Thesis for the Degree of Master of Science

Anti-cancer activity of biologically active substances from a Seaweed and a marine algae associated microorganism

by
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by

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Induction of Apoptosis accompanied by Cell Cycle Arrest and Endoplasmic Reticulum Stress by extract of *Saccharina japonica* in SK-Hep1 Human Hepatocellular Carcinoma Cells

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ABSTRACT

Saccharina japonica is a marine algae which belongs to family of Phaeophyceae (brown algae) and extensively cultivated in China, Japan and Korea. Brown algae contain bioactive compounds which can serve as anti-oxidant, anti-diabetic, anti-amnesia and anti-cancer material. The potential anti-cancer effect of n-hexane fraction of *S. japonica* was evaluated in SK-Hep1 human hepatocellular carcinoma cells. N-hexane fraction reduced the viability of what and increased the numbers of apoptotic cells in both dose- and time-dependent manner. The effect of n-hexane fraction on apoptosis was activated by both caspase-dependent and independent pathways. The caspase-dependent cell death pathway was mediated by cell surface death receptors and activated caspase-8 amplified the apoptotic signal either by direct activation of downstream caspase-3 or pro-apoptotic protein (Bad, Bax and Bak) subsequently led to the release of cytochrome c. On the other hand, caspase-independent apoptosis was mediated by the disruption of the mitochondrial membrane potential and the translocation of AIF to nucleus, where they induce chromatin condensation and/or large-scale DNA fragmentation. In addition, n-hexane extracts induced endoplasmic reticulum (ER)-stress and cell cycle arrest. The results suggested that the potential anti-cancer effect of n-hexane extract from *S. japonica* on SK-Hep1 cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem, accounting for more than 626,000 new cases per year in the world. The incidence of hepatocellular carcinoma is increasing in many countries, and it is the third leading cause of cancer-related death globally, behind lung and stomach cancers (Parkin et al., 2002). HCC is diagnosed in 30 to 40% of all patients at early stages and is amenable to potentially curative treatments, such as surgery, liver transplantation etc. Five-year survival rates of up to 60 to 70% can be achieved in well-selected patients. However, disease diagnosed at an advanced stage or with progression after locoregional therapy has a dismal prognosis, owing to the underlying liver disease and lack of effective treatment option (Bruix et al., 2001; Llovet et al., 2003).

Recent studies have been carried out to find cancer chemo-preventive and/or chemo-therapeutic agents from edible and natural resources such as fruits, vegetables, and terrestrial plants (Park et al., 2002). Some studies have reported that natural products have positive effects against cancer compared with chemotherapy. Therefore, many vegetables, fruits and medicinal herbs have been examined to identify new and effective anticancer compounds (Aherne et al., 2000).

Most recently, pharmaceutical companies have started their search for new drugs from marine organisms including seaweeds. Seaweeds are one of the natural resources in the marine ecosystem. It contains various biologically active compounds which have been used as source of food, feed and medicine (Lincoln et al., 1991). Recent findings evidenced that seaweeds contained antiviral (Matsuhiro et al., 2005), antibacterial (Xu et al., 2003), antifungal (Li et al., 2006), and antitumoral (Xu et al., 2004) potentials. Therefore, we selected *Saccharina japonica* which belongs to family of Phaeophyceae (brown algae) and extensively cultivated in China, Japan and Korea, to analyze its effect against hepatocellular carcinoma.

Brown algae contain several compounds with biological activities. Among other traditional food products, marine algae contain polysaccharides and

iodine organic products, mannitol, macro- and micro elements, vitamins, unsaturated fatty acids, and other biogenic compounds.

In the previous studies, researchers have found that brown algae and its extracts inhibited the proliferation of breast tumor cells, lung metastases, and leukemia in animal models (Ohigashi et al., 1992, Itoh et al., 1995, Riou et al., 1996, Funahashi et al., 1999, Funahashi et al., 2001). Funahashi et al. (1999) have shown that wakame extracts (wakame soaked in animals' drinking water) have a potent inhibitory effect on the progression of mouse mammary tumors. Similar extracts produced an equally profound apoptotic effect on breast cancer cells in vitro while the extracts were nontoxic to normal breast cells. When brown seaweed was included in the animals' diet, it was very clear that there was an anticancer effect in ingestion although the active components have not been determined (J. Helen Fitton., 2003).

The apoptosis is a process of cell death that was originally described by its morphological characteristics including cell shrinkage and chromatin condensation (Kerr et al., 1972). Apoptosis is essential for normal development and homeostasis in multicellular organisms and also serves as a defense mechanism to eliminate harmful cells, such as tumor cells and cells infected by viruses (Jacobson et al., 1997). It has shown that mitochondria play essential roles in apoptosis (Zamzami et al., 1996, Kroemer et al., 1997, Green et al., 1998). Cytochrome c, an essential component of the respiratory chain of the mitochondria, is released in response to various apoptotic stimuli (Kluck et al., 1997, Bossy-Wetzel et al., 1998), and binds to apoptotic protease activating factor 1 (Apaf1), leading to the formation of apoptosome. Apoptosome then proteolytically activates caspase-9, and the activated caspase-9 cleaves the downstream caspases including caspase-3, 6, and 7, bringing about apoptotic cell death by digesting essential cellular proteins (Zou et al., 1997, Li et al., 1997). On the other hand, mammalian cells in a certain circumstance can undergo caspase-independent apoptosis that is mediated by the disruption of the mitochondrial membrane potential and the translocation of AIF and endonuclease G to nucleus where they induce chromatin condensation and/or large-scale DNA fragmentation (Cregan et al.,

2004).

The ER is a continuous membrane system that consists of multiple domains that perform different functions (Sitia, R et al., 1992). These include translocation of secretory proteins across the ER membrane, integration of proteins into the membrane, folding and modification of proteins in the ER lumen, synthesis of phospholipids and steroids, detoxification, storage of calcium ions in the ER lumen and their release in the cytosol as well as segregation of nuclear contents from the cytoplasm (Voeltz, G.K et al., 2002).

Proper function of the ER is essential to cell survival and any perturbation of its function induces cellular damage and results in apoptosis. Various conditions can disturb ER functions, events collectively termed "ER-stress". These stresses include inhibition of protein glycosylation, reduction of formation of disulfide bonds, calcium depletion from the ER lumen, impairment of protein transport from the ER to the Golgi, expression of misfolded proteins, etc. The ER is regulated by signaling pathways that respond to accumulation of unfolded or misfolded proteins in the organelle. To survive and adapt under ER-stress conditions, cells have a self-protective mechanism against ER-stress, which has been termed the ER-stress response (Mori K., 2000, Kaufman RJ, 1999).

To combat the deleterious effects of ER-stress, cells have evolved various protective strategies, collectively termed the unfolded protein response (UPR). This concerted and complex cellular response is mediated through three ER transmembrane receptors: pancreatic ER kinase(PKR)-like ER kinase(PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) (Oyadomari S et al., 2002, Mori K., 2000). At least four functionally distinct responses have been identified. The first one involves upregulation of the genes encoding ER chaperone proteins to increase protein folding activity and to prevent protein aggregation. The second consists of translational attenuation to reduce the load of new protein synthesis and to prevent further accumulation of unfolded proteins. The third is degradation of proteins misfolded in the ER and this is called ER-associated degradation (ERAD). The fourth is apoptosis which occurs when functions of the ER are

extensively impaired (Oyadomari S et al., 2002).

In this study, we evaluated the potential anti-cancer activities of *S. japonica* n-hexane extract in SK-Hep1 hepatocellular carcinoma cells.

MATERIALS AND METHODS

Seaweed material.

S. japonica was harvested from Kijang aquaculture farm in Korea on May 2010. The samples were dried in cold-air drier (60°C) for 40 hr and ground with a hammer mill. The dried powder was stored at -20°C until used.

Extraction and fractionation.

The dried powder (2 kg) of *S. japonica* was refluxed with ethyl alcohol (95%, v/v) for 3 hr. The extract (446.0 g) was suspended in H_2O:ethyl alcohol (9:1, v/v) and partitioned with n-hexane, dichloromethane, ethyl acetate (EtOAc), n-butanol (n-BuOH), and water in sequence, yielding the n-hexane (135.5 g), dichloromethane (18.1 g), EtOAc (39.6 g), n-BuOH (55 g), and water (162.8 g) fractions. The n-hexane fraction was subjected to preparative size exclusion column of Shim-pack PREP-ODS (500 mm x 21.2 mm, Shimadzu Co., Tokyo, Japan). An exclusion HPLC apparatus consisted of a pump (Shimadzu LC-6AD), a photodiode array detector (Shimadzu SPDM20A), an online degasser (Shimadzu DUG-20A3), an autosampler (SIL-20A), a fraction collector (Shimadzu FRC-10A), a system controller (CBM-20A), and a Shimadzu LCsolution (ver. 1.22sp). The n-hexane fraction was chromatographed on a Shim-pack PREP-ODS column eluting with methanol at a flow rate of 5.0 mL • min^{-1} and monitored at 240 nm. The fraction was separated into four fractions (GS1 ~ GS4). The GS3 fraction was chromatographed over Phenomenex C18-ODS (Phenomenex Co., Tokyo, Japan). A preparative ODS HPLC system was similar to the exclusion HPLC system except for a binary pump (Phenomenex LC-6AD) and a column oven (Phenomenex CTO-20A). The separation of GS3 fraction was conducted using mobile phase water (solvent A) and methanol (solvent B). The elution profile consisted of a linear gradient from 20 to 70% solvent B for 90 min. The flow rate was 7.0 mL • min^{-1}, and detection was performed at 216 nm. The fraction gave fifteen subfractions (GS3-ODS1 ~ GS3-ODS15).

Cell culture.

All the cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Human hepatocellular carcinoma SK-Hep1 cells were maintained in Minimum Essential Medium with Earle's balanced salts (MEM/EBSS) (Hycolne, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), and 1% penicillin-streptomycin (PAA Laboratories GmbH, Pasching, Austria) at 37°C in humidified atmosphere at 95% air and 5% CO_2. The human embryonic kidney HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin at 37°C in humidified atmosphere at 95% air and 5% CO_2. THLE-3 cells were cultured in flask coated with 0.01 mg • mL^{-1} fibronectin, 0.03 mg • mL^{-1} bovine collagen type 1 and 0.01 mg • mL^{-1} bovine serum albumin for 24 h and cultivated in Bronchial Epithelial Cell Basal Medium (BEBM) with BEGM SingleQuot kit except GA-1000 and Epinephrine (Lonza Group Ltd., Basel, Switzerland), 10% heat inactivated fetal bovine serum (Lonza Group Ltd.) and 1% penicillin-streptomycin (PAA Laboratories) at 37°C in a humidified atmosphere of 5% CO_2.

