

Anti-cancer effects of enzyme-digested fucoidan extract from seaweed *Mozuku*

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ABSTRACT

Fucoidan is a uniquely-structured sulfated fucose-rich polysaccharide derived from brown algae. Recently, the abalone glycosidase-digested fucoidan extract (fucoidan extract) derived from seaweed *Cladosiphon novae-caledoniae* Kylin (*Mozuku*) draws much attention because of its clinical anti-cancer effect in Japan. Here, we report the cancer cells-specific apoptosis inducing effects of the fucoidan extract. The fucoidan extract suppressed the growth of various anchorage-dependent and -independent cancer cells. The fucoidan extract contained low molecular weight components, which induced apoptosis of human leukemic HL 60 cells but not of human lymphocytes. It was shown that the fucoidan extract lead caspase 3/7 activation and loss of mitochondrial membrane potential in HL 60 cells. Another function of the fucoidan extract was also observed. It has been known that sugar chain expression on the surface of cancer cell membrane changes dependent on their malignancy. The analysis on sugar chain expression profiling using FITC-labeled lectins revealed that the expression of concanavalin A (Con A) binding sugar chain was enhanced by the treatment of human lung adenocarcinoma A549, human uterine carcinoma HeLa and human fibrosarcoma HT1080 cells with the fucoidan extract. Con

Received: Sept. 8, 2008; Revised: Jan. 29, 2009; Accepted: Jun. 3, 2009

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A-induced apoptosis of cancer cells was stimulated in a dose- and time-dependent manner by the treatment with the fucoidan extract but not of human normal fibroblast TIG-1 cells.

Keywords: apoptosis, cancer, concanavalin A (Con A), fucoidan, seaweed

INTRODUCTION

Fucoidan is a uniquely-structured sulfated polysaccharide found in the cell walls of several types of brown seaweed. Recently, fucoidan has attracted a lot of clinical attention due to its strong anti-tumor potential, which has been intensively investigated. Fucoidan suppresses the growth of tumor cells *in vivo* and activates the immune system against tumors [1-6]. Sulfation of fucoidan enhanced its antitumor activity [2]. Koyanagi *et al.* reported that fucoidan inhibited tube formation following migration of human umbilical vein endothelial cells [7]. Ye *et al.* reported that enzyme-digested fucoidan extract inhibited invasion and angiogenesis of tumor cells [8]. Aisa *et al.* reported that fucoidan induced apoptosis HS-Sultan cells accompanied by caspase-3 activation and down-regulation of ERK pathway [9].

In this study, we examined that anti-cancer effects of enzyme-digested fucoidan extract derived from *Mozuku* of *Cladosiphon novae-caledoniae* Kylin, which originates in the Kingdom of Tonga, inducing the apoptosis of tumor cells.

MATERIALS AND METHODS

Preparation of Fucoidan and Treatment

The abalone glycosidase-digested fucoidan extract prepared from seaweed *Mozuku* of *Cladosiphon novae-caledoniae* Kylin from the Kingdom of Tonga, commercially available as a product named 'Power fucoidan', was donated for the study by the Daiichi Sangyo Corporation (Osaka, Japan) [8]. An undiluted solution (pH3.7) was neutralized to pH 7.0 with NaOH. The precipitants were removed by centrifugation at 2,200×g for 15 min. The supernatants were then autoclaved, and stored as 'fucoidan extract (2.8mg/ml)' at 4°C. The fucoidan extract was mixed with culture medium, and treatments to various cells were performed.

Cell Culture

The human fibrosarcoma cell line HT1080, human uterine carcinoma cell line HeLa, human lung adenocarcinoma and human normal fibroblast TIG-1 were cultured in Minimum Eagle's medium (MEM: Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 100 units/ml penicillin and 100 µg/ml streptomycin (10% FBS/MEM). Human leukemic cell lines

HL60, U937 and K562 cells were cultured in RPMI 1640 medium (Nissui) supplemented with heat-inactivated 10% FBS, 10 mM HEPES, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. All cell culture was performed in a humidified atmosphere with 5% CO_2 at 37°C.

Anti-proliferative Assay

Cells were inoculated at a final concentration of 1×10^5 cells/ml in culture medium in 35 mm culture dishes. After 24 h pre-incubation, the cells were exposed to various concentrations of the fucoidan extract. After cultivation, the cell number in each culture dish was measured using automated hematology analyzer (Sysmex Co., Kobe, Japan).

