Thesis for the Degree of Master of Science

Effect of stigmasterol and 5β-hydroxysitostanol isolated from microalgae *Navicula incerta* on apoptosis in human hepatoma HepG2 cells

by

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미세조류 *Navicula incerta* 로부터 분리된 stigmasterol 및 5β-hydroxysitostanol 의 인간 간암 HepG2 세포에서의 세포사멸 효과

Advisor: Prof. Se-Kwon Kim

by

Young-Sang Kim

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Abstract

Marine microalgae are the primary producers of the marine ecosystem and they produce large array of metabolic products. Therefore, marine microalgae is used in different fields of application including live stock feed, oceanic life raising feed, cosmetic ingredient and nutraceutical. Besides, recently many ongoing research have focused on producing biomass fuel using microalgae. However, there are less reports in use of microalgae on isolating active metabolites. Therefore, this study has focused on investigating the apoptosis inductive effects of active compounds isolate from marine microalgae *Navicula incerta* extracts. Stigmasterol and 5β-hydroxysitostanol, belonging to the sterol group were isolated from the methanol : dichloromethane (1:1) extract of *Navicula incerta*. There are several reports on anti-cancer effects of plant sterols on breast cancer and prostate cancer, but no reports on liver cancer. Thus, we investigated the apoptosis inductive effects of stigmasterol and 5β-hydroxysitostanol on hepatocarcinoma cells HepG2. According to the results obtained stigmasterol induce the apoptosis on HepG2 cell via intrinsic pathway by down regulating Bcl-2 and extrinsic pathway by upregulating caspase-8, 9 and FASL. On the other hand, the apoptosis inductive effects of 5β-hydroxysitostanol was not prominently seen. In conclusion, we can conclude that the stigmasterol isolated from marine microalgae shows potent apoptosis inductive effects and could be developed as a anti-cancer therapeutic against liver cancer.

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List of Abbreviations

Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
Caspase	Cysteine-dependent aspartate-directed proteases
DIP/MS	Direct insert probe mass spectroscopy
DMEM	Dulbecco's Modified Eagle Medium
EI/MS	Electron ionization mass spectroscopy
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GC/MS	Gas chromatography/mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear Magnetic resonance
PBS	Phosphate buffered saline
PI	Propidium iodide
RT-PCR	Reverse transcription polymerase chain reaction
TBS-T	Tris-buffered saline-Tween 20
TLC	Thin layer chromatography
XIAP	X-linked inhibitor of apoptosis protein

1. Introduction

The first use of microalgae by humans dates back 2000 years to the Chinese, who used *Nostoc* to survive during famine. However, microalgal biotechnology only really began to develop in the middle of the last century. There are numerous commercial applications of microalgae are available. Microalgae are potential source of single-cell protein, a means of sequestering carbon dioxide from stack gas, and can be used in effluent purification and for producing biofuels. Owing to their diverse chemical properties (carotenoids, polyunsaturated fatty acids, and phycocolloids), they can use as human and animal nutritional supplement or represent a source of natural food colorants (Spolaore et al., 2006). It is reported that pure molecules have been isolated from microalgae (Spolaore et al., 2006). Valuable products like fatty acids, pigments and stable isotope biochemicals were isolated from marine microalgae in previous studies (Borowitzka, M. A. et al., 1995; Metting, F. B. et al., 1996). Further, microalgae are ideal source of stable isotopically labeled compounds.

As photoautotrophs microalgae, their simple growth requirements make them attractive for bioprocesses aimed at producing high added-value compounds that are in large demand by the pharmaceutical industry. Microalgae also rich in pigments like chlorophyll (0.5% to 1% of dry weight), carotenoids (0.1% to 0.2% of dry weight on average and up to 14% of dry weight for β-carotene of *Dunaliella*) and

phycobiliproteins. Thus, their composition gives microalgae interesting qualities, which can be applied in human and animal nutrition.

Benthic diatom *Navicula incerta* is the major component of phytoplankton and also relatively easy to cultivate. The protein fraction of *Navicula incerta* have been studied for its anti-oxidant activity and alcohol-induced liver protection on the HepG2 cells (Kang et al., 2011). However there are no detailed repots on bioactive compounds isolation from *Navicula incerta*. Therefore in this study we have purified and isolated anti-cancer compounds from *Navicula incerta*.

All sorts of cancers show the highest incidence rates among the Korean mortality every year (Fig. 1). Among the cancer types, the liver cancer is second major cause of Korean cancer death rate in 2005. The prevention of liver cancer therefore represents one of the most important aspects of any cancer control strategy around the world. Cancer can be treated by surgery, chemotheraphy, radiation theraphy, immunotherapy and monoclonal antibody therapy. Unlike surgery, most of the cancer therapy mothod seize upon inhibition of cancer cells growing. However cancer cells undergoing apoptosis play an active role in their own death, so that apoptosis can be referred to as cell suicide (Hetz et al., 2005). Therefore, cell death via induction of apoptosis in cancer cells in considered as one of the cancer preventive and therapeutic strategies. The death of the cell is classified into the necrosis and apoptosis. And this is classified by the morphological and the

biochemical characters of the cell. The necrosis is the physiological and death of the cell by the chemical trauma. In addition, the necrosis occurs by DNA damage. By removing the cell having the thus harmful mutation it seems to act on the multicellular creature. And advantageously it is made under the control of the various genes (Searle et al., 1982, Lieberthal et al., 1996 and Zimmermann et al., 2001).

Though, in the apoptosis, the cytoplasm and chromatin condensation, cell membrane blebbing phenomenon and DNA fragmentation, and etc. are accompanied. This phenomenon is controlled by the exquisite signal transmission of the pars intracellularis (Schulze-Osthoff et al., 1998). It is reported that role of the suppresses the apoptosis mechanism among the gene concerned in the apoptosis induction Bcl-2 and Bax promoting the apoptosis mechanism is the most important (Chiarugi et al., 1994., Jurgensmeier et al., 1998 and Antonsson et al.,2000). These control the activity of many factors which directly concerned in the apoptosis induction through the change, that is the quantitative.

In this study, bioactive compounds were isolated from *Navicula incerta* and their anticancer activity was evaluated in HepG2 cells. In present study, the steam volatile metabolites of the marine microalgae *Navicula incerta*, grown in sea water culture were isolated and analyzed using GC and DIP mass spectrometry to identify their components. Moreover, the apoptosis effect of stigmasterol and 5β-hydroxysitostanol on human hepatocarcinoma HepG2 cell line via intrinsic pathway and extrinsic

pathway was evaluated.