Cell viability assay.

The *S. japonica* n-hexane fraction was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). The final concentration of DMSO in the culture medium was not exceeded 0.04% (v/v), and the same concentration of DMSO was added to the control dishes. For the cell viability assay, $1x10^4$ cells were resuspended in 100 µL medium and seeded onto each well of a 96-well plate. The cells were then treated with 5, 10, 15 and 20 µg • mL^{-1} of the fraction and were incubated for 24 h. After the treatment, 10 µL of EZ-Cytox Cell Viability Assay Solution WST-1® (Daeil Lab service, Jong-No, Korea) was added onto each well and the cells were further incubated for 3 h and then read the absorbance at 460 nm with ELISA reader (Molecular Devices, siliconvalley, CA, USA).

Caspase inhibitor assay.

To compare the caspase-dependent and caspase-independent cell deaths, cells were treated as follows control, Z-VAD-FMK (20 μM), sample (10, 15, 20 μg • mL^{-1}) and sample (10, 15, 20 μg • mL^{-1}) containing Z-VAD-FMK (20 μM) group. After 24 h, cells were changed to a fresh medium. After incubation, 10 μL of WST-1 solution was added to each well and further incubated for 3 h. The absorbance of supernatant was determined at 460 nm using VersaMax microplate reader (Molecular Devices).

Western Blot analysis.

To confirm the protein level in a dose-dependent manner, SK-Hep1 cells were preincubated in 10% FBS/MEM for 48 h. The medium was then replaced with the medium containning different dose of n-hexane extract followed by further incubation for 24 h. To confirm the protein level in a time-dependent manner, SK-Hep1 cells were cultured and treated with 20 μg • mL^{-1} *S. japonica* n-hexane extract for 2, 4, 6, or 12 h. The harvested cells were lysed in ice-cold lysis buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS and cocktail of protease inhibitors (Intron biotechnology, Gyeonggi, Korea)]. After incubation on ice for 30 min, the insoluble materials were removed by centrifugation at 14,000 rpm for 20 min at 4°C. The protein content of the cell lysates were determined by a Protein Quantification Kit (CBB solution®) (Dojindo Molecular Technologies, Rockville, MD, USA) with bovine serum albumin (BSA) as standard. Each sample in Laemmli buffer was boiled for 5 min, and then resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrotransferred onto a nitrocellulose membrane (PALL Life Sciences, MI, USA) and blocked in PBST buffer (135 μM NaCl, 2.7mM KCl, 4.3mM $NaPO_4$, 1.4mM KH_2PO_4, 0.5% Tween-20) containing 5% skim milk. After blocking, the membrane was probed with primary antibodies (Cell Signaling Technology Inc., Danver, MA, USA) and then washed three times with PBST buffer followed by incubation for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG

as second antibodies (Cell Signaling Technology Inc.). The blots were then washed in PBST buffer and visualized by enhanced chemiluminescent (ECL) detection solution (Pierce, Rockford, IL, USA).

Detection of intracellular Ca^{2+}.

SK-Hep1 cells were seeded in a 35×10 mm coverglass bottom dish (SPL life sciences, Gyeonggi, Korea) and cultured at 37°C. After 24 h, the cells were treated with 20 μg • mL^{-1} *saccharina japonica* n-hexane extract for 0 h, 1 h, 2 h, 3 h and 4 h. Then the cells were incubated with 1.5 μM Fluo-3AM (Invitrogen, Eugene, OR, USA) at room temperature for 30 min in dark. These cells on the slides were mounted in Prolong Gold Antifade Reagent (Invitrogen, Eugene, OR, USA) followed by observation under a Nikon ECLIPS 50i microscope equipped with charged-coupled device (CDD) camera. The fluorescence intensity indicating the concentration of Ca^{2+} was captured and processed with High-Content Analysis Software (Cambridge Healthtech institute, Needham, MA USA).

FACS analysis.

SK-Hep1 cells were treated with different concentrations of *S. japonica* n-hexane extract for 24 h. The cells were harvested by trypsinization, then washed with PBS and fixed in 70% ethanol at 4°C for overnight. Cells were stained with 40 μg • mL^{-1} propidium iodide for 30 min and analyzed using a FACS Calibur apparatus (Becton Dickinson, Mountain View, CA, USA).

Immunofluorescence of cleaved caspase-3 protein.

Cells were cultured on coverglass bottom dishes (SPL lifesciences, Gyeonggi, Korea) for 24 h and fixed with 4% formaldehyde (Sigma) for 15 min at room temperature and then blocked for 1 h in 5% mouse and rabbit normal serum (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Fixed and blocked cells were incubated with primary antibodies (cleaved caspase-3 and β-actin) (Cell Signaling Technology Inc.) for 2 h and then with 0.1 μg • mL^{-1} of anti-mouse IgG (H+L), F (ab′) 2 Fragment (Alexa Fluor® 555

Conjugate) and anti-rabbit IgG (H+L), F (ab′) 2 Fragment (Alexa Fluor® 488 Conjugate) (Cell Signaling Technology Inc.) for 1 h. Stained cells on the slides were mounted in Prolong Antifade Reagent (Invitrogen, Eugene, OR, USA) and observed in fluorescent microscope Nikon ECLIPS 50i microscope equipped with charged-coupled device (CDD) camera (Nikon, Tokyo, Japan). Images were captured and processed with High-Content Analysis Software (Cambridge Healthtech Institute, Needham, MA, USA).

TUNEL assay.

For *in situ* detection of apoptotic cells, the terminal deoxynucleotidyl transferase-mediated deoxy-uridine triphosphate (dUTP) nick-end labeling (ApopTag® Plus *In Situ* Apoptosis Fluorescein Detection Kit) (Millipore, Billerica, MA, USA) was used to detect the DNA fragmentation. Cells were cultured on coverglass bottom dishes (SPL lifesciences, Gyeonggi, Korea) in MEM medium (Hycolne) containing 10% FBS and penicillin-streptomycin (PAA Laboratories) for 24 h then fixed in 1% paraformaldehyde (pH 7.4) for 10 min at room temperature. The fixed cells were then incubated with cold ethanol and acetic acid mixture for 5 min at -20°C and then washed twice with ice cold PBS (135 μM NaCl, 2.7 mM KCl, 4.3 mM $NaPO_4$, 1.4 mM KH_2PO_4, 0.5% Tween-20). After that, the cells were incubated with terminal deoxynucleotidyl transferase (TdT) for 1 h at a humidified atmosphere and were immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin conjugate solution for 30 min in a humidified chamber to avoid exposure to light. For the counterstain, a mounting medium containing 0.5 $\mu g \cdot ml^{-1}$ of Propidium Iodide was applied.

Statistical analysis.

The GraphPad Prism 5.0 for Window was used to determine the statistical significance of the differences between the values of various experimental and control groups. Determinations were performed in triplicates and the results were presented as means±S.E.M. In cases where there was no error

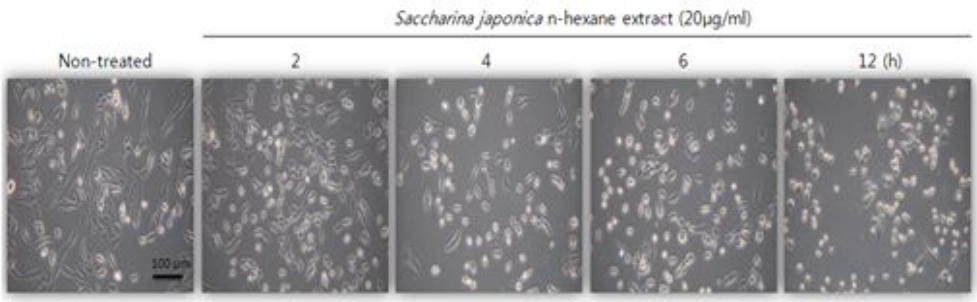
bar seen in the graph, it means the variation is small and thus, the bar is hidden behind. ANOVA post hoc test and subsequently, Dunnett's multiple comparison tests were used for statistical analysis.

RESULTS

Cell death of SK-Hep1 by n-hexane extract of *S. japonica*.

To determine whether the extract of *S. japonica* n-hexane exerts anticancer effects on the proliferation of SK-Hep1, THLE-3 and HEK 293 cell lines, dose-dependent and time-dependent studies were conducted. The result of the cell viability assay showed that the n-hexane extract of *S. japonica* inhibited proliferation of SK-Hep1 cells in a dose-dependent manner. However, the same dose of *S. japonica* n-hexane extract exhibited less anti-proliferation effect on both THLE-3 and HEK 293 cells compared to SK-Hep1 cells (Fig. 1B). The treated cells were shrinked and were floated on the medium as time increases (Fig. 1A). The results confirm that the extract of *S. japonica* exert anticancer activity on SK-Hep1 with less effect on non-cancer THLE-3 and HEK 293 cells (Fig. 1).

