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Anti-proliferation and induction of apoptosis by extract of *Turbinaria conoides* (J. Agardh) Kützing on human cervical cancer cell line

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Introduction : *Several types of brown seaweeds have shown to suppress cancer cells by inducing apoptosis and arresting the cell cycle. The brown seaweed from the east coast of the Gulf of Thailand may have potential effects on cervical cancer as well as ability to induce apoptosis.*

Objective : *To study the anti-proliferative effect and induction of apoptosis by Turbinaria conoides (J. Agardh) Kützing (TCK) on human cervical cancer cell line.*

Setting : *Burapha University*

Research design : *An in vitro study*

Target Population : *Human cervical cancer cell line (Hela).*

Method : *Fresh samples of Turbinaria conoides (J. Agardh) Kützing (TCK) were extracted and treated HeLa cells. The viable cell number is based on MTT colorimetric assay. The morphology of apoptotic nuclei were quantified by staining both DAPI and propidium iodide (PI). DNA agarose gel electrophoresis was used for qualitative analysis.*

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Result : *The extract of TCK inhibited viable HeLa cells in a dose-dependent manner with IC_{50} of 20.92 ± 3.15 μg/ml associated with rounding and blebbing cells. The apoptotic nuclei in TCK-treated cells were 34.92 ± 7.29% (early apoptosis) and 19.56 ± 6.34% (late apoptosis) compared to those in normal cells (45.42 ± 5.97%). In addition, the DNA fragmentation in agarose gel was observed in a dose-dependent manner.*

Conclusion : *The TCK extract induced cell death via morphological and biochemical events with particular reference to the induction of apoptosis that may have a major impact on the therapy of cancer. Further investigation is needed to confirm and elucidate detailed mechanisms underlying the apoptosis.*

Keywords : *Turbinaria conoides, HeLa cells, Anti-proliferation, Apoptosis.*

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บทนำ : *สาหร่ายสีน้ำตาลหลายชนิดมีประสิทธิภาพยับยั้งการเจริญของเซลล์มะเร็ง โดยเหนี่ยวนำให้เกิดอะโพโทซิสและยับยั้งวงจรการแบ่งตัวของเซลล์ ดังนั้น สาหร่ายสีน้ำตาลจากชายฝั่งทะเลภาคตะวันออกของประเทศไทย น่าจะมีศักยภาพสูงต่อเซลล์มะเร็งปากมดลูกและอะโพโทซิส*

วัตถุประสงค์ : *เพื่อศึกษาการยับยั้งเซลล์มะเร็งปากมดลูกและอะโพโทซิสของสารสกัดจาก Turbinaria conoides (J. Agardh) Kützing (TCK)*

สถานที่ : *มหาวิทยาลัยบูรพา*

ลักษณะการวิจัย : *การทดลอง*

สัตว์ทดลอง : *เซลล์มะเร็งปากมดลูก (HeLa)*

วิธีการทดลอง *ตัวอย่างสดถูกนำมาสกัดและบ่มกับเซลล์มะเร็งปากมดลูก นับเซลล์มีชีวิตด้วยเทคนิค colorimetric MTT ศึกษารูปร่างและนับจำนวนนิวเคลียสที่เป็นอะโพโทซิส โดยการย้อยสี DAPI และ Propidium iodide (PI) ศึกษาการแตกของ DNA โดย agarose gel electrophoresis*

ผลการทดลอง : *สารสกัดหยาบ TCK ยับยั้ง HeLa เซลล์ที่มีชีวิต โดยประสิทธิภาพการยับยั้งเพิ่มขึ้นตามขนาดความเข้มข้นของสารสกัด และมีค่า* IC_{50} *เท่ากับ* 20.92 ± 3.15 $\mu g/ml$ *ซึ่งสัมพันธ์กับเซลล์มีลักษณะกลม เยื่อหุ้มเซลล์เป็นตุ่มพอง เซลล์ที่ถูกบ่มด้วยสาร สกัดหยาบ TCK พบนิวเคลียสมีลักษณะอะโพโทซิส* $34.92 \pm 7.29\%$ *มีลักษณะอะโพโทซิสระยะหลัง* $19.56 \pm 6.34\%$ *เมื่อเทียบกับเซลล์ปกติมี* $45.42 \pm 5.97\%$ *นอกจากนี้ยังพบการแตกของ DNA เพิ่มขึ้นตามขนาดความเข้มข้นของสารสกัด*