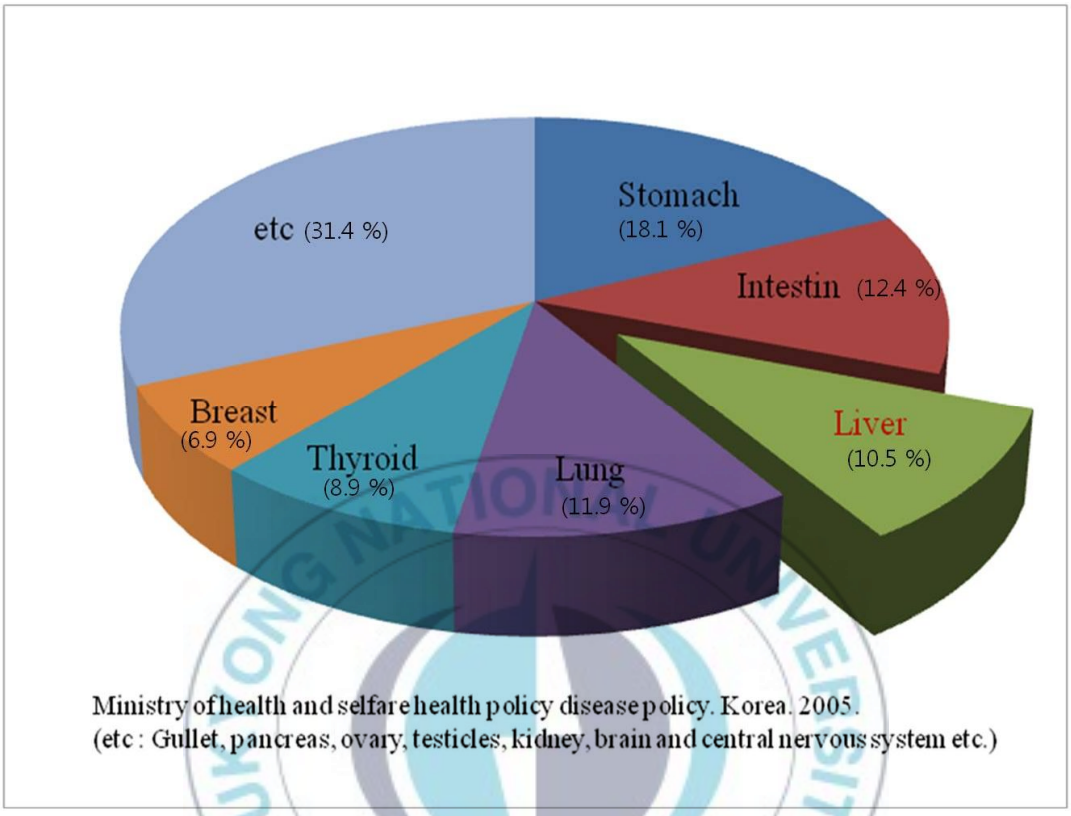


Figure 1. Cancer occurence rates among the Korea mortality in 2005 year.

2. Material and methods

2.1. Materials and chemicals

The benthic diatom, *Navicula incerta* (strain KMMCC B-001) used in this study was generously provided by Korea Marine Microalgae Culture Center (Busan, Korea). Methanol, dichloromethane, chloroform, acetone, hexane and ethyl acetate (95%, SK chemicals) were used as extract solvents in this study. For NMR spectroscopy (^{1}H 400MHz, ^{13}C 100MHz, JEOL JNM-ECP 400 NMR spectrometer), chloroform-D (Cambridge Isotope Laboratories, Inc., USA) was used for dissolved compounds. Open column chromatography was performed to isolate compounds with silica gel (silica gel 60, 0.062-0.200 mm, 70-230 mesh ASTM). Analytical TLC plates (TLC silica gel 60 $F_{254,}$ 20 x 20 cm) were purchased from Merck (Frankfurt, German). All chemicals used in this study were in ananlytical grade. Human liver hepatocellular carcinoma cell line (HepG2) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as monolayer in 10 cm^2 dish. Cell culture media Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS) were purchased from Gibco BRL, Life Technology (NY, USA). (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) reagent was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary and secondry antibodies used for Western blot analysis were purchased from Santa Cruz

Biotechnology Inc. (Santa Cruz, CA, USA) and Amercham Pharmacia Biosciences (Piscataway, NJ, USA). Other chemicals and reagents used of analytical grade commercially available.

2.2 Preparation of *Navicula incerta*

The bentic diatom, *Navicula incerta* (strain KMMCC B-001) used in this experiment was generously provided by Korea Marine Microalgae Culture Center. *Navicula incerta* culture was maintained in standard F/2 (Guillard's) medium. Culture conditions of Navicula incerta was in 20°C, 33 ‰, pH 8.3 ± 0.5, 12:12 L:D, and 250 μmol $m^{-2}·s^{-1}$, respectively. A 50 g of lyophilized *Navicula incerta* (Fig. 2) was percolated in methanol (Fraction 1), methanol and dichloromethane (1:1 ratio, Fraction 2), and dichloromethane (Fraction 3). *Navicula incerta* was used 50 g.

First, 50 g of *Navicula incerta* was percolated for 24 h in the room temperature with 3 L methanol. After that, methanol extract was filtered (filter paper No.20, size 300 mm, pore size 5 μm, Hyundai Micro co. Ltd.)(Fr. 1). Later, 3 L of methanol : dichloromethane (1:1) was added, and filtered (Fr. 2). Finally, 3 L of dichloromethane was added and filtered (Fr. 3). All fractions were evaporated in a rotary evaporator to prepare a solid state powder. In this experiment, Fr. 1 was around 20 g, fraction 2 was 20 g, Fr. 3 was around 3 g. Later, Fr. 2 was selected for isolation of compounds.

Chloroform : methanol (10:1 ratio) was added Fr. 2 for column chromatography as a sample.

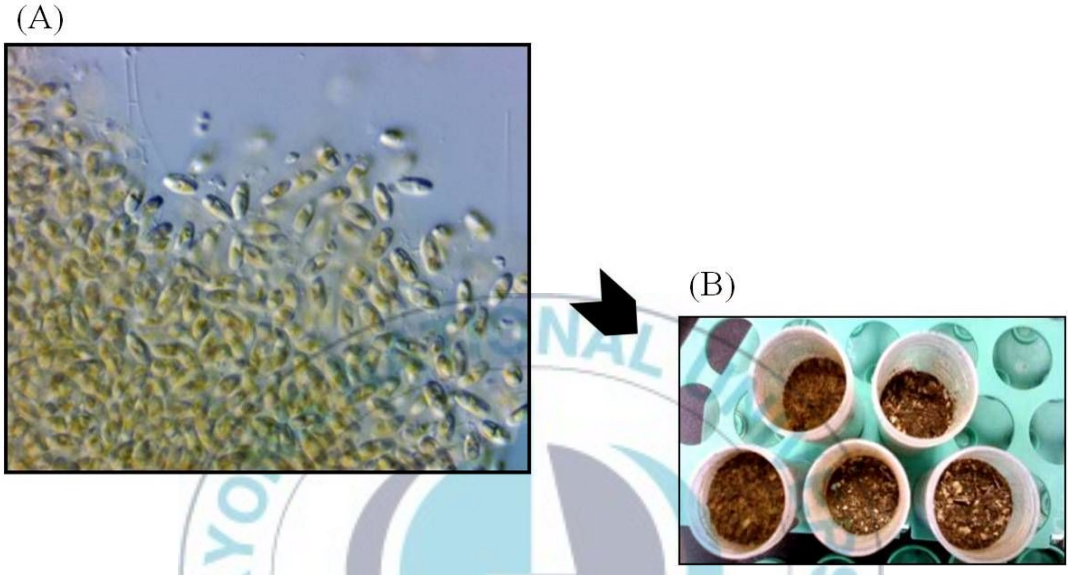


Figure 2. Morpology of cultured (A) and freeze-dried *Navicula incerta* (B)

