ฤทธิ์ยับยั้งการเจริญและกระตุ้นอะโพโทซิส ของสารสกัดจาก *Sargassum binderi* Sonder ที่มีต่อเซลล์มะเร็งปากมดลูก

Antiproliferative and Apoptosis-Inducing Activities of Extracts from *Sargassum binderi* Sonder on Human Cervical Cancer Cells

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บทคัดย่อ

สาหร่ายสีน้ำตาลมีส่วนประกอบหลายชนิดที่ออกฤทธิ์ยับยั้งเซลล์มะเร็งและกระตุ้นอะโพโทซิส การศึกษานี้ได้ทดสอบฤทธิ์ของสารสกัดจากสาหร่ายสีน้ำตาล *Sargassum binderi* Sonder (SB) บริเวณชายฝั่งทะเลอ่าวไทย ต่อการยับยั้งเซลล์มะเร็งปากมดลูก (HeLa) และอะโพโตซีส ตัวอย่างสดของ SB ถูกนำมาสกัดด้วย dichloromethane และ ethyl acetate (1:1) ได้เป็นสารสกัดหยาบ นำมาบ่มกับเซลล์ที่ความเข้มข้นแตกต่างกันนาน 72 ชั่วโมง ศึกษาการเจริญเติบโตของเซลล์ โดย MTT assay นับจำนวนนิวเคลียสที่มีลักษณะอะโพโทซิสโดยการย้อมสี 4'-6-Diamidino-2-phenylindole (DAPI) และ Propidium iodide (PI) จากนั้นศึกษาการแตกของ DNA โดย agarose gel electrophoresis สารสกัด SB ทำให้เซลล์ตาย โดยการตายเพิ่มขึ้นตามขนาดความเข้มข้นที่เพิ่มขึ้น ค่าความเข้มที่ยับยั้งการเจริญของเซลล์ได้ 50% คือ 90 ± 6.35 µg/ml โดยเซลล์ที่ตายหลุดจากพื้นผิวง่าย มี apoptotic body เซลล์มีลักษณะกลม เมื่อเทียบกับกลุ่มควบคุมที่เหยียดเกาะพื้นเป็นรูปกระสวย การประเมินเชิงปริมาณโดยย้อมสีนิวเคลียสด้วย DAPI และ PI พบโครมาตินหนาแน่น นิวเคลียสแตก เมื่อเทียบกับกลุ่มควบคุมที่มีลักษณะกลมติดสีเรียบเนียน พบเซลล์มีชีวิตที่มีลักษณะอะโพโตซีส 36.66 ± 3.2%, เซลล์ตายแบบอะโพโตซีสระยะหลัง 17.01 ± 1.82% และเซลล์ปกติ 46.33 ± 4.27% นอกจากนี้ยังพบการแตกของ DNA ฟุ้งกระจายใน agarose gel ผลการทดลองครั้งนี้แสดงให้เห็นว่าสารสกัด SB ทำให้เซลล์ตายร่วมกับการเปลี่ยนแปลงโครงสร้างที่เป็นลักษณะเฉพาะของอะโพโตซีส เช่น ผนังเซลล์เป็นตุ่ม โครมาตินหนาแน่น นิวเคลียสและ DNA แตก ซึ่งอะโพโตซีสเป็นกลยุทธิ์สำคัญที่ใช้รักษาโรคมะเร็ง ในการศึกษาครั้งต่อไปควรศึกษาเชิงลึกถึงกลไกของอะโพโทซิส

คำสำคัญ : *Sargassum binderi* Sonder HeLa cells ยับยั้งการเจริญเติบโต อะโพโทซิส การแตกของ DNA

