ORIGINAL ARTICLE

Anti-tumour activities of fucoidan from the aquatic plant *Utricularia aurea* lour

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Abstract

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Fucoidan, a sulfated polysaccharide with several biological activities, is usually isolated from marine seaweeds or from echinoderms. Here, we report on the anti-tumour activity of fucoidan isolated from the aquatic plant *Utricularia aurea* Lour (Lentibulariaceae). A crude extract (CE) prepared by incubating *U. aurea* with hot water at 95ºC for 12 hr was partially purified by Sephadex G-50, eluting with a 50mM sodium acetate buffer, at pH 5.0, containing 0.5M NaCl. Partially purified fucoidan (PPF) had a 3- fold increase in fucose content when compared with the CE and a molecular weigÄt of 11.6 kDa as determined by Sephadex G-200. Chemical analysis showed that CE consisted of 62.5% glucuronic acid, 5.0% fucose, 1.7% sulfate and 12.0% proteins while PPF consisted of 65.0% glucuronic acid, 15.3% fucose, 2.1 % sulfate and 8.3% proteins.

The anti-tumour activity of the CE and PPF was determined by the MTT test. The CE at 125 μg/mL fucoidan and PPF at 250 μg/mL inhibited the growth of KB cells (a nasopharynx tumour cell line), but did not inhibit that of normal fibroblast cells. The inhibition was postulated to occur via apoptosis as significantly more apoptotic cells were found after treatment than in the untreated KB cells ($P<0.05$) by the TUNNEL (TdT-mediated dUTP Nick-End Labelling) assay.

Key words : anti-tumour, aquatic plant, fibroblast, fucoidan, KB cell

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การยับยั้งเซลล์มะเร็งโดยฟูคอยแดนจากสาหร่ายข้าวเหนียว (*Utricularia aurea* Lour)

ว. สงขลานครินทร์ วทท. ธ.ค. 2548 27(ฉบับพิเศษ 3) : 799-807

ฟูคอยแดน เป็นสารซัลเฟตโพลีแซคคาไรด์ที่มีคุณสมบัติทางชีวภาพหลายด้าน พบได้ทั่วไปในสาหร่ายทะเลและสิ่งมีชีวิตในกลุ่ม Echinoderm จากการศึกษาครั้งนี้ได้ทำการศึกษาคุณสมบัติต้านการเจริญของเซลล์มะเร็งของสารฟูคอยแดนจากพืชน้ำ ที่สกัดด้วยน้ำกลั่นอุณหภูมิ 95°C เป็นเวลา 12 ชม. และทำบริสุทธิ์โดยใช้ Sephadex G-50 ชะด้วย 50 mM sodium acetate buffer, pH 5.0 ที่มี 0.5 M NaCl ด้วยอัตราการไหล 5 มล./ชม. สารฟูคอยแดนกึ่งบริสุทธิ์ที่ได้มีปริมาณ fucose สูงกว่าสารสกัดหยาบฟูคอยแดน 3 เท่า มีขนาดโมเลกุล 11.6 kDa โดยใช้ Sephadex G-200 สารสกัดหยาบฟูคอยแดน ประกอบด้วย 62.5% glucuronic acid, 5.0% fucose, 1.7% ซัลเฟต และ 12.0% โปรตีน ในขณะที่สารฟูคอยแดนกึ่งบริสุทธิ์ ประกอบด้วย 65.0% glucuronic acid, 15.3% fucose, 2.1% ซัลเฟต และ 8.3% โปรตีน

การศึกษาคุณสมบัติต้านการเจริญของเซลล์มะเร็งของสารสกัดหยาบฟูคอยแดนและสารฟูคอยแดนกึ่งบริสุทธิ์ด้วยวิธี MTT พบว่าสารสกัดหยาบที่ระดับความเข้มข้นของฟูคอยแดน 125 ไมโครกรัม/มล. และสารฟูคอยแดนกึ่งบริสุทธิ์ที่ระดับความเข้มข้น 250 ไมโครกรัม/มล. สามารถยับยั้งการเจริญของเซลล์มะเร็ง KB cell (nasopharynx tumour cell line) ได้ โดยไม่มีผลยับยั้งการเจริญของเซลล์ไฟโบบลาสต์ปกติ สารฟูคอยแดนสามารถยับยั้งเซลล์มะเร็งได้โดยกระตุ้นกระบวนการ apoptosis ซึ่งศึกษาด้วยวิธี TUNNEL (TdT-mediated dUTP Nick-End Labelling) assay พบว่าเซลล์มะเร็งในกลุ่มที่ได้รับสารฟูคอยแดนมีจำนวนเซลล์ที่เกิด apoptosis มากกว่าเซลล์มะเร็งที่ไม่ได้รับสาร โดยมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติที่ระดับความเชื่อมั่น 95% ($P<0.05$)