Caspase-3/7 Activity Assay

To examine caspase activity, HL60 cells were inoculated at a density of 2.0×10^4 cells in 100 μl culture medium with the fucoidan extract and cultured in a 96-well microplate. After 24 h culture, caspase-3/7 activity was measured with Apo-ONE™ Homogeneous Caspase-3/7 Assay kit (Promega Co., Madison, WI). Briefly, Apo-ONE™ reagent, profluorescent substrate containing the DEVD sequence, was added to the microplate. The sample plate was incubated for 4 h, and then a fluorescent dye rhodamine 110 was released through cleavage of the profluorescent substrate by the caspases-3/7 activity in the samples. The fluorescence was measured by a fluorometer (excitation at 498nm; emission at 521nm). The amount of fluorescent

product generated is representative of the amount of active caspase-3/7 present in the sample.

Analysis of Cell Cycle and Apoptosis

Cell cycle was determined by PI staining and flow cytometry as described [10]. Briefly, HL60 cells (2×10^5 cells/ml) were incubated in 10% FBS/RPMI 1640 containing the fucoidan extract for 24 h. After one gentle rinse by PBS, the cells were collected by trypsinization and centrifugation at $200 \times g$ for 5 min. Then the cell pellets were gently resuspended in 150 μl of PBS, and added 350 μl of cold ethanol for 30 min at 4°C. Fixed cells were collected by centrifugation at $200 \times g$ for 5 min, and were resuspended in 500 μl of PBS. The cell pellets were incubated in 500 μl of PI/RNase A/PBS staining solution (10 $\mu\text{g}/\text{ml}$ PI and 10 $\mu\text{g}/\text{ml}$ RNase A in PBS) at 25°C for 20 min in a dark place. Stained cells were analyzed by a flowcytometer (excitation: 488 nm and emission: 610nm). Ten thousand cells were evaluated for each sample. In some experiments, HL60 cells was pretreated with 20 μM Z-VAD-FMK (Promega) for 5 h followed by a 24 h incubation with the fucoidan extract before analysis of apoptosis. Mitochondrial membrane potential was determined by Rhodamine 123 staining using a flowcytometer, as previously reported [11]. Briefly, the cells were washed twice with PBS and incubated with 1 $\mu\text{g}/\text{ml}$ rhodamine 123 (Sigma-Aldrich Inc., St. Louis, MO) at 37°C for 30 min to assess the mitochondrial membrane potential by a flowcytometer. A higher intensity of

rhodamine 123 indicated a higher mitochondrial membrane potential [11].

Sugar Chain Expression Profiling Using FITC-Lectins

Lectin-staining of cells was performed. Briefly, Cells were inoculated at a density of 2.0×10^5 cells into a 6-well culture plate. After pre-incubation, the cells were treated with the fucoidan extract. After treatment, cells were washed with PBS and incubated in $50 \mu\text{l}$ of PBS containing $2 \mu\text{g}$ of FITC-conjugate lectins (J-OIL MILLS Inc., Tokyo, Japan) at 25°C for 15 min. After wash with PBS, cells were suspended in $500 \mu\text{l}$ of PBS, and then the analysis was performed by a flowcytometer.

Stimulation of Con A-induced Apoptosis of Cancer Cells

Cells were inoculated at a density of 1.0×10^5 cells into a 6-well culture plate. After pre-incubation, the cells were treated with the fucoidan extract for 48 h. Then, cells were washed with PBS and incubated in $50 \mu\text{l}$ of PBS containing $2 \mu\text{g}$ of Con A at 25°C for 30 min. After wash with PBS, cell cycle analysis was performed.

RESULTS

Anti-proliferative activity of the fucoidan extract to cancer cells

The treatment of the enzyme-digested fucoidan

extract suppressed the proliferations of various cancer cells (Figure 1). The fucoidan extract suppressed the proliferations of anchorage-dependent human cancer cells such as cervix adenocarcinoma HeLa, connective tissue fibrosarcoma HT1080 and lung adenocarcinoma A549 cells. Anchorage-independent human cancer cells, typical leukemic cells, such as acute promyelocytic leukemia HL-60, histiocytic lymphoma U937 and chronic myelogenous leukemia K562 cells were also suppressed the proliferations by the fucoidan extract treatment. The anti-proliferation effect of cancer cells was enhanced by treatment with the fucoidan extract in a dose-dependent manner.

