Master's Thesis

Effect of Fucoidan from *Undaria pinnatifida* on the Induction of Apoptosis in A549 Human Lung Cancer Cells and PC-3 Human Prostate Cancer Cells

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폐암 세포 A549와 전립선암 세포 PC-3에 유도된 세포사멸에 *Undaria pinnatifida*로부터 분리된 Fucoidan의 효과

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This thesis has been examined and approved.

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LIST OF ABBREVIATIONS

Abbreviations	Full name
PARP	Poly-(ADP-ribose) polymerase
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinases
PI3K	Phosphatidylinositol 3-kinase
Wnt	Wingless-type MMTV integration site family member
GSK-3β	Glycogen synthase kinase-3β
MMP-9	Matrix metalloproteinase-9
NF-κB	Nuclear factor-κB
TGF-β1	Transforming growth factor-β1
HO-1	Heme oxygenase-1
MDR	Multidrug resistance
MRP-1	Multidrug resistance-associated protein-1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SD	Standard deviation

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PART Ⅰ

Fucoidan from *Undaria pinnatifida* Induces Apoptosis in A549 Human Lung Carcinoma Cells

1. ABSTRACT

Fucoidan, a sulfated polysaccharide, has various biological activities, such as anti-cancer, anti-angiogenic, and anti-inflammatory effects; however, the mechanisms of action of fucoidan on anti-cancer activity have not been fully elucidated. We examined the anti-cancer effects of fucoidan from *Undaria pinnatifida* on A549 human lung carcinoma cells. Treatment of A549 cells with fucoidan resulted in potent anti-proliferative activity. Also, some typical apoptotic characteristics, such as chromatin condensation and an increase in the population of sub-G_1 hypodiploid cells, were observed. With respect to the mechanism underlying induction of apoptosis, fucoidan reduced Bcl-2 expression, but the expression of Bax was increased in a dose-dependent manner compared to controls. Furthermore, fucoidan induced caspase-9 activation, but decreased the level of procaspase-3. Cleavage of poly-ADP-ribose polymerase (PARP), a vital substrate of effector caspase, was found. We further investigated the role of the MAPK and PI3K/Akt pathways with respect to the apoptotic effect of fucoidan, and showed that fucoidan activates ERK1/2 pathway in A549 cells. Unlike ERK1/2, however, treatment with fucoidan resulted in the reduction of phosphorylation of p38. In addition, fucoidan resulted in the reduction of phosphorylation of

Akt. Together, these results indicate that fucoidan induces apoptosis of A549 human lung cancer cells through down-regulation of p38 MAPK and PI3K/Akt signaling pathway, and the activation of the ERK1/2 MAPK signaling pathway.

***Key words*:** A549, apoptosis, fucoidan, MAPK, PI3K/Akt

2. INTRODUCTION

Lung cancer is a leading cause of cancer deaths, with more than 1,180,000 deaths annually (Parkin *et al*., 2005); thus new and more effective treatments are needed. Fucoidan has anti-inflammatory, anti-angiogenic (Koyanagi *et al*., 2003), anti-coagulant (Durig *et al*., 1997), anti-HIV (Mcclure *et al*., 1992), and anti-cancer (Asia *et al*., 2005) activities, but the mechanisms underlying anti-cancer activity have not been fully elucidated.

Fucoidan is a sulfated polysaccharide in the cell wall matrix of brown algae, such as *Fucus vesiculosus*, *Ascophyllum nodosum*, *Laminaria angustata*, *Ecklonia kurome*, *Hizikia fusiforme,* and *Undaria pinnatifida*. Fucoidan contains a considerable percentage of $_{L}$-fucose and sulfated ester groups, along with lesser amounts of $_{D}$-xylose, $_{D}$-galactose, $_{D}$-mannose, and glucuronic acid (Gideon and Rengasamy, 2008). According to a previous study, the major framework of fucoidan is known to be α1,3-linked-$_{L}$-fucose-4-sulfate (Kim *et al*., 2008). In the case of fucoidan from *Undaria pinnatifida,* among several kinds of fucoidans, the main sulfated polysaccharide has been characterized as galactofucan sulfate (Lee *et al*., 2004). Until now, fucoidan from *Fucus vesiculosus* is commercially available (Holtkamp *et al*., 2009).

Mitogen-activated protein kinase (MAPK) pathways modulate gene expression, differentiation, cell cycle arrest, mitosis, proliferation, motility, metabolism, and apoptosis (Wada *et al*., 2004). Extracellular signal-regulated kinases (ERKs) affect differentiation, cell survival, and proliferation, whereas c-Jun N-terminal kinases (JNKs) and p38 MAPKs are activated by stress (Yang *et al*., 2007) to modulate cell growth, cell differentiation, cell cycle, inflammation, and cell death (Bassi *et al*., 2008). The phosphatidylinositol 3-kinase (PI3K)/Akt pathway also regulates cell survival, cell growth, and apoptosis (Jin *et al*., 2007), and promotes proliferation and survival in cancer cells (Toker *et al*., 2006). Herein we examined the anti-cancer effects and molecular mechanisms of action of fucoidan from *Undaria pinnatifida* in A549 human lung carcinoma cells.

3. MATERIALS AND METHODS

3.1. Reagents

Fucoidan (from *Undaria pinnatifida*), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, and propidium iodide (PI) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Mouse monoclonal anti-human Bcl-2, rabbit polyclonal anti-Bax, anti-caspase-3 and anti-poly (ADP-ribose) polymerase (PARP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotech, CA, USA); Rabbit polyclonal anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38, anti-Akt, anti-phospho-Akt and anti-cleaved caspase-9 antibodies were purchased from Cell Signaling Technology (Cell signaling Technology, Beverly, MA, USA); monoclonal β-actin was purchased from Sigma; HRP-conjugated goat anti-rabbit and horse anti-mouse IgGs were purchased from Vector (Vector Laboratories, Burlingame, MA, USA); Aprotinin, leupeptin, Nonidet P-40 were obtained from Roche (Roche Applied Science, Indianapolis, IN); Western blotting reagent, West-zol enhanced chemilumin, was obtained from Intron (iNtROn Biotechnology, Korea).

3.2. Cell culture

A549, a human lung cancer cell line, was obtained from the Korean Cell Line Bank (KCLB) and cultured in RPMI 1640 (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, UT, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO Inc, Grand Island, NY, USA) at 37°C in a humidified atmosphere with 5% CO_2.

3.3. Cell viability assay

The effect of fucoidan on the growth of A549 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Scudiero *et al*., 1988). Cells were seeded at 5×10^4 cells/mL in 200 ㎕ on 96-well microplates. After 18 h at 37°C in 5% CO_2 gas to allow cell attachment, cells were treated with fucoidan (10, 50, 100 and 200 ㎍/mL) for 96 h. At the end of experimental incubation, cells were treated with 50 ㎕ (5 ㎎/mL) MTT dye and incubated 37°C for 4 h. The medium was aspirated and replaced with 150 ㎕/well dimethyl sulfoxide to dissolve the formazan solution. Cell viabilities were determined by measuring the absorbance at 540 nm using a micro plate ELISA reader (Amersham Pharmacia Biotech, NY, USA). Each experiment was repeated at least three times.

3.4. Flow cytometric analysis of apoptosis

The effect of fucoidan on cell cycle distribution was analyzed by flow cytometry after staining the cells with propidium iodide (PI) (Fried *et al*., 1976). A549 cells (5×10^4 cells/mL) were treated with fucoidan (0, 50, 100 and 200 ㎍/mL) and cultured for 24 h. The treated cells were trypsinized, washed two times with PBS (phosphate-buffered saline) and fixed with 70% ethanol 30 min at 4°C. The fixed cells were washed twice with cold PBS, incubated with 50 ㎍/mL RNase A at 37°C for 30 min, stained with 50 ㎍/mL PI in the dark for 30 min at 37°C. The stained cells were analyzed using an EPICS-XL FACScan flow cytometer (Beckman Coulter, Miami, FL, USA). The proportion of cells in G_0/G_1, S, G_2/M phases was represented as DNA histograms. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G_1 peak in the cell cycle pattern. For each experiment, 10,000 events per sample were analyzed, and experiments were repeated three times.

3.5. Morphological analysis of apoptosis by Hoechst 33342 staining

For detection of apoptosis, cells were seeded at 5×10^4 cells/mL in 1 mL on 24-well microplates. After 18 h of incubation to allow cell attachment, cells were treated with fucoidan (0, 50, 100 and 200 ㎍/mL) for 24 h. The cells were incubated in a Hoechst 33342 (10 ㎍/mL medium, final) staining solution at 37°C for 20 min. The stained cells were

observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (magnification × 200).

3.6. Western blot analysis

A549 cells were treated with fucoidan (0, 50, 100 and 200 μg/mL) for 24 h. After treatment, the cells were harvested and washed two times with cold PBS. The cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM Phenylmethylsulfonylfluoride, 25 μg/mL aprotinin, 25 μg/mL leupeptin, 1 mM DTT, 1% Nonidet P-40) and kept on ice for 30 min at 4°C. The lysates were centrifuged at 15,000 rpm at 4°C for 15 min. The supernatants were stored at -20°C until use. Protein content was determined by the Bradford assay (Bradford, 1976). The same amount of lysates were separated on 10~15% SDS-PAGE gels and then transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, Hercules, CA, USA) by glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.8], and 20% MeOH [v/v]) at 100 V for 2 h. After blocking with 5% nonfat dried milk, the membrane was incubated with primary antibody against PARP (1:1000), procaspase-3 (1:1000), cleaved caspase-9 (1:1000), Bcl-2 (1:1000), Bax (1:5000), ERK1/2 (1:1000), phospho-ERK1/2 (1:1000), p38 (1:1000), phospho-p38 (1:1000),

Akt (1:1000), phospho-Akt (1:1000), and β-actin (1:1000) antibodies and incubated with a secondary HRP antibody (1:5000; Vector Laboratories, Burlingame, VT, USA) at room temperature. The membrane was exposed on X-ray films (AGFA, Belgium), and protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON., Gyeonggi-do, Korea).

3.7. Statistical analyse

Results are expressed as means ± standard deviation (SD) from representative of three independent experiments. Student's *t*-test was used to evaluate the data with the following significance levels: $*p<0.05$, $**p<0.01$, $***p<0.001$. All assays were performed in at least 3 independent experiments.

Table 1. Antibodies used in Western blot analysis on A549 cells.

Antibody	Origin	Company
poly-(ADP-ribose) polymerase (PARP)	rabbit polyclonal	Santa Cruz Biotechnology
Procaspase-3	rabbit polyclonal	Santa Cruz Biotechnology
Bcl-2	mouse monoclonal	Santa Cruz Biotechnology
Bax	rabbit polyclonal	Santa Cruz Biotechnology
ERK1/2	rabbit polyclonal	Cell signaling Technology
phospho-ERK1/2	rabbit polyclonal	Cell signaling Technology
p38/MAPK	rabbit polyclonal	Cell signaling Technology
phospho-p38/MAPK	rabbit polyclonal	Cell signaling Technology
Akt	rabbit polyclonal	Cell signaling Technology
phospho-Akt	rabbit polyclonal	Cell signaling Technology
Cleaved caspase-9	rabbit polyclonal	Cell signaling Technology
β-actin	mouse monoclonal	Sigma

4. RESULTS

4.1. Effect of fucoidan on the growth of A549 cells

To evaluate the effect of fucoidan on the growth of A549 cells, cell viability was assessed using the MTT assay. Fucoidan (10, 50, 100 and 200 ㎍/mL) treatment for 96 h induced dose-dependent cell death (Fig.1; 10 ㎍/mL, 17.8%; 50 ㎍/mL, 27.6%; 100 ㎍/mL, 41.2%; and 200 ㎍/mL, 52.1%).

4.2. Effect of fucoidan on cell cycle in A549 cells

To determine whether apoptosis induced by fucoidan is related to changes in cell cycle progression, cell cycle analysis was determined using propidium iodide (PI) DNA staining. Flow cytometry was used to measure the induction of apoptosis and cell cycle distribution. Fucoidan treatment increased the abundance of the sub-G_1 fraction in a dose-dependent fashion (Fig.2; 0 ㎍/mL, 1.59%; 50 ㎍/mL, 40.3%; 100 ㎍/mL, 51.5%; 200 ㎍/mL, 64.9%) for 24 h.

4.3. Effect of fucoidan on morphological change of apoptosis in A549 cells

Nuclear morphological changes, such as chromatin condensation, membrane blebbing, and cell shrinkage, are critical markers of cell apoptosis. Thus, to determine nuclear morphological change of apoptosis induced by fucoidan, we performed morphological study. Fucoidan induced the production of these apoptotic bodies in Hoechst-stained cells (Fig. 3).

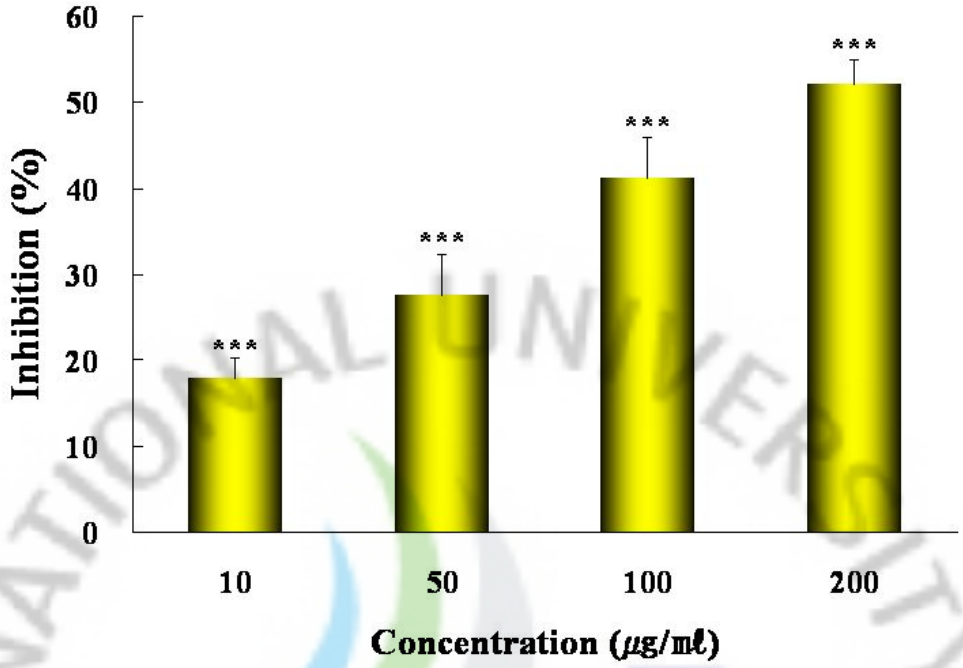


Figure 1. Fucoidan inhibited the growth of A549 cells. A549 cells (5×10^4 cells/mL) were treated with fucoidan for 96 h. Cell growth inhibition was measured by MTT assay. Data are presented as mean ± SD from representative of three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

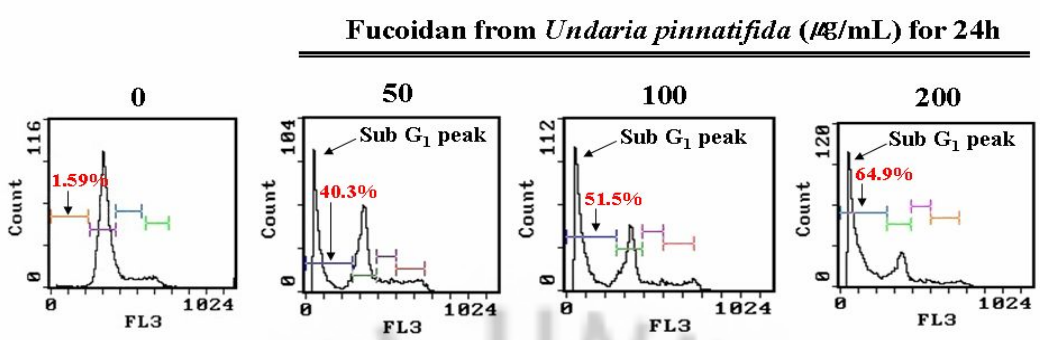


Figure 2. Fucoidan induced apoptosis via increase of sub-G_1 peak in A549 cells. A549 cells (5×10^4 cells/mL) were treated with fucoidan for 24 h. After treatment, cells were collected, fixed with 70% ethanol, and stained with propidium iodide. Changes in cell cycle phase distribution were measured by DNA flow cytometric analysis. Data represent the percentage of cells in sub-G_1 phases of the cell cycle.