(A)



(B)

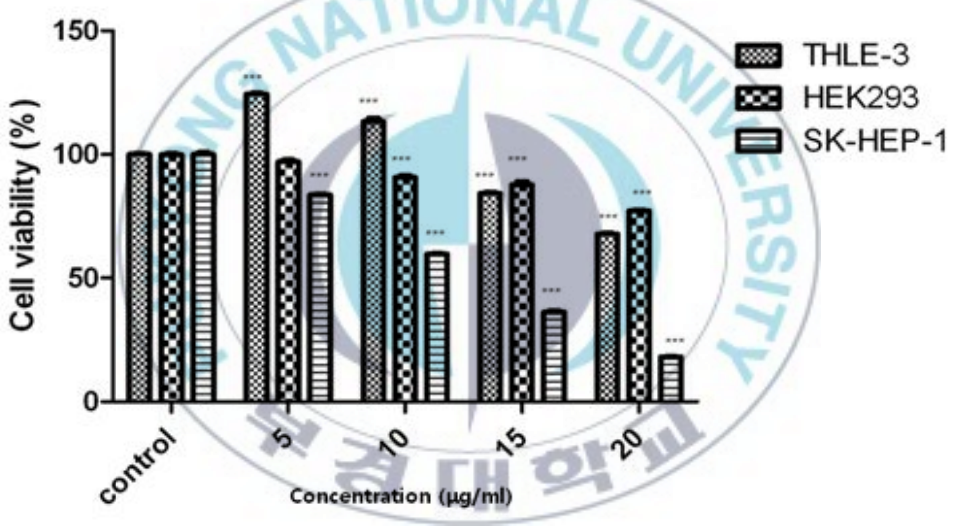
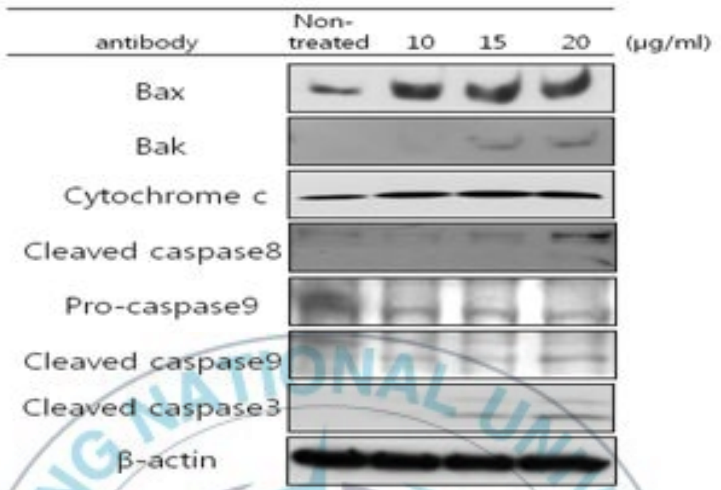


Fig. 1. Morphological changes and the cell viability by n-hexane extract of *S. japonica*. After treatment of n-hexane extract, morphological changes of SK-Hep1 cells were examined at various times. Morphology of the cells was visualized (×100) using an inverted microscope (A). Cell viability was examined by WST-1 assay after treatment of 5~20 μg • mL^{-1} *S. japonica* n-hexane extract on SK-Hep1 (human hepatocellular carcinoma cell), THLE-3 (human normal liver cell) and HEK 293 (human normal embryoinc kidney cell) for 24 h (B). For the control, non-treated cells were used. The bars represent means±S.E.M. of three experiments (***$p < 0.001$).

Induction of caspases mediated aoptosis in the extract treated cells.

As shown in Fig. 2A, western blot analysis revealed that n-hexane extract of *S. japonica* releases cytochrome c with the possible involvement of the increased expression of both Bax and Bak as an initial signal to induce apoptosis. Caspase-9 was activated by cytochrome c released from the mitochondria and the activated caspase-9 cleaves caspase-3. Cellular death receptors may also initiate caspase cascade by the activation of caspase-8. As cleaved caspase-8 was dramatically increased in a dose-dependent manner, induced expression levels of Bad and cleaved caspase-3 by the extract was detected in a time-dependent manner (Fig. 2B). In addition, we observed a time dependent increase in the expression level of cleaved caspase-3 in SK-Hep1 cells using immunofluorescence (Fig. 2C). Both Western blot analysis and immunofluorescence suggest that the *S. japonica* n-hexane extract induces not only mitochondria but also death receptor mediated apoptosis in SK-Hep1 cells.

(A)



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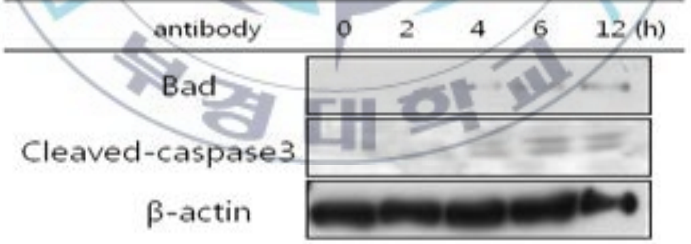


(C)

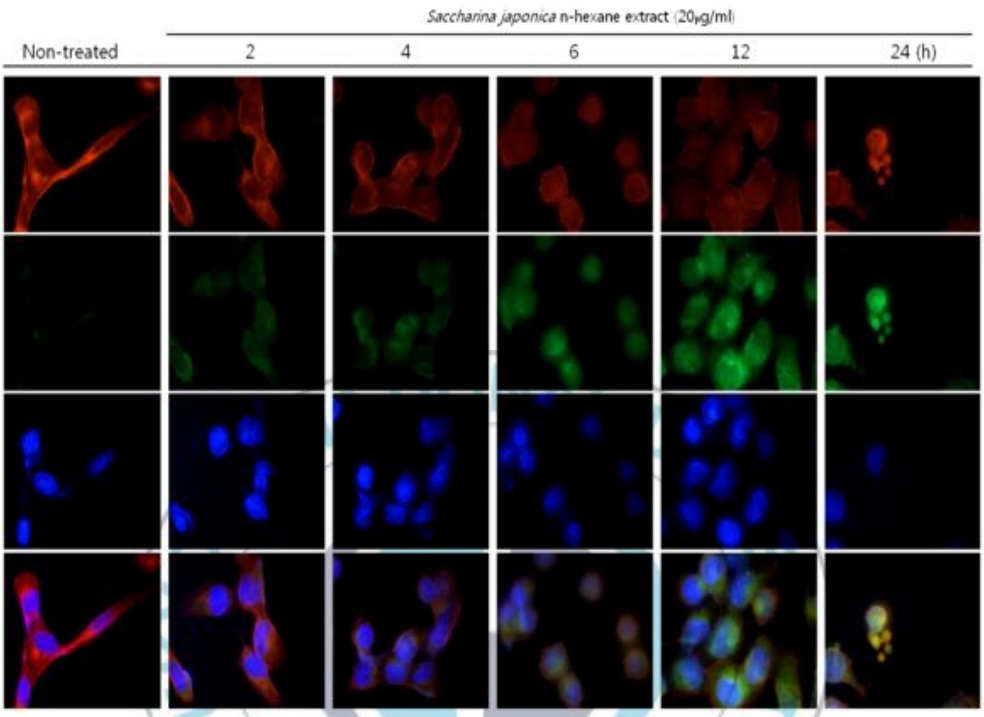
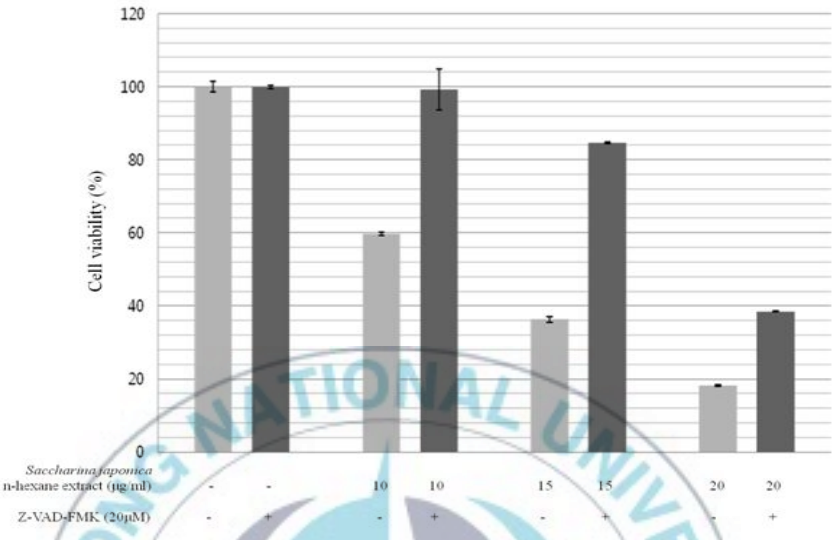


Fig. 2. The effects of *S. japonica* n-hexane extract on the expression levels of apoptosis related proteins in SK-Hep1 cells. Cell were treated with 0, 10, 15 and 20 μg • ml^{-1} *S. japonica* n-hexane extract for 24 h. Equal amounts of cell lysates (25 μg) were subjected to SDS-electrophoresis and analyzed by Western blot against Bax, Bak, Cytochrome c, cleaved caspase-8, procaspase-9, cleaved caspase-9, and cleaved caspase-3. β-actin was used as control for showing the same amounts of protein been loaded (A). The expression levels of Bad and cleaved caspase-3 induced by *S. japonica* n-hexane extract were increased in a timely manner (B). Cell were exposed to 20 μg • ml^{-1} concentration of n-hexane extract for 0, 2, 4, 6, 12 and 24 h. Cells were then incubated with antibody against cleaved caspase-3 followed by labeling with the Alexa Fluor 488 and 555 conjugated secondary antibodies. Nuclei were stained with DAPI (C).

Apoptosis induced by *S. japonica* n-hexane extract is mediated by both caspase-dependent and independent pathways.

In order to verify the involvement of caspases in *S. japonica* n-hexane extract induced cell death, we examined the cell viability with the treatment of Z-VAD-FMK, a caspase inhibitor. The cells were divided into two groups. One is treated with different concentration of the extract and the other is with a combination of both *S. japonica* n-hexane extract and Z-VAD-FMK (20 μM). In SK-Hep1 cells, between 40% and 60% of cell death was induced by the treatment of 15 $\mu g \cdot mL^{-1}$ n-hexane extract. Whereas, the combination of Z-VAD-FMK and *S. japonica* n-hexane extract showed less effect on the viability of SK-Hep1 cells. There was no difference in non-treated and Z-VAD-FMK only treated samples. The results suggest that *S. japonica* n-hexane extract partly enhances caspase-independent cell death (fig. 3A). Furthermore, PARP cleavage and AIF activation were also demonstrated by western blot analysis in a dose-dependent manner in SK-Hep1 cells (Fig 3B). Caspase-independent apoptosis is mediated by the disruption of the mitochondrial membrane potential and the translocation of AIF to nucleus where they induce chromatin condensation and/or large-scale DNA fragmentation. Our result suggest that the increased expression of cleaved PARP and AIF reveals the partial involvement of caspase independent cell death in SK-Hep1 cells treated with *S. japonica* n-hexane extract.

(A)



(B)

Fig. 3. Effect of *S. japonica* n-hexane extract in SK-Hep1 cells. Z-VAD-FMK enhances the caspase inhibition in SK-Hep1 cells. SK-Hep1 cells were divided into eight groups: non-treated, Z-VAD-FMK (treated with 20 μM Z-VAD-FMK), *S. japonica* n-hexane extract (treated with 10 μg • mL^{-1}, 15 μg • mL^{-1} and 20 μg • mL^{-1}) and combination of both (treated with extract of different concentration and 20 μM Z-VAD-FMK). The cell viability was measured with WST-1 assay (A). increase of cleaved PARP and caspase-independent AIF pathway mediated apoptosis induced by *S. japonica* n-hexane extract through the western blot analysis (B).

Induction of ER-stress and cell cycle arrest by *S. japonica* n-hexane extract.