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Fucoidan is one of the sulfated polysaccharides produced by marine seaweeds, especially brown seaweeds. Several biological activities of fucoidan have been reported. For example, a complex sulfated polysaccharide from the algae *Fucus vesiculosus* was found to inhibit HIV (human immuno-deficiency virus) *in vitro* and had a synergistic effect with AZT (azidothymidine) (Sugawara *et al*., 1989). Fucoidan from the brown seaweed *Adenocystis utricularia* was reported to inhibit the action of the type I&II herpes simplex viruses (Ponce *et al*., 2003). Some extracts from marine algae also have antibacterial properties (Rao and Perekh, 1981).

An extract from the brown seaweed *Sargassum thunbergii* has shown anti-tumour activity (Zhuang *et al*., 1995), and the inhibition of tumour metastasis of the rat mammary adenocarcinoma cell (13762 MAT) has also been reported (Coombe *et al*., 1987). Moreover, low molecular weight fucoidan isolated from *Ascophyllum nodosum* showed an anti-proliferative effect on both normal and malignant cells, including fibroblasts (Hamster Kidney Fibroblast CCL39), sigmoid colon adenocarcinoma cells (COLO320 DM), and smooth muscle cells (Vischer and Buddecka, 1991; Ellouali *et al*., 1994). The inhibitory effect appears to depend on the sulfate content of the fucoidan (Haroun-Bouhedja *et al*., 2000). It has been proposed that the inhibition of metastasis from some tumours are caused by sulfated polysaccharides interfering with the passage of tumour cells across the capillary wall (Coombe *et al*., 1987). Furthermore, an oversulfated polysaccharide prevents the tube formation of HUVEC (human umbilical vein endothelial cells)

whereby OSF (oversulfated fucoidan) induced PAI-1 (plasminogen activator inhibitor-1), which was released from HUVEC by increasing the binding of bFGF (basic fibroblast growth factor) to the heparan sulfate (HS) on the cell surface (Soeda *et al*., 2000). The heparan sulfate (HS) molecules are required for binding to the high affinity receptors with tyrosine kinase activity (Soeda *et al*., 2000). In addition, fucoidans are also reported to be involved in the adhesion, attachment and spreading of certain human melanoma cell lines (Ginsburg and Roberts, 1988).

In this investigation, we examined a fucoidan preparation from *Utricularia aurea* Lour which has shown anti-tumour activity. A possible mechanism of its activity was determined.

Materials and Methods

Preparation of fucoidan from *U. aurea*

Fucoidan was extracted from *U. aurea* (100 g dry weight) with hot water at 95°C for 12 hr. This crude extract (CE) was filtered, dialyzed against water, lyophilized and analyzed for its fucose content (Winzler, 1971). L-fucose was used as the standard. The amount of fucoidan was calculated according to Doner and Whisler (1973) whereby 1 µg of fucoidan is equivalent to 1.75x fucose (µg).

The CE was then partially purified as described by Zierer and Mouroa (2000). 100 mg of CE was applied to a Sephadex G-50 (1x60 cm) column which had been equilibrated at a flow rate of 5 mL/hr and eluted with 50mM sodium acetate buffer, containing 0.5M NaCl, pH 5.0. Consecutive 2 mL fractions were collected, checked for fucose by the cysteine-sulfuric method (Winzler, 1971) and for glucuronic acid by the carbazole reaction (Disch, 1947). Metachromatic properties were determined for the presence of sulfate molecules by using 1, 9 dimethylmethylene blue (Farndale *et al*., 1986). The appropriate fractions were pooled, dialyzed, lyophilized and stored at 4°C. This fucoidan preparation was named 'partially purified fucoidan' (PPF).