สรุป : *สารสกัดหยาบ TCK เหนี่ยวนำให้เซลล์ตายร่วมกับการเปลี่ยนแปลงทางรูปร่างและชีวเคมี ที่เป็นลักษณะเฉพาะแบบอะโพโทซิส ซึ่งเป็นกลยุทธ์หนึ่งที่ใช้รักษาโรคมะเร็ง ในการศึกษาครั้งต่อไปควรเพิ่มรายละเอียดในกลไกการเกิดอะโพโทซิส*

คำสำคัญ : *Turbinaria conoides, HeLa cells, ยับยั้งการเจริญเติบโต, อะโพโทซิส.*

Cervical cancer is one of the main life-threatening diseases that are primary causes of death among women. The incidence of cervical cancer continues to rise with tremendous trauma. At present, the conventional treatment such as chemotherapeutic agents, surgery and radiation have not been fully effective and cause unacceptable side effects. This has led to researches for more natural products that can suppress tumor development. The strategy of anticancer therapy is to provoke apoptosis selectively in cancer cells. [1]

Seaweeds are one of nature's biosources of proteins, carotenes, chlorophyll, vitamins, iodine and minerals and offer wonderful health benefits. The composition of seaweeds is quite different from land plants. Several new metabolites from seaweeds have shown pharmacological properties, and they are now being confirmed by modern scientific research. [2] Various kinds of brown seaweeds contain major carotenoids (fucoxanthin, neoxanthin) and polysaccharides (fucoidan, alginic acids, fucans). Medical potentials of bioactive components of brown seaweed are anti-herpes simplex viral,[3] antioxidative,[4] antiadhesive, [5,6] antivasculogenic,[7] and anticoagulant. [1]

Studies on anticancer effects of brown seaweed species have been reported by a number of researchers in studies with cultured cells as well as experimental animals. For example, an extract from a brown seaweed *Sargassum thunbergii* has shown anti-tumor activity. [9] Fucoidan, a sulfated polysaccharide present in brown seaweed, has been reported to exhibit antitumor and anti-metastatic activities in rat mammary adenocarcinoma cells. [10,11] Moreover, low molecular weight fucoidan isolated from *Ascophyllum nodosum* showed an anti-proliferative effect on both normal and malignant cells. [12] 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 receptors. The increased number of sulfate groups in the focoidan molecules contributes to the effectiveness of its anti-angiogenic and antitumor activities. [13,14] Fucoxanthin found in edible brown seaweed has also been shown on as anticancer carotenoids. For example, fucoxanthin causes cell growth inhibition and apoptosis in human leukemia cells[15,16] and colon cancer cells. [17] Fucoxanthin induced apoptosis in human leukemia cells via the reduction of mitochondrial membrane potential followed by caspase-9 and -3 activation. [16] Moreover, fucoxanthin from edible seaweed *Undaria pinnatifida* induced apoptosis and enhanced the anti-proliferative effect of troglitazone on colon cancer cells. [17] Furthermore, water extract of Mekabu activated caspaspes 3, 6, and 8 to induce apoptosis in human breast cancer cell line. [18]

These accumulated data raise the possibility that brown seaweed may have clinical value in the treatment of cancer as well as the ability to induce apoptosis. Thailand is a tropical country with diverse seaweed flora which is recognized as being an important source of medicinal plants for developing novel drugs. To date, there have been no studies of the effect of *Turbinaria conoides* (J. Agardh) Kụ̈tzing (TCK) on human cervical cancer cells. In this study, we examined pharmacological evidence regarding the anti-proliferative effects and its molecular mechanisms of TCK on cervical cancer. A better understanding of the mechanisms of these anti-cancer

candidates will facilitate the development of novel anti-cancer drugs derived from these valuable medicinal plants.

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) 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, *Turbinaria conoides* (J. Agardh) K tzing (TCK), was collected from Chonburi, a 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.[19] The voucher specimens are deposited in Bangsaen Institute of Marine Science, Burapha University. Fresh samples of the plant were extracted with methanol. Each organic residue was partition with dichloromethane and ethyl acetate (1:1) to afford crude extract after removal of solvents. The crude extract of TCK were dissolved in absolute ethanol, filtered through a 0.22 μm cellulose nitrate membrane and then stored at -20 °C. The dilution was dissolved in phosphate buffer saline (PBS).