On the other hand, the fucoidan extract did not induce apoptosis to human peripheral blood lymphocytes even at the highest dose of $560 \mu\text{g/ml}$. At the highest dose, HL-60 and U937 cells were induced 33.2% and 34.5% sub-G1 population, respectively (data not shown). It was suggested that the fucoidan extract specifically suppressed the proliferation of cancer cells.

The fucoidan extract induced apoptosis in HL60 cells

The cell cycle analysis of human leukemic HL60 cells was analyzed using a flowcytometer. The percentage of sub-G1 fraction was increased in a time- and dose- dependent manner after treatment with the fucoidan extract (data not shown). Pretreatment of HL60 cells with pan-caspase inhibitor, Z-VAD-FMK, inhibited the

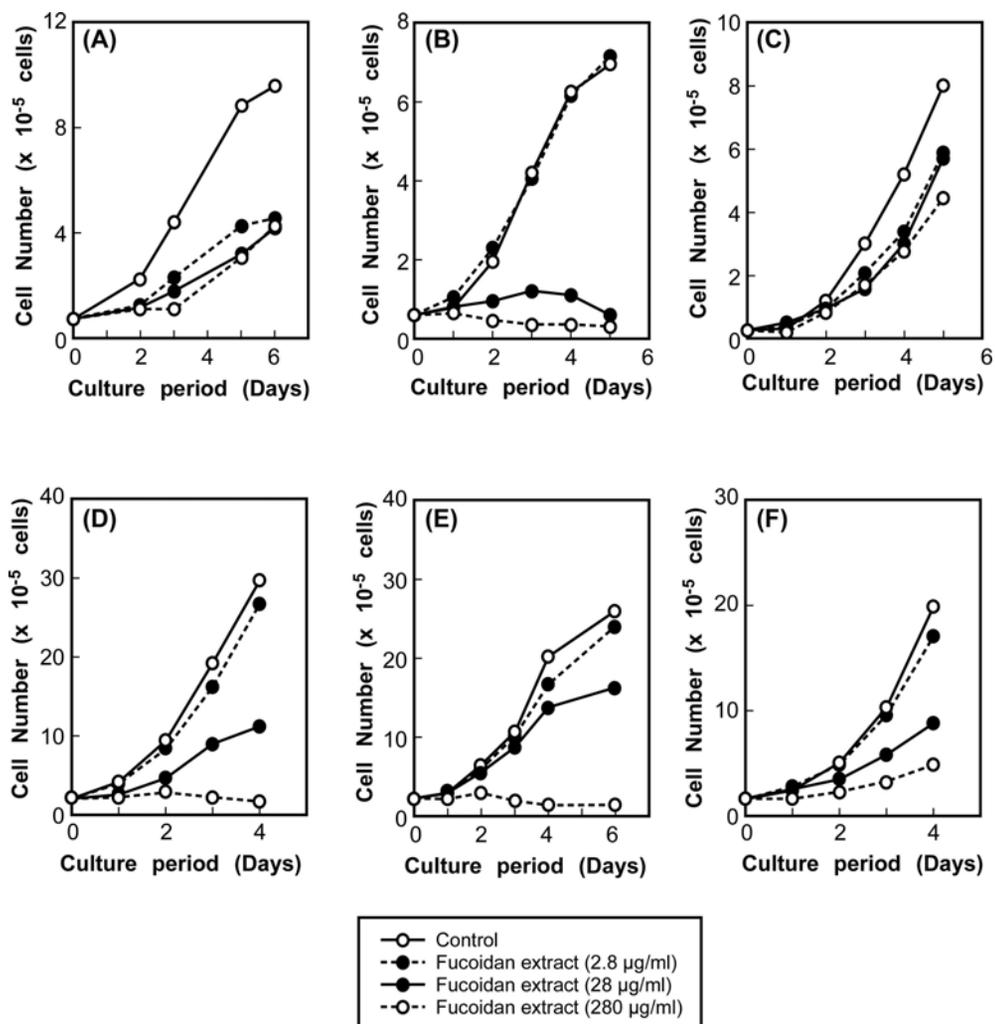


Figure 1. Anti-proliferative activity of the fucoidan extract to human cancer cells. HeLa (cervix adenocarcinoma), (B) HT1080 (connective tissue fibrosarcoma), (C) A549 (lung adenocarcinoma), (D) HL-60 (acute promyelocytic leukemia), (E) U937 (histiocytic lymphoma), (F) K562 (chronic myelogenous leukemia).