In order to study the effect of *S. japonica* on ER-stress, we used fluo-3/AM to measure the relative Ca^{2+} concentration. Disruption of calcium homeostasis induces endoplasmic reticulum stress. As shown in Fig. 4A, the fluorescence density gradually increased in SK-Hep1 cells treated with *S. japonica* n-hexane extract in a time-dependent manner. The degree of inducted Ca^{2+} density at 2 h closely increased. To confirm the ER-stress in protein level, we performed western blots for a number of ER-stress-associated proteins. The obtained data demonstrated that time dependent expression of ER-stress associated proteins Bip, TRAF2, p-JNK, ATF6α, CHOP, Calnexin and p-eIF2α (Fig. 4B). CHOP proteins were delayed somewhat compared with the onset of the expression of ATF6α. This is consistent with the known placement of these proteins downstream of ATF6α. Additional evidence for activation of the ER-stress response is the induction of the ER chaperone protein BiP and calnexin. The Phosphorylation of eIF2α is a stress signal and is a negative regulator, thereby attenuating translation of most mRNAs while selectively increasing translation of the ATF4 (Harding et al. 2000). Also, expression of TRAF2 and p-JNK is increased for stress responses as shown Fig. 4B. Together, our observations indicate that *S. japonica* n-hexane extract triggers ER-stress.

On the other hand, *S. japonica* n-hexane extract induces cell cycle arrest. The results revealed that when the cells were treated with 10, 15 and 20 μg • mL^{-1} of *S. japonica* n-hexane extract for 24 h, it leads to changes in cell cycle progression and the expression of cell cycle related proteins such as CDK2, phospho-Rb and phosphor-cdc2. As shown Fig. 4C, down-regulation of CDK-2 resulted in expression of p-cdc2 following inhibition of phosphorylaion of Rb. Suggesting that *S. japonica* n-hexane extract may induce cell cycle inhibition by affecting the levels of CDK2 in SK-Hep1 cells.

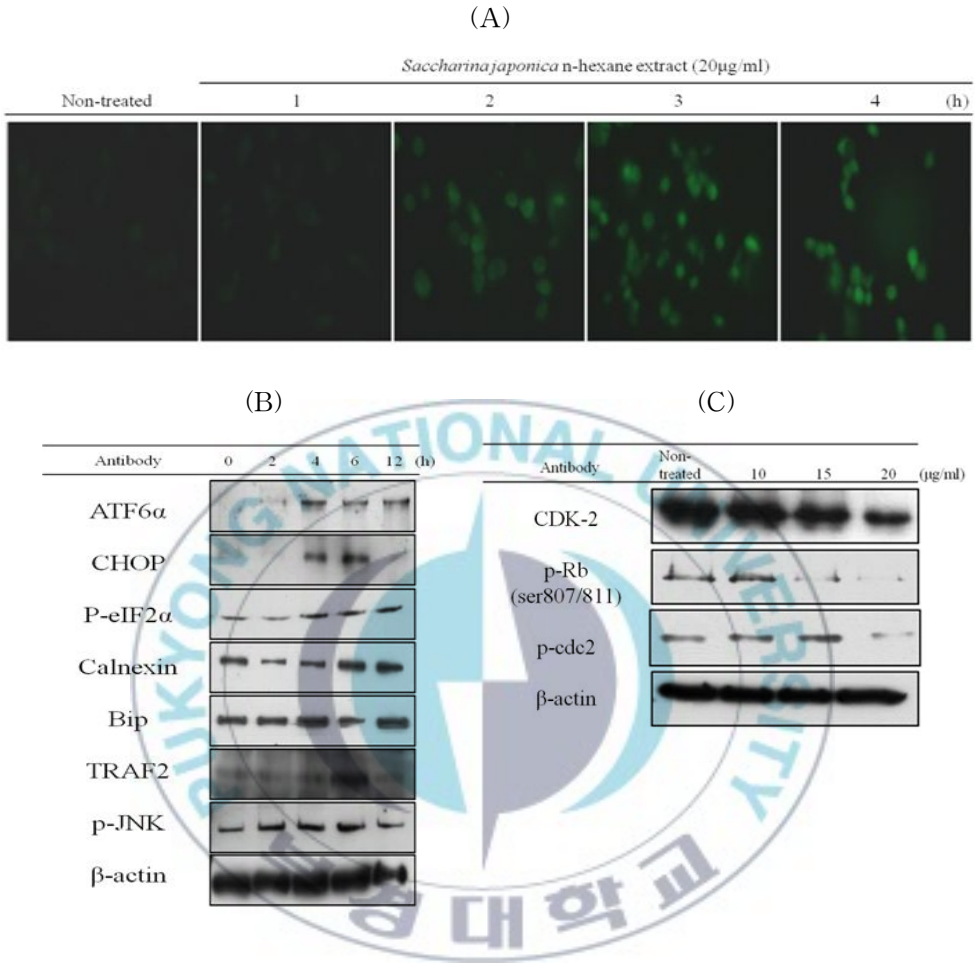


Fig. 4. Endoplasmic reticulum (ER) stress was involved in *S. japonica* n-hexane extract induced apoptosis. concentration of Ca^{2+} was gradually increased in SK-Hep1 cells treated with 20 μg • mL^{-1} *S. japonica* n-hexane extract (A). Western blot analysis of proteins involved in ER-stress signaling from cell extracts of SK-Hep1 cells treated with *S. japonica* n-hexane extract 20 μg • mL^{-1} for indicated time periods (h) (B). Expression levels of proteins in cell cycle were examined by western blot analysis. Down-regulation of CDK-2 resulted in expression of p-cdc2 following inhibiting phosphorylaion of Rb (C).

Effect of DNA fragmentation on SK-Hep1 cells by *S. japonica* n-hexane extract.

The quantification of apoptosis was confirmed using flow cytometry analysis through estimation of sub-G1 DNA content in *S. japonica* n-hexane extract treated SK-Hep1 cells. The result indicates that the presence of sub-G1 DNA in control around 2.14%, but in treated cells increased up to 33.99%, indicates that the subgenomic content was gradually increased in dose dependent manner (Fig. 5A). To determine whether the *S. japonica* n-hexane extract induced nuclear DNA fragmentation, SK-Hep1 cells were treated *S. japonica* n-hexane extract (20 $\mu g \cdot mL^{-1}$) and the numbers of DNA fragmented cells were assessed using the apop tag plus fluorescein *in situ* apoptosis detection kit. Exposure of SK-Hep1 cells to *S. japonica* n-hexane extract for 24 h resulted in a significant increase of TUNEL-positive (Fig. 5B).

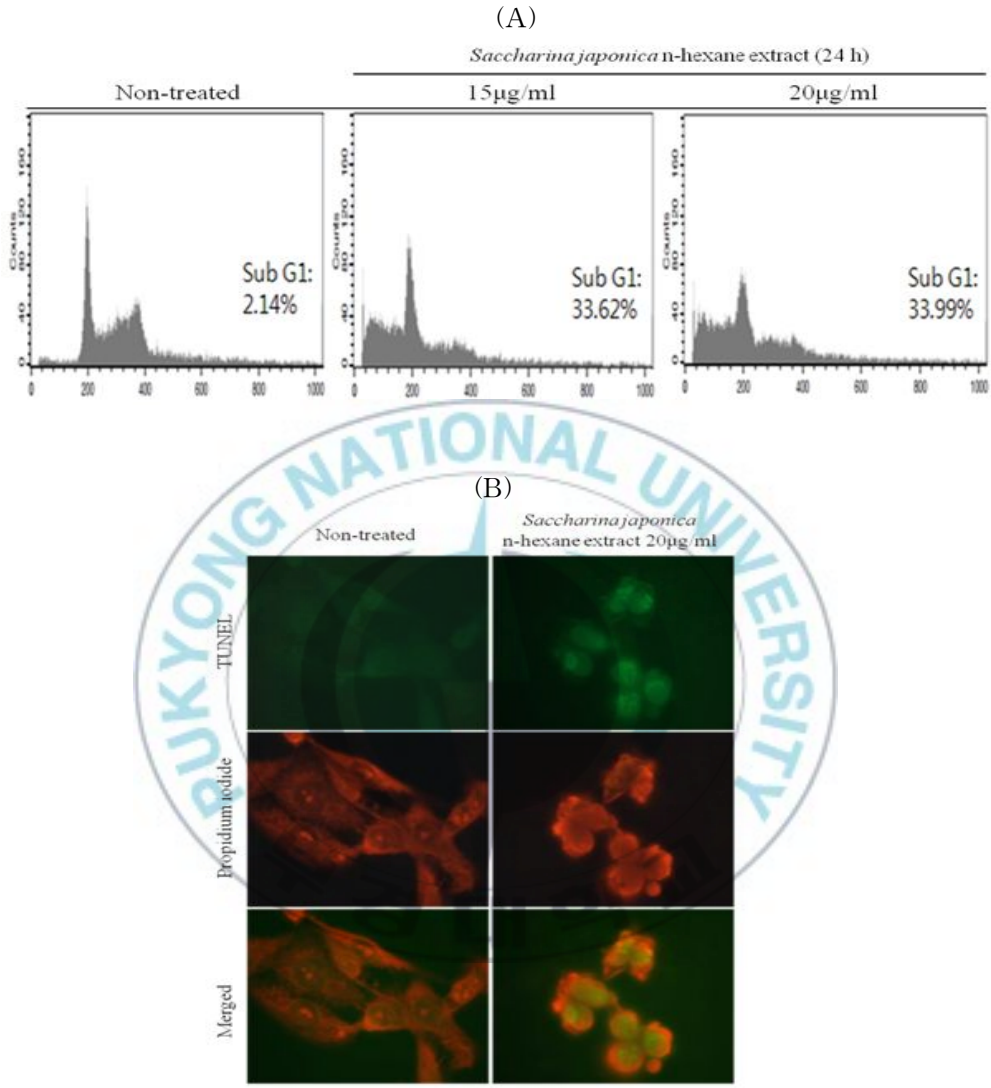


Fig. 5. Induction of apoptosis by *S. japonica* n-hexane extract on SK-Hep1 cells. Flow cytometry analysis for quantification of sub-G1 DNA content of SK-Hep1 cells treated with 15 μg • mL^{-1} and 20 μg • mL^{-1} for 24 h (A). Detection of nuclear DNA fragmentation in SK-Hep1 cells by *in situ* TUNEL assay. Top column TUNEL images, middle column propidium iodide images, bottom column merged images (B).