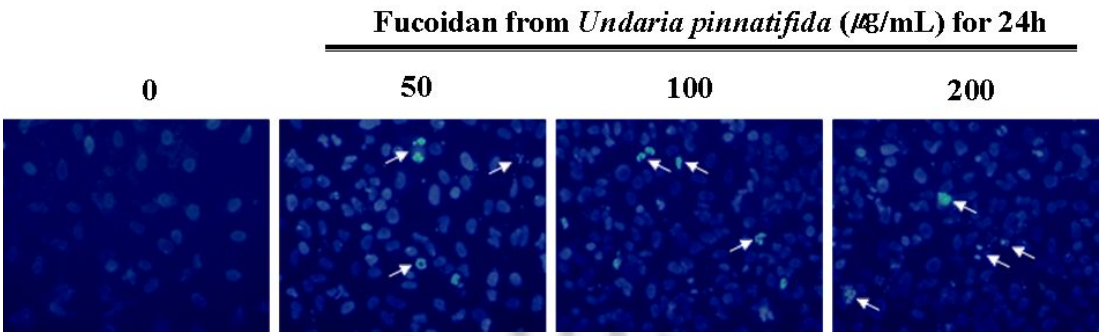


Figure 3. Fucoidan induced apoptosis through morphological change in A549 cells. A549 cells (5×10^4 cells/mL) were treated with fucoidan for 24 h. Cells were stained with DNA-specific fluorescent dye, Hoechst 33342 (10 ㎍/mL, concentration of final). Apoptotic bodies were observed by an inverted fluorescent microscope equipped with an IX-71 Olympus camera (magnification×200).

4.4. Effect of fucoidan on the expressions of Bcl-2 and Bax in A549 cells

Mitochondria integrate apoptotic signals from both extrinsic and intrinsic apoptotic pathways. We therefore measured the expression of mitochondrial anti- and pro- apoptotic proteins by western blot analysis. Fucoidan reduced Bcl-2 expression, but increased Bax expression in a dose-dependent manner (Fig. 4).

4.5. Effect of fucoidan on activation of caspase-9 and -3 in A549 cells

To determine the effect of fucoidan on caspase pathway, A549 cells were incubated with fucoidan at diverse concentration (0, 50, 100 and 200 μg/mL) for 24 h. Fucoidan decreased the procaspase-3 expression, but induced the caspase-9 activation. Furthermore, fucoidan induced poly (ADP-ribose) polymerase (PARP) cleavage, a vital caspase substrate (Fig.5)

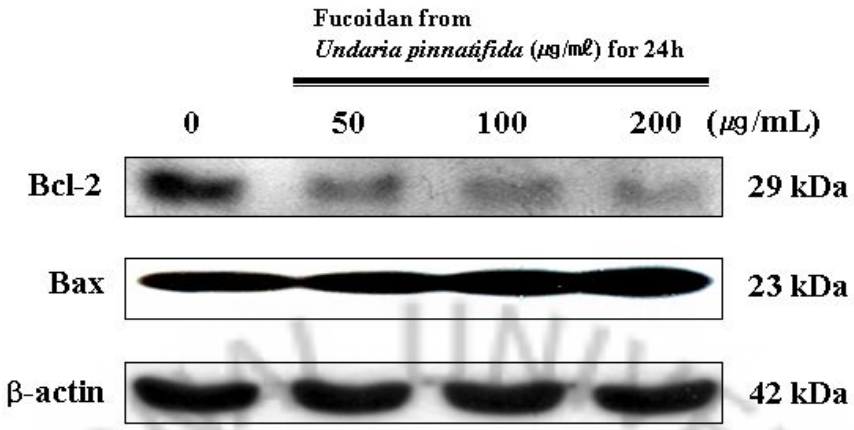


Figure 4. Effect of fucoidan on the expressions of Bcl-2 and Bax in A549 cells. A549 cells (5×10^4 cells/mL) were treated with the indicated concentration of fucoidan for 24 h. Lysates were prepared from these cells, and then analyzed the expressions of Bax and Bcl-2 by western blot analysis using specific antibodies.

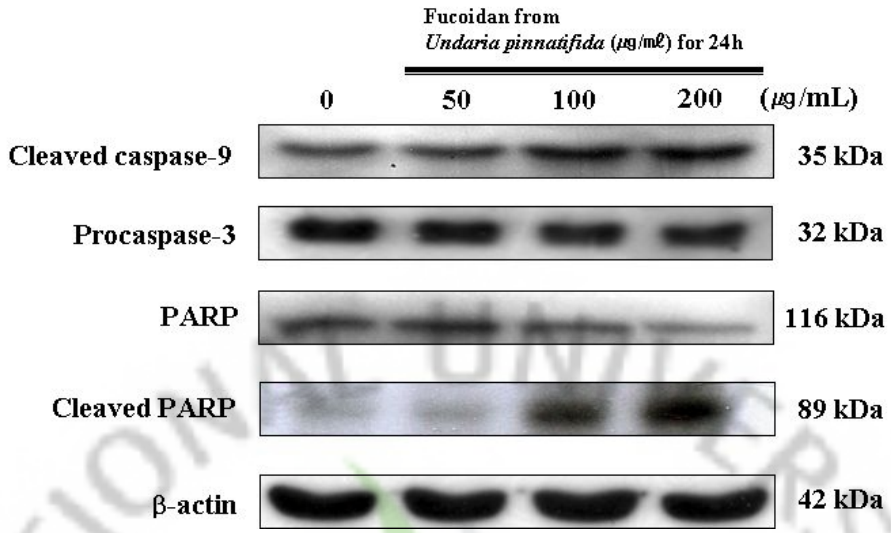


Figure 5. Effect of fucoidan on the expressions of caspase-9 and procaspase-3 in A549 cells. A549 cells (5×10^4 cells/mL) treated with the indicated concentration of fucoidan for 24 h. Lysates were prepared from these cells, and then analyzed the expressions of caspase-9 and procaspsae-3, and PARP cleavage by western blot analysis using specific antibodies.

4.6. Effect of fucoidan on MAP kinase signaling in A549 cells

In order to establish the MAP kinase mechanism of apoptosis induced by fucoidan, the expression ERK1/2 and p38 following fucoidan treatment was examined by western blot analysis. A549 cells were treated with fucoidan at difference concentration (0, 50, 100 and 200 μg/mL) for 24 h. Fucoidan treatment activated phospho-ERK1/2, but down-regulated phospho-p38 expression (Fig. 6).

4.7. Effect of fucoidan on PI3K/Akt signaling in A549 cells

To evaluate the PI3K/Akt mechanism of apoptosis induced by fucoidan, A549 cells were incubated with fucoidan at diverse concentration (0, 50, 100 and 200 μg/mL) for 24 h. Fucoidan treatment resulted in decreased phospho-Akt levels (Fig. 7).

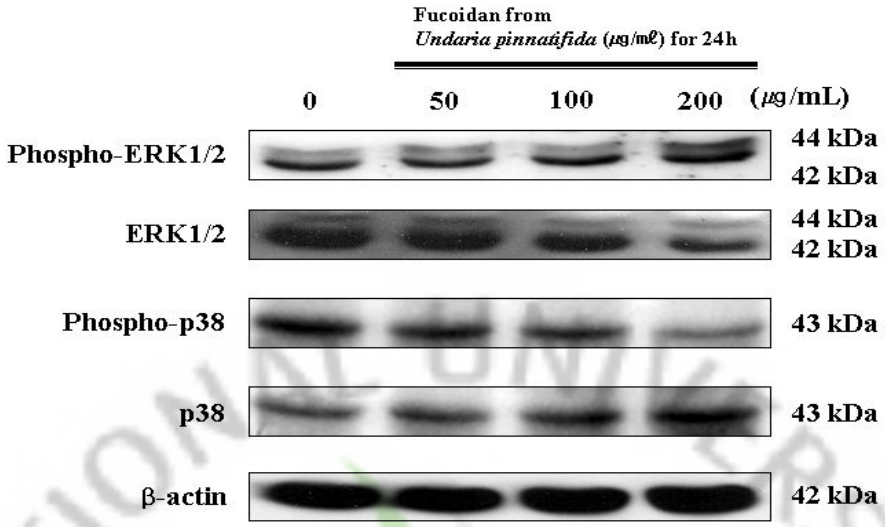


Figure 6. Effect of fucoidan on MAP kinase signaling in A549 cells. A549 cells (5×10^4 cells/mL) were treated with the indicated concentration of fucoidan for 24 h. Lysates were prepared from these cells, and then analyzed the expressions of MAP kinase (ERK1/2, p38) by western blot analysis using specific antibodies.

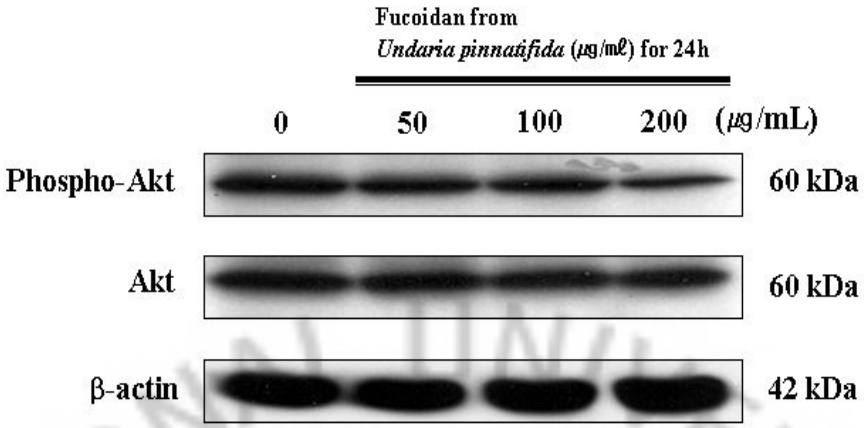


Figure 7. Effect of fucoidan PI3K/Akt signaling in A549 cells. A549 cells (5×10^4 cells/mL) were treated with the indicated concentration of fucoidan for 24 h. Lysates were prepared from these cells, and then analyzed the expression of PI3K/Akt by western blot analysis using specific antibodies.

5. DISCUSSION

In the present study, we demonstrated that fucoidan treatment inhibited the growth of A549 lung carcinoma cells by inducing apoptosis via the up-regulation of ERK1/2 MAPK signaling pathway and the down-regulation of p38 MAPK and PI3K/Akt signaling pathway.

Fucoidan can attack cancer cells directly, as well as forestall cancer cells by enhancing immunity. Such an induction of apoptosis function is not a reported characteristic of other polysaccharides. Other polysaccharides, such as β-glucan (Chan *et al*., 2009), avemar (Boros LG *et al*., 2005), and AHCC (Hirose *et al*., 2007), do not attack cancer cells directly, even if they kill cancer cells by improve immunity by increasing NK-cell, B lymphocyte, and macrophage functions. Previous studies have suggested that fucoidan directly inhibits the proliferation of some cancer cells, such as HS-Sultan (Asia et al., *2005*) and U937 (Teruya *et al*., 2007) cells. This study confirms that fucoidan can directly inhibit the proliferation of A549 cells. Fucoidan inhibited the growth of A549 cells (Fig. 1) and HCT-15 colon cancer cells (Hyun *et al*., 2009) in a dose-dependent fashion, but fucoidan is not toxic to normal RAW 264.7 cells (Chen *et al*., 2009). Indeed, fucoidan is cytotoxic in a specific manner against cancer cells without cytotoxicity against normal cells. Therefore, we examined the

mechanism of induction of apoptosis in A549 cells.

Apoptosis is a complex physiologic process that permits the reduction of harmful cells during development, tissue homeostasis, and disease (Porter and Janicke, 1999). Apoptotic cell death has typical characteristics, such as chromatin condensation, membrane blebbing, cell shrinkage, and an increased population of sub-G_1 hypodiploid cells (Yang *et al*., 2007). Fucoidan treatment (200 μg/mL; approximately 1.5 μM) increased the sub-G_1 fraction by 64.9% in A549 for 24 h (Fig. 2). Fucoidan also induced nuclear morphologic changes as occur during apoptosis (Fig. 3). On the other hand, polyporenic acid C (PPAC) from *Poria cocos* is known to induce apoptosis of A549 cells. When A549 cells are treated with PPAC (60 μM for 48 h), the sub-G1 hypodiploid cell population increased by 47% (Ling *et al*., 2009). 5-hydroxy-3,7-dimethoxy-1,4-phenanthraquinone (Denbinobin), which is isolated from several *Dendrobium* or *Ephemerantha* species, is another agent that induces apoptosis. Denbinobin treatment (20 μM) of A549 cells for 48 h also increased the population of sub-G_1 hypodiploid cells by 38.5% (Kuo *et al*., 2008). Compared with other apoptosis-inducing compounds, such as PPAC and denbinobin, fucoidan appears to be more effective in induction of apoptosis in A549 cells. Apoptosis occurs as interplay of pro- and anti-apoptotic molecules. The Bcl-2 family is separated into two members that either inhibits apoptosis (anti-apoptosis proteins [Bcl-2 and Bcl-xL]) or promotes apoptosis (pro-apoptosis proteins

[Bax, Bid, and Bak]). Pro-apoptotic members of the Bcl-2 family induce apoptosis through the release of cytochrome c, which in turn activates 'initiator' caspases. One caspase activation pathway (extrinsic apoptotic pathway) involves the triggering of the death-receptor superfamily, such as the TNF receptor-1 or Fas receptor with its ligand. The second pathway is the mitochondria-dependent pathway (intrinsic apoptotic pathway) and is induced by changes in the expression of pro- and anti-apoptotic proteins in the cell (Zimmermann and Green, 2001). In contrast, mitochondria integrate apoptotic signals from both the extrinsic and intrinsic apoptotic pathways. We therefore examined the expression of the apoptosis-related proteins, Bcl-2, Bax, caspase-3, and caspase-9. Fucoidan reduced Bcl-2 expression, but increased Bax expression in a dose-dependent fashion (Fig. 4). Fucoidan also decreased the pro-caspase-3 levels and increased the caspase-9 levels and PARP cleavage (Fig. 5). Thus, fucoidan induces apoptosis of A549 cells through the mitochondrial pathway.

MAPK pathways regulate apoptosis (Keyse, 2008). Although ERKs are known to promote cell survival, differentiation and cell proliferation (Seger and Kerbs, 1995), some reports have suggested that ERKs induce cell death (Cheung and Slack, 2004). Cis-diamminedichloroplatinum (CDDP; Cisplatin) induces apoptosis in HeLa cells through activation of the ERK pathway (Wang *et al*., 2000). p38 MAPK can promote cancer cell growth and survival (Junttila *et al*., 2008), and docosahexaenoic acid (DHA) induces

apoptosis in A549 cells by down-regulating p38 (Serini *et al*., 2008). In the current study, fucoidan activated ERK1/2 MAPK signaling pathway, but decreased p38 MAPK signaling pathway (Fig. 6). The PI3K/Akt pathway inhibits apoptosis (Jin *et al*., 2007); fucoidan treatment decreased the phosphorylation of PI3K/Akt (Fig. 7). Magnolol induces apoptosis in human prostate cancer cells by blocking Akt activation (Lee *et al*., 2009). These results suggest that fucoidan induced apoptosis of A549 cells via the activation of ERK1/2 MAPK singaling pathway, as well as down-regulation of PI3K/Akt and p38 MAPK signaling pathway.

In conclusion, fucoidan from *Undaria pinnatifida* induced apoptosis in A549 cells through the down-regulation of Bcl-2 and activation of the caspase pathway. Fucoidan also down-regulated p38 MAPK and PI3K/Akt signaling pathway and activated the ERK1/2 MAPK signaling pathway. These results suggest that fucoidan might have therapeutic potential for lung cancer treatment.