2.3 Isolation of stigmasterol and 5β-hydroxysitostanol

Methanol : dichloromethane (1:1) extracted (Fr. 2) *Navicula incerta* (20.313 g) was separated by silica gel column and eluted by gradient solution with increasing polarity. Methanol is widely used to isolated compounds from extracellular systems and dichloromethane is widely used to isolated compounds from intracellular systems (kim et al., 2010). Dichloromethane : acetone (30:1 to 1:2) and acetone was used to give 11 fractions. First, chloroform : acetone, 30:1 ratio was used 3 L in 100 mL erlenmeyer flask, second, chloroform : acetone, 15:1 ratio was used in 100 ml erlenmeyer flask. This solvent gradient was changed to 1:2 ratio, and last, only acetone was used as extract solvent. Total 110 erlenmeyer flasks were used to getting extract solution. Fr. 2.1 (221.3 mg), Fr. 2.2 (303.2 mg), Fr. 2.3, Fr. 2.4 (Fr. 2.3/4, 256.9 mg), Fr. 2.5 (726 mg), Fr. 2.6, Fr. 2.7 (Fr 2.6/7, 244.2 mg), Fr. 2.8 (285 mg), Fr. 2.9, Fr. 2.10 (Fr 2.9/10, 397.5 mg), Fr. 2.11 (878.5 mg) (Fig. 3). Fr. 2.3/4 and Fr. 2.5. were selected for isolate compounds because we target kinds of steroid compound which cannot detected under UV light (Fig. 4). Fraction 2.3/4 was selected for further. For isolation this fraction, solvent gradient, hexane : ethyl acetate was used at 10:1 ratio. Total volume was 770 mL and total 54 Erlenmeyer flasks were used. Fraction 2.5 was selected for further isolation. For this fraction, solvent gradient was hexane : ethyl acetate was changed 10:1 to 5:1. Total volume of solvent was 1,030 mL, and total 55 erlenmeyer flaks were used.

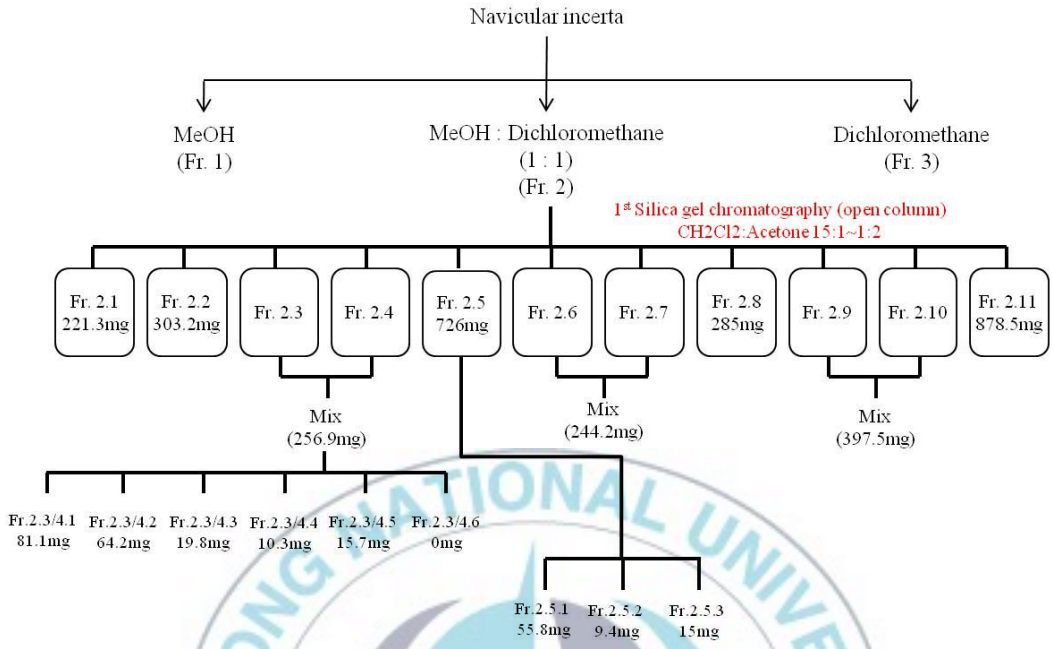


Figure 3. Isolation of Stigmasta-5,22-dien-3-ol and Stigmasta-3,5-diol from *Navicula incerta* extract

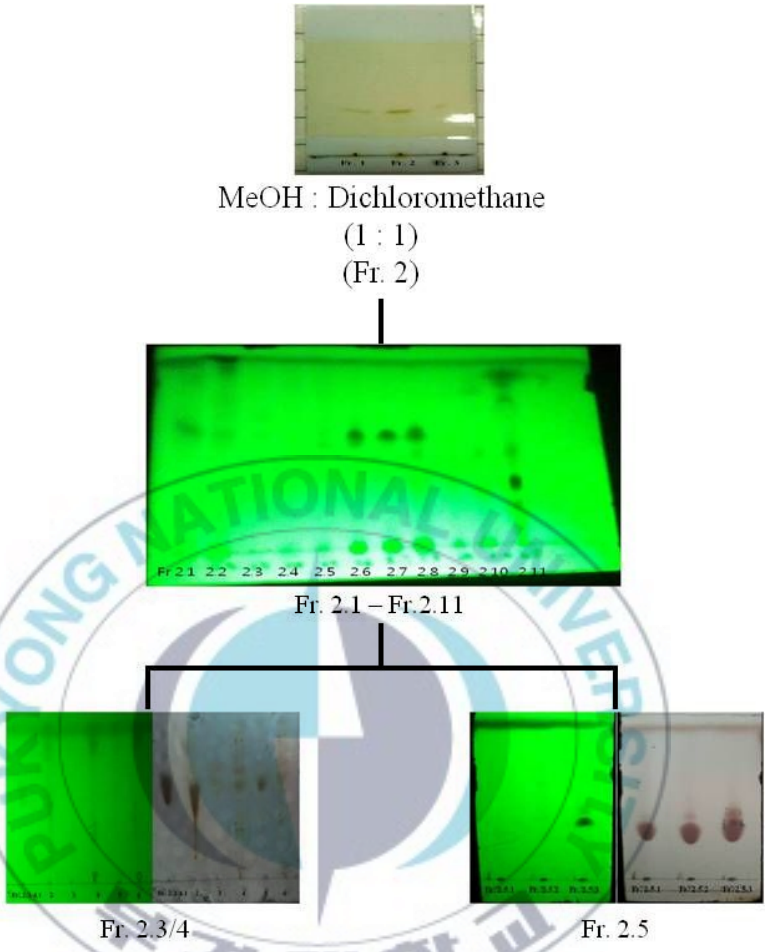


Figure 4. Thin layer chromatography (TLC) for the separation of compounds from *N. incerta*. Three fractions were used for first separate

.

2.4. Cell culture

HepG2 cell line was cultured in 10 cm^2 dish and 6 cm^2 dish, 12, 96 well flat-bottom transparent plates and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 μg streptomycin/penicillin per mL at 37°C in huminified atmosphere of 5% CO_2. Cells were subcultured by detaching with trypsin-EDTA solution 1-2 times every week at about 70-80% confluency.

2.5. Cell viability assay

Cytotoxic levels of the enzymatically hydrolysis cultured cells were measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Denizot and Lang, 1986).

The HepG2 cells were grown in 96-well plates at a density of 2 x 10^4 cells/well. After 24 h, cells were washed with fresh medium and treated with different concentrations of stigmasterol and 5β-hydroxysitostanol. After incubation for 24 h, the cells were rewashed and incubated with 100 μL of MTT (1 mg/mL) for 4 h at 37°C. Finally, a 100 μL of DMSO was added to solubilize the formed formazan crystals and the amount of formazan crystal was determined by measuring the absorbance at 550 nm using a multidetection microplate reader (GENios® microplate reader, Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan crystal. Viability of cells was quantified as a percentage compared to the control and dose response curves were developed.