Cell line and cell culture

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

Cell anti-proliferation assay

HeLa cells in logarithmic growth phase were collected. After digestion with trypsin-EDTA, cells (5×10^4/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 TCK (0-200 μg/ml) were added. Cells were also treated with 0.35% 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.[20] 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 cell viability. Under these conditions, 0.35% absolute ethanol was not toxic and cell survival in vehicle control was assumed 100%. The percentage of cell viability in relation to control was determined by the following equation:

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

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 was quantified using fluorescence dye staining. Briefly, at the 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 ^{O}C. After removing unbound dye, cells were mounted on a glass slide with mounting solution (1: 9 PBS: glycerol), the edges were sealed with nail polish and then visualized with fluorescence microscope (Olympus BX51) at 100ื 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 are 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 μ l) at 60 ^{O}C. RNase A (20 mg/ml, 10 μl) was added and incubated for 10 min at 37 ^{O}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 125 V for 30 min in TBE buffer. 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) from independent 3 - 4 experiments and analyzed with the software Microcal™ Origin 6.

Result

In this study, a human cervical cancer cell line was used to investigate the capability of TCK extracts to induce growth inhibition through apoptosis. HeLa cells were exposed to 0 - 200 μg/ml of TCK extracts for 72 h. The TCK extracts induced cell death in a dose dependent manner as determined using MTT assay (Figure 1). It was found that absolute ethanol (0.35%) was not toxic to the cells. At 100 μg/ml of TCK extract, the viability of HeLa cells was

reduced to less than 10%. The IC_{50} values of the TCK extracts and doxorubicin were 20.92 ± 3.15 and 2.13 ± 0.06 μg/ml. The cells were observed by phase contrast inverted microscope. The morphological evidence in HeLa cells treated with TCK extract and doxorubicin indicated a diminished size, rounded and detached from the monolayer, condensed cytoplasm accompanied by transforming cells into clusters of membrane-bound bodies when compared with cuboids and polygonal adherent cells in normal shape. The IC_{50} determinations were selected as the optimal concentrations to use for subsequent regimens.

The nuclear morphological features were examined by fixing the cells remaining in the cover slide and staining with both DAPI and PI. By treatment with TCK extracts (20.92 μg/ml) and doxorubicin (2.13 μg/ml) for 72 h, the majority of the cells were detached and had floated away into the medium. Almost all of the remaining cells on the cover slide were shown in Figure 2. Quantization of surface morphological changes (bright field), nuclear fragmentation in living cells (DAPI) and necrotic cells (PI) are shown in Table 1. TCK extracts-treated HeLa cells exhibited rounding and blebbing cells with apoptotic body formation, chromatin condensation and fragmentation of nuclear chromatin into irregular size as compared to control cells with prominent round nuclei and defined plasma membrane contours.

In the control group, the quantitative estimation of normal cells was 97.4 ± 6.32%, the viable cells with apoptotic nuclei was 1.4 ± 0.17% and the necrosis or late apoptotic nuclei was 1.2 ± 0.06%. When HeLa cells were treated with TCK (20.92 μg/ml) for 72 h, the quantitative estimation of normal cells was 45.42 ± 5.97%, the viable cells with apoptotic nuclei was 34.92 ± 7.29% and the necrosis or late apoptotic nuclei was 19.56 ± 6.34%. In the treatment with 2.13 μg/ml doxorubicin for 72 h, the quantitative estimation of normal cells was 17.45 ± 2.5% the viable cells with apoptotic nuclei was 21.25 ± 4.1% and the necrosis or late apoptotic nuclei was 61.3 ± 5.89%.

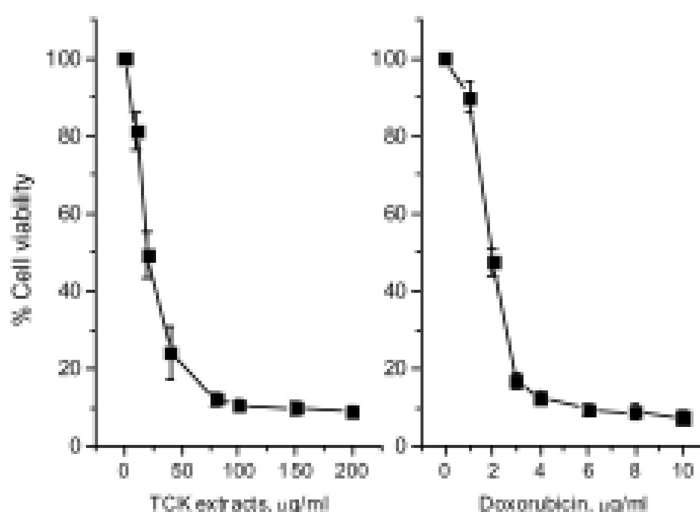

Figure 1. Antiproliferative effects of TCK extract and Doxorubicin to HeLa cells. Cells ($2x10^5$ cells/well) were exposed to TCK extract (0-200 μg/ml) or Doxorubicin (0-10 μg/ml) for 72 h. Viable cell number was measured with MTT assay. Data are expressed as mean ± S.E.M of n = 4 samples.