PART Ⅱ

Anti-cancer Activity of Fucoidan from *Undaria pinnatifida* in PC-3 Human Prostate Cancer Cells

1. ABSTRACT

Fucoidan, a sulfated polysaccharide, has various biological activities such as anti-cancer, anti-angiogenic and anti-inflammation effects. However, the action mechanisms of fucoidan on anti-cancer activity have not been fully elucidated. We examined the anti-cancer effect of fucoidan from *Undaria pinnatifida* in PC-3 cells, human prostate cancer cells. Fucoidan exhibited potent anti-proliferation activity against PC-3 cells. When treated with fucoidan, some typical apoptotic characteristics, such as chromatin condensation and the increase of the population of sub-G_1 hypodiploid cells, were observed. The fucoidan activated extrinsic and intrinsic pathways of apoptosis via up-regulation of DR-5, the cleavage of procaspase-8, down-regulation of Bcl-2, up-regulation of Bax, the cleavage of procaspase-3, and cleavage of PARP. In addition, we found the activation of ERK1/2 MAPK signaling pathway, and down-regulation of p38 MAPK and PI3K/Akt signaling pathway. Furthermore the fucoidan induced up-regulation of p21 and down-regulation of E2F-1, cell-cycle-related proteins. Wnt/β-catenin is known to be up-regulated in PC-3 cells; we examined the role of Wnt/β-catenin pathway in the apoptosis-inducing effect of fucoidan. The fucoidan could activate GSK-3β in PC-3 cells and resulted in the decrease of c-myc and cyclin D1 expressions,

target genes of β-catenin via the decrease of β-catenin level. On the other hand, PC-3 cells are reported to be able to induce metastasis and MDR. The fucoidan treatment decreased the levels of NF-κB, MMP-9, TGF-β1, HO-1 and MRP-1. The data indicated that fucoidan might be able to inhibit metastasis and MDR. The results suggested that fucoidan treatment could induce apoptosis of PC-3 prostate cancer cells via up-regulation of ERK1/2 MAPK signaling pathway and down-regulation of p38 MAPK and PI3K/Akt signaling pathway as well as activation of extrinsic apoptosis pathway and intrinsic apoptosis pathway; furthermore, the induction of apoptosis by fucoidan was accompanied by down-regulation of Wnt/β-catenin signaling pathway. The fucoidan treatment could also inhibit metastasis via down-regulation expressions of NF-κB and MMP-9; and down-regulation of MDR-related proteins level through down-regulation expression of TGF-β1, p38, HO-1, NF-κB and MRP-1 expressions. Thus, fucoidan might have therapeutic potential for prostate cancer treatment.

Key words: PC-3, Apoptosis, fucoidan, Wnt/β-catenin, MAPK

2. INTRODUCTION

Fucoidan is a sulfated polysaccharide found in the cell wall matrix of brown seaweed, including *Ascophyllum nodosum*, *Laminaria angustata*, *Ecklonia kurome*, *Hizikia fusiforme*, *Fucus vesiculosus* and *Undaria pinnatifida* (Li *et al*., 2008). The structure of fucoidan is known as heparin-like molecule. Fucoidan includes a substantial percentage of $_{L}$-fucose and sulfated ester groups, as well as small proportions of $_{D}$-xylose, $_{D}$-galactose, $_{D}$-mannose, and glucuronic acid (Gideon and Rengasamy, 2008). Previous studies have suggested that the major structure of fuoidan is known to be α1, 3-linked-$_{L}$-fucose-4-sulfate (Kim *et al*., 2008). Among several kinds of fucoidans, the main sulfated polysaccharide of fucodian from *Undaria pinnatifida* has been described as galactofucan sulfated (Lee *et al*., 2004). Fucoidan has various biological activities such as anti-cancer (Asia *et al*., 2005), anti-inflammatory, anti-angiogenic (Koyanagi *et al*., 2003), anti-coagulant (Durig *et al*., 1997) and anti-HIV (McClure *et al*., 1992) activities. However, the action mechanisms of fucoidan on anti-cancer activity have not been fully elucidated.

Apoptosis is a ubiquitous and highly regulated mechanism that permits individual cells to kill and remove harmful cells during development, tissue homeostasis and disease (Porter

and Janicke, 1999). Apoptosis, or programmed cell death, is typical characteristics including membrane blebbing, cell shrinkage, chromatin condensation and the increase of the population of sub-G_1 hypodiploid cells. In general, the two main apoptosis pathways are the "extrinsic pathway" and "intrinsic pathway". The extrinsic pathway can be initiated at the plasma membrane upon ligation of death receptors, such as CD95/Fas/Apo1, tumor necrosis factor (TNF) receptor 1 (TNFR1), TNF receptor 2 (TNFR2) and death receptor 3-6 (DR3-6). Binding to the receptors results in activation of the initiator caspase-8, which can subsequently induce activation of the effector caspase-3. The intrinsic pathway, mitochondrial pathway, can be initiated by the release of apoptogenic factors including cytochrome c, apoptosis inducing factor, second mitochondria-derived activator of caspase (Smac/Diablo), Omi/HtrA2, endonuclease G, caspase-2 and caspase-9 from the mitochondrial intermembrane space. This pathway is activated via various stimuli including DNA damage, hypoxia, cell detachment, cellular distress and cytotoxic drugs. The release of cytochrome c into the cytosol induces caspase-3 activation via formation of apoptosome complex, such as cytochrome c/Apaf-1/caspase-9 (Ashkenazi *et al*., 2008).

Up-regulation of the Wnt/β-catenin pathway has been found in a large portion of prostate cancer patients (Zi *et al*., 2005). The Wnt ligands, a family of secreted cysteine-rich glycoproteins, have been described in accordance with their roles in early development and

tumorigenesis (Verras *et al*., 2004). The increased expression of β-catenin plays a pivotal role in many cancer. The level of free β-catenin is strongly regulated by a β-catenin degradation complex. In the absence of a Wnt signal, β-catenin is constitutively down-regulated by a β-catenin degradation complex including axin, adenomatous polyposis coli (APC), casein kinase Ⅰ and GSK-3β. These proteins promote the phosphorylation on the serine and threonine residues in the amino-terminal region of β-catenin and thereby target it for ubiquitination and degradation via ubiquitin proteasome pathway by βTrCP or Siah leading to degradation in the 26S proteasome. In the presence of a Wnt signal this process is prevented, which leads to cytoplasmic stabilization of β-catenin, a key component of the canonical Wnt signaling pathway, and its resulting accumulation of β-catenin in the nucleus, is a characteristic of the Wnt signaling pathway activation. The nuclear accumulation of β-catenin promotes the formation of transcriptionally activity of Lymphoid Enhancer-binding Factor (LEF)/T-cell factor (TCF) transcription factors in the nucleus, thereby Wnt target genes (c-myc, cyclin D1, and MMP-7 etc.) are activated. Among proteins of destruction complex, GSK-3β plays a pivotal role in the Wnt pathway by regulating the degradation of β-catenin. GSK-3β is also prevented via Wnt signaling, which may contribute to progression of the prostate cancer. The activity of GSK-3β is diminished through the phosphorylation of serine 9 and previous studies have shown that serine 9 in GSK-3β is phosphorylated by Akt

(Morin, 1999).

Prostate cancer is the most common diagnosed cancer and second leading cause of mortality in males in industrialized countries (Dehm and Tindall, 2006). The incidence of prostate cancer in Asian countries is lower than that in Western countries; but, the mortality of prostate cancers is increasing most rapidly in males in Asia because of westernization of dietary life (Nieto *et al*., 2007). Prostate cancer advances slowly and can be treated efficiently when discovered early. However, metastasis of other internal organs appears a major barrier to improve survival rate and treatment efficiency (Lim *et al*., 2005). Most of prostate cancer patients present bone metastasis (Hall *et al*., 2006). Therefore, metastasis has an important role for an efficient prostate cancer treatment. Metastasis of cancer cells involves multi-stepwise process that relies on the activity of many proteins (Vijayababu *et al*., 2006). Proteolytic degradation of the extracellular matrix (ECM) components is one of the characteristics of cancer cell invasion and migration (Hung *et al*., 2009). The metastasis is involving several classes of proteinases, such as serine proteinaes, cysteine proteinases and matrix metalloproteinases (MMP's). Among these various proteins, MMP's are a family of zinc-dependent proteases that can degrade components of the ECM (Vijayababu *et al*., 2006). Especially, MMP-9 has been found to be particularly involved with metastasis of prostate cancer and the expression of MMP-9 is up-regulated by NF-κB (Lindholm *et al*., 2000).

Drug resistance to therapeutic agents frequently occurs in androgen-independent prostate cancer. This multi-drug resistance is a main obstacle to cancer treatment and leads to high mortality for patients. Previous study has demonstrated that early response genes involved to nuclear factor-κB (NF-κB) contribute to metastasis and development of hormone-refractory prostate cancer (HRPC) (Uzzo *et al*., 2008). Several proteins including MDR gene and its product, P-glycoprotein, breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP) are related to resistance of chemotherapy (Kim *et al*., 2006). Among these proteins, MRP is found in multidrug-resistant prostate cancer cell lines (Cole *et al*., 1992). Especially, among of MRPs, MRP1 is expressed in prostate cancer cell line (Van *et al*., 2001; Sullivan *et al*., 1998).

In the current study, we demonstrate the anticancer activity of fucoidan from *Undaria pinnatifida* in PC-3 human prostate cancer cells on the apoptosis induction, metastasis inhibition and the regulation of MDR-related proteins level.

3. MATERIALS AND METHODS

3.1. Reagents

Fucoidan (from *Undaria pinnatifida*), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, propidium iodide (PI), GSK-3β inhibitor, lithium chloride (LiCl), were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Mouse monoclonal anti-human Bcl-2, anti-c-myc, anti-MMP-9, anti-Smad-4, rabbit polyclonal anti-Bax, anti-procaspase-3, anti-β-catenin, anti-E2F-1, anti-TGF-β1 and anti-HO-1, goat polyclonal anti-MRP-1, anti-NF-κB-p50, anti-DR5, anti-GSK-3β, anti-phospho-GSK-3β and anti-Smad2/3 were purchased from Santa Cruz Biotechnology (Santa Cruz Biotech, CA, USA); Rabbit polyclonal anti-p38, anti-phospho-p38, anti-Akt, anti-phospho-Akt, anti-ERK1/2, anti-phospho-ERK1/2, anti-cleaved poly (ADP-ribose) polymerase (PARP) and anti-cleaved caspase-9 antibodies were purchased from Cell Signaling Technology (Cell signaling Technology, Beverly, MA, USA); Rabbit polyclonal anti-cleaved caspase-8, mouse monoclonal anti-p21 and cyclin D1 were purchased BD Biosciences (BD Biosciences, San Diego, CA, USA); monoclonal β-actin was purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA); HRP-conjugated goat anti-rabbit and horse anti-mouse IgGs were purchased from Vector (Vector Laboratories, Burlingame, MA, USA); HRP-conjugated

mouse anti-goat IgGs was purchased from Santa Cruz Biotechnology (Santa Cruz Biotech, CA, USA); Aprotinin, leupeptin, Nonidet P-40 were obtained from Roche (Roche Applied Science, Indianapolis, IN); Western blotting reagent, West-zol enhanced chemilumin, was obtained from Intron (iNtROn Biotechnology, Korea).

3.2. Cell culture

PC-3, a human prostate cancer cell line, was obtained from the Korean Cell Line Bank (KCLB) and cultured in RPMI 1640 (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, UT, USA), 100 U/mL penicillin and 100 ㎎/mL streptomycin (GIBCO Inc, Grand Island, NY, USA) at 37°C in a humidified atmosphere with 5% CO_2.

3.3. Cell viability assay

The effect of fucoidan on the growth of PC-3 cells was evaluated using the MTT assay (Scudiero *et al*., 1988). Cells (1×10^5 cells/mL) were seeded at into each well in 200 ㎕ on 96-well microplates. After 18 h at 37°C in 5% CO_2 gas to allow cell attachment, cells were treated with fucoidan (10, 50, 100 and 200 ㎍/mL) for 72 h. At the end of experimental incubation, cells were treated with 50 ㎕ (5 ㎎/mL) MTT dye and incubated 37°C for 4 h. The medium was aspirated and replaced with 150 ㎕/well dimethyl sulfoxide to dissolve the

formazan solution. Cell viabilities were determined by measuring the absorbance at 540 nm using a micro plate ELISA reader (Amersham Pharmacia Biotech, NY, USA). Each experiment was repeated at least three times.

3.4. Flow cytometric analysis of apoptosis

The effect of fucoidan on cell cycle distribution was analyzed by flow cytometry after staining the cells with propidium iodide (PI) (Fired *et al*., 1976). PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan and cultured for various times (0, 12, 24 and 48 h). The treated cells were trypsinized, washed two times with PBS (phosphate-buffered saline) and fixed with 70% ethanol 30 min at -20°C. The fixed cells were washed twice with cold PBS, incubated with 50 μg/mL RNase A at 37°C for 30 min, stained with 50 μg/mL PI in the dark for 30 min at 37°C. The stained cells were analyzed using an FACS caliber flow cytometry (Becton Dickinson, USA). The proportion of cells in G_0/G_1, S, G_2/M phases was represented as DNA histograms. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G_1 peak in the cell cycle pattern. For each experiment, 10,000 events per sample were analyzed, and experiments were repeated three times.

3.5. Morphological analysis of apoptosis by Hoechst 33342 staining

For detection of apoptosis, cells were seeded at 1×10^5 cells/mL in 1 mL on 24-well microplates. After 18 h of incubation to allow cell attachment, cells were treated with 100 μg/mL of fucoidan and cultured for various times (0, 12, 24 and 48 h). The cells were incubated in a Hoechst 33342 (10 μg/mL medium, final) staining solution at 37°C for 20 min. The stained cells were observed with an inverted fluorescent microscope equipped with an IX-71 Olympus camera and photographed (magnification × 200).

3.6. Western blot analysis

PC-3 cells were treated with 100 μg/mL of fucoidan and cultured for various times (0, 12, 24 and 48 h). After treatment, the cells were harvested and washed two times with cold PBS. The cells were lysed with lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM Phenylmethylsulfonylfluoride, 25 μg/mL aprotinin, 25 μg/mL leupeptin, 1 mM DTT, 1% Nonidet P-40] and kept on ice for 30 min at 4°C. The lysates were centrifuged at 15,000 rpm at 4°C for 15 min. The supernatants were stored at -20°C until use. Protein content was determined by the Bradford assay (Bradford, 1976). The same amount of lysates were separated on 6~10% SDS-PAGE gels and then transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, Hercules, CA, USA) by glycine transfer buffer [192 mM

glycine, 25 mM Tris-HCl (pH 8.8), and 20% MeOH (v/v)] at 150 V for 90 min. After blocking with 5% nonfat dried milk, the membrane was incubated with primary antibody against Akt (1:1000), phospho-Akt (1:1000), p38 (1:1000), phospho-p38 (1:1000), ERK1/2 (1:500), phospho-ERK1/2 (1:500), cleaved caspase-9 (1:500), cleaved PARP (1:1000), β-catenin (1:1000), c-myc (1:500), E2F-1 (1:500), MMP-9 (1:500), Smad-4 (1:500), Bax (1:1000), MRP-1 (1:250), procaspase-3 (1:1000), TGF-β1 (1:500), Bcl-2 (1:1000), DR5 (1:500), HO-1 (1:1000), NF-κB-p50 (1:1000), Smad2/3 (1:500), GSK-3β (1:250), phospho-GSK-3β (1:250), cleaved caspase-8 (1:500), p21 (1:500), cyclin D1 (1:500) and β-actin (1:1000) antibodies and incubated with a secondary HRP antibody (1:10000) at room temperature. The membrane was exposed on X-ray films (AGFA, Belgium), and protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON., Gyeonggi-do, Korea).

3.7. Nuclear extract preparation

PC-3 cells were treated with 100 ㎍/mL of fucoidan and cultured for various times (0, 12, 24 and 48 h). After treatment, the cells were harvested and washed two times with cold PBS. The cells were lysed with 1 mL of lysis buffer (1 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, and 1% NP-40) for 5 min. After 10 min of centrifugation at 3,000×g, the pellets were

re-suspended in 50 ㎕ of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF), and kept on ice for 30 min. The cell lysates were centrifuged at 14,000×g for 15 min. The supernatants were then harvested as nuclear protein extracts and stored at -70℃.

3.8. Wound healing assay

For cell migration determination, PC-3 cells (1×10^5 cells/mL) were seeded in 6-well plate and grown to 80~90% confluence. After aspirating the media, in the center of the cell, monolayers were scraped with a sterile micropipette tip to create a denuded zone (gap) of constant width. Subsequently, cellular waste was washed with PBS, and PC-3 cells were exposed to 100 ㎍/mL of fucoidan. The wound closure was monitored and photographed at 0, 12, 24 and 48 h with an IX-71 Olympus camera (magnification × 100).

3.9. Statistical analyse

Results are expressed as means ± standard deviation (SD) from representative of three independent experiments. Student's *t*-test was used to evaluate the data with the following significance levels: *$p<0.05$, **$p<0.01$ and ***$p<0.001$. All assays were performed in at least three independent experiments.

Table 2. Antibodies used in Western blot analysis on PC-3 cells.