DISCUSSION

Apoptosis is the best characterized form of programmed cell death and it is the major strategy for the development of anti-cancer drugs. Mitochondria are one of the most susceptible organelles to apoptotic stimulus. In the present study we have evaluated the potential anti-cancer activity of *S. japonica* n-hexane extract in heptocellular carcinoma. The observed result demonstrate that *S. japonica* n-hexane extract induces both caspase-dependent and independent cell death and also induce ER-stress and cell cycle arrest. The caspase-dependent cell death is mediated by cell surface death receptors such as Fas. This receptor recruits the adaptor protein Fas-associated death domain (FADD) and caspase-8, leads to cleavage and activation of capase-8. Activated caspase-8 induces the mitochondrial release of cytochrome C or activation of caspase-3 directly (Ashkenazi A et al.,1998, Jin Z et al., 2005). Our result indicates that the induction of caspases (caspase-8,-9 and-3) by *S. japonica* n-hexane extract attest the possibility of caspase-dependent apoptotic effect on SK-Hep1 hepatoma cells. The Bcl-2 family comprises a group of structurally related proteins that plays a vital role in the regulation of intrinsic apoptosis. Bcl-2 and Bcl-xL maintains the integrity of the mitochondrial outer membranes and prevent apoptosis. Our data demonstrated that *S. japonica* n-hexane extract increases the expression of pro-apoptotic Bcl-2 family protein Bad, Bax and Bak. The *S. japonica* n-hexane extract also induce caspase-independent cell death by activation of AIF. AIF (a mitochondrial flavoprotein) translocates to the nucleus when it induce caspase independent chromatin condensation and DNA fragmentation. The release of AIF from mitochondria is largely depends on activation of PARP (Zhao YJ et al., 2009). Our results reveal that *S. japonica* n-hexane extract induces the higher expression of cleaved PARP and AIF. The large-scale DNA fragmentation and cell cycle arrest were confirmed by FACS and western analysis. These results indicate that the n-hexane extract of *S. japonica* induces the caspase-independent cell death and cell cycle inhibition in hepato

cellular carcinoma SK-Hep1 cells.

In addition to mitochondria, other organelles like ER, Golgi and lysosome are also involved in the apoptotic initiation. Recently, many researches have focused on ER stress induced apoptosis in tumor cells. Two distinct involved in ER stress induced apoptosis: the accumulation of unfolded protein and Ca^{2+} signaling. The disruption of calcium homeostasis induces ER-stress, resulting in the upregulation of ER-stress related genes Bip, TRAF2, P-JNK, ATF6α, and CHOP. Our results show that the extract of *S. japonica* alters the calcium homeostasis and induce ER-stress that leads to the upregulation of many ER specific mediator that induces apoptosis in hepatocellular carcinoma cells.

In summary, the n-hexane fraction of *S. japonica* induces ER-stress by disturbing the calcium homeostasis and activation both caspase-dependent and independent cell death in hepatocellular carcinoma SK-Hep1 cells.

Saccharina japonica 추출물에 의한 SK-Hep1에서의 세포 주기 억제와 소포체 stress를 통한 세포 사멸유도

정현일

부경대학교 대학원 미생물학과

요약

*Saccharina japonica*는 갈조류에 속하는 해양조류이며 중국, 일본, 한국에서 광범위하게 재배되어지고 있다. 갈조류는 항산화, 항당뇨 그리고 항암에 대한 생리활성 물질들을 다양하게 함유하고 있다. 따라서 본 연구에서 *S. japonica* n-hexane 추출물의 항암효과를 human hepatocellular carcinoma 세포인 SK-Hep1에서 측정하였다. N-hexane fraction은 농도-시간 의존적으로 SK-Hep1 세포의 생존율을 감소시키고 apoptotic 세포의 수를 증가시키는 효과를 나타낸다. 이러한 apoptosis 기전에서 n-hexane 추출물은 caspase 의존적 또는 비 의존적 신호전달을 통해 활성화 된다. Caspase 의존적 세포사멸 기작은 세포 표면 death receptor에 의해서 매개되어지는데 활성화된 caspase-8는 그 down stream에 있는 caspase-3의 활성을 직접적으로 증폭시키거나 pro-apoptotic 단백질 (Bad, Bax 그리고 Bak)을 활성화 시켜 최종적으로 cytochrome c의 방출을 일으켜(mitochondria로 부터) 세포사멸을 유도하게 된다. 반면에 caspase 비 의존적 apoptosis는 mitochondria membrane potential의 붕괴로부터 매개되어 지며, AIF 단백질이 핵 (nucleus)으로의 전좌를 통해 caspase 비 의존적 세포사멸이 일어나게 된다. AIF 단백질은 염색질을 응축시키거나 대규모 DNA 절단을 유발시키게 된다. 따라서 이러한 apoptosis를 유도하는 기전이 어떤 원인에 의해서 일어나는지 확인 해보고자 stress 관련 assay 및 western blot 실험을 통하여 소포체내 calcium 항상성 파괴에 의한 ER stress임을 확인 할 수 있었으며, 그와 동시에 cell cycle arrest가 일어나는 것을 확인 할 수 있었다. 따라서 본 연구를 통해 *S. japonica* n-hexane 추출물이 SK-Hep1 간암 세포에서 ER stress와 cell cylce arrest에 의한 세포 내 기작과 동시에 death receptor stimulation인 세포 외 기작에 의한 apoptosis임을 알 수 있었다.

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Anti-angiogenic effect of Toluhydroquinone, a Secondary Metabolite from Marine fungi *Phoma herbarum* isolated from a Marine Algae *Hypnea saidana* on Human Umbilical Vein Endothelial Cells

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ABSTRACT

Many of fascinating marine natural products have been reported and some of them are originated from secondary metabolites of marine microorganisms. Some of these secondary metabolites have a potential as chemotherapeutic agents for the treatment of a wide variety of diseases. Toluhydroquinone (2-methylbenzene-1,4-diol), a secondary metabolites, was been isolated from a marine fungi *Phoma herbarum* (Class: *Dothideomycete*). Activity of toluhydroquinone is not known. In this study, the anti-angiogenic effects of toluhydroquinone were investigated in vitro. Angiogenesis is a crucial step in the growth and metastasis of cancer. We analyzed the effect of toluhydroquinone on HUVEC cells. Toluhydroquinone had no effect on human umbilical vein endothelial cells (HUVECs) viability. However, toluhydroquinone treatment resulted in a dose-dependent inhibition of tube formation after plating HUVECs on Matrigel and induced inhibition of HUVEC migration in a time dependent manner. More importantly, the invasion activity of HUVECs was significantly inhibited by toluhydroquinone. In conclusion, The results indicated that toluhydroquinone has an ability to inhibit diverse cell behaviours of HUVECs and the effects may be mediated at different levels of the tumor growth. Therefore, toluhydroquinone is proposed to be a potent anti-angiogenesis drug candidate.

INTRODOUCTION

The increasing needs for drugs able to control new illnesses or resistant strains of microorganisms stimulated search for unconventional new sources of bioactive natural products. The oceans turned out to be an attractive field. Since then, giant efforts have been accomplished worldwide aiming the isolation of new metabolites from marine organisms. Although in many cases the functions of these secondary metabolites in the marine species themselves are unclear, other compounds play well-defined roles-for example as trail markers, sexual attractants, antifouling substances or antifeedants. What is clear is that many of the most interesting marine secondary metabolites have potent activities largely unrelated to their *in situ* roles. Examples include abound of antitumour, antiviral, immunosuppressive and antimicrobial agents, as well as neurotoxins, hepatotoxins and cardiac stimulants [1].

Toluhydroquinone secondary metabolites have been isolated from *Phoma herbarum* (calss: *Dothideomycetes*) of marine fungi. Most marine *Dothideomycetes* are intertidal and they are frequently found as saprobes of decaying woody materials in the marine environment. The species that occur completely submerged in the sea are mostly parasites or symbionts of seagrasses or marine algae [2]. *Phoma herbarum* Westend. (Fungi imperfecti) is a ubiquitous saprophyte and toxigenic pathogen to plants and animals [3], including humans under some occasions [4]. *P. herbarum* possesses strong adaptability to diverse environments including salty and chilly surroundings [5]. *P. herbarum* is also a versatile producer of many natural products with potent activities [6].

Angiogenesis represents an essential step in tumor proliferation, expansion and metastasis [7]. It is generally accepted that there are two stages of tumor progression regarding its vasculature [8, 9]. During the initial avascular stage of tumor growth (tumor mass<1.5 mm), nutrition and oxygen supplementation can be achieved by diffusion. When tumor mass grows larger than 0.5mm, nutrition through diffusion is no longer sufficient

and formation of new vasculature is necessary for further growth (vascular stage) [10, 11]. The tumor remains in a dormant state until it can stimulate blood vessel growth from nearby pre-existing capillaries by a process known as angiogenesis [12-14]. Therefore, the growth of tumors is dependent on their capacity to induce angiogenesis, to induce the growth of blood vessels to supply them with oxygen and nutrients. Therefore, tumor angiogenesis is essential for cancer growth and metastasis.

There is substantial preclinical and clinical evidence that angiogenesis plays a role in the development of tumors and the progression of malignancies. Inhibiting angiogenesis has been considered as an important anticancer strategy to suppress tumor growth and metastasis [15].

In this study, we attempted to identify and characterize whether toluhydroquinone could induce anti-angiogenesis *in vitro*. We investigated the effects on the angiogenic progression (migration invasion, tube-like structure formation).

Our results suggested that toluhydroqiunone has the ability to inhibit diverse cell behaviours of human umbilical vein endothelial cells (HUVECs), and the effects may be mediated at different levels of the tumor growth presenting a potential anti-angiogenic drug candidate.

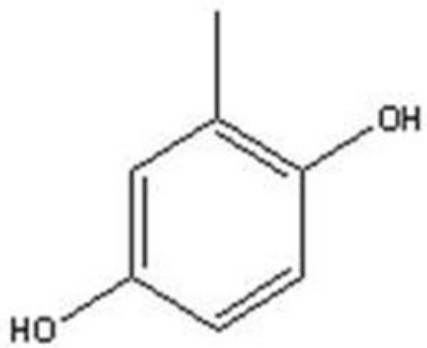


Figure 1. Structure of toluhydroquinone isolated from *Phoma herbarum*

MATERIALS AND METHODS

Fungal isolation and culture

The fungal strain, *Phoma herbarum* was isolated from the surface of the marine red alga *Hypnea saidana* collected in the Tongnyeong, Gyeongnam province, Korea in 2008 and identified based on the morphological evaluation. A voucher specimen is deposited at Pukyong National University with the code MFA301-1.