Characterization of partially purified fucoidan (PPF)

The molecular weight of PPF was determined with a Sephadex G200 (1X100 cm) column using dextran sulfate (8 kDa), chrondroitin sulfate A (15 kDa) and chondroitin sulfate C (60 kDa) as standard molecular weight markers. The CE was applied to a column which had been equilibrated at a flow rate of 5 mL/hr and eluted with 50mM sodium acetate buffer, containing 0.5M NaCl, pH 5.0. Consecutive 2 mL fractions were collected and fractions containing the PPF were pooled and assayed for fucose by the cysteine-sulfuric method (Winzler, 1971), glucuronic acid by the carbazole reaction (Disch, 1947) and sulfate by the gelatin method (Dogson, 1961).

Anti-tumour activity of fucoidan from *U. aurea*

Cell culture

KB cells (a nasopharyn tumor cell line, ATCC CCL 17) and normal fibroblast cells, isolated from the foreskin of 8-12 yr old patient's penises, at Songklanagarind hospital, were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal calf serum, 2% penicillin-streptomycin (penicillin G 1000 units/mL, streptomycin sulfate 10,000 µg/mL) and amphotericin B (25 µg/mL) at 37°C in a humidified 5% CO_2 atmosphere.

Antiproliferative assay of fucoidan by MTT (methythiazolyldiphenyl tetrazolium bromide) assay (Freshney, 1994)

KB cells and fibroblast cells ($1.5x10^2$ cell) were seeded into 96 well plates and allowed to attach to the well bottom for 24 hr. The attached cells were then treated for 48 hr in the medium containing the CE or PPF at a concentration of 1,000, 500, 250 and 125 µg fucoidan/mL. After treatment, the medium in each well was replaced with 50 µL of 5 mg/ml MTT and further incubated for 4 hr at 37°C in a humidified 5% CO_2 atmosphere. MTT was removed from each well and then 200 µL DMSO (Dimethyl sulfoxide) and 50 µL of 0.1 M glycine containing 0.1 M NaCl were added.

Absorption was measured by microplate reader (Titertek® Multiskan) at 570 nm. Each experiment was carried out on 2 occasions and each sample was performed in triplicate wells and used to calculate the percentage inhibition of cell growth by fucoidan. The percentage of inhibition was presented as mean±SD. One way analysis of variance, Anova in SPSS 10.0, was used to analyse the data.

Apoptosis test by Tunnel (TdT-mediated dUTP Nick-End Labelling) assay

KB cells ($2x10^6$ cell) were seeded into T-75 for 24 hr. The medium was then supplemented with PPF at 250 µg fucoidan/mL and, after a further 48 hr, cells were trypsinized and washed twice with PBS (phosphate buffered saline). The cells were fixed in 10% formaldehyde on a poly-L-lysine coated slide, soaked in 0.2% Triton-x 100 for 10 min and washed twice with PBS, 5 min each. The cells on the slide were assayed using the DeadEnd TM Tunnel procedure (Promega, USA). Briefly, the slide was equilibrated with 100 µL of equilibration buffer (200 mM potassium cacodylate, pH 6.6, 25 mM Tris-HCl, 0.2 mM DTT (dithiothreitol), 0.25 mg/mL BSA (bovine serum albumin) and 2.5 mM $CoCl_2$). The slide was then incubated in a 100 µL reaction mixture (98 µL equilibration buffer, 1 µL biotinylated nucleotide and 1 µL TdT enzyme) at 37°C for 1 hr and in 2xSSC (0.3 M NaCl, 0.03 M $Na_3C_6H_5O_7$, pH 7.0) for 15 min. The slide was washed 3 times with PBS, soaked in 0.3% H_2O_2 for 10 min, washed as previously described and incubated with Streptavidin-HRP (horseradish-peroxidase labeled streptavidine) for 30 min at room temperature. The slide was washed and the color was developed with DAB (diaminobenzidine) solution for 10 min. The reaction was stopped with distilled water, the slide was soaked in ethanol and xylene, and mounted. The nucleus-stained cells showing apoptosis were counted under the microscope. Each tunnel assay was performed twice in a triplicate assay and the percentage of apoptosis was presented as mean±SD. One way analysis of variance, Anova in SPSS 10.0, was used to analyse the data.