augmentation of sub-G1 population induced by the fucoidan extract (Figure 2 A). This result indicated that the caspase activity played the important role for the augmentation of sub-G1 population in the fucoidan extract treated HL60

cells. To evaluate the involvement of the activation of caspase-3/7, the activity of caspase-3/7 was determined. After 3 h treatment of the fucoidan extract, the caspase-3/7 activity reached the maximum value (Figure 2 B). The

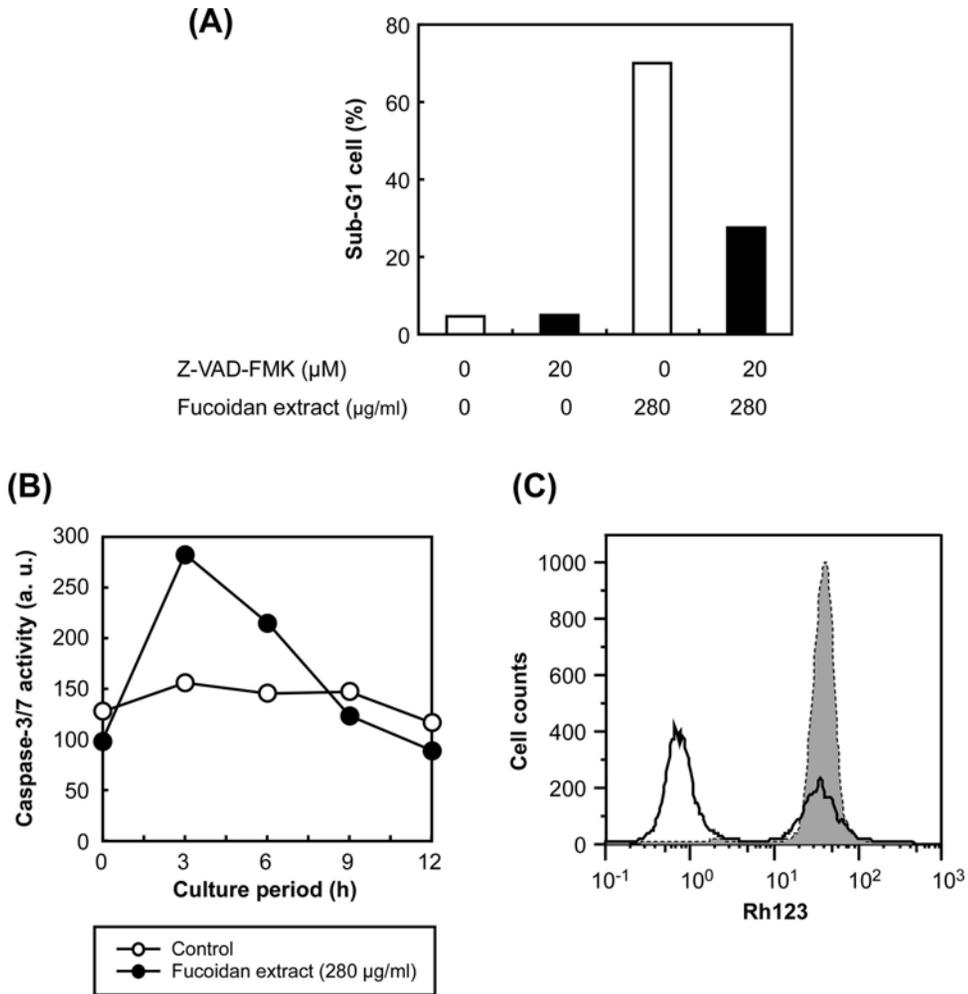


Figure 2. The fucoidan extract induced apoptosis in human leukemic HL60 cells. (A) Detection of sub-G cells treated with or without the fucoidan extract and pan-caspase inhibitor Z-VAD-FMK. (B) Detection of caspase-3/7 activation in the fucoidan extract treated HL60 cells. (C) Analyses of mitochondrial electric potential. Control; a dotted line and gray filling. Fucoidan extract treatment (280 μg/ml, 24 h); a solid line.

mitochondrial membrane potential of HL60 cells was decreased by the fucoidan extract treatment for 24 h (Figure 3 C). These results showed that the treatment of the fucoidan

extract induced apoptosis in HL60 cells via caspase-3/7 activation and the mitochondrial depolarization.

