO_2 incubator overnight. The spent medium was then discarded on the next day by pipette it out from each well. Next, 200µL of different concentrations of *E. cottonii* crude extracts were added to each well accordingly. The 96 wells plate was then incubated in CO_2 incubator for 24h. After 24h, 50µL of MTT reagent was added to each well and the 96 wells plate wrapped with aluminium foil was again returned to CO_2 incubator for another 4h until purple precipitate is visible [14]. After that, all the solution in each well was discarded and 200µL of absolute DMSO was added into each well to dissolve the formazan crystal. Then, the plate

cover was removed and the absorbance in each well was measured at 570nm with a reference filter of 630nm using an ELISA reader. The absorbance readings were recorded. All the steps were repeated with incubation time for 48h.

Induction and detection of apoptosis in MCF-7 cells

MCF-7 cells were counted with hemocytometer by trypan blue exclusion assay. A total volume of $5x10^5$ cells were plated into a T-25 flask with 5mL of EMEM medium. The T-25 flask was then incubated in CO_2 incubator overnight. After that, the spent medium was discarded on the next day and 1mL of 5x 0.1mg/mL *E. cottonii* crude extracts and 4mL of EMEM were added into the T-25 flask. Then, the T-25 flask was returned to CO_2 incubator for 24h. All the steps were repeated for 5x 0.3mg/mL, 5x 0.5mg/mL, 5x 0.7mg/mL, 5x 0.9mg/mL, 5x 1.0mg/mL, 5x 1.5mg/mL, 5x 2.0mg/mL, 5x 2.5mg/mL and 5x 3.0mg/mL.

After 24h, apoptosis was induced in MCF-7 cells and the control without induction was incubated concurrently. The cultured medium with non-viable cells was collected in a 15mL falcon tube. Then, the non-viable cells were pellet down by centrifugation for 5 min at 200xg. The supernatant was discarded carefully with 1mL of supernatant left in the falcon tube. The pellet was resuspended and the cell suspension was transferred into a 1.5mL microcentrifuge tube. After that, the non-viable cells were pellet down for another 1 minute at 13,000rpm. The supernatant was pipetted out carefully. Cells were lysed with 35µL of TE Lysis Buffer by repeat pipetting. A total volume of 5µL of Enzyme B Solution was added to the crude lysate. The lysate was incubated at 50°C in a water bath for 30 min. Then, 5µL of ammonium acetate solution was added into the sample and mixed well by repeat pipetting. Isopropanol that kept at -20°C was taken out and a total volume of 50µL isopropanol was transferred into the sample. The sample was incubated at -20°C for 10 min. After 10 min, the sample was centrifuged for 10 min at 13,000rpm to precipitate the DNA. The supernatant was discarded carefully. A total volume of 0.5mL of 70% cold ethanol was added in to wash the DNA pellet and the sample was pellet down for another 10 min at 13,000rpm. The supernatant was discarded and the DNA pellet was air-dried at room temperature. Then, 20µL of DNA Suspension Buffer was added and the suspension was mixed well by repeat pipetting to dissolve the DNA pellet. After that, 5µL of each samples were mixed with 2µL of 6 X loading dye and loaded on a 1.8% agarose gel electrophoresis at 60V for analysis purpose. The gel was allowed to run until the yellow dye ran to the edge of the gel. Ethidium bromide was used to stain the DNA in this study. The stained DNA ladder was visualized by illumination of 254nm UV wavelength and the gel image was printed [15].

RESULTS AND DISCUSSION

Cell viability test with MTT assay

In this study, *in vitro* cytotoxic activity of *E. cottonii* seaweed extract at various concentrations against MCF-7 cancer cell line was carried out using MTT assay. MTT cell viability test was carried out in this study with a range of concentration of *E. cottonii* crude extract from 0.1mg/mL to 15.0mg/mL. The percentage of MCF-7 cells viability for 24h and 48h incubation time were stated in Tables 1 and 2 respectively. From the results, it shows that growth of the MCF-7 cells was successfully inhibited by the crude extracts from *E. cottonii* after incubated for 24h and 48h. Based on results in Table 1 and Table 2, the effect of *E. cottonii* crude extract to MCF-7 cells was in a dose dependent manner in both time lengths.

Table 1. The effect of *E. cottonii* extract towards cell viability of MCF-7 (24h).

Samples	Doses	Percentage of cell viability (%)
Solvent control		100.0
Crude extracts of *E. cottonii* (mg/mL)	0.1	74.8
	0.3	67.5
	0.5	62.7
	0.7	61.3
	0.9	57.8
	1.0	55.6
	3.0	51.2
	5.0	44.9
	10.0	5.0
	15.0	4.1

Table 2. The effect of *E. cottonii* extract towards cell viability of MCF-7 (48h).