Results and Discussion

Preparation and characterization of fucoidan from *U. aurea*

Extraction of 100g U. aurea by hot water gave *U. aurea* CE in the amount of 15.3±0.6 g dry weight (n=3). The amount of fucoidan was calculated according to the fucose which was measured by the cysteine-sulfuric method (Winzler, 1971). We found that 100g dried weight of *U. aurea* CE contains 8.75 g (n=3) fucoidan and 100g dried weight of *U. aurea* plant contains 1.3 g fucoidan. One hundred milligram of the CE was separated on a Sephadex G-50 column (1 x 100 cm) collecting consecutive 2 mL fractions. Fucose and metachromatic activity which indicated fucoidan and sulfate, respectively were found in fractions 17-23 while glucuronic acid was found in fractions 17-38 (Figure 1). Fractions 17-23 were pooled, dialyzed and dried for further study. The fucoidan had a 3 fold increase in fucose content after purification (Table 1). The PPF had a molecular weight of 11.6 kDa as estimated by Sephadex G200 (1x100 cm column). Low molecular weight fucoidans are known to have anti-coagulation activity, for example the anti-coagulant fucoidans from *Porana volubilis* and *Pelvetica canaliculata* with molecular weights of 10 and 30 kDa respectively (Colliec *et al*., 1994; Yoon *et al*., 2002). Also, a fucoidan with a molecular weight of 18 kDa from *Ascophylum nodosum* showed anti-coagulation activity and anti-tumour activity (Haroun-Bouhedja *et al*., 2000).

The compositions of the CE and PPF were determined. The CE consisted of 62.5% glucuronic acid, 5.0% fucose, 1.7% sulfate and 12.0% proteins while PPF consisted of 65.0% glucuronic acid, 15.3% fucose, 2.1% sulfate and 8.3% proteins (Table 1). Fucoidans from several sources have a high glucuronic acid content, for example fucoidans from *Larminaria digitata, Pelvetia canaliculata, Fucus vesiculosus* and *Sargassum muticum* consist of 46.4%, 28.1%, 28.2% and 27.9% glucuronic acid, respectively. The fucose content of the fucoidans from these algae are 2.3%, 13.1%, 9.7% and 3.2%, and the sulfate content are 11.6%,

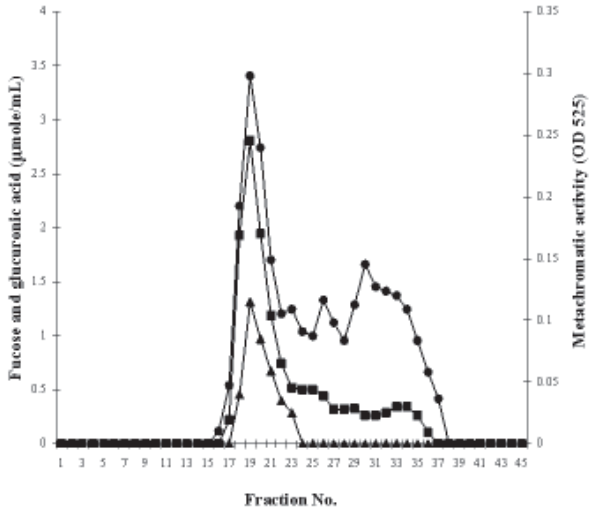

Figure 1. Purification of crude extract by sephadex G-50 (1x 60 cm.), the column was equilibrated and eluted with 50 mM sodium acetate containing 0.5 M NaCl, pH 5.0, at a flow rate of 5 mL /hr and collection of 2 mL fractions.
—■— Fucose (µmole/mL), —●— glucuronic acid (µmole/mL),
—▲— metachromatic activity.

Table 1. Composition of the crude extract and the partially purified fucoidan from *U. aurea*

Sample	Fucose(g)/fucoidan(g) (mean±SD, n=3)	Glucuronic acid(g) (mean±SD, n=3)	Sulfate(g) (mean±SD, n=3)	Proteins(g) (mean±SD, n=3)
Crude Extract (100 g) (CE)	5.0±0.3/8.75	62.5±19.0	1.7±0.2	12.0±0.3
Partially purified fucoidan (100g) (PPF)	15.3±0.9/26.8	65.0±7.0	2.1±0.8	8.3±0.8

11.6%, 6.9% and 5.0%, respectively (Mabeau *et al*., 1990). Fucoidans that contain a high glucuronic acid content are known as U-fucoidans, and most of the U-fucoidans containing fucose and sulfate in the molecule have an ability to inhibit the metastasis of tumours (Rocha *et al*., 2001). Although the fucoidan isolated in this study has a high content of glucuronic acid, the sulfate content is quite low when compared with other samples (Mabeau *et al*., 1990; Haroun-Bouhedja *et al*., 2000).