2.6. Observation of morphological changes of HepG2 cells

Cells were grown in 12-well plates at a density of $2x10^4$ cells/well. After overnight incubation, cells were treated with different concentrations of stigmasterol and 5β-hydroxysitostanol that were prepared by dissolving them in cell culture medium and incubated for about 24 h. Following, medium was discarded and cells were washed with PBS. After washing, cells were fixated with 4% (v/v) formaldehyde solution in PBS for 1 h at room temperature. The fixed cells were washed with PBS and their morphological changes were detected by a light microscope (CTR 6000; Leica, Wetzlar, Germany).

2.7. Hoechst 33342 cell staining

HepG2 cells were grown in 12-well plates at a density of $2x10^4$ cells/well. After overnight incubation, cells were treated with different concentrations of stigmasterol and 5β-hydroxysitostanol that were prepared by dissolving them in cell culture medium and incubated for about 24 h. Following, medium was discarded and cells were washed with PBS. After washing, cells were fixated with 4% formaldehyde in PBS for 1 h at room temperature. The fixed cells were washed with PBS and cells were stained with 1 μg/mL of th fluorescent DNA-binding dye, Bisbenzimide Hoechst 33342 (Sigma-Aldrich Corp., St. Louis, MO, USA) and incubated for 1 h at

room temperature to reveal nuclear condensation/aggregation. The Hoechst 33342-stained cells were visualized and photographed under fluorescence microscope ((CTR 6000; Leica, Wetzlar, Germany).

2.8. Flow cytometry analysis for measurement of cell cycle arrest

The cells were harvested and washed once with PBS, fixed in ice-cold 70% ethanol and stored at 4°C. Prior to analysis, the cells were washed once again with PBS, suspended in 1 mL of a cold propidium iodide (PI, Sigma) solution containing 50 µg/mL RNase A, 250 µg/mL PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 20 min in the dark. Flow cytometric analyzes were carried out using a flow cytometer (FACS Calibur, BD Sciences, Heidelberg, Germany) and CellQuest software was used to determine the relative DNA content based on the presence of a red fluorescence.

2.9. RNA extraction

Total RNA was extracted from HepG2 cells for 24 h treated with and without stigmaterrol and 5β-hydroxysitostanol. Cells in 6 cm^2 dishes were lyzed with 100 µL of TRIzol® reagent for each dish and the lysate was passed through a pipette several times. Cell lysates were transferred to microtubes and incubated 2 min at room temperature. Incubation was followed by adding 200 µL of chloroform to each tube

and vortexing. Microtubes were centrifuged at 12,000 xg for 15 min at 4°C. after centrifugation, colorless supernatant phase was transferred to a new microtube carefully without mixing with lower protein phase. The RNA in the aqueous phase was precipitated by mixing with isopropanol at the ratio of 1:1, incubation for 10 min at room temperature and centrifugation at 12,000 xg for 10 min at 4°C. supernatant was discarded and RNA pellet was washed with 1 mL of 75% ethanol, followed by centrifugation at 12,000 xg for 15 min at 4°C. Following removal of ethanol, RNA pellet was suspended in DEPC-treated water and incubation at 55°C for 10 min. Dissolved RNA pellet was kept at -20°C for further experiments. Purity of extracted RNA was determined by measuring OD of each tube at 260 nm and 280 nm using a microplate reader (GENios Tecan Austria GmbH, Austria).

2.10. Reverse-transcriptase polymerase chain reaction

RT-PCR was performed to check specific mRNA expression in differentiated cells. Two µg of total RNA was mixed DEPC-treated water to reach the total volume of 13 µL in 0.5 µL PCR microtubes. Two µL of oligo(dT) was added to this mixture and RNA was denaturated by incubation at 70°C for 5 min. after denaturation microtubes were placed on ice immediately for primer annealing. Later, RT-PCR mastermix containing chemicals was added to microtubes and RT-PCR reaction was carried out with the indicated incubation times by a thermal cycler. Resulted mixture

containing complementary DNA (cDNA) was kept at 4°C for further experiments. cDNA synthesized from RT-PCR was used as a template for normal PCR. PCR reaction mixture was prepared by mixing the chemicals and reaction was carried out with incubation conditions by a thermal cycler. Sequences of the gene specific primers used in these reactions were shown in table 1. PCR products were electrophoresed on 1.5% agarose gel and visualized under UV light after ethidium bromide staining.

Table 1. Sequences of the gene specific primers

primer	sequence	
Bax	Forward	5′-TGC-CAG-CAA-ACT-GGT-GCT-CA-3′
	Reverse	5′-GCA-CTC-CCG-CCA-CAA-AGA-TG-3′
Caspase-8	Forward	5′-CAT-CCA-GTC-ACT-TTG-CCA-GA-3′
	Reverse	5′-GCA-TCT-GTT-TCC-CCA-TGT-TT-3′
Caspase-9	Forward	5′-AAG-ACC-ATG-GCT-TTG-AGG-TG-3′
	Reverse	5′-CAG-GAA-CCG-CTC-TTC-TTG-TC-3′
p21	Forward	5′-CTG-TCA-CAG-GCG-GTT-ATG-AA-3′
	Reverse	5′-TGT-GCT-CAC-TTC-AGG-GTC-AC-3′
p53	Forward	5′-GCG-CAC-AGA-GGA-AGA-GAA-TC-3′
	Reverse	5′-CTC-TCG-GAA-CAT-CTC-GAA-GC-3′
FAS	Forward	5′-TTG-CTG-GCA-CTA-CAG-AAT-GC-3′
	Reverse	5′-AAC-AGC-CTC-AGA-GCG-ACA-AT-3′
FASL	Forward	5′-CAC-TAC-CGC-TGC-CAC-CCC-3′
	Reverse	5′-CCA-GAG-AGA-GCT-CAG-ATA-CGT-TG-3′
XIAP	Forward	5′-GAA-GAC-CCT-TGG-GAA-CAA-CA-3′
	Reverse	5′-CGC-CTT-AGC-TGC-TCT-TCA-GT-3′
Bcl-2	Forward	5′-ATA-CCT-GGG-CCA-CAA-GTG-AG-3′
	Reverse	5′-TGA-TTT-GAC-CAT-TTG-CCT-GA-3′
β-actin	Forward	5′-GCC-ACC-CAG-AAG-ACT-GTG-GAT-3′
	Reverse	5′-TGG-TCC-AGG-GTT-TCT-TAC-TCC-30′

2.11. Western Blot Analysis

Western blotting was performed according to standard procedures. Briefly, cells were cultured at a density of 1 x 10^4 cells mL^{-1} in 10 cm^2 culture dishes with serum-free medium. After incubation for 24 h, the cells were treated with different concentrations of stigmasterol and 5β-hydroxysitostanol for 24 h. Cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 0.4 % Nonidet P-40, 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 80 μg mL^{-1} leupeptin, 3 mM NaF and 1 mM DTT at 4ºC for 30 min. Total protein was extracted and 100 μg mL^{-1} of protein were separated using a 10 % SDS-polyacrylamide gel and 5% stacking gels and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech., England, UK). The membrane was blocked for 1.5 hr at 37ºC using TBS-T buffer containing 0.1% Tween-20 and 3% BSA. After washing the membrane with TBS-T twice, the blots were incubated for 1 h with suitable antibodies at 25ºC. The respective proteins were detected with a chemiluminescent ECL assay kit (Amersham Pharmacia Biosciences, NJ, USA) according to the manufacturer's instructions. The western blot bands were visualized using a LAS-3000 system and quantified by Multi Gauge V3.0 software (Fujifilm Life Science, Tokyo, Japan).

2.12. Statistical Analysis

Each value was expressed as as means ± S.E.M (n = 3). The statistical significance of differences was analyzed by ANOVA using SAS (Chicago, IL, USA).