Antibody	Origin	Company
Bcl-2	mouse monoclonal	Santa Cruz Biotechnology
c-myc	mouse monoclonal	Santa Cruz Biotechnology
MMP-9	mouse monoclonal	Santa Cruz Biotechnology
Smad4	mouse monoclonal	Santa Cruz Biotechnology
Bax	rabbit polyclonal	Santa Cruz Biotechnology
Procaspase-3	rabbit polyclonal	Santa Cruz Biotechnology
β-catenin	rabbit polyclonal	Santa Cruz Biotechnology
E2F-1	rabbit polyclonal	Santa Cruz Biotechnology
TGF-β1	rabbit polyclonal	Santa Cruz Biotechnology
HO-1	rabbit polyclonal	Santa Cruz Biotechnology
MRP-1	rabbit polyclonal	Santa Cruz Biotechnology
NF-κB p50	mouse monoclonal	Santa Cruz Biotechnology
DR5	goat polyclonal	Santa Cruz Biotechnology
GSK-3β	goat polyclonal	Santa Cruz Biotechnology

Phospho-GSK-3β	goat polyclonal	Santa Cruz Biotechnology
Smad2/3	goat polyclonal	Santa Cruz Biotechnology
p38/MAPK	rabbit polyclonal	Cell signaling Technology
Phospho-p38/MAPK	rabbit polyclonal	Cell signaling Technology
Akt	rabbit polyclonal	Cell signaling Technology
Phospho-Akt	rabbit polyclonal	Cell signaling Technology
ERK1/2	rabbit polyclonal	Cell signaling Technology
Phospho-ERK1/2	rabbit polyclonal	Cell signaling Technology
Cleaved-poly-(ADP-ribose) polymerase (PARP)	rabbit polyclonal	Cell signaling Technology
Cleaved caspase-9	rabbit polyclonal	Cell signaling Technology
Cleaved caspsae-8	rabbit polyclonal	BD Biosciences, USA
p21	mouse monoclonal	BD Biosciences, USA
Cyclin D1	mouse monoclonal	BD Biosciences, USA
β-actin	mouse monoclonal	Sigma

4. RESULTS

4.1. Inhibitory effect of fucoidan on the growth of PC-3 cells

To examine the effect of fucoidan on the growth of PC-3 cells, cell viability was evaluated using the MTT assay. The cells were treated with various doses (10, 50, 100 and 200 ㎍/mL) of fucoidan for 72 hour. Fucoidan treatment induced a dose-dependent cell death (10 ㎍/mL, 15.2 %; 50 ㎍/mL, 29.8 %; 100 ㎍/mL, 39.3 %; 200 ㎍/mL 45.1 %) (Fig. 8). This result exhibited that fucoidan could inhibit the growth of PC-3 cells in a dose-dependent manner.

4.2. Fucoidan induced apoptotic characteristics in PC-3 cells

Nuclear morphological change is a crucial biochemical characteristic of apoptosis. We examined nuclear morphological changes by fucoidan in PC-3 cells. Apoptotic bodies were observed by Hoechst 33342 staining in fucoidan-treated cells but not in without fucoidan treatment (Fig. 9A). This result demonstrated that fucoidan induced the morphological changes of apoptosis including chromatin condensation, membrane blebbing and cell shrinkage.

To evaluate whether fucoidan could increase the sub-G_1 peak, cell cycle analysis was determined using PI (propidium iodide) DNA staining. Fig. 9B showed that the percentage of sub-G_1 fraction (12 h, 22.99%; 24 h, 18.33%; 48 h, 26.27%) was increased after stimulation with 100 μg/mL of fucoidan for various times (0h, 12h, 24h and 48h) (Fig. 9B and C). These results showed that fucoidan induced the apoptosis of PC-3 cells.

4.3. Fucoidan induced apoptosis through extrinsic apoptosis pathway in PC-3 cells

Extrinsic apoptosis pathway is one of the critical molecular signaling pathways that lead to apoptotic cell death (Debatin, 2004); thus, we examined whether fucoidan could induce apoptosis via the extrinsic pathway. As results, fucoidan treatment resulted in the activation of extrinsic apoptosis pathway-related proteins, DR5 and caspase-8, which were followed by the activation of caspase-3 and the cleavage of PARP (Fig. 10A, B, 11A and B).

4.4. Fucoidan induced apoptosis via intrinsic apoptosis pathway in PC-3 cells

Intrinsic apoptosis pathway is one of the important molecular signaling pathways that induce apoptotic cell death (Debatin, 2004). We examined whether fucoidan could induce apoptosis via the intrinsic pathway. As results, fucoidan treatment resulted in the activation of intrinsic pathway through the decrease of bcl-2, the increase of bax, and the activation of

caspase-9, which were followed by the activation of caspse-3 and the cleavage of PARP (Fig. 11A and B).

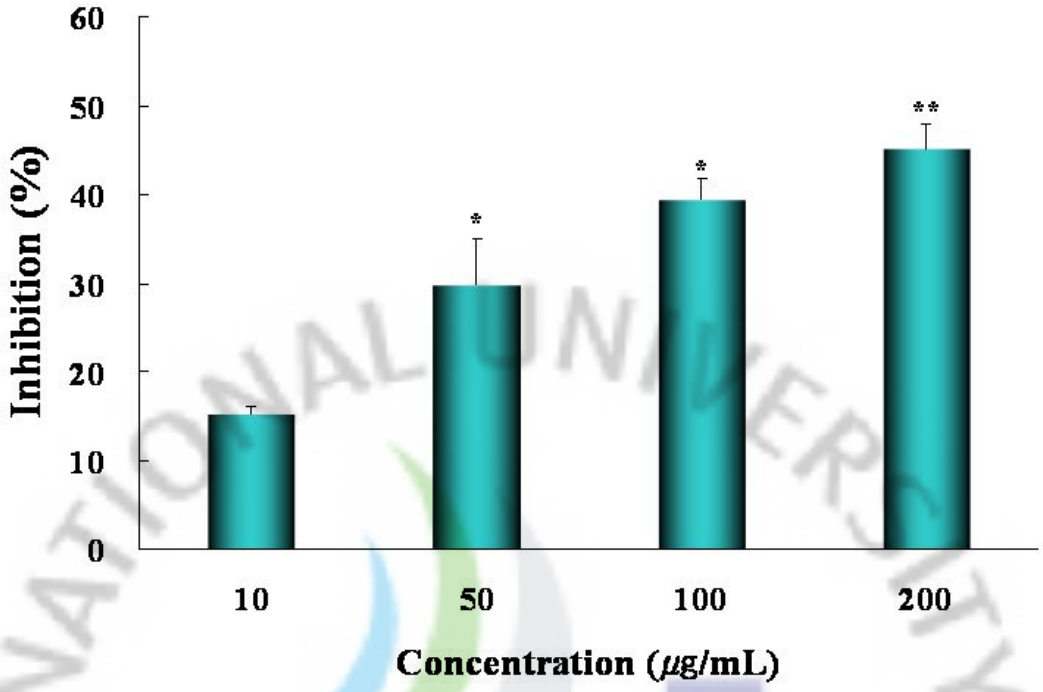


Figure 8. Fucoidan induced growth inhibition in PC-3 cells. The cells (1×10^5 cells/mL) were treated with various concentrations (0, 10, 50, 100 and 200 μg/mL) of fucoidan for 72h. Cell growth inhibition was measured by MTT assay. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

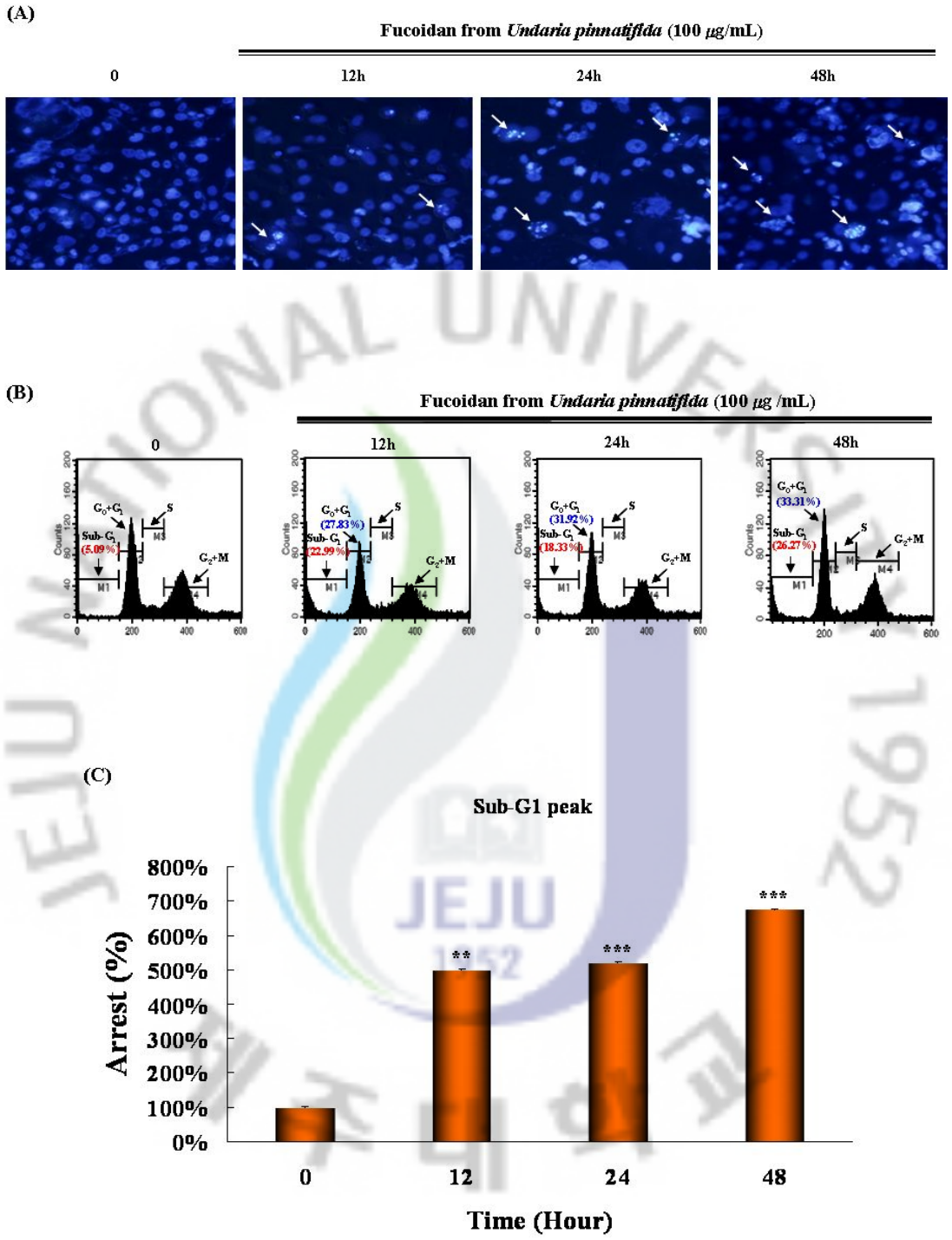


Figure 9. Fucoidan led to apoptotic characteristics in PC-3 cells.

(A) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Cells were stained with DNA-specific fluorescent dye, Hoechst 33342 (10 μg/mL, final concentration). Apoptotic bodies were observed by an inverted fluorescent

microscope equipped with an IX-71 Olympus camera (magnification × 200). (B) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). After treatment, cells were harvested, fixed with 70% ethanol, and stained with propidium iodide. Changes in cell cycle phase distribution were measured by DNA flow cytometric analysis. (C) Data represent the percentage of sub-G_1 peak in the cell cycle. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

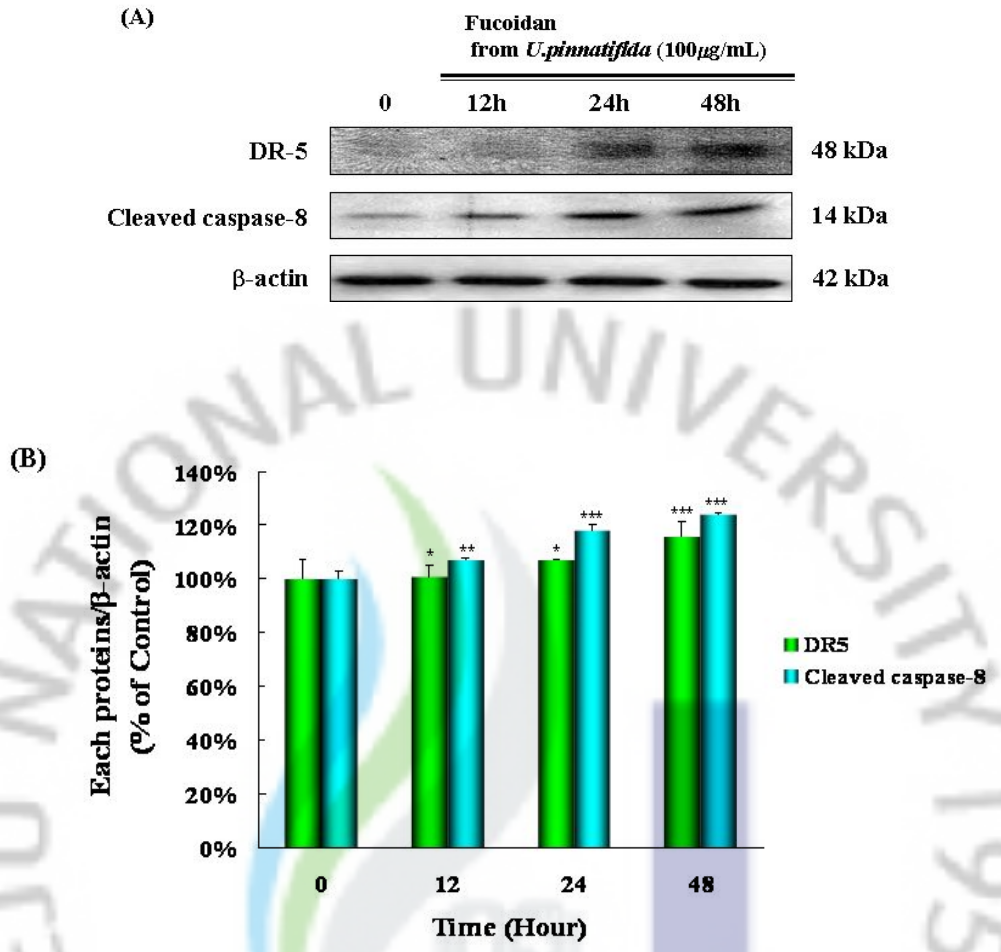


Figure 10. Effect of fucoidan on the expression of extrinsic apoptosis pathway-related proteins in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expressions of DR5 and cleaved caspase-8 by western blot. β-actin used as loading control of whole protein. (B) Data represent the percentage of DR5 and cleaved caspase-8 expressions in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.01$, **$p<0.05$, and ***$p<0.001$ compared with the control (control; without fucoidan).