The fucoidan extract changed sugar chain expression on the surface of cancer cell membrane and stimulated Con A-induced apoptosis of cancer cells

We treated various cancer cells with the fucoidan extract, Con A binding was enhanced in human cancer-derived A549, HeLa and HT1080 cells, but human normal fibroblast TIG-1 cells did not (Table 1). The binding profiles with other lectins were also changed. The binding of 3 cancer cells with Con A was increased depending upon their malignancy (data not shown). The malignancy is considered to become stronger in order of A549, HeLa and HT1080 cells.

When HT1080 cells were pretreated with the fucoidan extract, apoptosis-inducing ability of Con A was enhanced. However, normal TIG-1

cells were not (Figure 3). Con A-induced apoptosis of cancer cells was enhanced by treatment with the fucoidan extract in a dose- and time-dependent manner (data not shown).

DISCUSSION

Fucoidan is reported to induce apoptosis of tumor cells [9, 12-13]. However, the mechanism by which of fucoidan induces the apoptosis of tumor cells has not been clearly elucidated. We used fucoidan derived from the seaweed *Cladosiphon novae-caledoniae* Kylin and digested with abalone glycosidase [8]. The fucoidan extract was found to consist mostly of fucose (73%) by sugar composition analysis, and digested small MW fraction (72%, MW: <500) by size-

Table 1. Sugar chain expression profiling using FITC-labeled lectins after treatment with the fucoidan extract

Lectin		Normal	Cancer		
		TIG-1	A549	HeLa	HT1080
ConA	α -D-Man	-	↑	↑	↑
PHA-E4	D-GalNAc	-	-	-	-
LCA	Fuca1→6G1cNAc	↑	-	-	-
PNA	Gal β 1→3GalNAc	-	-	↓	↑
DBA	GalNAca1→3GalNAc	-	-	↑	↓
UEA-I	α -L-Fuc	↑	-	↑	-
RCA120	Gal β 1→4G1cNAc	-	↓	-	↑
WGA	D-G1cNAc	↑	↓	↑	↑

Con A; Concanavalin A (Lectin from *Canavalia ensiformis*, jack beans), PHA-E4; *Phaseolus vulgaris* agglutinin (Erythroagglutinin, Lectin from *Phaseolus vulgaris*, red kidney bean), LCA; *Lens culinaris* agglutinin (Lectin from *Lens culinaris*, lentil), PNA; Peanut agglutinin (Lectin from *Arachis hypogaea*, peanut), DBA; *Dolichos biflorus* agglutinin (Lectin from *Dolichos biflorus*, horse gram), UEA-I; *Ulex europaeus* agglutinin-I (Lectin from *Ulex europaeus*, gorse, furze), RCA120; *Ricinus communis* Agglutinin (Lectin from *Ricinus communis*, castor bean), WGA; Wheat germ agglutinin (Lectin from *Triticum vulgaris*, wheat). -; no change, ↑; increase of expression, ↓; decrease of expression.