The fungus was cultured (20L) for 21 days (static) at 29°C in SWS medium: soytone (0.1%), soluble starch (1.0%), and seawater (100%).

Extraction and isolation

The culture broth and mycelium were seperated, and the broth (10 L) was extracted with ethyl acetate to provide a crude extract (640 mg) which was subjected to silica gel flash chromatography and progressively eluted with n-hexane/EtOAc (5:1), n-hexane/EtOAc (1:1), n-hexane/EtOAc (1:5), n-hexane/EtOAc (1:10) and finally with EtOAc. Each collections (30 mL each) were combined on the basis of their TLC profiles to yield five major fractions. Medium pressure liquid chromatography (MPLC) of each fractions on ODS by elution with MeOH afforded compounds 1-5, respectively. The isolated compounds were further purified by HPLC (YMC ODS-A, MeOH) utilizing a 30 min gradient program of 50% to 100% MeOH in H2O to furnish (+)-epoxydon (1, 5.0 mg), (+)-epoxydon monoacetate (2, 12.0 mg), gentisyl alcohol (3, 10.0 mg), 3-chlorogentisyl alcohol (4, 20.0 mg), and methylhydroquinone (toluhydroquinone) (5, 5.0 mg), respectively. The isolated fraction such as methylhydroquinone (toluhydroquinone) was used in this study.

Physicochemical data of compound

Common name : Toluhydroquinone (methylhydroquinone). $CH_3C_6H_3(OH)_2$ (M.W.124.14) (Fig. 1). ^1H NMR (400 MHz, DMSO-d_6) 8.44 (1H, s, 2-OH),

6.53 (1H, d, J = 8.5 Hz, H-3), 6.36 (1H, dd, J = 8.5, 2.8 Hz, H-4), 8.48 (1H, s, 5-OH), 6.45 (1H, d, J = 2.8 Hz, H-6), 2.02 (3H, s, H_3-7); ^{13}C NMR (100 MHz, DMSO-d_6) 124.3 (s, C-1), 147.6 (s, C-2), 117.1 (d, C-3), 115.0 (d, C-4), 149.5 (s, C-5), 112.6 (d, C-6), 16.1 (q, C-7).

Drugs and reagents

Stock concentration of Toluhydroqinone (50 mM) was prepared with dimethyl sulfoxide (DMSO) and stored at -20°C. The stock solution was further diluted with the appropriate assay medium immediately before use. The maximum final concentration of DMSO (< 0.1%) did not affect cell proliferation and did not induce cytotoxicity on the cell lines (data not shown). Vascular endothelial growth factor (VEGF), methanol and DMSO were perchased from Sigma-aldrich (St Louis, MO, USA), formaldehyde (Junsei, Tokyo, Japan), Giemsa (Gurr-Giemsa, BDH Merk Ltd, Poole, England) and WST-1® (Daeil Lab service, Jong-No, Korea) were used. EBM-2 medium was obtained from Lonza (Walkersville, MD, USA) and fetal bovine serum (FBS) was obtained from cellgro (Manassas, VA, USA).

Cell culture

Cell cultures were prepared and maintained according to standard cell culture procedures. Human endothelial cells (HUVECs) were cultured in an endothelial basal medium-2 (EBM-2) supplemented with EGM-2 singleQuots kit (Lonza Group Ltd, walkersville, MD, USA), 1% penicillin-streptomycin (PAA Laboratories GmbH, PA Austria) and 10% fetal bovin serum (FBS) At 37°C and 5% CO_2. HUVECs between P2 and P5 were used for all experiment.

Cell proliferation assay

The proliferative activity of HUVECs was measured using the WST-1 cell proliferation assay. For the cell viability assay, 1×10^4 cells were resuspended in 100μl standard medium and seeded onto each well of a 96-well plate for 24 h incubation, and then sufficient volumes of toluhydroquinone (0-30 μM)

was added to the culture medium, in order to obtain different treatment doses. After 24 h, 10μl of WST-1 solution was added to each well, and the cells were incubated at 37°C in an atmosphere of 5% CO_2 for 3 h. The absorbance of each well was measured at 460 nm with ELISA reader (Molecular Devices, siliconvalley, CA, USA). Experiments were performed in triplicate.

Tube-like structure formation assay (Morphogenesis assay)

The tube formation assay was used to investigate the effect of toluhydroquinone on angiogenesis *in vitro*. Lab-Tek chamber slide (Thermo Fisher Scientific, Rochester, NY, USA) was coated with 200 μl liquid Matrigel (BD Biosciences, Two Oak Park, Bedford, MA USA) per well, which was allowed to solidify at 37°C for 30 min. HUVECs were trypsinized, and resuspended, and seeded at a density of 5×10^4 cells in culture medium containing different concentration of toluhydroquinone (0-15 μM). The cells were incubated for 12 h at 37°C and 5% CO_2 (sufficient for formation of an intact network in the control group), and the tube-like structure formation was observed using phase contrast inverted microscopy (Olympus CKX41; Olympus Optical Co. Ltd, Tokyo, Japan), and total vessel lengths were counted with Wimasis imaging analysis software (Wimasis GmbH, Munich, Germany).

Migration assay

HUVECs (3.5×10^4 cells per each well) were plated to confluence in the culture-insert in 35 mm μ-dishes (Ibidi GmbH, Amkloferspitz19, 82152Martinsried, Germany) placed in EBM-2 containing 10% fetal bovin serum for 24 h. After incubation, the inserts were detached and cells were cultured with medium to facilitate cell migration. Cells were treated with 5 μM, 10 μM and 15 μM of toluhydroquinone and non-treated cell was used as negative control. Cell migration was recorded by phase contrast microscopy over a 2 h time course after treating toluhydroquinone.

Matrigel invasion assay

In vitro invasion of HUVEC cells was measured by the invasion of cells through 24 well multiple well plate with transwell separated by a microporous polycarbonate membrane with 8 μm pore size (Corning, NY, USA). To further confirm the effects of toluhydroquinone on cell invasion, transwell invasion assay was performed using the transwell coated with 15% Matrigel and incubated at 37°C for 1 h. The bottom chambers were filled with standard medium (600 μl) containing several concentration of toluhydroquinone (0-20 μM) and 4 nmol/L VEGF. The top chambers were seeded with several concentration toluhydroquinone (0-20 μM) and cells re-suspended to a final concentration of 3.5×10^4/ml in 200μl pure medium, and then plate incubated at 37°C and 5% CO_2 atmosphere for 6 h. After incubation, HUVECs that had invaded the underside of the membrane were fixed with 4% formaldehyde, permeabilized with 100% methanol, and stained with Giemsa. The cells on the upper surface of the membrane were removed by wiping with cotton swabs. Images were taken using inverted microscope at a magnification of ×40, and each assay was performed in triplicate.

Statistical analysis.

The GraphPad Prism 5.0 for Window was used to determine the statistical significance of the differences between the values of various experimental and control groups. Determinations were performed in triplicates and the results were presented as means±S.E.M. In cases where there was no error bar seen in the graph, it means the variation is small and thus, the bar is hidden behind. ANOVA post hoc test and subsequently, Dunnett's multiple comparison tests were used for statistical analysis.

RESULTS

The effect of toluhydroquinone on HUVECs viability

To examine whether toluhydroquinone was capable to inhibit the proliferation of HUVEC, we performed cell viability assay with a different concentration of toluhydroquinone. HUVECs were treated with 0, 5, 10, 15, 20, 25 and 30 μM toluhydroquinone for 24 h. Despite increased toluhydroquinone concentration, there was no change in cell viability severely. From this result, we concluded that toluhydroquinone in range of concentration was non-toxicity on HUVEC cells.

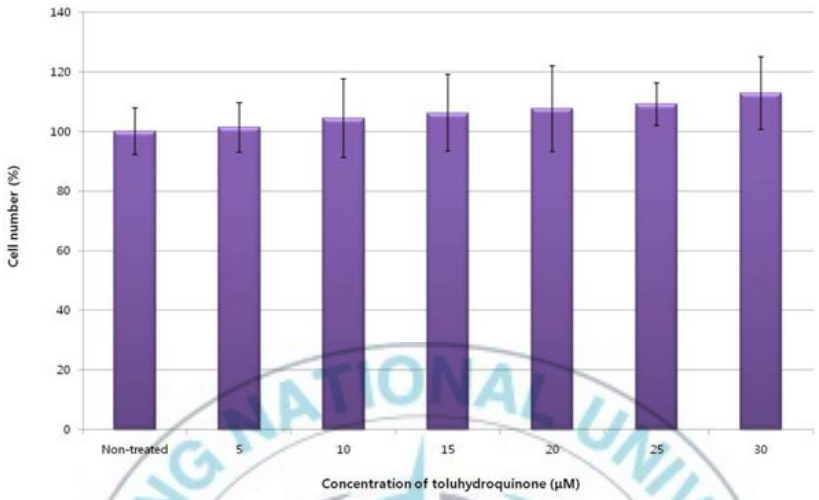
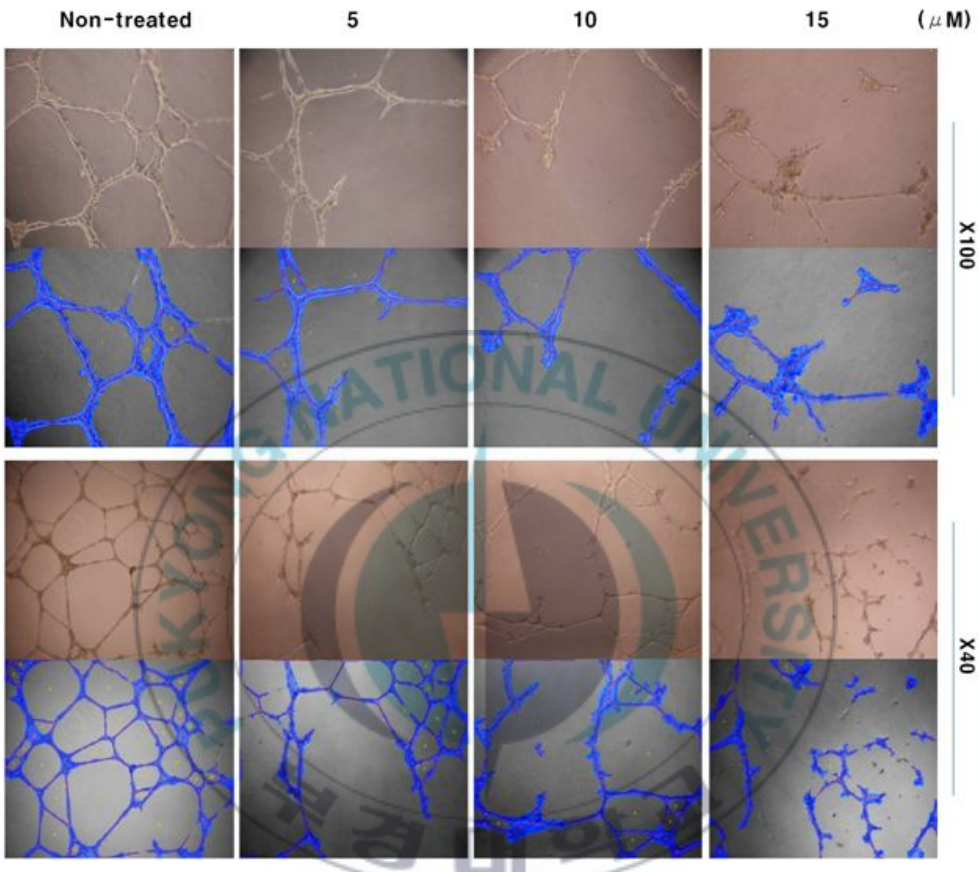