3. Result and Discussion

3.1 Extraction of stigmasterol and 5β-hydroxysitostanol from *Navicular incerta*

Benthic diatom, *Navicula incerta* is the major component of phytoplankton and also relatively easy to cultivate. After mass culture, a lyophilized *Navicula incerta* was percolated in methanol (Fr. 1), methanol and dichloromethane (1:1 ratio, Fraction 2), and dichloromethane (Fr. 3). First, *Navicula incerta* was percolated for 24 h in the room temperature with 3 L methanol. And then filtered with filter paper (methanol extract, Fr. 1). Later, *Navicula incerta* was percolated for 24 h at the room temperature with 3 L methanol : dichloromethane (1:1 ratio) (methanol : dichloromethane 1:1 extract, Fr. 2). Last, *Navicula incerta* was percolated for 24 h at the room temperature with 3 L dichloromethane only (dichloromethane extract, Fr. 3). After that, thin layer chromatography was performed to separate compounds. As shown in figure 4A, fraction 2 has a clear spot in TLC plate. As compared with these three spots, middle of TLC plate was the thickest and clear. Therefore fraction 2 spot was easier to isolated than other fractions. Fraction 2 (20.313 g) was evaporated until dryness by rotary evaporater (Buchi, R-215, Rotavapor). Then, it was separated by silica gel column chromatography and eluted by dichloromethane : acetone (30:1 to 1:2). After that, spectroscopy studies were done using NMR and DIP/MS or GC/MS.

3.2 Purification and elucidation of stigmasterol and 5β-hydroxysitostanol

Fraction 2 (20.313 g) was fractionated by silica gel open column chromatography (dichloromethane : acetone, 30:1 to 1:2, acetone), and generated 11 fractions (Fr.1 – Fr. 11) containing active compounds. As shown in figure 3, fraction 2 was easier to isolate with organic solvent than those of other fractions. Among these fractions, Fr. 2.3/4 and Fr. 2.5 were select to be deployed in cell viability assay to evaluate the cell toxicity.

Fraction 2.3/4 (256.9 mg, from dichloromethane : acetone, 15:1 to 10:1 elution) was separated on silica gel, eluting by hexane : ethlyacetate (10 : 1) to give 5 fractions; Fr 2.3/4.1 (81.1 mg), Fr 2.3/4.2 (64.2 mg), Fr 2.3/4.3 (19.8 mg), Fr 2.3/4.4 (10.3 mg), Fr 2.3/4.5 (15.7mg). Fraction 2.3/4.1 was further isolated by preparative thin layer chromatography (PTLC) eluted by chloroform only. Recrystallization of Fr 2.3/4.1 from chloroform gave white solid (17.2 mg) of **stigmasterol (Stigmasta-5,22-dien-3-ol) (Fig.5A):** white solid; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.67-2.32 [44H, m, containing 1.01 (3H, s, CH_3), 0.81 (3H, s, CH_3)], 5.36 (1H, d, J = 5.2 Hz, H-6), 5.15 (1H, m, one of 22-H or 23-H), 5.02 (1H, m, one of 22-H or 23-H), 3.53 (1H, m, 3-H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 140.7 (C-5), 138.3 (C-23), 129.3 (C-22), 121.7 (C-6), 71.8 (C-3), 56.8 (C-14), 55.9 (C-17), 51.2 (C-24), 50.1 (C-9), 42.3 (C-13), 42.2 (C-20), 40.5 (C-4), 39.7 (C-12), 37.2 (C-10), 36.5 (C-2), 31.9 (C-8), 31.9 (C-25), 31.9 (C-1), 31.6 (C-7), 28.9 (C-15), 25.4 (C-16), 24.4 (C-28), 21.2 (C-11), 21.2 (C-18),

21.1 (C-27), 19.4 (C-19), 19.0 (C-21), 12.2 (C-26), 12.0 (C-29); LREIMS *m/z* 412 $[M]^+$ (2). $C_{29}H_{48}O$ (M.W: 412.31). ^{13}C NMR data were given in table 2.

Fraction 2.5 (726 mg, from dichloromethane : acetone, 10:1 ~ 5:1 elution) was separated on silica gel, eluting by hexane : ethlyacetate (10 : 1 ~ 5 : 1) to give 3 fractions; Fr 2.5.1 (55.8 mg), Fr 2.5.2 (9.4 mg), Fr 2.5.3 (15 mg). Recrystallization of Fr 2.5.1 from methanol : dichloromethane (1:1) extract gave white solid (55.8 mg) of **5β-hydroxysitostanol (Stigmasta-3,5-diol) (Fig. 5B):** white solid; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.67-2.32 [44H, m, containing 1.01 (3H, s, CH_3), 0.81 (3H, s, CH_3)], 5.36 (1H, d, J = 5.2 Hz, H-6), 5.15 (1H, m, one of 22-H or 23-H), 5.02 (1H, m, one of 22-H or 23-H), 3.53 (1H, m, 3-H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 75.6 (C-3), 74.2 (C-5), 56.6 (C-14), 56.1 (C-17), 55.6 (C-24), 52.9 (C-9), 46.0 (C-13), 42.5 (C-20), 40.0 (C-4), 36.6 (C-12), 36.3 (C-10), 36.2 (C-2), 34.9 (C-8), 33.9 (C-25), 32.5 (C-1), 28.9 (C-7), 28.3 (C-15), 27.2 (C-16), 26.4 (C-28), 25.3 (C-11), 24.2 (C-6), 23.0 (C-23), 20.6 (C-22), 19.6 (C-18), 19.0 (C-27), 18.8 (C-19), 14.0 (C-21), 12.3 (C-26), 12.1 (C-29); LREIMS *m/z* 414 $[M\text{-}H_2O]^+$ (1). $C_{29}H_{52}O_2$ (M.W: 432.31). ^{13}C NMR data were mentioned in table 2.

Stigmasterol is the kind of the phytosterol. It has a double bond on the side chain and consists of three cyclohexane rings and one cyclopentane ring. As to the chemical formula, the $C_{29}H_{48}O$ is distinguished from the cholesterol. 5β-hydroxysitostanol is also a kind of phytosterol, but it has no double bond on the carbon chain but consist

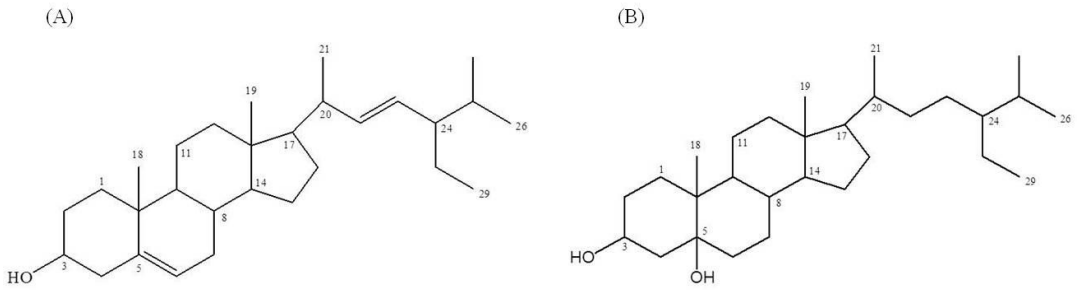
extra hydroxyl group on C-5 position. The structures of stigmasterol and 5β-hydroxysitostanol were elucidated by the comprehensive analyses of their chemical, gas chromatography mass chromatography and NMR spectroscopic data. These compounds, especially sterols, exhibit significant and multiple activities such as anti-cancer effect and cholesterol reducing effect in vascular cells.