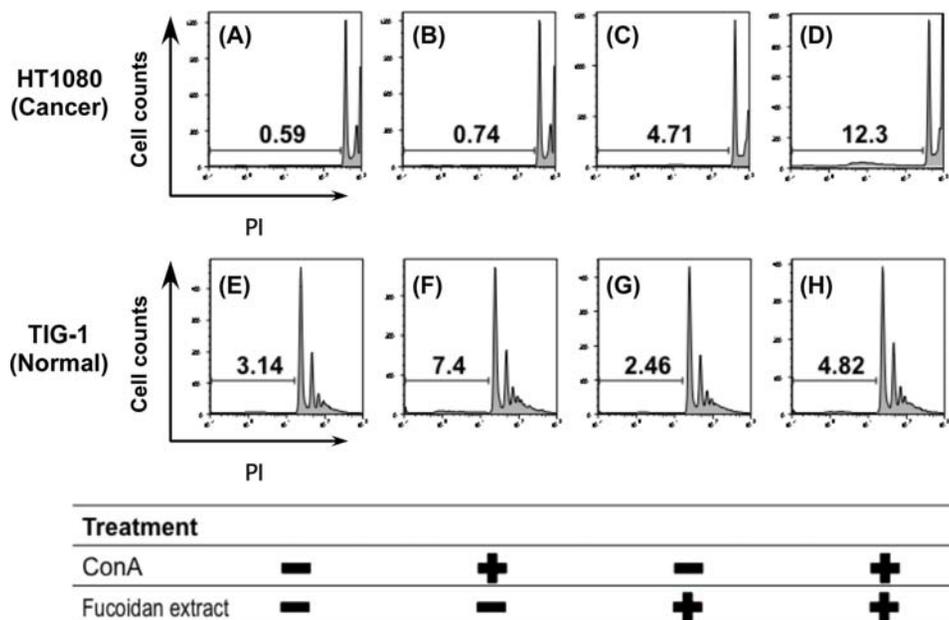


Figure 3. Con A-induced apoptosis of cancer cells was stimulated by the treatment with the fucoidan extract. (A) and (E); controls. (B) and (F); treatment with Con A only. (C) and (G); treatment with the fucoidan extract only. (D) and (H); treatment with both the fucoidan extract and Con A. A percentage of Sub-G1 population was indicated in each histogram.

exclusion chromatography; therefore, fucoidan extract consists mostly of digested fucoidan. In this study, we tried to evaluate the apoptosis inducing activity of the fucoidan extract as one of the anti-tumor effects.

First, anti-proliferative activity of the fucoidan extract was indicated. It was shown that the fucoidan extract inhibited the proliferation of all human cancer cell lines we tested. The fucoidan extract suppressed the proliferations of anchorage-dependent and anchorage-independent human cancer cells in a dose-dependent manner (Figure 1), and an enzymatic digestion of

fucoidan was not an obstacle to suppress the proliferation of cancer cells. In another experiment, a cell death was not induced in human peripheral blood lymphocytes even at the highest dose of 560 $\mu\text{g}/\text{ml}$. At the highest dose, HL-60 and U937 cells exhibited 33.2% and 34.5% sub-G1 population, respectively. It was suggested that the fucoidan extract specifically suppressed the proliferation of cancer cells. The cell cycle analysis revealed that the percentage of sub-G1 fraction was increased after treatment with the fucoidan extract. The augmentation of sub-G1 fraction was suppressed

by pan-caspase inhibitor Z-VAD-FMK. The treatment of the fucoidan extract stimulated the caspase-3/7 activation and the mitochondrial membrane depolarization (Figure 2). These results strongly suggested that the suppression of cell proliferation in HL60 cells induced by the fucoidan extract was the apoptosis.

Next, we are interested in the mechanism why the fucoidan extract induces cancer-specific apoptosis. It is well known that cancer cells changes the membrane surface sugar chains depending upon their malignancy. Although normal cells cannot be aggregated by concanavalin A (Con A), one of lectins, cancer cells are easily aggregated by Con A. This phenomenon suggested that sugar chain synthesis pathways in cancer cells are different from those in normal cells. The treatment with the fucoidan extract enhanced Con A binding to human cancer-derived A549, HeLa and HT1080 cells. However, human normal fibroblast TIG-1 cells did not enhanced. Con A-induced apoptosis in HT1080 cells were augmented by pretreatment with the fucoidan extract in a dose- and time-dependent manner, however, normal TIG-1 cells was not. Philchenkov *et al.* described that fucoidan sensitized human malignant lymphoid cells to etoposide [14]. Our result suggested that the fucoidan extract had enhancing effect of Con A-inducing apoptosis in HT1080 cells. These suggested the fucoidan extract had 2 types of anti-tumor effect *via* apoptosis. One is induction of apoptosis to cancer cells directly; another is enhancement of

apoptosis in cancer cells *via* various stimuli.

In conclusion, the enzyme-digested fucoidan extract suppressed the growth of anchorage-dependent and anchorage-independent cancer cells. The enzyme-digested fucoidan extract stimulated the caspase-3/7 and mitochondrial pathways to induce apoptosis. The enzyme-digested fucoidan extract enhanced Con A binding of cancer cells and Con A-induced apoptosis.

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