Figure 2. Effect of toluhydroquinone on HUVECs growth in a concentration-dependent manner. Cell was treated with toluhydroquinone (5, 10, 15, 20, 25 and 30 μM) or without toluhydroquinone for 24 h. Graphs showing the absorbance at 460 nm after the WST-1 cytotoxicity assay. The graph shows the dose-dependent cytoxic effects of toluhydroquinone on HUVECs. Values were expressed as percent of control, which was defined as untreated cells. Toluhydroquinone showed nontoxic in HUVECs in range of high concentration.

The effect of toluhydroquinone on HUVECs tube-like structure

The production of tubular structures is another important step in angiogenesis. We investigated whether toluhydroquinone decreased the tube formation of HUVEC cells. Figure 3A shows that, all HUVECs, plated on Matrigel and incubated with standard medium. Non-treated HUVECs formed a mesh of tubes within 24 h, whereas HUVECs treated with toluhydroquinone did not. HUVECs cultures, treated with various concentrations of toluhydroquinone, formed fewer tubes and weaker anastomoses in a dose dependent manner. HUVECs treated with low concentration of toluhydroquinone (5 μM) were formed to the semblance of Non-treated. Whereas those treated with higher concentration (10-15 μM) remained short nets and length, low branching point, total tubes and loops or dotted on the Matrigel without obvious morphological changes.

(A)



(B)

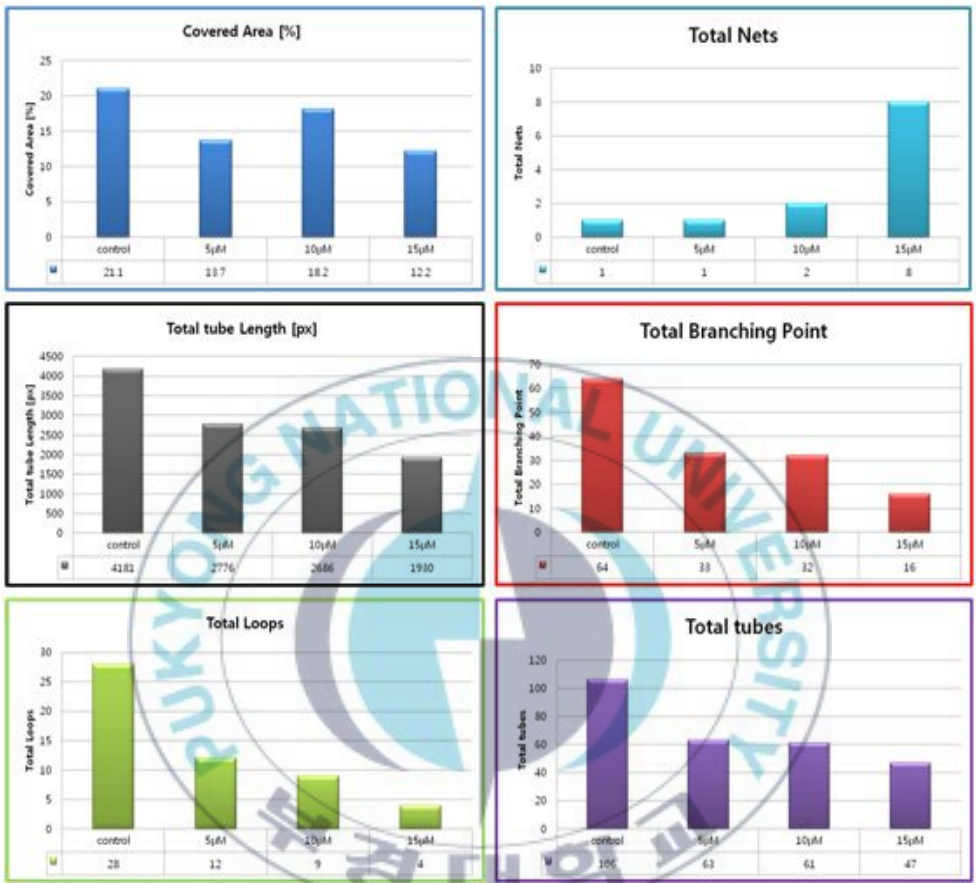


Figure 3. The effects of toluhydroquinone on tube formation of HUVECs. HUVECs were plated on the surface of Matrigel in complete media with 5, 10 and 15 μM of toluhydroquinone or without toluhydroquinone, and tube formation was evaluated 12 h later. The angiogenic stuctures was inhibited by toluhydroquinone obviously especially above concentration of 15 μM. Tube formation was evaluated by phase contrast microscopy with 100× magnification (top) and 40× magnification (bottom) (A). Quantification of covered area, total nets, total tube length, total branching point, Total loops and total tubes were performed using WIMASIS imaging analysis software and expressed in μm/field (B).

Toluhydroquinone inhibited HUVECs migration

Endothelial cell migration is important standard and the one of major processes underlying tube formation for angiogenesis, we examined the effects of touluhydroquinone on the migration of HUVECs. On HUVECs, toluhydroquinone induced a time dependent decrease in cell migration (figure 4). HUVECs migrated into the clear area after insert was detached. However, migration of HUVECs were suppressed in the present of toluhydroquinone of 5 μM and inhibited more effective than non-treated groups. This concentration is non-toxic as is evident from cell viability assay and hence the inhibitory effect could not be attributed to cytotoxic activity.

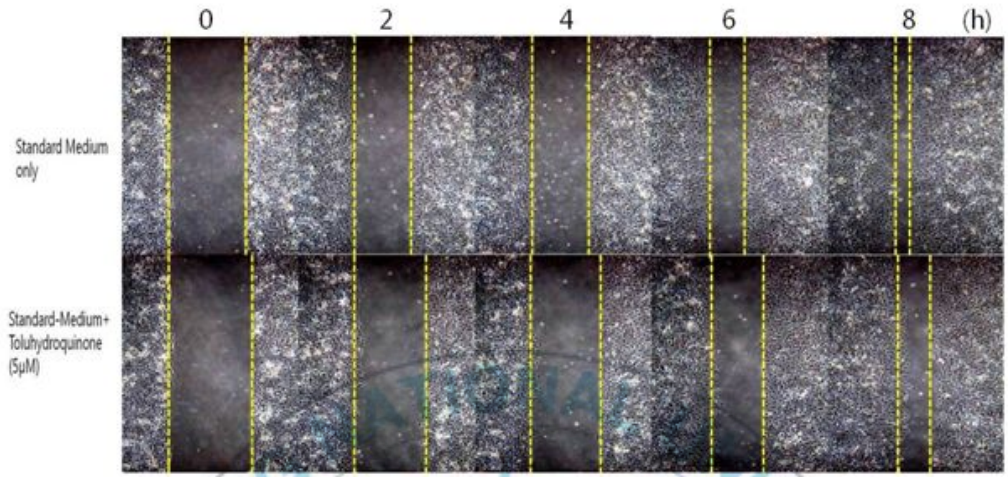


Figure 4. Migration assay. Wound assay was done to determine whether toluhydroquinone inhibits HUVECs migration. After treatment with 5 μM of toluhydroquinone HUVECs were allowed to migrate into the denuded area for 8 h. HUVECs migration were visualized by phase contrast microscope. Typical photomicrographs (final magnification, ×40) were shown in untreated in 0-8 hour and toluhydroquinone treated in 8 h at the concentration of 5 μ M.

Suppression of HUVECs invasion by toluhydroquione

To further confirm the effect of toluhydroquinone on cell invasion, we examined the invasion of HUVECs through Matrigel-coated transwell inserts was tested. As shown in figure 5. The invasion ability of HUVEC cells were significantly different from that of the non-treated group. After the treatment of the cells with 5, 10, 15, and 20 μM toluhydroquinone for 6 h, the number of invading cells decreased as the concentration of toluhydroquinone increased. The average number of invading HUVEC cells decreased to 80.63 ± 2.20%, 66.67 ± 1.64%, 54.29 ± 1.22% and 39.05 ± 1.22% after the addition of 5, 10, 15 and 20 μM toluhydroquinone, respectively, compared to the non-treated group (Figure 5F). Statistical analysis revealed that invasion of HUVEC was significantly suppressed by toluhydroquinone (***$p < 0.0001$). Especially, high concentration of toluhydroquinone (20 μM) strongly inhibited HUVECs invasion without cytotoxicity. Because no significant difference in cell viability rates was observed between non-treated and toluhydroquinone treated cells (figure 1.).