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Abstract

Brown seaweeds contain a wide variety of compounds that inhibit cell proliferation and stimulate apoptosis. In this study, we investigated the antiproliferative and apoptotic properties of *Sargassum binderi* Sonder (SB) from the east coast of the Gulf of Thailand using human cervical cancer cell line (HeLa) as a model system. The fresh samples were extracted with dichloromethane and ethyl acetate (1:1) to afford crude extracts. The SB extractions were treated with HeLa cells (72 hours) and cell proliferation was determined by MTT assay. The quantitation of apoptotic nuclear morphology was counted using fluorescent double staining: 4'-6-Diamidino-2-phenylindole (DAPI) and Propidium iodide (PI). Qualitative analysis of DNA fragmentation by agarose gel electrophoresis was observed. The SB extractions inhibited the proliferation of HeLa cells in a dose-dependent manner with an IC_{50} of 90 ± 6.35 µg/ml. Morphological alteration in SB-treated HeLa cells were detached from the surface and rounded with apoptotic body when compared with cuboid and polygonal in control cells. Nuclear morphology stained with DAPI and PI exhibited chromatin condensation and nuclear fragmentation as compared to control with rounded nuclei. Quantitative estimation were 36.66 ± 3.2% (apoptotic nuclei), 17.01 ± 1.82% (late apoptotic nuclei), and 46.33 ± 4.27% (normal nuclei). Qualitative DNA fragmentation by agarose gel electrophoresis showed undefined outline due to DNA diffusing into agarose. These results indicated that SB-induced cell death via morphological changes typical of apoptosis including membrane blebbing, chromatin condensation, nuclear and DNA fragmentation. Because apoptosis may have a major impact on the therapy of cancer, further investigation is needed to confirm and characterize the apoptotic pathway.

Keywords : *Sargassum binderi* Sonder, HeLa cells, Antiproliferation, Apoptosis, DNA fragmentation

Introduction

Seaweed has long been used in traditional medicine in the treatment of cancer and now is being confirmed by modern scientific research as a challenge worldwide. Based on epidemiological data, consumption of seaweeds is the best source of proteins, carotenes, chlorophyll, vitamins, iodine and minerals and contributes the anticancer activities (Fujimoto *et al*., 1979). Seaweeds sometimes yield new compounds which are not possible in land plants. A lot of seaweeds and their metabolites showed potent cytotoxic activities which played an important role for antitumor drugs (Jha & Zi-rong, 2004). Brown seaweeds are known to be rich in carotenoids and sulphated polysaccharides with tremendous pharmacological actions especially anti-herpes simplex viruses (Ponce *et al*., 2003) antioxidative (Sachindra *et al*., 2007), antiadhesive (Liu *et al*., 2000; Rocha *et al*., 2001), antivasculogenic (Dias *et al*., 2008), anticoagulative (Silva *et al*., 2005) activities.

The edible brown seaweed, *Undaria pinnatifida* (Wakame in Japanese), inhibited the proliferation of human breast cancer cells by inducing apoptosis (Funahashi *et al*., 1999) and also reported a strong preventive effect against the development of breast cancer induced by 7,12 dimethylbenz anthracene (DMBA) in rats (Funahashi *et al*., 2001). The water extract of Mekabu induced apoptosis in a human breast cancer cell line (MDA-MB231) via caspase activation and DNA fragmentation in agarose gel. The main components of Mekabu proteins, carbohydrate, minerals and vitamins but a well-known special component is fucoidan. However, this extract is the natural foodstuff and it is safe for humans making it a very attractive substance (Sekiya *et al*., 2005).

Fucoidan isolated from *Ascophyllum nodosum* showed an anti-proliferative effect on both normal and malignant cells, including fibroblasts, sigmoid colon adenocarcinoma cells (Ellouali *et al*., 1993). The sulfate fucoidan from Korean brown seaweeds have anticancer activity and it seem affect on DNA replication (Park *et al*., 2002). The antitumor action of fucoidan is due to its anti-angiogenic potency by preventing the binding of vascular endothelial growth factor 165 ($VEGF_{165}$) to its cell surface receptor. Furthermore, the increasing the number of sulfate groups in the focoidan molecules contributes to the effectiveness of its anti-angiogenic and antitumor activities (Koyanagi *et al*., 2003). Additionally, fucoidan isolated from brown seaweeds Fucus evanescens is able to enhance apoptosis by combining this fucoidan with etoposide that may also be alternative tools to control cancer diseases (Philchekov *et al*., 2007).

Fucoxanthin found in edible brown seaweed has also been focused on as anticancer carotenoids. Apoptosis induced by fucoxanthin in leukemia cells (HL-60) was associated with a loss of mitochondrial potential followed by caspase-9 and -3 activation (Hosokawa *et al*.,1999; Kotake-Nara *et al*., 2005). Fucoxanthin from Undaria pinnatifida remarkably reduced the viability of human cancer cell lines (Caco-2, HT-29 and DLD-1) via apoptosis and enhances the antiproliferative effect of troglitazone on colon cancer cells (Hosokawa *et al*., 2004). Fucoxanthin may act as a chemopreventive and/or chemotherapeutic carotenoid and may have clinical value in the treatment of cancer.