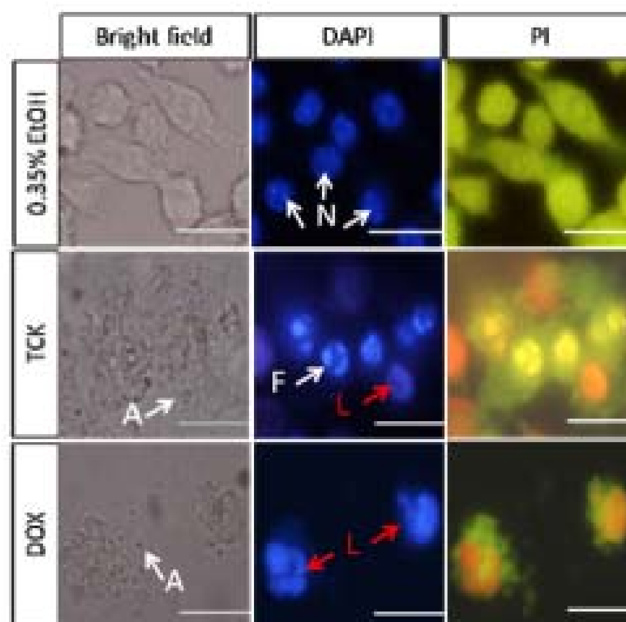

Figure 2. Correlation of surface morphologies with nuclear features. HeLa cells were treated with 0.35% EtOH or TCK extract (20.92 μg/ml) or Doxorubicin (2.13 μg/ml) for 72h and then fixed and stained using both DAPI and PI. Cells were detected by bright field, green filter (PI) and blue filter (DAPI) at the same view point. A: apoptotic body, N: normal nuclei, F: nuclear fragmentation and L: late apoptosis. Data are expressed as mean ± S.E.M of n = 4 samples. Scale bar is 20 μM.

Table 1. Percentage of nuclei staining with DAPI and PI. HeLa cells were treated with 0.35% EtOH or TCK extract (20.92 μg/ml) or Doxorubicin (2.13 μg/ml) for 72h and then fixed and stained using both DAPI and PI. Cells were detected by green filter (PI) and blue filter (DAPI) at the same view point. Data are expressed as mean ± S.E.M of n = 4 samples.

	% Normal cells (Homogenous DAPI staining)	**% Apoptotic cells (Condensed or fragmented DAPI staining)**	**% Late apoptosis or necrotic cells (PI staining)**
0.35% EtOH	97.4 ± 6.32	1.4 ± 0.17	1.2 ± 0.06
TCK 20.92 μg/ml	45.42 ± 5.97	34.92 ± 7.29	19.56 ± 6.34
DOX 2.13 μg/ml	17.45 ± 2.5	21.25 ± 4.1	61.3 ± 5.89

In addition to quantitative estimation, qualitative DNA fragmentation analysis was carried out by agarose gel electrophoresis. DNA of HeLa cells treated with EtOH or TCK extracts or doxorubicin was extracted and analyzed on 1.5% agarose gel as illustrated in Figure 3. TCK extracts and doxorubicin treated cells showed a hazy or undefined outline due to DNA fragmentation in a concentration-dependent manner that is in good accordance with the result obtained by anti-proliferative analysis. The intensity of banding was more prominent at 55μg/ml compared to that of control.

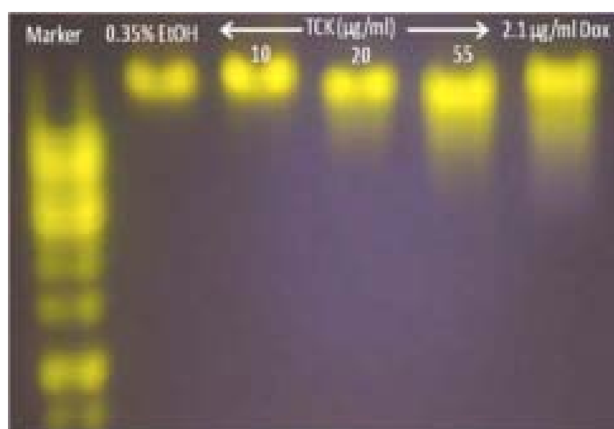

Figure 3. A photograph of the SYBER Gold-stained agarose gel, which is representative of three independent experiments, is shown. HeLa cells were exposed to 0.35% EtOH or TCK extract or Doxorubicin for 72 h and then DNA was extracted and electrophoresed.