Samples	Doses	Percentage of cell viability (%)
Solvent control		100.0
Crude extracts of *E. cottonii* (mg/mL)	0.1	70.4
	0.3	62.5
	0.5	58.0
	0.7	57.1
	0.9	47.1
	1.0	39.0
	3.0	34.1
	5.0	30.3
	10.0	11.1
	15.0	7.2

According to Table 1 and 2, the cell viability for 1.0 mg/mL was 55.6% and 39.0% respectively. The difference in cell viability for same dosage can be explained by the difference in cell passage number as the MTT cell viability test for 48h was repeated only after a few weeks from the first MTT cell viability test. In other word, the MCF-7 cells used for the repeat test have been subcultured for few times before the second MTT cell viability test was carried out. Furthermore, there is also evidences stated that responses to drug treatment can be influenced by the occurrence of complex changes in a variety of cell cycle kinetic and biochemical parameter during *in vitro* growth [16].

Based on the results, growth of MCF-7 cells was found to be suppressed by the crude extract of *E. cottonii* in a dose dependent manner of IC_{50} at 3.5mg/mL (24h) and 0.85mg/mL (48h). Cells viability percentage was found to decrease gradually when the doses are increasing from the lowest concentration to the highest concentration for both

24h and 48h. This infers the existence of dose dependent properties of *E. cottonii* extract against the human breast carcinoma, MCF-7 cells. Previously, there is evidence showing that *E. cottonii* did not cause any significant differences on the HeLa cell viability which was treated for 24h and 48h [17].

Induction and detection of apoptosis in MCF-7 cells

In this study, detection of DNA fragmentation has been chosen as a way to show and analyse the induction of apoptosis in MCF-7 cells by crude extracts of *E. cottonii*. Cell apoptosis was then detected using GeneTex Enhanced Apoptotic DNA Ladder Detection Kit and the results were visualized using gel electrophoresis. Results of apoptosis induced by various concentrations of *E. cottonii* crude extracts were shown as in Figure 1.

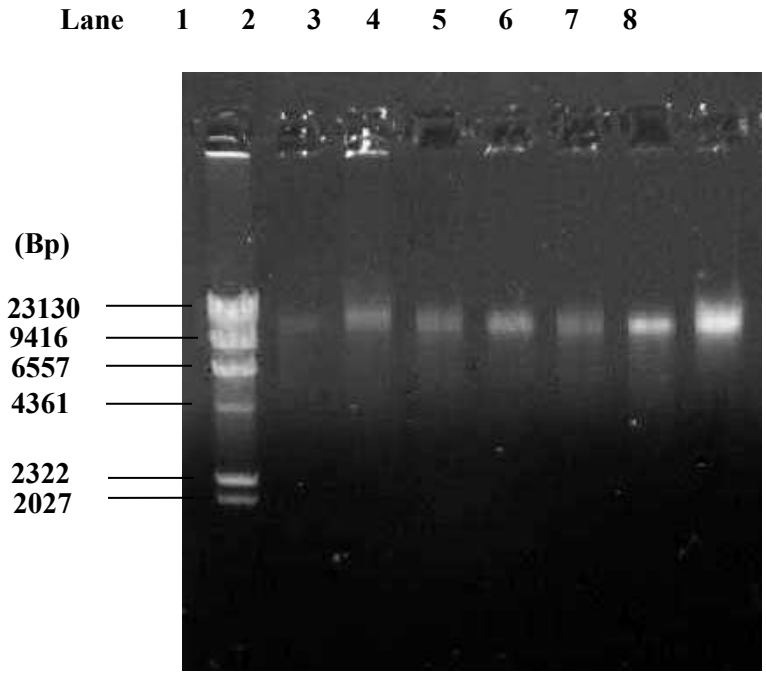

Figure 1. DNA gel electrophoresis of DNA fragmentation in 1.8% agarose gel after treating with crude extracts of *E. cottonii*. Various concentrations of *E. cottonii* crude extracts were used to induce apoptosis. (1) DNA lambda marker (2) 0.9mg/mL (3) 1.0mg/mL (4) 1.5mg/mL (5) 2.0mg/mL (6) 2.5mg/mL (7) 3.0mg/mL (8) Negative control without treatment.

Apoptosis in MCF-7 was induced with various concentrations of crude extracts of *E. cottonii*. From the gel image as shown in Figure 1, lower concentrations such as 0.1mg/mL, 0.3mg/mL, 0.5mg/mL, and 0.7mg/mL were not included in the result due to the absence of cells pellet after the centrifugation step. Absence of cells pellet can be explained by low amount of dead cells which were not enough for the detection of apoptosis. According to the results of MTT assay, it shows that the percentage of MCF-7 cell viability for 24h does not reach IC_{50} even though a total of 1.0mg/mL of *E. cottonii* crude extracts was used. In addition, the minimum concentration of *E. cottonii* crude extracts to cause 50% inhibition of biological activity of MCF-7 cells was 3.5mg/mL. Therefore, the results of MTT assay supported the inference which stated that MCF-7 cells viability did not decreased much at low concentration like 0.1mg/mL, 0.3mg/mL, 0.5mg/mL, and 0.7mg/mL and thus, only little amount of dead cells can be obtained.