However, all studies were confined to a few species. A wide variety of other seaweeds in different regions still remain to be investigated for their anti-cancer activity. Thailand is a tropical country with a diverse seaweed flora that may have it own significance for the anti-cancer activity. To date, there has been no study of the effect of Sargassum binderi Sonder (SB) on human cervical cancer cells. In this study, we examined pharmacological evidence of SB regarding the morphological and biochemical features of apoptosis on human cervical cancer cells (HeLa).

Materials and methods

Chemicals

The following chemicals were purchased from the following suppliers: propidium iodide (PI), 4'-6-Diamidino-2-phenylindole (DAPI) and SYBER Gold from invitrogen, Ltd. (Paisley, UK); dimethyl sulfoxide (DMSO) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT), proteinase K and RNase A from Sigma Chemical Co. (St Louis, MO, USA); Cell culture media or materials were purchased from Gibco BRL (Gaithersburg, MD, USA) and InVitromex (Grevenbroich, Germany).

Plant materials and preparation of extracts

The brown seaweed, *Sargassum binderi* Sonder (SB), was collected from Chonburi province along the east coast of the Gulf of Thailand from November 2007 to January 2008 by SCUBA diving. The identification was based on taxonomic references (Ajisaka, 2002). The voucher specimens are deposited in Bangsaen Institute of Marine Science, Burapha University. The fresh samples were extracted with methanol. Each organic residue was partition with dichloromethane and ethyl acetate (1:1) to afford crude extracts that contained almost all of polar and non-polar molecules. The crude extracts of SB were dissolved in absolute ethanol, filtered through a 0.22 µm cellulose nitrate membrane and then stored at -20 ˚C. Dilution was dissolved in phosphate buffer saline (PBS).

Cell line and cell culture

Human cervical cancer cell line (HeLa) was obtained from National Cancer Institute of Thailand. HeLa cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 1mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell cultures were maintained in humidified atmosphere of 95% air and 5% CO_2 at 37 ˚C.

MTT assay

HeLa cells in logarithmic growth phase were collected. After digestion with trypsin-EDTA, cells (5 x 10^4 /well) in growth media (100 µl) were inoculated into each well of a 96-well flat-bottom plate. After 24 h of incubation, the crude extracts of SB (0-400 µg/ml) were added. Cells were also treated with 0.45% absolute ethanol (vehicle control), Doxorubicin 0–10 µg/ml (positive control) under the same conditions. After 72 h of incubation, cell proliferation was determined using with MTT (20 µl of 5 mg/ml) for another 3 h (Alley *et al*., 1976). The supernatant fluid was removed and 100 µl DMSO was added per well. The absorbance at 570 nm was measured with a microplate reader (Cecil Bioquest 2000 Series). This assay is based on cleavage of the tetrazolium salt by mitochondrial dehydrogenase of viable cells to formazan dye. At least three separate experiments for each sample were used to determine the cell viability. Under these conditions, 0.45% absolute ethanol was not toxic and cell survival in vehicle control was assumed 100%. The percentage of cell viability in relation to control according to the following equation:

$$\%\ Cell\ viability = \frac{Absorbance\ at\ 570\ nm\ of\ treated\ cells}{Absorbance\ at\ 570\ nm\ of\ control\ cells} \times 100$$

IC_{50} is the extract concentration under which a 50% inhibition of cell proliferation occurred.

Quantitative analysis of nuclear morphology

HeLa cells were placed in the six-well plate attached with cover slide. Following various treatments, the morphology of apoptotic nuclei were quantified using fluorescence dye staining (Saengkhae *et al*., 2010). Briefly, at designated time points, media were removed and cells were fixed in 2.5% glutaraldehyde. RNase A treatment was performed at 20 mg/ml, 5 µl in the dark for 30 minutes at room temperature. After washing with PBS, the cells were then incubated with DAPI (5 µg/ml) used to identifying nuclear fragmentation, and propidium iodide (PI) (5 µg/ml) used to identifying non-viable cells for 30 min at 37 ˚C. After remove unbound dye, cells were mounted on a glass slide with mounting solution containing PBS:glycerol (1:9), sealed the edges with nail polish and then visualized with fluorescent microscope

(Olympus BX51) at 100 × magnification. Cells were detected by bright field, green filter (PI) and blue filter (DAPI) at the same view point. Normal nuclei can be identified by glowing bright and homogeny only in DAPI staining. Apoptotic nuclei are condensed chromatin and fragmented morphology of nuclear bodies observed in DAPI staining. Late apoptotic nuclei were stained both DAPI and PI. For each treatment group, approximately 500 different nuclei were counted in random microscopic fields. Data were expressed as percentage of nuclei in different phases. At least three separate experiments for each sample were performed.