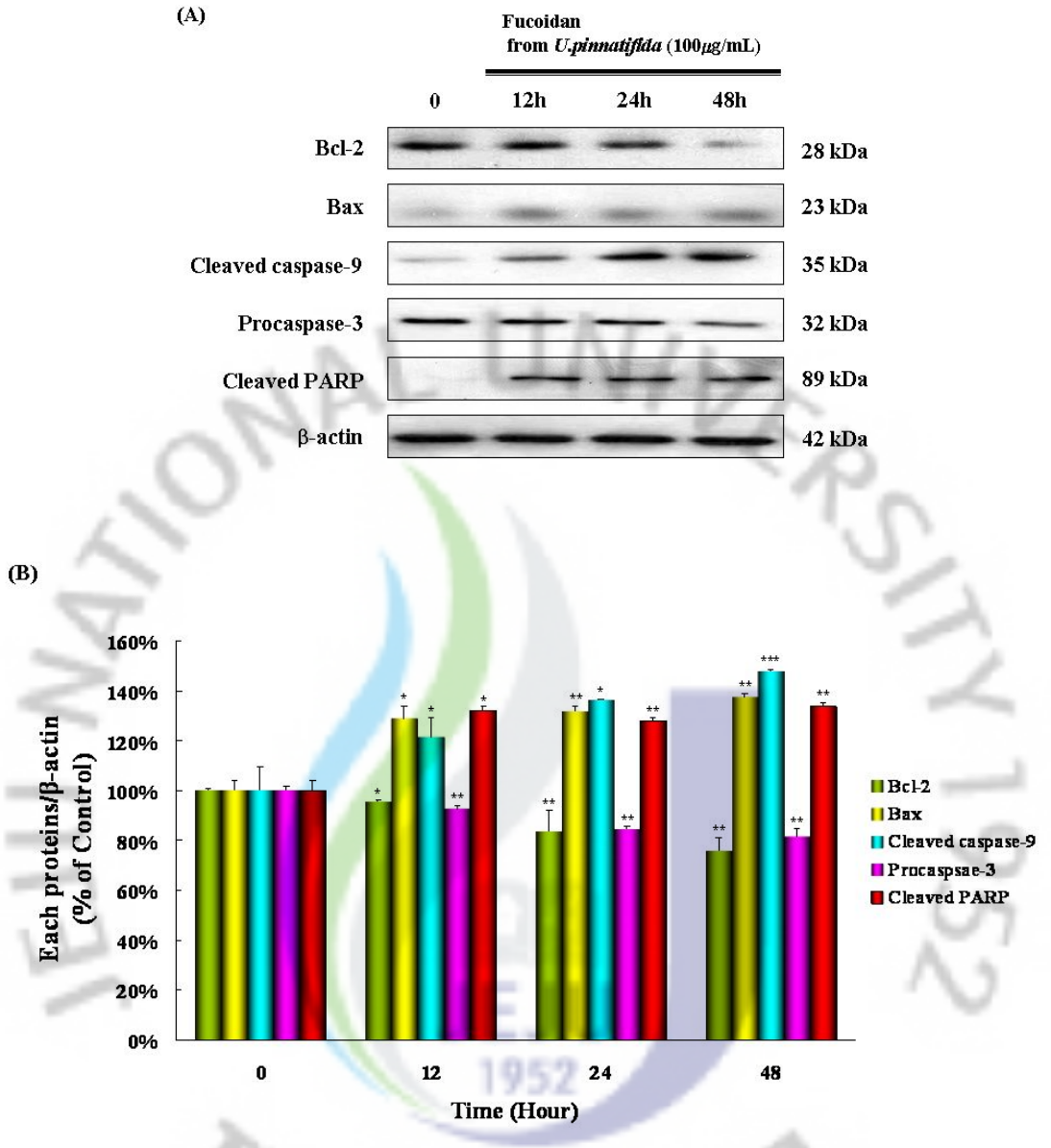


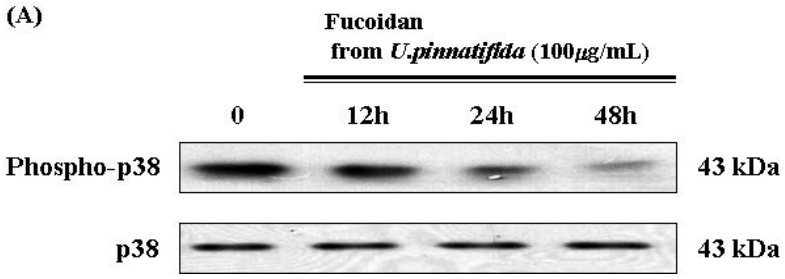
Figure 11. Effect of fucoidan on the expression of intrinsic apoptosis pathway-related proteins in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expressions of Bcl-2, Bax, cleaved caspase-9, procaspase-3 and cleaved PARP by western blot. β-actin used as loading control of whole protein. (B) Data represent the percentage of Bcl-2, Bax, cleaved caspase-9, procaspase-3 and cleaved PARP expressions in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.01$, **$p<0.05$, and ***$p<0.001$ compared with the control (control; without fucoidan).

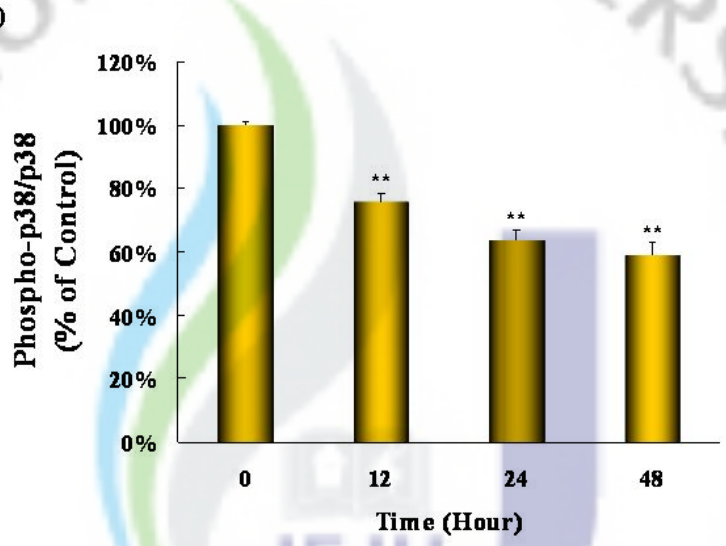
4.5. Effect of fucoidan on MAP kinase signaling in PC-3 cells

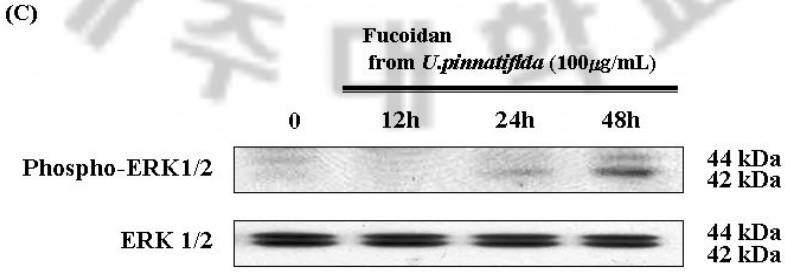
Mitogen-activated protein kinase (MAPK) pathways regulate differentiation, mitosis, proliferation, and apoptosis (Wada and Penninger, 2004). In order to establish the MAP kinase mechanism of apoptosis induced by fucoidan, the expressions ERK1/2 MAPK and p38 MAPK following fucoidan treatment were examined by western blot. As results, fucoidan treatment activated phospho-ERK1/2, but resulted in down-regulation of phospho-p38 (Fig. 12A-D). These results suggested that fucoidan induced apoptosis via the up-regulation of ERK1/2 MAPK pathway and down-regulation of p38 MAPK pathway.

4.6. Effect of fucoidan on PI3K/Akt signaling in PC-3 cells

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway also regulates cell survival, cell growth, and apoptosis (Jin *et al*., 2007), and promotes proliferation and survival in cancer cells (Toker *et al*., 2006). To evaluate the PI3K/Akt mechanism of apoptosis induced by fucoidan, we performed western blot analysis using specific antibody. As a result, fucoidan resulted in decreased expression of phospho-PI3K/Akt (Figure 13A and B). These results indicated that fucoidan induced apoptosis via the down-regulation of PI3K/Akt pathway.







(D)

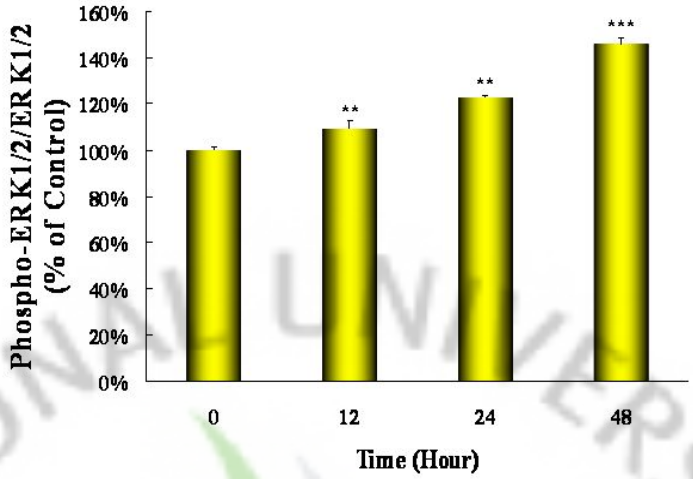


Figure 12. Effect of fucoidan on MAP kinase signaling in PC-3 cells. PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). (A) Lysates were analyzed for the expressions of phospho-p38 and p38 by western blot. (B) Data represent the percentage of phospho-p38 and p38 expressions in PC-3 cells. (C) Lysates were analyzed for the expressions of phospho-ERK1/2 and ERK1/2 by western blot. (D) Data represent the percentage of phospho-ERK1/2 expression in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

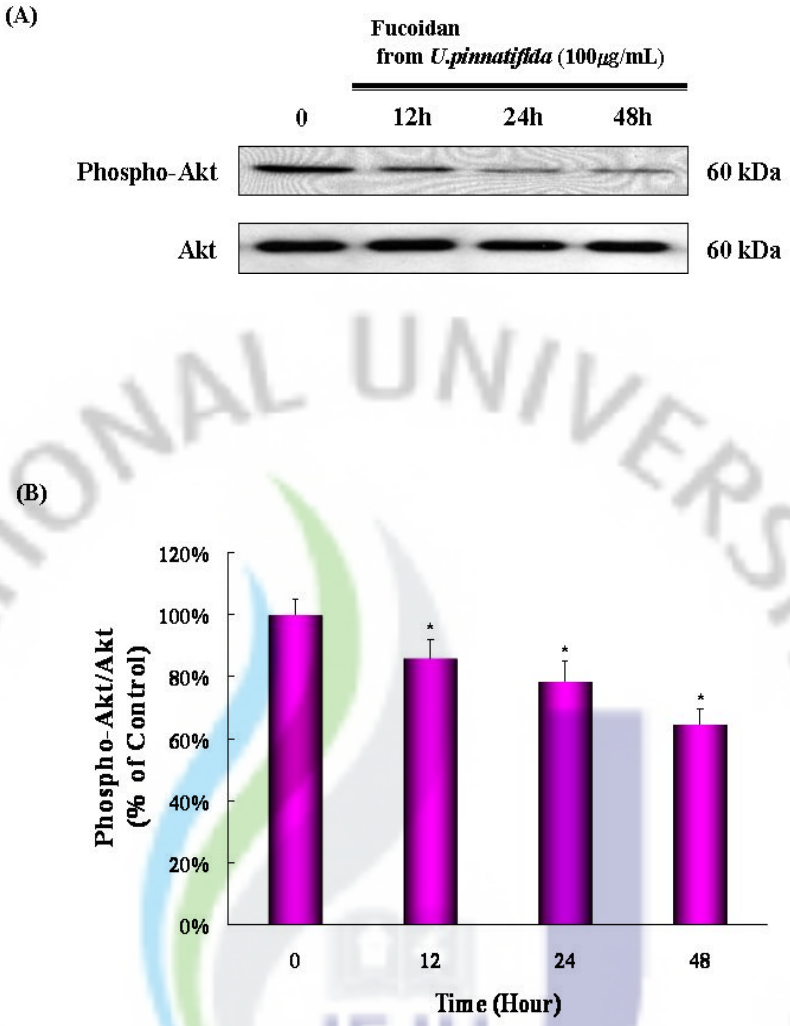


Figure 13. Effect of fucoidan on PI3K/Akt signaling in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were pretreated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expressions of phospho-Akt and Akt by western blot. (B) Data represent the percentage of phospho-Akt expression in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

4.7. Fucoidan caused cell-cycle arrest via regulation of cell-cycle-related proteins in PC-3 cells

Fig.9B showed that the percentage of G_0+G_1 fraction (12 h, 27.83%; 24 h, 31.92%; 48 h, 33.31%) increased after stimulation with 100 ㎍/mL of fucoidan for various times (0, 12, 24 and 48 h) and Fig.14 showed that the percentage of each phase (G_0+G_1, S and G_2+M phase). These results indicated that fucoidan could induce arrest of G_0+G_1 phase in PC-3 cells.

E2F-1 is an important transcription factor in cell cycle progression from G_1 to S phase and DNA synthesis (Kaseb *et al*., 2007); and p21Cip1/Waf is known to regulate the entry of cells at the G_1-S-Phase transition checkpoint and induce apoptosis (Cayrol *et al*., 1998). We examined the effect of fucoidan on the level of E2F-1 and p21Cip1/Waf. As a result, fucoidan treatment caused a significant reduction in expression of E2F-1 (Fig. 15A and B). Fucoidan treatment resulted in a significant increased expression of p21 compared with the control (Fig. 15A and B). These results indicated that down-regulation of E2F-1 and up-regulation of p21 by fucoidan might contribute to failure to enter from G_1 to S phase.

4.8. Fucoidan induced apoptosis via down-regulation of Wnt/β-catenin signaling pathway in PC-3 cells

Up-regulation of Wnt/β-catenin signaling pathway has a pivotal role in the development

and progression of cancer (Lu *et al*., 2009). We examined expressions of Wnt/β-catenin signaling-related proteins by fucoidan. As shown Fig. 16A and B, fucoidan treatment resulted in down-regulation of β-catenin, a key molecule of Wnt/β-catenin signaling pathway; and fucoidan also activated expression of GSK-3β, a regulator of β-catenin.

4.9. Fucoidan induced down-regulation of β-catenin target genes expression in PC-3 cells

We investigated the expressions of β-catenin target genes, c-myc and cyclin D1 using western blot. As a result, fucoidan treatment also decreased expressions of c-myc and cyclin D1, β-catenin target genes (Figure 17A and B).

4.10. Fucoidan regulated β-catenin by Wnt/β-catenin pathway in PC-3 cells.

To more clarify whether fucoidan induces apoptosis via the Wnt/β-catenin signaling pathway, we inhibited GSK-3β, important upstream regulator of β-catenin, using GSK-3β inhibitor. Interestingly, following pretreatment with LiCl, a GSK-3β inhibitor, fucoidan caused an increase β-catenin level (Figure 18A and B). These data indicated that fucoidan might regulate β-catenin through GSK-3β activation and induction of apoptosis by fucoidan was associated with down-regulation of Wnt/β-catenin signaling pathway.

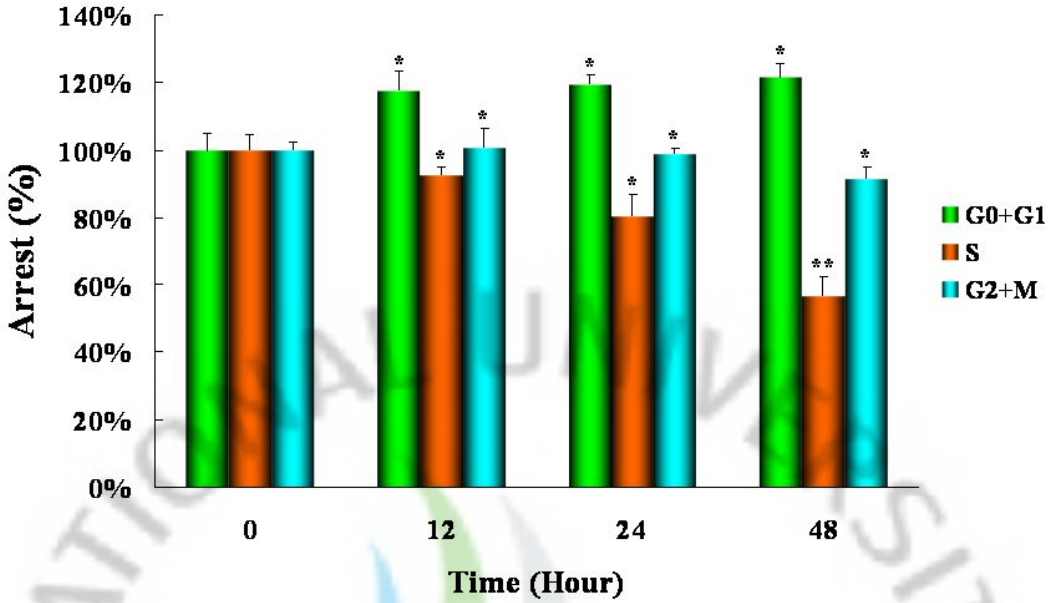


Figure 14. Fucoidan induced arrest of G_0+G_1 phase in PC-3 cells. PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). After treatment, cells were harvested, fixed with 70% ethanol, and stained with propidium iodide. Changes in cell cycle phase distribution were measured by DNA flow cytometric analysis. Data represent the percentage of cells in each phase of the cell cycle. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