These kinds of Phtytosterols are known to have an inductive effect of apoptosis in breast cancer, prostate cancer, human colon cancer in HT-29 cells and U937 human monocytic cells, and in this study, phytosterol was used at 4-70 μM concentrations and cell viability of those cells were around 60 % (Awad et al., 2000; Holtz et al., 1998; Raicht et al., 1980; O'Callaghan et al., 2010).

In a previous study, about sterols, it could confirm that double bond of sterol has an effect in gene expression (Trouillas et al., 2005). Following this study, it is considered that interaction occurs with mRNA and protein, and double bond of C-5, C-22 position have effect on these expression levels. Stigmasterol and 5β-hydroxysitostanol have OH group, but this group has no effect on the apoptosis progression.

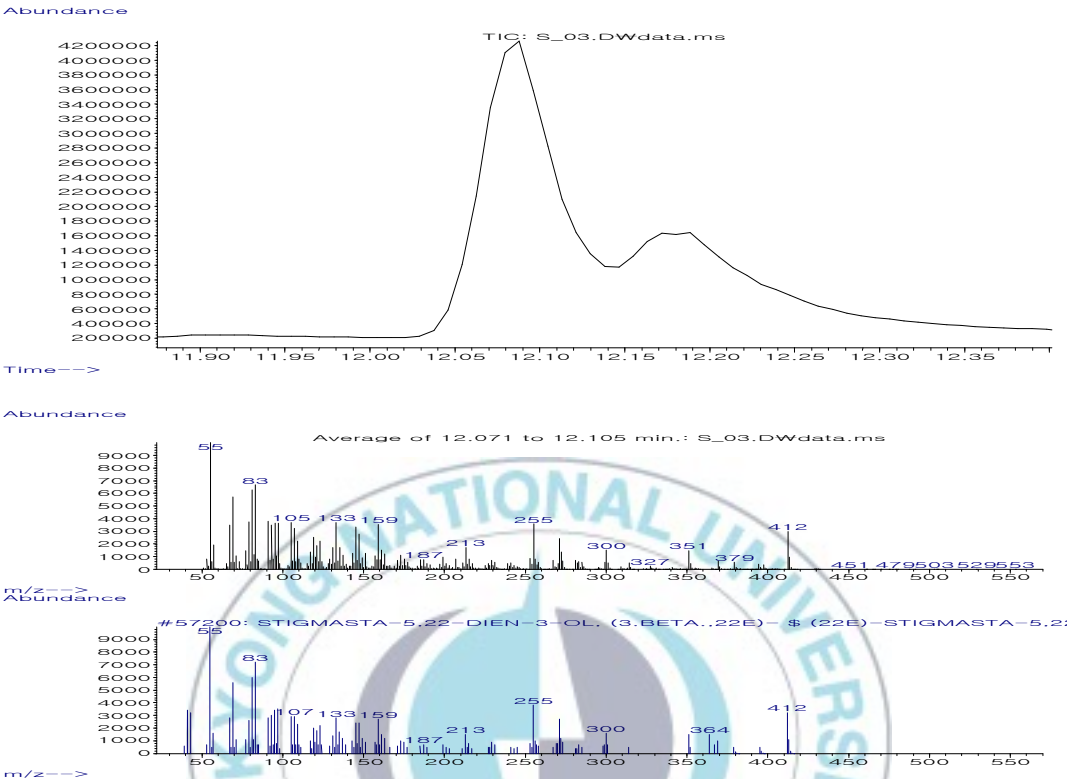
Table 2. Assignment of ^{13}C NMR data

Carbon No.	Stigmasterol	5β-hydroxysitostanol
	δ_c (multi)	δ_c (multi)
1	31.9 (CH_2)	32.5 (CH_2)
2	36.5 (CH_2)	36.2 (CH_2)
3	71.8 (CH)	75.6 (CH)
4	40.5 (CH_2)	40.0 (CH_2)
5	140.7 (C)	74.2 (C)
6	121.7 (CH)	24.2 (CH_2)
7	31.6 (CH_2)	28.9 (CH_2)
8	31.9 (CH)	34.9 (CH)
9	50.1 (CH)	52.9 (CH)
10	37.2 (C)	36.3 (C)
11	21.2 (CH_2)	25.3 (CH_2)
12	39.7 (CH_2)	36.6 (CH_2)
13	42.3 (CH)	46.0 (C)
14	56.8 (CH)	56.6 (CH)
15	28.9 (CH_2)	28.3 (CH_2)
16	25.4 (CH_2)	27.2 (CH_2)
17	55.9 (CH)	56.1 (CH)
18	21.2 CH_3	19.6 (CH_3)
19	19.4 CH_3	18.8 (CH_3)
20	42.2 (CH)	42.5 (CH)
21	19.0 (CH_3)	14.0 (CH_3)
22	129.3 (CH)	20.6 (CH_2)
23	138.3 (CH)	23.0 (CH_2)
24	51.2 (CH)	55.6 (CH)
25	31.9 (CH)	33.9 (CH)
26	12.2 (CH_3)	12.3 (CH_3)
27	21.1 (CH_3)	19.0 (CH_3)
28	24.4 (CH_2)	26.4 (CH_2)
29	12.0 (CH_3)	12.1 (CH_3)



Stigmasta-5,22-dien-3-ol **Stigmasta-3,5-diol**

Figure 5. Structures of stigmasterol (A) and 5β-hydroxysitostanol (B) isolated from *Navicula incerta* (microalgae)



Name STIGMASTA-5,22-DIEN-3-OL, (3BETA,22E)- $ (22E)-STIGMASTA-5,22-DIEN-3-OL #$ (22E)-STIGMASTA-5,22-DIEN-3-OL $(22E)-STIGMASTA-5,22-DIEN-3-OL (COMPUTER-GRENERATE NAME) $ (22E)-STIGMASTA-5,22-DIEN-3,BETA-OL $ (22E,24S)-24-ETHYLCHOLESTA-5,22-DIEN-3,BETA-O

CAS Numbe 000083-48-7

Entry Number 57200

Molecular Formula C29H48O

Misc information ORIGINALNO: 2_290167; WILEYID: 429058; QI=861, Source=EP-5554-0-0, WLN=L E5 B666 LUTJ A E FY&1U1Y2&Y OQ;, Database compilation Copyright© 2006 John Wiley & Sons, Inc. All Rights Reserved

Match Quality 98

Company ID WBN06

Retention Index 0

Melting point

Boiling Point

Molecular weight 412.00

Figure 6. EI-MS spectrum (GC-MSD, Agilent 5975C, Santa Clara, USA) of stigmasterol in $CHCl_3$