Qualitative analysis of DNA fragmentation

The GF-1 Tissue DNA Extraction Kit (Vivantis) was used according to the manufacturer's instructions. After treatments, floating and adherent cells were washed with PBS and then lysed with digestion buffer containing proteinase K (20 mg/ml, 20 µl) at 60 °C. RNase A (20 mg/ml, 10 µl) was added and incubated for 5 min at 37 °C. Genomic DNA was extracted with ice-cold absolute ethanol. Equal amounts of DNA samples (300 ng) were mixed with SYBER Gold (0.1 mg/ml, 1 µl) and loading buffer and then loaded onto pre-solidified 1.5% agarose. The agarose gels were run at 150 V for 60 min in TBE buffer (Saengkhae *et al*., 2010). Gels were observed and photographed under transilluminator (Clare Chemical Research).

Data processing

Data were expressed as mean ± standard error of the mean (S.E.M) of 3-4 different trials and analyzed with the software Microcal™ Origin 6.

Results and Discussion

HeLa cells were treated with various concentration of SB extract (0-400 µg/ml) in a 96-well tissue culture plate for 72 h. The viability was analyzed by MTT assay. The SB extract markedly decreased viable cell numbers in a dose-dependent manner (Figure 1). It was found that absolute ethanol (0.45%) used in vehicle control was not toxic for cells. At 200 µg/ml of SB extract, the cell viability was reduced to less than 20%. The SB extract was less toxic than Doxorubicin in that the cell number decreased to less than 10% at Doxorubicin concentrations 6 µg/ml. The IC_{50} values for SB extract and Doxorubicin on HeLa cells was 90 ± 6.35 µg/ml and 2.13 ± 0.25 µg/ml respectively.

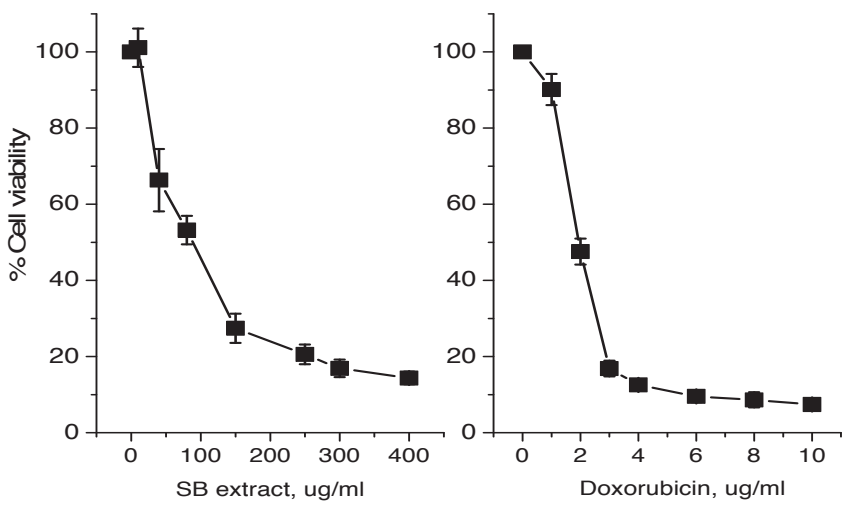

Figure 1 Dose-response curve on SB extract and Doxorubicin-treated HeLa cells. Cells (5×10^4 cells/well) were exposed to SB extract (0-400 µg/ml) or Doxorubicin (0-10 µg/ml) for 72 h. Viable cell number was measured with MTT assay. Data were expressed as mean ± S.E.M of 4 replicates.

Given these results, the IC_{50} values were selected for subsequent experiments. Marked morphological changes could be seen after 72 h treatment with SB extract and Doxorubicin characterized by cell rounding, surface blebbing with apoptotic body formation and lost of cell-to-cell contract when compared with cuboid and polygonal in normal shape. This result was in agreement with the finding of other studies, which found that fucoxanthin from natural brown seaweed has been shown to have growth arrest on various cell lines such as prostate cancer PC-3, DU 145, and LNCaP cells (Kotake-Nara *et al*., 2001), leukemia HL-60 cells (Hosokawa *et al*., 1999), colon cancer HT-29, Caco-2, and DLD-1 cells (Hosokawa *et al*., 2004).