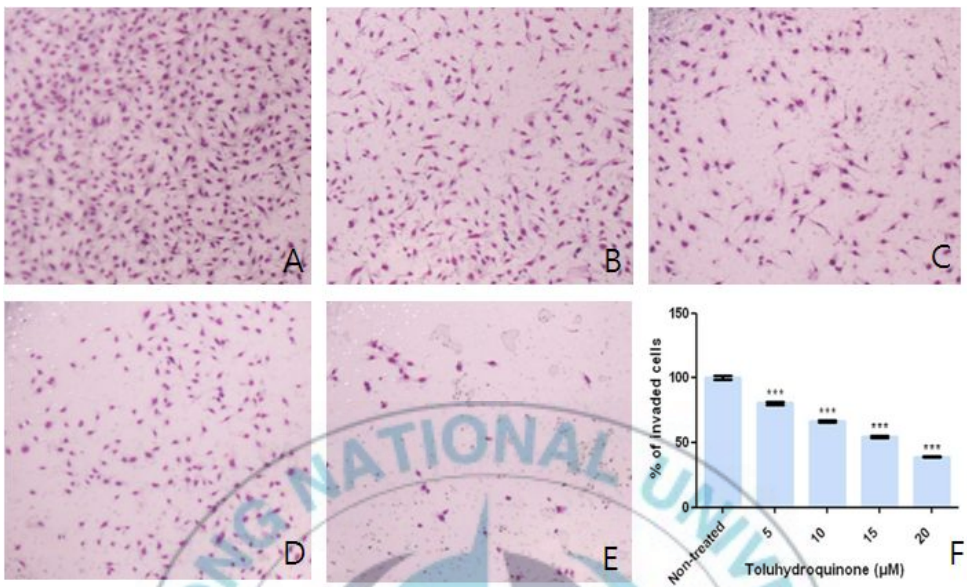


Figure 5. The shown photographs are representative data of invasion assay of HUVECs. Cells on the lower face of the membrane were confirmed by the method described under materials and method. The number of invading HUVEC cells were compared with the non-treated (control). The number of invading cells decreased as the concentration of toluhydroquinone increased. Statistically significant differences were observed between the toluhydroquinone treated samples and the non-treated (control) (***$p < 0.0001$). A. Control; B. toluhydroquinone 5 μM; C. toluhydroquinone 10 μM; D. toluhydroquinone 15 μM; E. toluhydroquinone 20 μM; F. statistical results.

DISCUSSION

Chemotherapeutic agents do not specifically target tumor cells, but rather interfere with cell division or inhibit enzymes involved DNA replication or metabolism. These drugs therefore also damage the normal dividing cells of rapidly regenerating tissues. Cancer chemotherapy is limited by a lack of specificity, resulting in damage not only cancer cells but also normal cells. Considering the side effects associated with traditional chemotherapies and the possibility of interrupting a tumor's supply of oxygen and nutrient, there has been great interest in the targeting of tumor vasculature and much effort has been directed towards the development of anti-angiogenic agents that could disrupt this angiogenesis [16]. Tumor induced angiogenesis, the formation of neovessels from preexisting ones, is critical for supporting tumor growth and progression not only by providing the necessary blood supply but also by allowing metastatic cells into the circulation [17]. Angiogenesis is an exciting target for novel anticancer therapies because of the many advantaged that it may offer, including accessibility to tumors, independence of tumor cell resistance mechanisms and broad applicability to many tumor types [18]. The potential use of natural and synthetic angiogenesis inhibitors is currently being studied intensively by many laboratories [19, 20]. Inhibitors of angiogenesis block any of the steps in the angiogenic cascade, including attachment of endothelial cells to the extracellular matrix proteins, migration and invasion through the matrix, which is required for the capillary sprouting and morphogenesis in a thin tube meshwork and differentiation and stabilization [21-23].

In this study, the cell toxicity, tube-like structure formation, cell migration, and invasion were the main points of investigation. Nontoxic doses of toluhydroquinone that did not affect cell viability significantly (figure 2). The assay of tube formation on Matrigel mimics the final events during angiogenesis when endothelial cells become organized in a three-dimentional network of capillaries [24]. However, after exposed to various concentration of toluhydroquinone (0-15 μM) for 24 h, HUVEC cells inhibited tube

formation in a dose-dependent manner on Matrigel (figure 3). Endothelial cell migration is an important component of angiogenesis for the new vasculature. After being exposed to 5 μM of toluhydroquinone for 8 h, we determined that toluhydroquinone significantly reduced HUVECs migration. These findings suggest that inhitibion of cell migration could contribute partially to endothelial tube formation inhibition induced by toluhydroquinone treatment (figure 4). Recent studies suggest that angiogenesis and invasion cooperate in tumour development and involve similar biological mechanisms [25]. Our study showed that toluhydroquinone at a several concentration did not cause any cell death in HUVECs, suggesting that the toluhydroquinone induced HUVECs invasion inhibition (figure 5). In conclusion, this study has provided evidence of anti-angiogenesis activities of toluhydroquinone *in vitro*. The anti-angiogenic activity may be a novel mechanism that contributes to the cancer chemopreventive activity of toluhydroquinone. Further studies are necessary to elucidate the mechanisms of anti-angiogenic action of toluhydroquinone.

해양 조류 *Hypnea saidana*로 부터 분리된 *Phoma herbarum* 2차대사산물인 toluhydroquinone의 신생혈관형성 억제 효과

정현일

부경대학교 대학원 미생물학과

요약

최근에 해양생물 유래 생리활성 물질의 다양성이 보고되고 있으며 그 중에 해양 미생물로부터 유래한 물질인 2차 대사산물에 대해서 지속적으로 보고되어지고 있다. 특히 2차 대사산물은 특이한 분자구조를 가지고 다양한 생리활성을 가지고 있어 각종 질병에 대한 화학 치료제로써 개발되어 지고 있다. 그 중 해양조류인 *Hypnea saidana*의 공생미생물인 *Phoma herbarum* (Class: *Dothideomycete sp.*)의 2차 대사산물 Toluhydroquinone (2-methylbenzene-1,4-diol)을 이용하여 연구를 수행하였으며, 최근까지 알려진 toluhydroquinone의 생리활성에 대해서는 항산화 활성 외에는 알려진 바가 없다. 따라서 본 연구에서는 *in vitro* 상에서 toluhydroquionone의 신생혈관형성 억제 효과에 대해 연구를 진행하였다. 신생혈관형성은 암의 전이와 성장에서 결정적 단계로 작용하게 된다. 암 (tumor)은 크기가 커지게 되면 산소와 영양분이 부족하게 되고, 이에 암 (tumor)은 VEGF와 같은 growth factor를 분비함으로써 endothelial cell의 신생혈관 형성을 유도하게 되고, 결과적으로 angiogenesis를 통하여 영양분을 획득하여 증식 및 metastasis를 유도하게 된다. 이에 본 연구에서는 toluhydroquinone을 이용하여 human umbilical vein endothelial 세포인 HUVEC에서 어떠한 활성을 나타내는지 확인하였다.

그 결과 toluhydroquinone은 HUVEC 세포의 생존율에는 영향을 미치지 않으면서 혈관 신생 과정이 진행되는데 중요한 단계인 invasion과 migration 활성억제, 그리고 tube like structure 형성을 toluhydroquinone 농도 의존적으로 감소시키는 것을 확인 할 수 있었다. 이러한 결과로부터 toluhydroquinone은 HUVEC 세포의 신생혈관형성을 위한 behaviour을 억제하고 결론적으로 암의 성장 및 전이를 억제 시킬 수 있는 새로운 물질로서 가능성을 가지고 있음을 확인 할 수 있었다.

Acknowledgement

어느 덧 2년이라는 시간이 흘러 석사과정의 종지부를 찍으려 합니다. 힘들고 어려웠던 시간도 있었지만 즐거움과 감사한 것이 더 많았던 대학원 생활은 저의 인생에서 가장 중요하고 소중한 시간으로 기억 될 것입니다. 본 논문이 완성되기 까지 언제나 옆에서 용기와 힘을 주신 분들 덕분에 무사히 마칠 수 있었기에 고마운 마음을 전하고자 합니다.

먼저 언제나 저를 믿어주시고 아낌없는 조언과 많은 가르침을 주신 김군도 교수님께 감사의 말씀을 드립니다. 그리고 바쁘신 와중에도 논문수정을 위해 많은 도움을 주신 최태진 교수님, 언제나 어머니가 자식 걱정하듯 학생들의 앞날을 위해 좋은 말씀해주신 이명숙 교수님, 항상 격려해주시고 “정도령“ 이라고 웃으시며 불러주시던 이훈구 교수님, 따뜻한 관심과 많은 가르침을 주셨던 김영태 교수님, 송영환 교수님, 저에게 많은 조언으로 격려해주신 김경호 교수님께 무한한 감사의 말씀 올립니다.

바쁘신 와중에도 저의 건강을 많이 챙겨주시던 성영애 선생님, 힘든 타지 생활을 하면서도 항상 웃음을 잃지 않고 저를 반겨주던 kasin형, 멀리 계시지만 조언과 격려를 아낌없이 베풀어주신 영원한 방장 철우 행님, 많은 시간을 함께 실험실 생활을 하며 지내왔지만 평소에 고마움과 미안함을 전하지 못한 진수에게 진심으로 감사의 말을 전합니다. 2년 동안 같이 고생하며 석사 생활을 한 절대 매력남 상보에게 수고했다는 말을 전합니다. 그리고 나의 영원한 동반자이자 활력소 조미에게 모든 걸 떠넘기고 가게 되서 정말 미안하고 정신적, 육체적으로 힘이 돼줘서 너무 고맙다는 말 전합니다. 멀리서도 항상 내가 옆에 있는 것 같다며 쪽 웃음을 선사 해준 든든한 지원자 뭠쥐, 뒤 돌아 보면 이마를 까고 나의 장난을 받아주던 성자, 조용히 자기 할 일을 다 하면서도 궂은일을 마다하지 않는 순진, 항상 웃는 얼굴로 사람을 즐겁게 만들어 주는 은수, 꿈을 위해 한시도 쉬지 않고 노력하는 나의 술 벗 우석, 웃을 때 어깨 연기하는 덕현, 술 꼴통 난희, 초원 힘든 일도 많았을 텐데 불평 없이 많이 도와줘서 항상 미안하고 고맙습니다. 오바쟁이 사자후 coy, 묵묵히 자기 위치에서 최선을 다하는 지웅이, 창원이, 민석이, 민재, 재미없는 애들이지만 볼수록 상쾌해지는 동화, 보은, 지현이 그리고 지금까지 세포신호전달 실험실에서 함께 동고동락했던 많은 동기, 후배님들 모두 저에게 있어 평생 잊을 수 없는 소중한 인연들이며 앞으로 모두 좋은 결과 있으시길 바랍니다.

어느 대학원생들 보다 화목했던 10학번 미생물학과 동기 현수, 아름 상보에게 감사의 말씀 드리며 각자의 길에서 최선을 다하고 있는 나의 03학번 동기들 도형, 세원, 정훈, 윤배, 명석, 진영, 현수, 대근, 혁, 태혁, 그리고 키 큰 동생 민재, 한번씩 부산에 찾아와 나의 기분을 전환시켜줬던 사각멤버, 화롯가 추억을 선사해준 옥동기, 종시기에게도 감사의 마음을 전합니다.

마지막으로 저를 아껴주시고 걱정해주시는 모든 분들께 감사드리며, 아들을 믿고 옆에서 항상 응원해 주신 부모님, 누나에게 이 조그만 결실을 바칩니다.

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