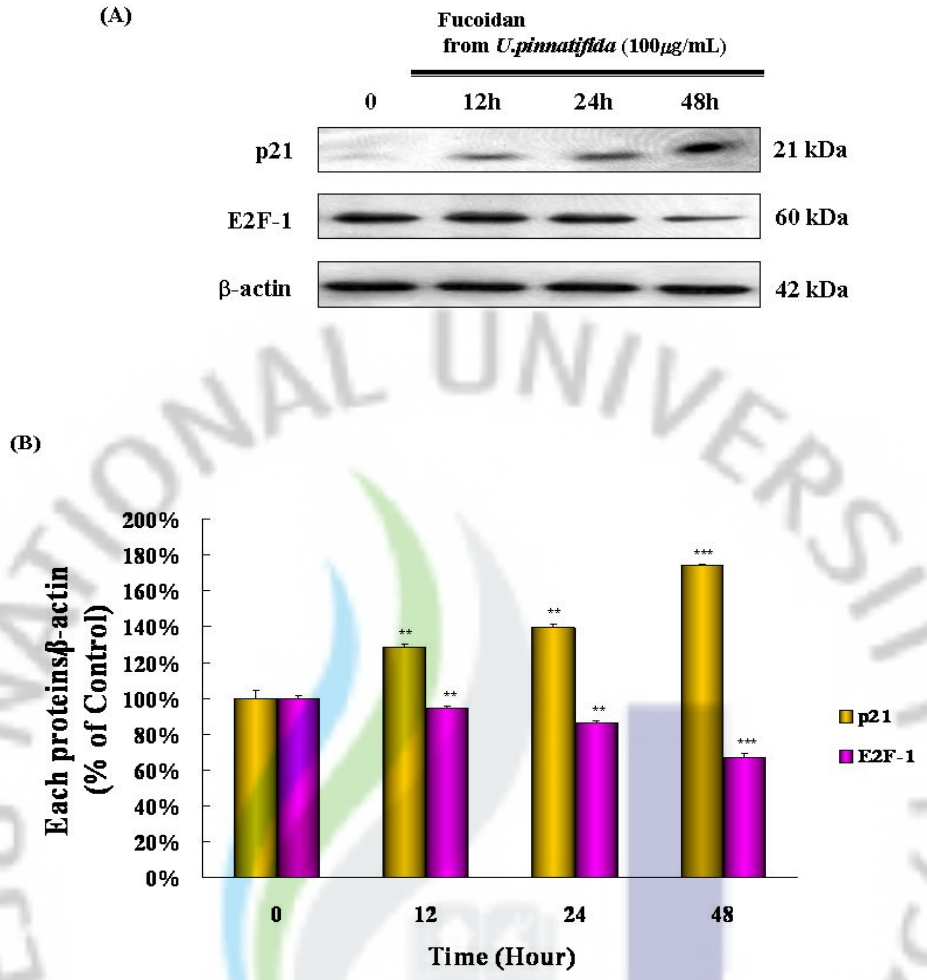


Figure 15. Effect of fucoidan on the expressions of cell cycle-related proteins in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expressions of p21 and E2F-1 by western blot. β-actin used as loading control of whole protein. (B) Data represent the percentage of p21 and E2F-1 expressions in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

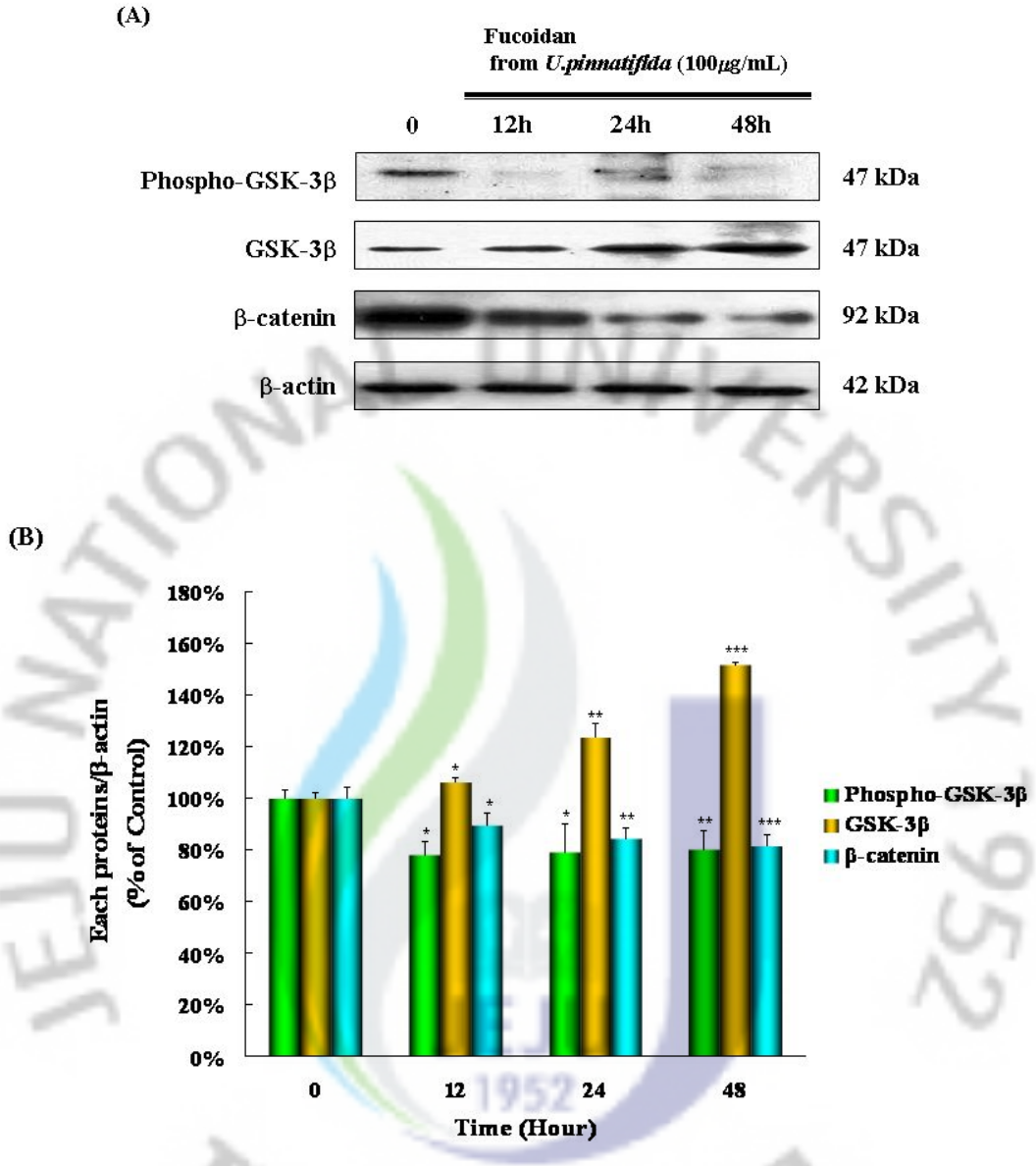


Figure 16. Effect of fucoidan on the expressions of Wnt/β-catenin signaling pathway-related proteins in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg /mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expressions of phospho-GSK-3β, GSK-3β and β-catenin by western blot. β-actin used as loading control of whole protein. (B) Data represent the percentage of various proteins (phospho-GSK-3β, GSK-3β and β-catenin) expressions in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

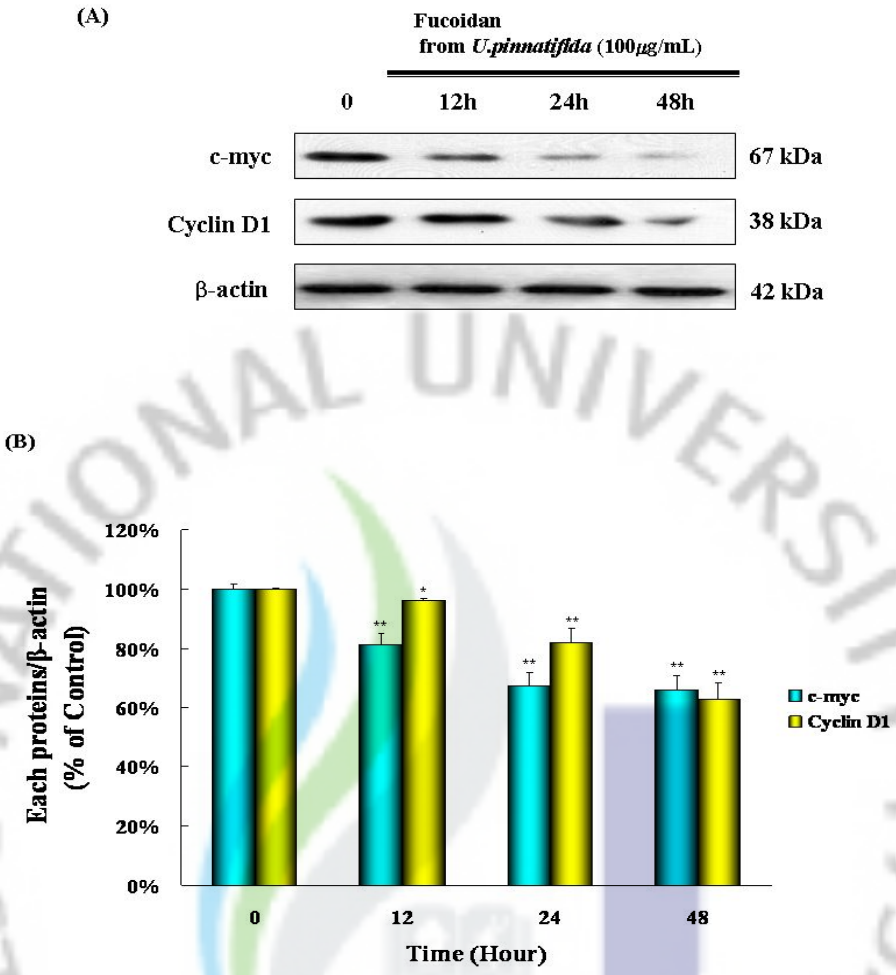


Figure 17. Effect of fucoidan on the expressions of β-catenin target genes in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expressions of c-myc and cyclin D1 by western blot. β-actin used as loading control of whole protein. (B) Data represent the percentage of c-myc and cyclin D1 expressions in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

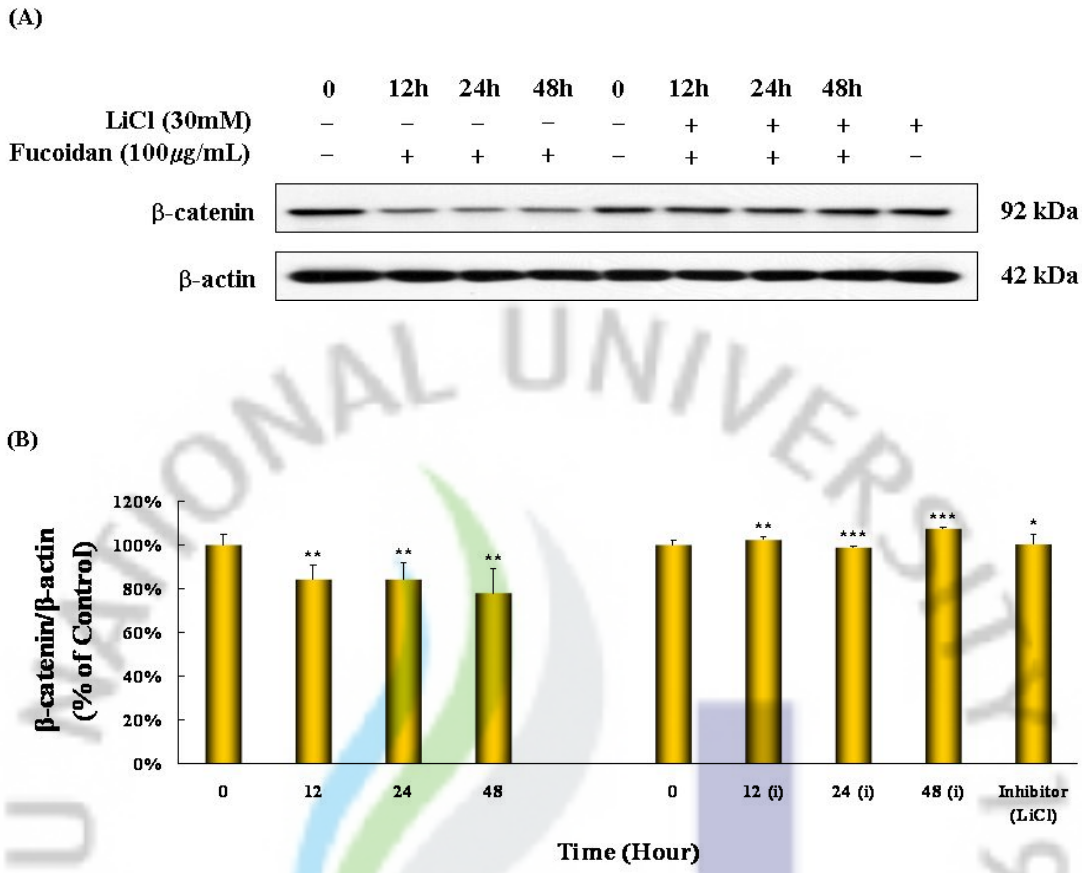


Figure 18. Effect of fucoidan on the regulation of β-catenin via Wnt/β-catenin signaling pathway in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were pretreated with 30mM of GSK-3β inhibitor (LiCl) for 1hr and then PC-3 cells were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expression of β-catenin by western blot. β-actin used as loading control of whole protein. (B) Data represent the percentage of β-catenin expression in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

4.11. Inhibition of metastasis by fucoidan in PC-3 cells

Among various prostate cancer cell lines, the PC-3 cell line is well-known as human prostate cancer bone metastasis (Mizutani *et al*., 2009). According to previous study, MMP-9 is known to be involved with metastasis of prostate cancer and the expression of MMP-9 is highly regulated by NF-κB (Lindholm *et al*., 2000; Lin *et al*., 2009). To evaluate the effect of fucoidan on the metastasis inhibition, the levels of MMP-9 and NF-κB following fucoidan treatment was examined by western blot. Fucoidan reduced MMP-9 and NF-κB levels in a time-dependent manner compared to the control (Fig. 19A and B). These results showed that fucoidan could inhibit the metastasis of PC-3 cells via the decrease of NF-κB and MMP-9 levels in PC-3 cells.

4.12. Fucoidan suppressed migration in PC-3 cells

To evaluate the effect of fucoidan on the migration suppression, we performed wound-healing assay; as the result, fucoidan could inhibit the migration of PC-3 cells in a time-dependent manner compared to the control (Fig. 20).

4.13. Regulation of MDR-related proteins level by fucoidan in PC-3 cells

According to previous study, among multidrug-related proteins, such as MDR, BCRP, and

MRP, evaluated expression of MRP1 was specifically observed in PC-3 cell line (Sgerbakova *et al*., 2008). Previous study has suggested that possible resistance mechanism in prostate cancer cell lines have discovered an increase in the expression of MRP1 encoded by the MRP gene (Zalcberg *et al*., 2000). Therefore, to evaluate whether the fucoidan could regulate MDR-related proteins level, the expression of MRP-1 following fucoidan treatment was examined by western blot. Fucoidan reduced MRP-1 protein expression in a time-dependent manner compared to the control (Fig. 21A and B). Furthermore, to investigate the proteins such as NF-κB and HO-1, which are known to be over-expressed in resistance cell lines (Kweon *et al*., 2006; Bentires-Alj *et al*., 2003), we performed western blot using specific antibodies. As the result, fucoidan diminished expression of NF-κB and HO-1 in a time-dependent manner (Fig. 19A, B, 21A and B); and according to report that NF-κB is regulated by TGF-β1 (Park *et al*., 2003), we examined expression of TGF-β1. As shown Fig. 21A and B, fucoidan decreased the TGF-β1 expression. These results showed that fucoidan could down-regulate MDR-related proteins level via the down-regulation of TGF-β1, which was followed by the decrease of HO-1, NF-κB and MRP-1 levels.