MTT assay of the antiproliferative action of fucoidan

The antiproliferative action of fucoidan was performed using MTT assay on KB cell line and normal fibroblast cells. Both cells were grown in the medium containing CE at the final concentrations of 1000, 500, 250, and 125 µg/mL. The percentage inhibition of KB cell proliferation, after being treated with fucoidan from the highest to the lowest was 92.6±2.4, 88.2±4.6, 64.2±4.7 and 28.0±5.8, respectively, while the percentage inhibition of normal fibroblast proliferation was 74.6±1.5, 35.5±8.4, 13.8±11.9 and 0, respectively (Figure 2).

When the culture medium was supplemented with PPF to give fucoidan concentrations of 1000, 500 and 250 µg/mL, KB cell proliferation was inhibited by 34.2±8.2, 25.1±7.3 and 20.2±10.3 percent respectively, and fibroblast proliferation

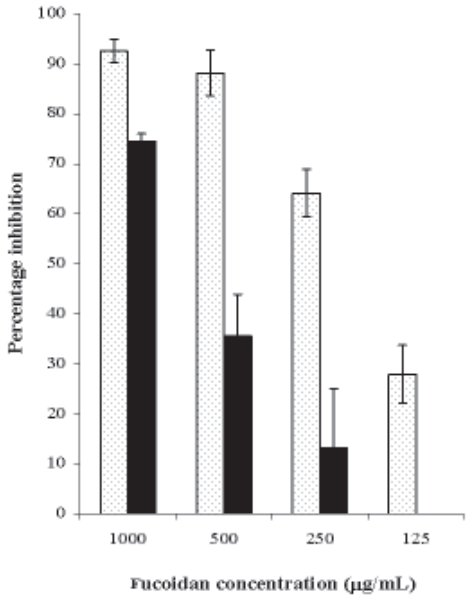

Figure 2. A percentage inhibition (mean±S.D, n=3) of the KB cells and the normal fibroblast cell proliferation cultured in medium supplemented with crude extract containing various concentrations of the fucoidan. ▫ KB cells ■ Normal fibroblast cell.

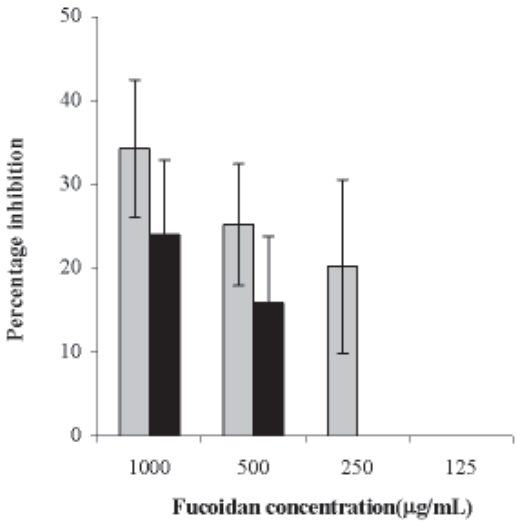

Figure 3. A percentage inhibition (mean±S.D, n=3) of the KB cells and the normal fibroblast cells proliferation cultured in medium supplemented with the partially purified fucoidan at various concentrations. ▫ KB cells ■ normal fibroblast cells.

was inhibited by 24.0±8.9, 15.8±7.9 and 0 percent (Figure 3). Hence the CE and the PPF containing the fucoidan at concentration of 125 µg/mL and 250 µg/mL, respectively was able to inhibited KB cells proliferation, but had no effect on the normal fibroblast cells. Fucoidan from other sources has different capacities to inhibit cell proliferation, for example fucoidan from *Ascophyllum nodusum* at a concentration of 1000 µg/mL inhibited the growth of the CCL39 fibroblast cell line between 90-100%

(Haroun-Bouhedja *et al*., 2000) while fucoidan from Fucus vesicolosus at a concentration of 100 µg/ml inhibited HUVEC proliferation about 40% (Koyanagi *et al*., 2002). These different capabilities of fucoidan from different sources to inhibit cell proliferation are may be due to structural differences and the cell type used in the experiments. In this study, PPF was expected to have higher inhibitory activity than crude fucoidan; however, the result occurred in the opposite direction. This may due to the structural changes during purification or the inhibitory effect may be caused not only by the fucoidan but by other factors in the crude extract that may enhance the effect.