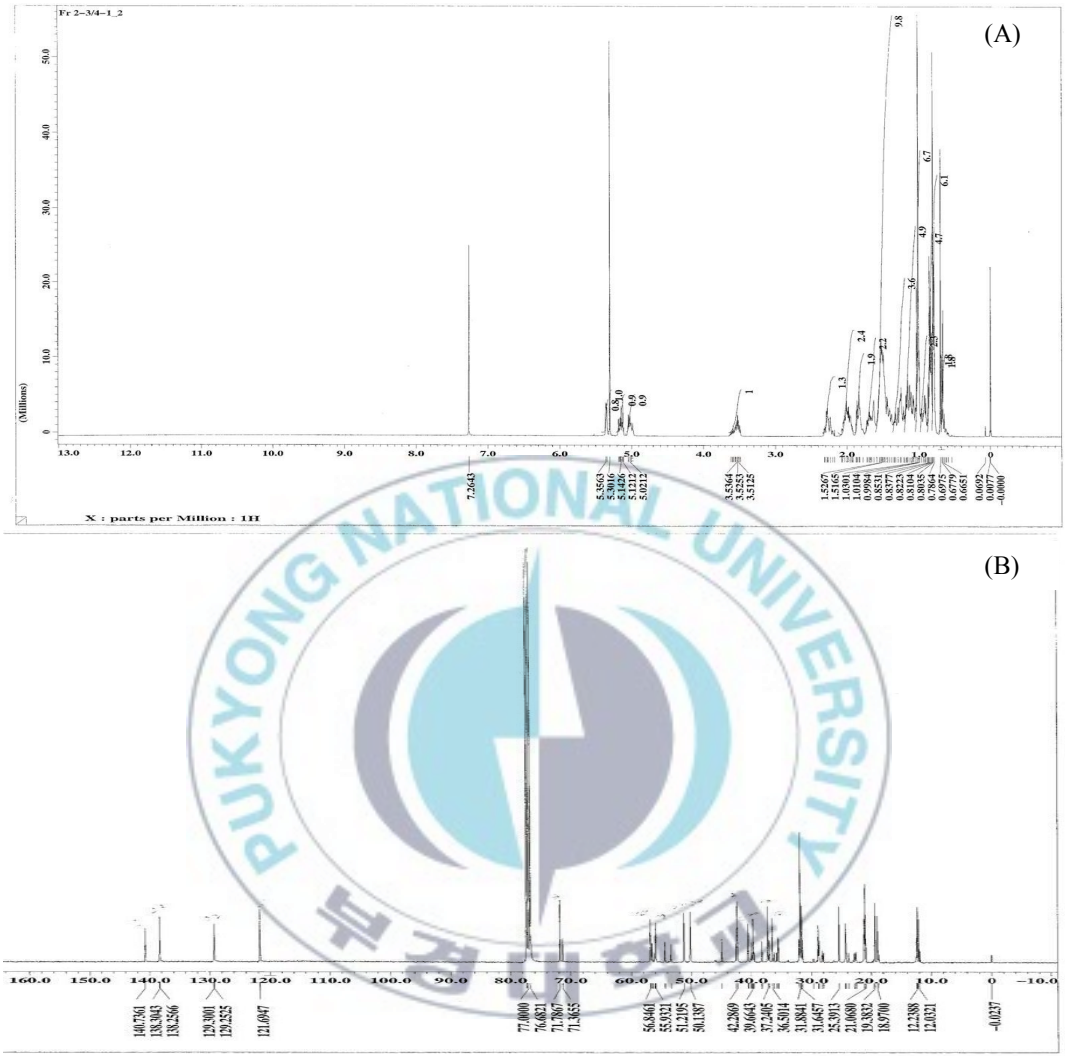


Figure 7. ^{1}H-NMR spectrum (A) and ^{13}C-NMR spectrum (B) of stigmasterol in $CDCl_3$