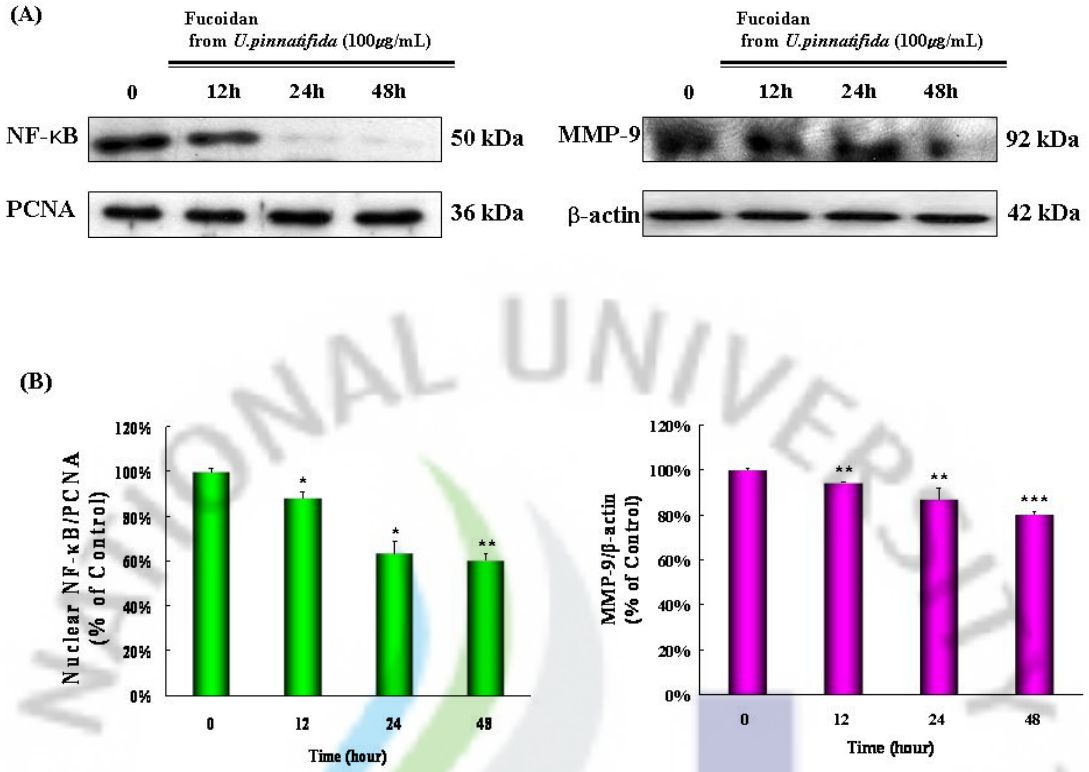


Figure 19. Effect of fucoidan on the expression of metastasis-related proteins in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expressions of MMP-9 and NF-κB by western blot. β-actin used as loading control of whole protein and PCNA used as loading control of nuclear protein. (B) Data represent the percentage of NF-κB and MMP-9 expressions in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

Figure 20. Suppression of migration by fucoidan in PC-3 cells. PC-3 cells (1×10^5 cells/mL) were seeded in 6-well plate. After attached, cells were scratched using sterile micropipette tip, and then PC-3 cells were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Cell migration was observed by an inverted fluorescent microscope equipped with an IX-71 Olympus camera (magnification × 100).

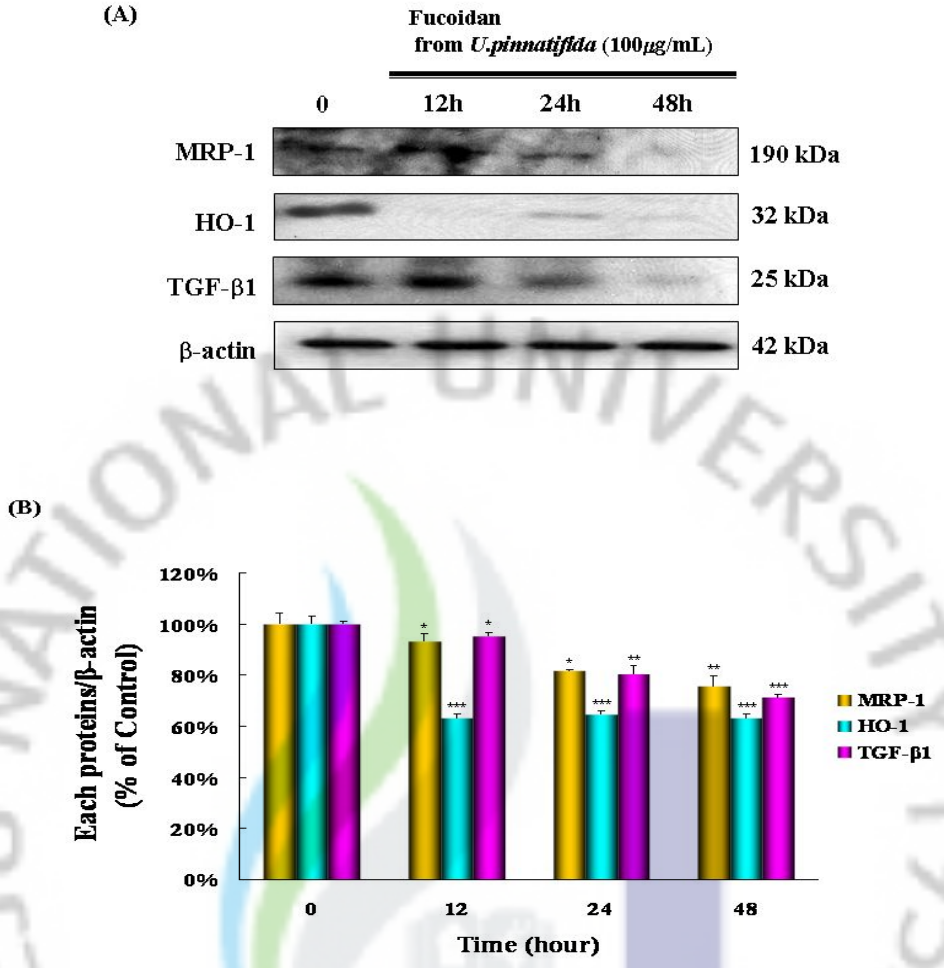


Figure 21. Down-regulation of MDR-related proteins by fucoidan in PC-3 cells. (A) PC-3 cells (1×10^5 cells/mL) were treated with 100 μg/mL of fucoidan for various times (0, 12, 24 and 48 h). Lysates were analyzed for the expressions of MRP-1, HO-1 and TGF-β1 by western blot. β-actin used as loading control of whole protein and PCNA used as loading control of nuclear protein. (B) Data represent the percentage of various proteins (MRP-1, HO-1 and TGF-β1) expression in PC-3 cells. Data are presented as mean ± SD from three independent experiments. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared with the control (control; without fucoidan).

5. DISCUSSION

In the present study, we demonstrated that fucoidan treatment inhibited the growth of PC-3 human prostate cancer cells by inducing apoptosis via the activation of extrinsic and intrinsic apoptosis pathways, the up-regulation of ERK1/2 MAPK signaling pathway, the down-regulation of p38 MAPK, PI3K/Akt and Wnt/β-catenin signaling pathway. Moreover, treatment of fucoidan inhibited metastasis and migration of PC-3 cells by down-regulating expression of NF-κB and MMP-9; and fucoidan regulated MDR-related proteins level by down-regulating TGF-β1, p38 MAPK pathway, HO-1, NF-κB and MRP-1 expressions.

Fucoidan can attack cancer cells directly, as well as prevent cancer cells by enhancing immunity. Other polysaccharides including β-glucan (Chan *et al*., 2009), avemar (Boros *et al*., 2005), and AHCC (Hirose *et al*., 2007) do not attack cancer cells directly, even if they prevent cancer cells by improve immunity by increasing B lymphocyte, NK-cell, and macrophage functions. Previous studies have indicated that fucoidan directly inhibited the proliferation of some cancer cells including HS-Sultan (Asia *et al*., 2005), U937 (Teruya *et al*., 2007), and HCT-15 (Hyun *et al*., 2009) cells. Indeed, this study confirmed that fucoidan could directly inhibit the proliferation of PC-3 cells; fucoidan inhibited the growth of PC-3

cells in a dose-dependent manner (Fig. 8), but fucoidan is not toxic to normal RAW 264.7 (Chen *et al*., 2009) and HEL-299 (Hyun *et al*., 2009) cells. These results suggested that fucoidan is cytotoxic in a specific manner against cancer cells without cytotoxicity against normal cells.

Apoptosis is a highly regulated physiologic mechanism of cell death during homeostasis, disease and development (Ashkenazi, 2008); morphologically characterized by chromatin condensation, membrane blabbing, cell shrinkage, and an increased population of sub-G_1 hypodiploid cells (Yang *et al*., 2007). This study indicated that nuclear morphologic changes as occur during apoptosis is induced by fucoidan treatment (Fig. 9A); treatment of fucoidan (100 ㎍/mL; approximately 0.75 μM) also increased the sub-G_1 fraction by 26.27% at 48 h (Fig. 9B) compared with the control. On the other hand, resveratrol is known to induce apoptosis of prostate cancer cells. When PC-3 cells are treated with resveratrol (25 μM for 96 h), the sub-G_1 hypodiploid cell population increased by 4.3% compared with the control (Hsieh and Wu, 1999). Compared with resveratrol, fucoidan appears to be more effective in induction of apoptosis in PC-3 cells.

Two key molecular signaling pathways lead to apoptotic cell death. The one is the extrinsic pathway, which is activated by death receptor from outside the cell; the other is the intrinsic pathway, which is activated by Bcl-2 protein family and downstream mitochondrial

signals from inside the cell (Debatin, 2004). We examined activation of extrinsic and intrinsic apoptosis pathways following to fucoidan treatment. The fucoidan treatment led to activation of DR5 and cleaved caspase-8, a critical component of extrinsic pathway; fucoidan treatment also led to down-regulation of bcl-2, up-regulation of Bax, and activation of caspase-9, an essential component of intrinsic pathway (Figure 10A, B, 11A and B). These results suggested that fucoidan induced apoptosis of PC-3 cells through activation of the extrinsic and intrinsic apoptosis pathway.

MAPK pathways are known to regulate apoptosis (Keyse, 2008). Among MAPK proteins, ERK1/2 MAPK is known to promote differentiation, cell survival and cell proliferation (Seger and Kerbs, 1995), but several reports have indicated that activation of ERK1/2 MAPK induce apoptosis (Cheung and Slack, 2004). For example, Cisplatin induced apoptosis in HeLa cells via activation of the ERK pathway (Wang *et al*., 2000). p38 MAPK is known to be activated by stress to modulate cell differentiation, cell cycle, cell growth, inflammation, and cell death (Bassi *et al*., 2008); whereas some reports have suggested that p38 MAPK can promote cancer cell growth and survival. For example, docosahexaenoic acid (DHA) induced apoptosis in A549 cells by down-regulation of p38 MAPK (Serini *et al*., 2008). This study confirmed that fucoidan treatment activated phospho-ERK1/2 MAPK, but diminished phospho-p38 MAPK expression (Figure 12A-D). PI3K/Akt pathway inhibits

apoptosis; in the present study, treatment of fucoidan decreased the phosphorylation of Akt (Figure 13A and B). Phosphorylation of Akt is reported to be regulated by p38; our results suggested that down-regulation of p38 MAPK signaling pathway by fucoidan treatment seems to lead to down-regulation of PI3K/Akt signaling pathway. These results suggested that induction of apoptosis by fucoidan was accompanied by activation of ERK1/2 MAPK signaling pathway, as well as down-regulation of p38 MAPK and PI3K/Akt signaling pathway.

Fucoidan treatment increased the G_0+G_1 fraction whereas % of S phase decreased (Figure 9C). Among cell-cycle-related proteins, E2F-1 and p21Cip1/Waf are known to an important role in cell cycle progression from G_1 to S phase. The present study indicated that decreased expression of E2F-1 and increased expression of p21 by fucoidan prevented to enter from G_1 to S phase (Figure 15A and B); these results also supported result of Fig. 14 that exhibited increase of G_1 phase and decrease of S phase by fucoidan. E2F-1 and p21 are regulated by β-catenin, an essential component of Wnt/β-catenin pathway.

Wnt/β-catenin signaling plays a pivotal role in the development and progression of prostate cancer. Furthermore, previous study suggested that highly invasive androgen-independent prostate cancer cell lines including PC-3 and DU-145 displayed the higher levels of Wnt/β-catenin signaling compared with the androgen-dependent cell line, LNCaP,

and non-cancerous PWR-1E and PZ-HPV-7 prostate cells (Lu *et al*., 2009). We examined levels of Wnt/β-catenin signaling by fucoidan treatment in PC-3 cells. This study confirmed that fucoidan treatment resulted in decreased expression of β-catenin, an essential component of Wnt/β-catenin pathway. According to previous study that activation of β-catenin is inhibited by GSK-3β, this study demonstrated that treatment of fucoidan inhibited β-catenin through activation of GSK-3β expression (Figure 16A and B). GSK-3β is known to be inhibited by phospho-Akt; thus, we examined expression of phospho-Akt by fucoidan. As a result, fucoidan treatment decreased expression of phospho-Akt (Figure 13A and B). These results suggested that fucoidan treatment inhibited β-catenin through down-regulation of phospho-Akt and activation of GSK-3β expression. We also found that the treatment with GSK-3β inhibitor, LiCl, led to increased level of β-catenin. This result supported the hypothesis that fucoidan could regulate the level of β-catenin via Wnt/β-catenin signaling pathway (Fugure 18A and B). c-myc and cyclin D1 are known to be β-catenin target genes; thus, we examined expressions of c-myc and cyclin D1 following to fucoidan treatment. As results, treatment of fucoidan led to down-regulation of c-myc and cyclin D1 with down-regulation of β-catenin (Figure 17A and B). c-myc and cyclin D1 are also known to cell-cycle-related proteins. These results might demonstrate that induction of apoptosis and cell cycle arrest by fucoidan were accompanied by down-regulation of Wnt/β-catenin signaling

pathway.

Among prostate cancer cell lines, PC-3 cells are known to migrate to bone; and overexpress of MRP1, multidrug-resistance protein. To treat prostate cancer efficiently, the metastasis and MDR (multidrug-resistance) must be regulated. We examined effect of fucoidan on the metastasis and MDR regulation. Fucoidan treatment reduced NF-κB and MMP-9 expressions (Figure 19A and B); result of wound healing assay also showed that treatment of fucoidan inhibited migration of PC-3 cells compared with the control (Figure 20). According to previous study that MMP-9, metastasis-related protein, is regulated by NF-κB, the results demonstrated that fucoidan treatment inhibited metastasis and migration by inhibiting MMP-9 expression via down-regulation of NF-κB expression in PC-3 cells. Treatment of fucoidan decreased MRP1, multidrug-related protein, expression; furthermore, HO-1 and NF-κB which are known to be over-expressed in resistant cell lines are reduced by fucoidan (Figure 19A, B, 21A and B). In accordance with previous study that TGF-β1 activates HO-1 through p38 MAPK (Ning *et al*., 2002), and NF-κB is regulated by TGF-β1 (Park *et al*., 2003) and HO-1 (Hsieh *et al*., 2008), and MRP1 is known as one of NF-κB target genes (Gazzaniga *et al*., 2007), the results demonstrated that fucoidan treatment regulated level of MDR-related protein by inhibiting MRP-1 expression through a series down-regulation of TGF-β1, p38 MAPK pathway, HO-1 and NF-κB expression in PC-3 cells

(Figure 21A, B, 12A and B).

In summary, fucoidan from *Undaria pinnatifida* induced apoptosis through the activation of ERK1/2 MAPK signaling pathway, down-regulation of p38 MAPK and PI3K/Akt signaling pathway, as well as activation of extrinsic and intrinsic apoptosis signaling pathways. Moreover, the induction of apoptosis by fucoidan was accompanied by down-regulation of Wnt/β-catenin signaling pathway. This study indicated that fucoidan inhibited metastasis and migration via down-regulation of NF-κB and MMP-9 levels; and fucoidan regulated level of MDR-related proteins by down-regulation TGF-β1, p38 MAPK pathway, HO-1, NF-κB and MRP-1 expressions. The results demonstrate that fucoidan might have therapeutic potential for prostate cancer treatment.

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Ⅳ. 요약문

Fucoidan은 황산화 L-fucose를 주성분으로 하는 황산화 다당류로서 D-xylose, D-galactose, D-mannose 등의 당과 sulfate, uronic acid 등이 결합한 구조를 가진다. 특히 *Undaria pinnatifida*로부터 분리된 fucoidan의 경우, 다른 종으로부터 분리된 fucoidan에 비하여, 황산기를 많이 함유하고 있어 생리활성 기능이 더 뛰어난 것으로 확인되었다. 이러한 황산기의 함량은 암세포 증식저해와 비례한다는 사실 또한 밝혀진 바 있다. Fucoidan의 생물학적 효능은 항바이러스 작용, 항혈액응고 작용, 항콜레스테롤 작용, 혈압 상승 억제, 혈당 상승 억제, 항산화작용, 항알레르기 작용 등과 같이 다양하게 알려져 있다. 그러나, fucoidan의 항암 작용에 대한 활성과 그 분자적 기전은 아직 충분히 밝혀지지 않은 실정이다. 현대 질병 중 "암"은 질병으로 인한 사망의 가장 주된 원인으로 꼽히고 있다. 국내에서 암 환자는 2000년에 84,000여 명, 2005년에는 142,1610여 명으로 발병률이 계속 증가하고 있는 추세이며, 암으로 인한 사망도 2000년에 58,000여 명을 넘어서고, 2006년에는 65,909명으로 늘어나고 있다. 또한 전체 사망자 중 암에 의한 사망이 2000년 23.5%, 2005년 25%, 2006년에는 27%로 우리나라 국민 4명 중 1명은 암으로 사망하고 있다는 통계가 나왔다. 암의 대표적인 특징으로는 여러 가지가 알려져 있으나, 특히 세포사멸의 회피와 조직의 침윤성 및 전이성이 중요한 특징으로 나타나게 된다. 세포사멸이란 외부인자의 자극이나 내적인 변화에 의해서 그 세포들이 자발적으로 죽음에 이르는 것을 말하며, 세포사멸 경로는 일반적으로 stress, UV들에 의해 유도되는 내인성 경로와, death-receptor를 통해 신호를 전달하는 외인성 경로가 알려져 있다.