The specific morphological changes including chromatin condensation, nuclear breakdown and DNA fragmentation were investigated in order to gain more insight whether apoptosis is involved in cell-death pathways caused by SB extract on HeLa cells. Both DAPI and PI staining are fluorescent nuclear dye that binds strongly to DNA. DAPI can pass through an intact cell membrane but PI is membrane impermeant that commonly used for identifying necrosis or late apoptotic cells. HeLa cells treated with 0.45% ethanol produced rounded nuclei with homogenous DAPI staining and defined plasma membrane contours (Figure 2). SB extract-treated HeLa cells (90 µg/ml for 72 h) exhibited condensation of chromatin, nuclear fragmentation. In vehicle control group, the quantitative estimation of nuclei was 98.2 ± 3.49% (normal cell), 1.88 ± 0.16% (viable cells with apoptotic nuclei) (Table 1). When the cells were treated with SB extract, the quantitative estimation of nuclei was 46.33 ± 4.27% (normal cell), 36.66 ± 3.2% (viable cells with apoptotic nuclei) and 17.01 ± 1.82% (necrosis or late apoptotic nuclei). In the treatment with Doxorubicin 2.13 µg/ml for 72 h, the quantitative

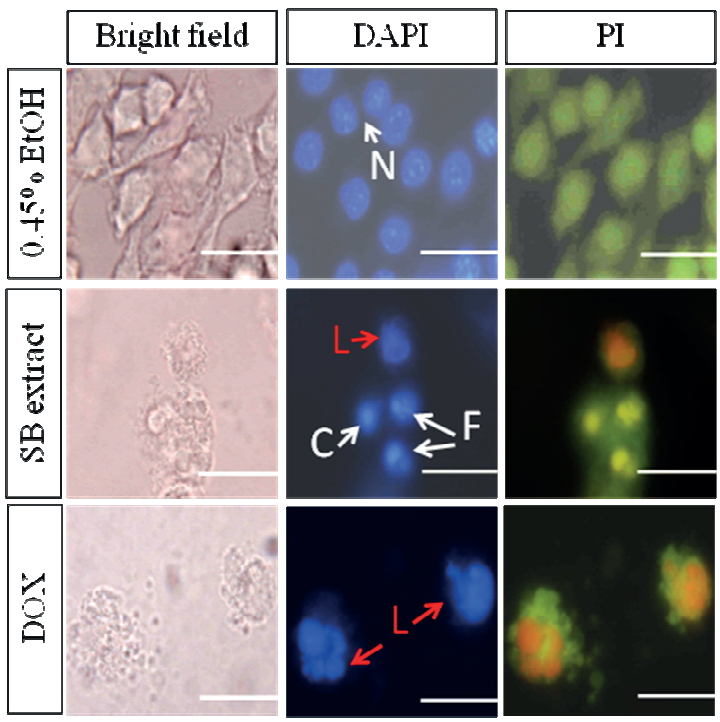

Figure 2 Morphological features of HeLa cells treated with 0.45% EtOH, SB extract 90 µg/ml and Doxorubicin 2.1 µg/ml for 72 hr. Cells were observed by bright field morphology, DAPI staining and PI staining. N: normal nuclei, C: chromatin condensation, F: nuclear fragmentation and L: late apoptosis

Table 1. Percentage of nuclei staining with DAPI and PI. HeLa cells were treated with SB extract (90 µg/ml) and Doxorubicin (2.1 µg/ml) for 72 h. The percentage of cells with a normal or condensed or fragmented nucleus was estimated by counted directly with fluorescent microscope. The values are mean ± S.E.M of 3 replicates.

	% Normal cells (Homogenous DAPI staining)	**% Apoptosis cells** (Condensed or fragmented DAPI staining)	**% Late apoptosis or necrotic cells** (PI staining)
EtOH 0.45%	98.2 ± 3.49	1.88 ± 016	-
SB extract 90 µg/ml	46.33 ± 4.27	36.66 ± 3.2	17.01 ± 1.82
Doxorubicin 2.1 µg/ml	17.45 ± 2.5	21.25 ± 4.1	61.3 ± 5.89

estimation of nuclei was 17.45 ± 2.5% (normal cell), 21.25 ± 4.1% (viable cells with apoptotic nuclei) and 61.3 ± 5.89% (necrosis or late apoptotic nuclei).