Discussion

TCK extract induced cytotoxicity to HeLa cancer cells with morphological changes including cell rounding, surface blebbing with apoptotic body formation. The anti-proliferating effect of TCK extract meets the criteria set by the National Cancer Institute for medicinal plants intended to be used as anticancer agents.[21] The nuclear morphological analysis of cells staining both with DAPI and PI displayed nuclear condensation and fragmentation. These are in good accordance with the results obtained by agarose gel electrophoresis of DNA extracted which is a progressive increase in fragmentation. These findings demonstrate the morphological and biochemical events of apoptosis *in vitro*. Because no phagocytic cells were present in culture, the apoptotic cells eventually reached a late stage similar to necrosis. At any time point, the cell asynchrony would demonstrate a mixture of apoptosis events together with necrosis or late apoptosis.

During tumor progression, cancer cells are conferred with capacity to proliferate independently. Thus, the anti-proliferative effect of medicinal plant on cancer cells is one of the mechanistic ways in chemoprevention and chemotherapy of other human malignancies. This result was in agreement with the findings of other studies. For example, fucoxanthin, a natural carotenoid in brown seaweeds, induces G1 arrest in HepG2 associated with down-regulation cyclin D.[22] Concomitantly, this compound has been shown to have growth arrest on various cell lines such as prostate cancer PC-3, DU 145, and LNCaP cells,[23] leukemia HL-60 cells,[15] colon cancer HT-29, Caco-2, and DLD-1 cells.[17]

The increase of apoptosis in tumors is also effective tactics for prevention of tumor growth. Previous studies have reported that fucoxanthin from the edible seaweed *Undaria pinnatifida*, along with β-carotene, induced apoptosis via DNA fragmentation on colon cancer cells. In addition, it enhanced the anti-proliferative effect when used in

combination with troglitazone which is known to inhibit cell growth and induce apoptosis.[17] Apoptosis induced by fucoxanthin in human promyelocytic leukemia (HL-60) cells was associated with a loss of mitochondrial membrane potential and caspase-3 activation.[16] Furthermore, Mekabu extract activates caspases 3, 6, and 8 and contributes to intracellular signaling to induce apoptosis in a human breast cancer cell line.[18] Fucoidan, isolated from brown seaweed *Fucus evanescensin*, enhances etoposide induced caspase-dependent pathway in human T-acute leukemia cell line.[24] Sargaquinoic acid and sargachromenol isolated from the brown alga *Sargassum sagamianum* enhanced apoptosis in combination with UVB irradiation, highlighting their potential use as a therapeutic agent against hyperproliferative diseases such as psoriasis.[25]

Taken together with this present study, the TCK extract inhibited the proliferation of cervical cancer cells via apoptosis. Because caspases are known to play the important mediators in apoptosis, the elucidation of the apoptosis-related intracellular targets of the TCK extract will be addressed in future studies. However, cancer is a multi-factorial disease, which demands multimodal therapeutic approaches. Brown seaweeds are known to be attractive as they sometimes yield active compounds showing several kinds of different bioactivities such as anti-adhesion to matrix proteins that are the crucial steps in the formation of metastasis,[6] anti-angiogenic and anti-tumor activities by deprive cancer cells of oxygen and other basic nutrients.[7,14]

The main components of brown seaweeds are proteins, carbohydrates, iodine and vitamins such as carotene. In addition, a well-known special component is fucoidan (polysaccharides) and fucoxanthin (carotenoid).[2] Recent studies have shown that many constituents from brown seaweeds have a wide range of biological actions. Although the chemical constituents of crude TCK extract were not identified in this study, considering that TCK extract contains many of these active substances, its apoptosis-induction potency may be due to the effects of an individual active agent or the combined effects of various agents. Further investigation is necessary to resolve the questions of the bioactive constituents contained in this extract and. There is still a need to examine whether these compounds are effective in inhibiting the progression of cervical cancer in animal models.

Acknowledgement

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