In comparison with other lanes (Lane 2 to 7), the result shown in Lane 2 (0.9mg/mL) was found to be fairer due to the lower amount of dead cells as compared to other concentrations. MCF-7 cells treated with different concentration of *E. cottonii* crude extracts were always observed under a

microscope before pellet down the non-viable cells by centrifugation. From the observation, the amount of floating cells that represented the dead cells after treating was found to be increasing with the concentration of *E. cottonii* crude extracts. However, the increase was not very obvious. This can be seen through the results shown in Figure 1, which does not gives an increasing result showing that higher dose of *E. cottonii* crude extract will deliver a more significant DNA fragmentation when compared to lower dose. Hence, it does not mean that the higher the doses added in to induce apoptosis, the more significant apoptosis or definitely the apoptosis to be occurred or to be induced.

In order to analyse the induction of apoptosis in MCF-7 cells by crude extracts of *E. cottonii*, detection of DNA fragmentation was used in this study, where DNA fragmentation was stated as one of the most frequently used techniques to detect and study apoptotic cells. Gel electrophoresis of genomic DNA is one of the traditional methods for demonstrating internucleosomal DNA degradation, whereby the resulting DNA fragmentation is the most classical features for apoptosis. Previously, research indicates that DNA in apoptotic cells is degraded specifically and the DNA will show a characteristic ladder pattern on the gel, whereas DNA in necrotic cells is degraded randomly by extracellular DNAse I or by lysosomal DNAse II, giving rise to a smear of DNA [18].

During apoptosis, DNA fragmentation was induced by caspase-mediated cleavage of inhibitor of caspase-activated DNase (ICAD), followed by the activation of caspase-activaed DNase (CAD) [19]. This event then leads to characteristic internucleosomal DNA double-strand breaks with fragments of multiples of 180 base pairs in size. As compared to necrotic cell death which is accompanied by late and random DNA fragmentation through the release of lysosomal, techniques that detect DNA fragmentation however, are not specific to apoptosis as it may sometimes detect DNA damage in variety types of cell death [20]

The occurrence of DNA fragmentation was shown in Lane 3, Lane 4, Lane 5, Lane 6, and Lane 7 (1.0mg/mL, 1.5mg/mL, 2.0mg/mL, 2.5mg/mL and 3.0mg/mL respectively) (Figure 1). According to the gel image, the result indicated that MCF-7 cells have undergone apoptosis as significant DNA fragmentation can be observed in Figure 1. As compared to the negative control (Lane 8) which gives a thicker band with a little smear, other lanes (Lane 2 to 7) show a thinner band with a longer smear and this was inferred as DNA fragmentation even though DNA ladder does not present. DNA fragmentation usually occurs in the later phase of apoptosis [21]. Therefore, the absence of a DNA ladder does not means that the cells do not undergo early apoptosis. Furthermore, DNA fragmentation might occur during preparation which making it difficult to generate a nucleosome ladder.

Previously, researchers have concluded that specific biochemical analyses such as DNA ladders, as well as the presence of proteolytically active caspases or the cleavage products of their substrates should not be used as an exclusive means to define apoptosis [22]. This is because apoptosis can sometimes occur without oligonucleosomal DNA fragmentation, and the presence of active caspases or the specific products of their enzymatic activity can sometimes related to non-lethal biological processes. However, the measurement of DNA fragmentation and measurement of caspase activation may be useful in diagnosing apoptosis.

CONCLUSION

E. cottonii was previously reported to have good potential in treating human breast adenocarcinoma (MCF-7 cells). This study aimed to determine the cytotoxic activity of breast cancer cell using the crude extracts *Eucheuma cottonii,* as well as the effective concentration of this crude extract that able to induce apoptosis on breast cancer cell line. Result of MTT assay showed satisfactory result indicated that cell viability percentage of MCF-7 after treated for 24h and 48h was found to decrease gradually when the doses are increasing. Growth of MCF-7 cells was suppressed by the crude extract of *E. cottonii* in a dose dependent manner of IC_{50} at 3.5mg/mL (24h) and 0.85mg/mL (48h). On the other hand, concentrations of 1.0mg/mL, 1.5mg/mL, 2.0mg/mL, 2.5mg/mL and 3.0mg/mL have shown an induced apoptosis to the MCF-7 cells. In contrast to necrosis, apoptosis is preferred in this study as it will not provoke any inflammation response to the neighbouring cells after the cells dead.

Since some of the substances in *E. cottonii* are still remains unknown, further research need to be explored to study the bioactive compounds of *E. cottonii* as well as for the successful implication of them to become a potent therapeutic tool against cancer. This study has shown that crude extracts of *E. cottonii* can inhibit the growth of the human

breast adenocarcinoma cells (MCF-7) and was through induction of apoptosis on MCF-7 cells. In regards to the significant results of this study, further investigations such as evaluation of *in vivo* anticancer activity of *E. cottonii* is recommended which may lead to finding new effective natural antitumor compound. Besides that, other assay can be carried out or same methods can be used to test on other types of cancer.

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