After examining hundreds of cells, there is remarkable heterogeneity of apoptotic events within a single culture. Since Doxorubicin is more toxic than SB extract, the stages of apoptosis occurred more rapidly and there have no phagocytic cells to destroy apoptotic cells in culture system. The apoptotic cells eventually reached a terminal stage that look very much like necrotic cells. Drastic decrease in apoptotic cells together with increase in late apoptotic cells was noticed in Doxorubicin-treated cells. In addition, the qualitative DNA fragmentation analysis was characterized biochemically utilizing an agarose gel electrophoresis. The fragmentation of the nuclear DNA into oligonucleosome fragments due to the activation of an intracellular endonuclease which is common to all processes of apoptosis (Compton, 1992). In SB extract and Doxorubicin-treated cells, the presence of degraded DNA is observed as a hazy or undefined outline due to DNA diffusing into agarose gel. DNA fragmentation became apparent at 30 µg/ml in SB extract-treated cell and it was dose-dependent manner. Control cells show a clear margin without any DNA diffusion as shown in Figure 3. This is in good accordance with its growth suppressive effects and nuclear fragmentation. However, further investigation of caspase activity which provides a direct link between endonucleases and apoptotic DNA fragmentation need to be further characterized.

Reseaech of recent years provides strong evidence that the extractions from brown seaweed have

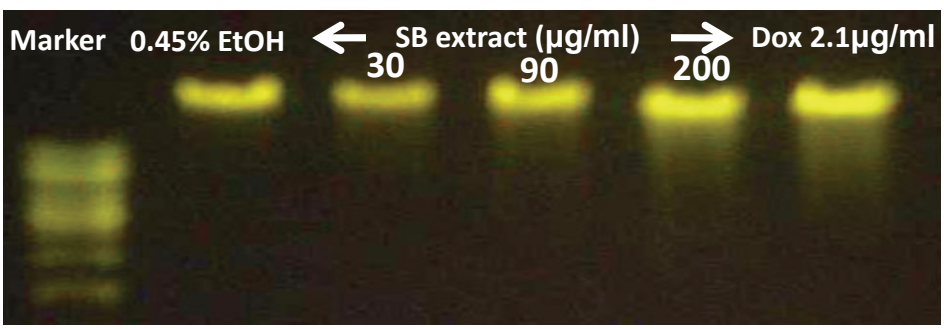

Figure 3 A photograph of the SYBER Gold–stained gel, which is representative of three independent experiments, is shown. DNA fragmentation of HeLa cells exposed to SB extract and Doxorubicin for 72 hr. Cells were lysed and DNA was extracted and electrophoresed on 1.5% agarose gels stained with SYBER GOLD for detection of DNA fragmentation.

been shown to have inhibitory effects on cell growth in various experimental models (Hosokawa *et al*., 1999; Hosokawa *et al*., 2004; Kotake-Nara *et al*., 2005; Sekiya *et al*., 2005). As cancer is a disorder of deregulated cell proliferation, the inhibiting cell proliferation and increasing apoptosis are effective tactics for control cancer growth (Sellers & Fisher, 1999). Although SB extract is much less toxic than most chemotherapeutic agents used to fight cancer, it may be seeing natural remedies combined with synthetic chemotherapeutic compounds that might improve efficacy and decrease side effects (Philchenkov *et al*., 2007; Hur *et al*., 2008). These accumulate finding raise the possibility that brown seaweed may have clinical value in cancer therapy.

The cervical cancer is highly-vascular tumors that allow blood vessels to offer a nourishing connection. Strategies that disrupt the blood vessel cells to die will starve the cancer cells to death. Thus, the antitumor action of fucoidan is due to anti-angiogenic potency (Koyanagi *et al*., 2003). The polysaccharide from brown seaweed *Sargassum stenophyllum* displays antivasculogenic properties that might interfere during microvessel formation (Dias *et al*., 2008). However, cancer is a multifactorial disease, which demands multimodal therapeutic approaches. The invasion of cancer cells and adhesion to matrix proteins are the crucial steps in the formation of metastasis. Therefore, the activity of brown seaweed *Spatoglossum schröederi* on inhibiting cell adhesion to several extracellular matrix proteins should theoretically prevent the formation of metastasis (Rocha. *et al*., 2001).

The SB extract is interesting brown seaweed because of the variety of biological affects especially its potent anticancer activity. However, it need to perform further studies whether the induction of apoptosis by SB extract was due to a single active agents or the combined effects of multiple agents contained in the extract.

Conclusion

In conclusion, the SB extract exhibits an *in vitro* antiproliferative effect against HeLa cells via typical morphological changes in the cells, such as plasma membrane blebbing, chromatin condensation and fragmentation of nuclear DNA. Extensive investigations into the identifying active constituents and molecular mechanisms underlying apoptosis cell death should be further elucidated.

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