Apoptosis test by TUNNEL (TDT-mediated dUTP Nick-End Labelling) assay

A Tunnel assay was performed to investigate apoptotic cells present among KB cells after treatment with 250 µg fucoidan/ml of PPF for 48 hr. In the presence of fucoidan 50.7±3.2% of the apoptotic cells were found and significantly increased comparing to 28.3±2% apoptotic cell found in untreated group ($p<0.05$, Figure 4). Apoptosis can be triggered by several factors. A copper-complex was reported to damage plasma membranes and trigger apoptosis in copper-complex treated leukemic cells (Dovovinov'a *et al*., 2002). Furthermore, apoptosis has been triggered by an increase in oxidative stress as well as by an increase of the glutathione level (Higuchi *et al*., 1998; Chandra *et al*., 2000; Mates' and Sa' nches-Jime'nez, 2000). The result from this study implies that fucoidan may trigger apoptosis in KB cells which, in turn, affects KB cell proliferation. This study differs from previous investigation done by Religa *et al*. (2000) in that fucoidan inhibits smooth muscle cell proliferation by inhibiting the mitogen-activated protein kinase pathway (Religa *et al*., 2000).

Moreover, low molecular weight fucoidans isolated from *Ascophyllum nodosum* had an anti-proliferative effect that depended on the sulfate content of the fucoidan (Vischer and Buddecke, 1991; Ellouali *et al*., 1993; Haroun-Bouhedja *et al*., 2000). It was suggested that the anti-tumour mechanism of the fucoidan was caused by the sulfated polysaccharides preventing the tumour from entering the blood vessels (Coombe *et al*., 1987). Koyanagi *et al*. (2002) demonstrated that a fucoidan from *Fucus vesiculosus* inhibited Sarcoma 180, Lewis lung carcinoma and B16 melanoma by binding to VEGF165 (vascular endothelial growth factor 165), and this prevented the VEGF165. to promote tumour-induced angiogenesis. Some fucoidans are involved in the adhesion, attachment and spreading of some human melanoma cell lines (Ginsburg and Roberts, 1988). In this study, the concentration of PPF fucoidan (250 µg/mL) required to inhibit proliferation of the KB cells was twice that of the concentration of the CE fucoidan (125 µg/mL). However, 250 µg/mL of the PPF

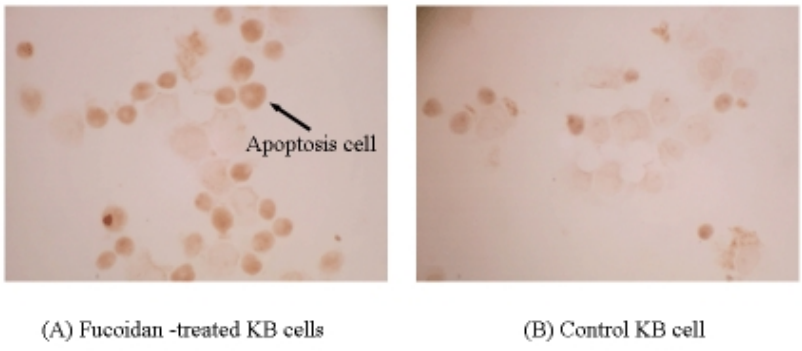

Figure 4. A Tunnel assay of the KB cells. (A) KB cells treated for 48 hr by DMEM containing partially purified fucoidan at 250 µg/mL (B) KB cell cultured in DMEM without PPF.

fucoidan and 125 µg/mL of the CE fucoidan have nearly the same amount of sulfate, 20 µg/mL and 25 µg/mL respectively (calculated from Table1). The fucoidan isolated from *U. aurea* in this report contains a low percentage of sulfate, therefore the anti-tumour activity of 20 µg PPF sulfate/mL and 25 µg CE sulfate/mL produced only 20% and 28% inhibition respectively. It would seem, therefore, that the inhibition of the cell growth depended on the sulfate content of the fucoidan. This suggestion is supported by the molecular compositions of the fucoidans that have been shown to have anti-tumour activity. Although they contain variable amounts of glucuronic acid with fucose and sulfate in the molecule, their inhibitory activity increases as the amount of sulfate increases (Haroun-Bouhedja *et al*., 2000).

Although this report describes a new source of fucoidan from *U. aurea* with anti-tumour activity that acts by inducing the apoptotic mechanism, there is still a need to elucidate the mechanism of fucoidan-initiated apoptosis. Investigating the effect of an oversulfate fucoidan from *U. aurea* could confirm the link between the anti-tumour activity and the sulfate content.

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