1. 폐암 세포인 A549에서의 fucoidan의 항암 효과

폐암은 최근까지도 남성 사망률의 1위, 여성에서의 사망률도 2위를 차지하고 있어, 폐암의 사망률을 낮추기 위한 연구가 여전히 중요시 되고 있다. 따라서 본 연구에서는 폐암세포 중 하나인 A549를 사용하여 fucoidan의 항암활성을 알아보고자 하였다.

fucoidan에 의한 A549세포의 성장 억제효과를 알아보고자 MTT assay를 수행한 결과, fucoidan은 농도 의존적으로 A549세포의 성장을 저해하였다. Fucoidan에 의해 유도되는 세포사멸을 apoptotic body들의 관찰과, sub-G_1 peak의 증가를 통해 확인하였다. 세포사멸을 유도하는 신호전달에 중요하게 작용하는 apoptosis 관련 단백질들 (Bcl-2, Bax)과 caspase 관련 단백질들 (Caspase-9, caspase-3, PARP)의 발현을 분석하였다. Fucoidan의 처리는 anti-apoptotic 단백질인 Bcl-2의 발현을 농도 의존적으로 감소시킨 반면, pro-apoptotic 단백질인 Bax의 발현은 농도 의존적으로 증가시키고 있음을 확인하였다. 또한 Caspase-9과 Caspase-3가 fucoidan의 처리에 따라 활성화되는 것을 확인하였고, PARP의 cleavage를 유도하였음을 관찰하였다. MAPK와 PI3K/Akt 경로는 세포의 생존과 죽음에 중요한 역할을 수행하는 것으로 알려져 있기에, fucoidan에 의해 유도된 세포사멸이 MAPK와 PI3K/Akt 경로와 연관되는지를 분석하였다. Fucoidan 처리에 따라 ERK1/2 MAPK의 인산화가 농도 의존적으로 증가된 반면, p38 MAPK와 Akt의 인산화가 감소되는 것을 확인하였다. 이러한 결과들로 미루어 보아, fucoidan은 A549 세포에서 Bcl-2를 감소시키고, Bax를 활성화시킴으로써 caspase-9과 caspase-3를 활성화시키고, PARP의 cleavage를 유도하였으며, ERK1/2 MAPK 경로의 활성화와 p38 MAPK 그리고 PI3K/Akt 경로의 감소를 통하여 A549 세포의 세포사멸을 유도할 수 있을 것이라 사료된다.

2. 전립선암 세포인 PC-3에서의 fucoidan의 항암 효과

서구적인 생활양식이 보편화되면서 전립선암의 발병률은 우리나라에서 남성암 중 빠르게 증가하고 있는 암으로 꼽히고 있으며, 그에 따른 사망률 또한 꾸준히 증가하고 있는 추세이다. 따라서 본 연구에서는 전립선암 세포인 PC-3에서 세포사멸의 유도, 전이의 저해, 그리고 다약물내성 관련 단백질들의 조절을 관찰하여 fucoidan의 항암활성을 알아보고자 하였다.

본 연구에서는 우선 fucoidan이 농도 의존적으로 PC-3 세포의 성장을 저해하는 것을 확인하였다. 그리고 fucoidan에 의해 유도되는 세포사멸을 apoptotic body들의 관찰과, sub-G_1 peak의 증가를 통해 확인하였다. Fucoidan에 의해 유도되는 세포사멸이 외인성 세포사멸 경로를 통해 유도되는지 분석한 결과, DR(death-receptor)5와 caspase-8이 fucoidan 처리에 따라 시간 의존적으로 증가하는 것을 확인하였다. 내인성 세포사멸 경로에 fucoidan의 효과를 분석한 결과, fucoidan 처리에 따라 Bcl-2는 시간 의존적으로 감소하였으나, Bax는 시간 의존적으로 증가하였다. 또한 caspase-9과 caspase-3가 활성화되는 것을 확인하였으며, PARP의 cleavage 또한 관찰하였다. 이러한 결과들로 미루어 보아, fucoidan은 외인성 세포사멸 경로와 내인성 세포사멸 경로를 통해 세포사멸을 유도하고 있는 것으로 사료된다. PC-3 세포에서 MAPK와 PI3K/Akt 경로에 fucoidan의 효과를 분석한 결과, ERK1/2 MAPK의 인산화가 시간 의존적으로 증가된 반면, p38 MAPK와 Akt의 인산화가 감소되는 것을 확인하였다. 정상세포는 세포분열을 마치면 간기의 G_1기에서 정지상태로 존재하지만, 암세포는 자기 조절능력을 잃어서 무한히 세포분열을 반복하기 때문에 세포수가 급격히 증가되어 악성종양을 형성하게 된다. 따라서, fucoidan에 의해 cell cycle arrest가 유도되는지 분석하였다. Fucoidan 처리 시, G_0/G_1 기는 증가된 반면, S 기는 감소한 것으로 보아, G_0/G_1 기에서 arrest가 일어나고 있음을 확인하였다. Cell-cycle arrest에 관련된

단백질들의 level에 대한 fucoidan의 효과를 확인한 결과, p21의 발현은 fucoidan의 처리에 따라 시간 의존적으로 증가하였으나, E2F-1, c-myc, cyclin D1의 level은 감소되는 것을 확인하였다. 이들 단백질들은 Wnt/β-catenin 경로의 표적 유전자들로 알려져 있으며, 또한 전립선암 환자들의 경우 Wnt/β-catenin 경로가 증가되어 있다고 알려져 있어 Wnt/β-catenin 경로 관련 단백질들의 level을 알아본 결과, fucoidan은 phospho-GSK3β를 감소시킴으로써, GSK-3β의 활성화를 유도하였으며, β-catenin을 시간 의존적으로 감소시키는 것을 확인하였다. 그리고 β-catenin이 Wnt/β-catenin 경로를 통해 조절되는지를 명확히 하기 위하여, β-catenin의 상위 조절자인 GSK-3β의 저해제를 사용하여 분석한 결과, 저해제를 처리하지 않은 군에서, β-catenin은 fucoidan 처리에 따라 시간 의존적으로 감소하였으나, 저해제를 처리한 군에서는 β-catenin이 감소되지 않는 것을 확인하였다. 전립선암은 다른 암에 비해 진행속도가 느림에도 불구하고, 많은 환자수와 높은 사망률을 나타내는 것은 뼈로 전이하는 비율이 약 70~80%나 되기 때문이다. NF-κB와 MMP-9이 전이된 세포에서 과발현 되어 있다는 사전보고에 따라 이들 단백질의 발현을 분석한 결과, fucoidan의 처리에 따라 NF-κB 와 MMP-9이 감소되는 것을 확인하였다. 또한 fucoidan이 PC-3 세포들의 migration 을 저해하고 있음도 확인하였다. MDR이란 하나의 항암제에 노출된 암세포가 전에 사용한 적이 없는 구조나 기능이 다른 다양한 항암제에 내성을 보이는 현상을 말한다. PC-3 세포의 경우, MDR 단백질 중 MRP1을 발현한다고 알려져 있어, MDR 단백질의 조절에 fucoidan의 효과를 알아보고자 하였다. MDR에 관련된 단백질의 발현을 확인한 결과, MDR 단백질인 MRP1이 fucoidan 처리에 따라 감소되는 것을 확인하였다. 내성 세포들에 과발현 된다는 HO-1과 NF-κB를 확인한 결과, fucoidan 처리에 따라 감소되는 것을 확인하였다. 또한 HO-1과 NF-κB가 TGF-β1에 의해 조절된다는 보고에 따라 TGF-β1의 발현을 분석한 결과, fucoidan 처리에 따라 TGF-β1이 감소되는 것을 확인하였다.

본 연구에서의 실험결과 및 이전 연구 보고들을 정리하면, Fucoidan은 다음과 같은 작용기전들의 조절을 통하여 PC-3 세포에서 항암활성을 나타내는 것으로 사료된다. 1) Fucoidan은 TGF-β1을 감소시킴으로써, p38 MAPK 경로와 Akt 경로를 감소시키고, 이로 인하여 GSK-3β의 활성화를 이끎으로써, β-catenin의 감소를 유도하여 Wnt/β-catenin 경로의 감소를 야기시켰다. 이로 인하여 Wnt/β-catenin 경로에 의하여 조절을 받는 E2F-1, c-myc, cyclin D1이 감소되고, p21이 증가됨으로써 cell cycle arrest와 세포사멸을 유도할 수 있을 것이라 사료된다. 2) Fucoidan은 DR5 의 활성화를 이끌어 외인성 세포사멸 경로를 통해 세포사멸을 유도할 수 있을 것이라 생각되며, Bax의 활성화를 이끌고 Akt 경로의 감소에 의해 Bcl-2의 감소를 이끌어, caspase-9을 활성화시킴으로써 내인성 세포사멸 경로를 통해 세포사멸을 유도할 수 있을 것이라 생각된다. 3) Fucoidan은 ERK1/2 MAPK 경로의 증가를 초래하였기에, ERK1/2 MAPK 경로의 증가로 인한 세포사멸 또한 유도될 수 있을 것이다. 4) Fucoidan은 TGF-β1과 p38 MAPK 경로의 감소로 인하여, HO-1과 NF-κB가 감소되어, MMP-9의 감소를 이끌 수 있을 것이라 생각되며, 이로 인하여 전립선암 세포의 전이를 저해할 수 있을 것이라 사료된다. 5) 또한 NF-κB의 감소에 의하여 MRP-1이 감소될 수 있을 것이라 생각되며, 이를 통하여 다약물내성 관련 단백질들의 감소를 이끌 수 있을 것이라 생각된다. 6) 그리고 MMP-9이 β-catenin의 직접적인 표적으로 알려져 있기에, Wnt/β-catenin 경로의 감소가 전립선암 세포의 전이를 저해하는데 기여했을 수도 있을 것이라 사료된다.

따라서 본 연구는 이러한 결과들을 바탕으로, fucoidan이 폐암과 전립선암을 치료하는 데 있어 새로운 치료 전략으로 이용 가능함을 시사하는 바이다.

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아무것도 모르는 저를 받아주시고, 원하는 실험과 공부를 마음껏 하게 아낌없이 지원해 주신 **강희경 교수님**께 감사의 말씀을 전하며, 교수님의 제자로 남게 될 수 있어 영광이라는 말씀 또한 함께 전하고자 합니다. 교수님의 작은 말씀 하나하나가 저에게는 매우 큰 힘이 되었고, 그로 인하여 제가 이만큼 성장할 수 있었습니다. 눈보라가 휘몰아치듯 아득하기만 했던 제 앞 길에 환한 불을 비춰주셔서 감사합니다. 어디서든 제자리에 멈춰 있는 저의 모습이 아닌, 항상 발전하고 성장하는 모습 보여드리겠노라 약속 드리며, 교수님의 큰 가르침 항상 잊지 않고 간직하며 살아가겠습니다. 항상 건강하시길 기원합니다. 그리고 저의 미흡한 논문을 성심 성의껏 심사해 주신 **유은숙 교수님**과, **고영상 교수님**께 감사의 말씀을 전합니다. 교수님들의 세심한 지도 덕분에 제 미흡한 논문이 한층 더 나아질 수 있었습니다. 감사합니다. 학위과정 동안 많은 가르침을 주시고, 폭 넓은 사고를 지닐 수 있도록 이끌어 주신 **박덕배 교수님**, **조문제 교수님**, **현진원 교수님**, **은수용 교수님**, **강현욱 교수님**, **이영기 교수님**, **정영배 교수님**, **정성철 교수님**, **이근화 교수님**, **김수영 교수님** 감사합니다.

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신 지난 날들을 잊지 않고 살아가겠습니다.

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제가 갈 곳을 잃어 헤매고 있을 때, 항상 자기 일처럼 이끌어주시는 저의 정신적 지주이신 **윤정언니**에게 감사하다는 말씀을 전합니다. 가장 힘들고 어려울 때, 제 고민 다 들어주시고 옆에서 조언해주시고 격려해주신 덕택에 한층 더 성장할 수 있었던 것 같습니다. 그 고마움 항상 간직하며 살아가겠습니다. 그리고 항상 저를 친동생처럼 아껴주시는 **민정언니**에게 감사의 말씀을 전합니다. 제가 갖고 있는 고민을 해결해 주고자 많은 조언을 해주신 **정용오빠**, **하나**, **긴내언니**, **여진언니**, **아름**이에게도 감사의 마음을 전합니다.

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언제나 저를 믿어주고, 존재 만으로도 큰 힘이 되어주는 **미연**, **민경**, **나루**, **얌전**, **미향**, **은지**, **선미**, **지나**에게 고마운 마음을 전합니다. 만날 때 마다 제 얘기를 귀담아 들어주고

큰 조언해주는 **보영**이에게 고마운 마음을 전하며, 하는 연구 잘 되기를 빈다는 말 또한 함께 전합니다. 그리고 멀리서도 잊지 않고 챙겨주는 **수지**에게도 고마운 마음을 전합니다. 내가 자주 연락 못해도 먼저 연락해서 많은 격려를 해주고 큰 힘을 주었던 **철호**에게도 고마운 마음을 전합니다.

항상 제 입장에 먼저 서서 생각해 주시고, 제 의견을 100% 다 수용해주시며, 지지해주시는 사랑하는 **아빠**, **엄마**에게 감사의 말씀을 전합니다. 아빠, 엄마의 좋은 말씀과 큰 믿음 덕분에 무사히 학위과정을 마칠 수 있었습니다. 큰 딸 노릇 제대로 못해 죄송하다는 말씀 전하며, 앞으로도 아빠, 엄마의 기대 져버리지 않도록 항상 반듯하게, 그리고 성실히 살아가는 모습 보여드리겠습니다. 사랑합니다. 바쁘다고 신경 못 써주지만 항상 착하고, 바르게 살아가는 사랑하는 나의 동생들 **혜민**, **혜인**이에게도 고마운 마음과 매 순간 최선을 다하며 살아가길 바란다는 말을 함께 전합니다. 그리고 언제나 든든한 버팀목이 되어 주시는 **할머니**께도 감사의 말씀을 전합니다. 항상 건강하시길 기원합니다.

학위과정 내내 제가 하고 싶은 실험과 공부를 하며 배움의 즐거움에 흠뻑 젖어 살았던 것 같습니다. 그 과정 중에 물론 눈물 나게 힘들 때도 있었고, 지칠 때도 있었지만 결과적으로 뒤돌아 봤을 때 힘들고 어려웠던 과정을 겪고 난 후의 설레고 벅찼던 제 마음이 더 컸던 것 같습니다. 제가 하는 일을 즐기며 할 수 있어 행복했으며, 하고 있는 일이 즐겁다는 것 만으로도 저는 가장 행복한 사람이 아닌가 싶습니다. 제가 즐기며 학위생활을 할 수 있었던 데는 많은 조언과 격려를 아끼지 않으셨던 많은 분들이 있었기 때문이며 그 분들께 감사의 말씀을 전합니다. 그리고 제 생활이 바쁘답시고 뒤돌아 보지 못했던 지난 날들에 상처받았을 많은 분들께 죄송하다는 말씀도 함께 전하고자 합니다. “처음”이라는 단어에는 항상 설렘과 함께 낯섦이 붙어 다니는 것 같습니다. 저는 이제 또 다른 “새로운 시작”을 하고자 첫 발걸음을 내딛고자 합니다. 많이 설레고 동시에 낯설지만 그 과정 중에 “Deserve the Desire (많이 갖추고 나서 소망하라)” Max Gallo의 말을 되새기며, 항상 제자리에 멈춰 있는 제가 아닌 목표를 향해 매 순간 최선을 다하며 정진하는 모습을 보여드리겠습니다.