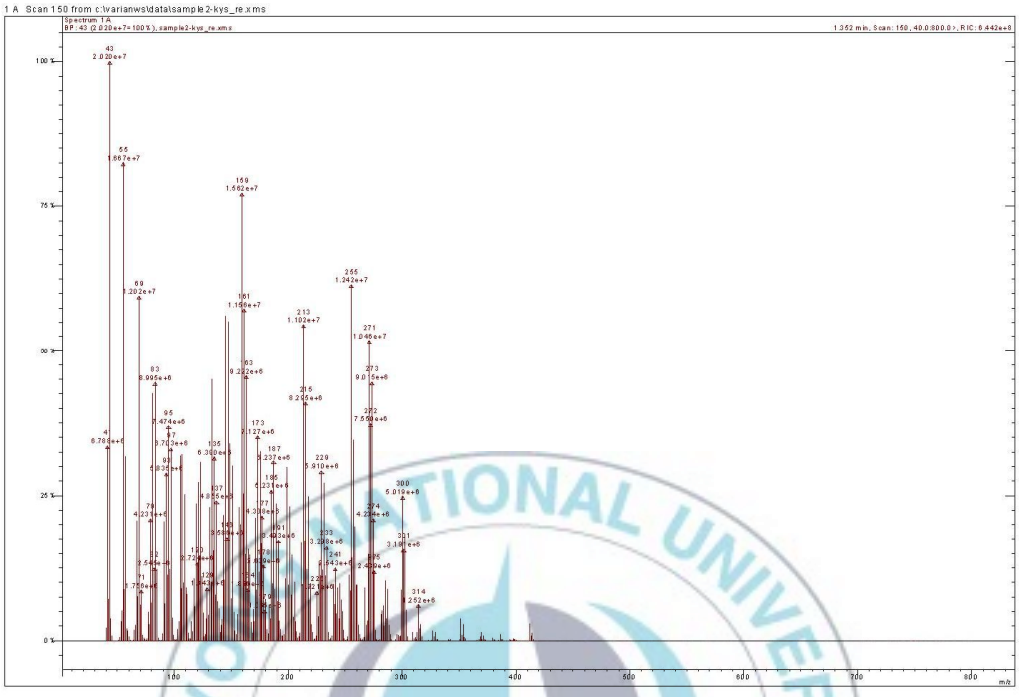


Figure 8. DIP-MS spectrum of 5β-hydroxysitostanol in $CHCl_3$

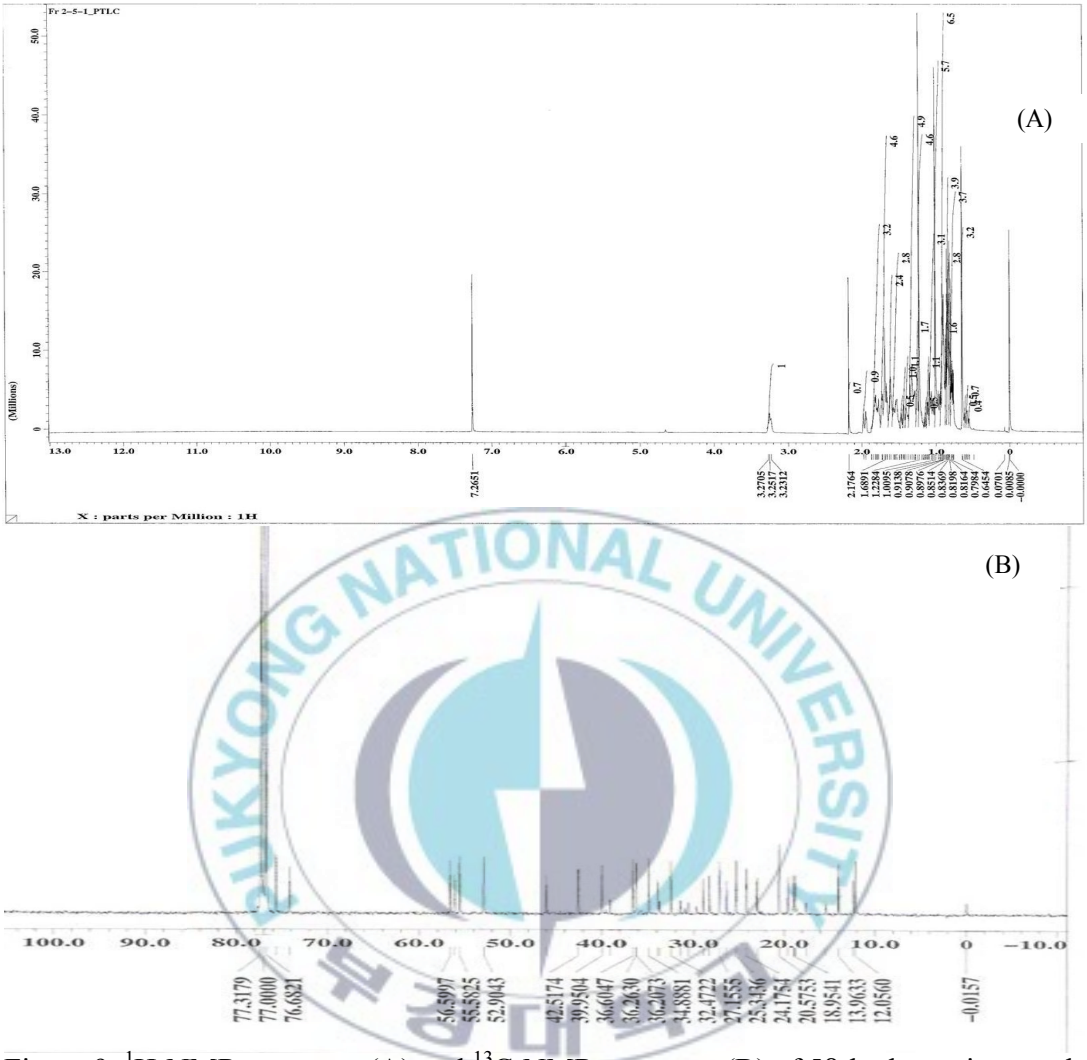


Figure 9. ^{1}H-NMR spectrum (A) and ^{13}C-NMR spectrum (B) of 5β-hydroxysitostanol in $CDCl_3$

3.3. Cell viability of stigmasterol and 5β-hydroxysitostanol

As mentioned in part 3.2, phytosterol has an inductive effect of apoptosis on some cancer cells. In this study, we evaluated whether stigmasterol and 5β-hydroxysitostanol have cytotoxicity or proliferation on the HepG2 cells.

A main application allows to assess the viability (cell counting) and the proliferation of cells. It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials. Those agents would stimulate the inhibition of cell viability and growth. Changes in metabolic activity can change MTT results while the number of viable cells are constant. Metabolically active cells are able to convert the dye to water-soluble dark blue formazan by reductive cleavage of tetrazolium ring. When the amount of purple formazan produced by cells treated with compounds compared with the amount of formazan produced by untreated control cells, the effectiveness of the compounds, can be deduced through the production of a dose-response curve.

In order to compare the effect of stigmasterol and 5β-hydroxysitostanol on cell viability, the cells were exposed to increasing concentrations of stigmasterol and 5β-hydroxysitostanol for 24 h and cell viability was examined by MTT viability assay (Figure 10). Exposure of HepG2 cells to increasing concentrations of stigmasterol and 5β-hydroxysitostanol resulted in a dose-dependent decreased in cell viability relative to control cells. Treatment with stigmasterol for 24 h inhibited the cell

viability with the rates of approximately 40, 43 and 54% at the concentrations of 5, 10 and 20 μM, respectively. Treatment with 5β-hydroxysitostanol for 24 h inhibited the cell viability with the rates of approximately 6, 9 and 15% at the concentrations of 5, 10 and 20 μM, respectively. compare to previous studies, cell viability effect of oxidized derivatives of stigmasterol is almost same with them. However in this paper, cells died at high concentrations. According to the results of MTT assay, among both samples, quiete different cell viability results were obtained. Stigmasterol has strong cytotoxicity effect a HepG2 cells, but 5β-hydroxysitostanol has no effect of cytotoxixity effect on HepG2 cells. On the other hand, stigmasterol has dose-dependent apoptosis effect on HepG2 cells.

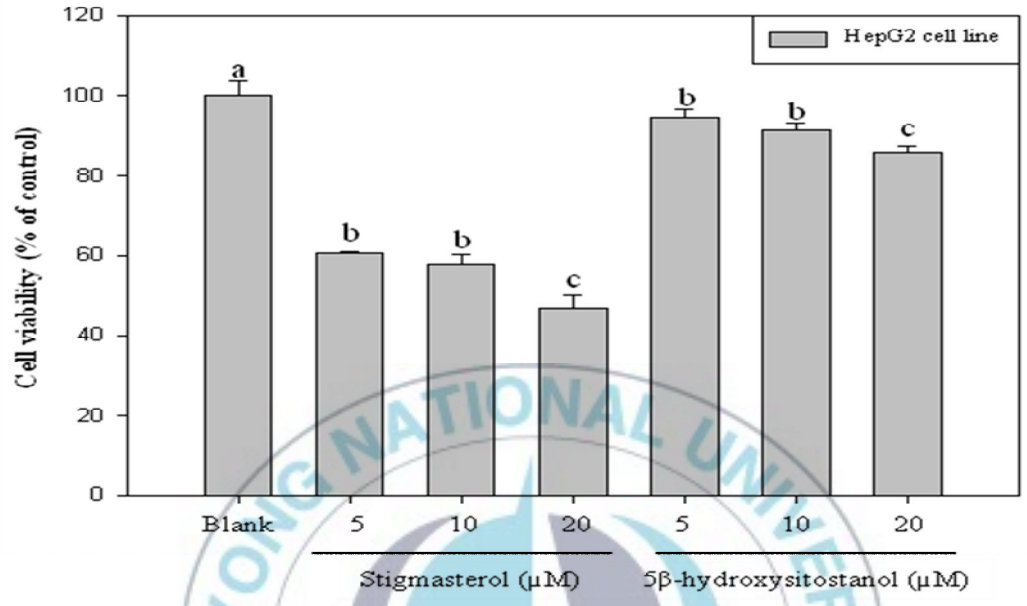


Figure 10. Cell viability of HepG2 cells treated with stigmasterol and 5β-hydroxysitostanol for 24 h. [a-c] Different letters an each bar shows significant different ($p<0.05$) by Duncan's multiple range test

3.4. Morphological changes and Hoechst 33342 staining of HepG2 cell line with stigmasterol and 5β-hydroxysitostanol

In the previous test, cell viability was decreased with dose-dependent manner. This means that cells died with the treatment of compounds. However it is cannot be exactly confirmed why cells died. In the morphological changes, we could observed how the cell death occured. When apoptosis is progress in cells, they display distinctive morphological features during the apoptotic process. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton. The breakdown of chromatin in the nucleus often leads to nuclear condensation and detached from bottom of cell culture dishes. Morphological changes and the cell death of HepG2 cells were characterized using light microscope. Figure 10 shows the morphological changes after 24 h exposure to various concentrations of stigmasterol and 5β-hydroxysitostanol. This technique allows us to observe attached cells which are alive. According to results, the number of attached cells was reamarkably reduced with increasing doses of stigmasterol and 5β-hydroxysitostanol. The changes of cell membrane of attached cells were observed. Treatment of stigmasterol and 5β-hydroxysitostanol caused shrinking the cell shape. The results showed that stigmasterol has the cytotoxicity effect on HepG2 cells compared to 5β-hydroxysitostanol.

In order to determine whether the inhibitory effect of stigmasterol and 5β-

hydroxysitostanol on cell viability was due to the apoptotic cell death, HepG2 cells were stained with Hoechst 33342 dye after 24 h sample treatment and the morphological changes of nuclear were observed under fluorescence microscope in figure 11-12. The nuclear degradation of HepG2 cells significantly observed by fluorescence lighting. Hence, the nuclei with chromatin concentration and apoptotic bodies were observed in the cells exposed to stigmasterol and 5β-hydroxysitostanol with increasing concentrations. This observations exhibit that stigmasterol induce cell death in HepG2 cells through a typical apoptotic pathway.

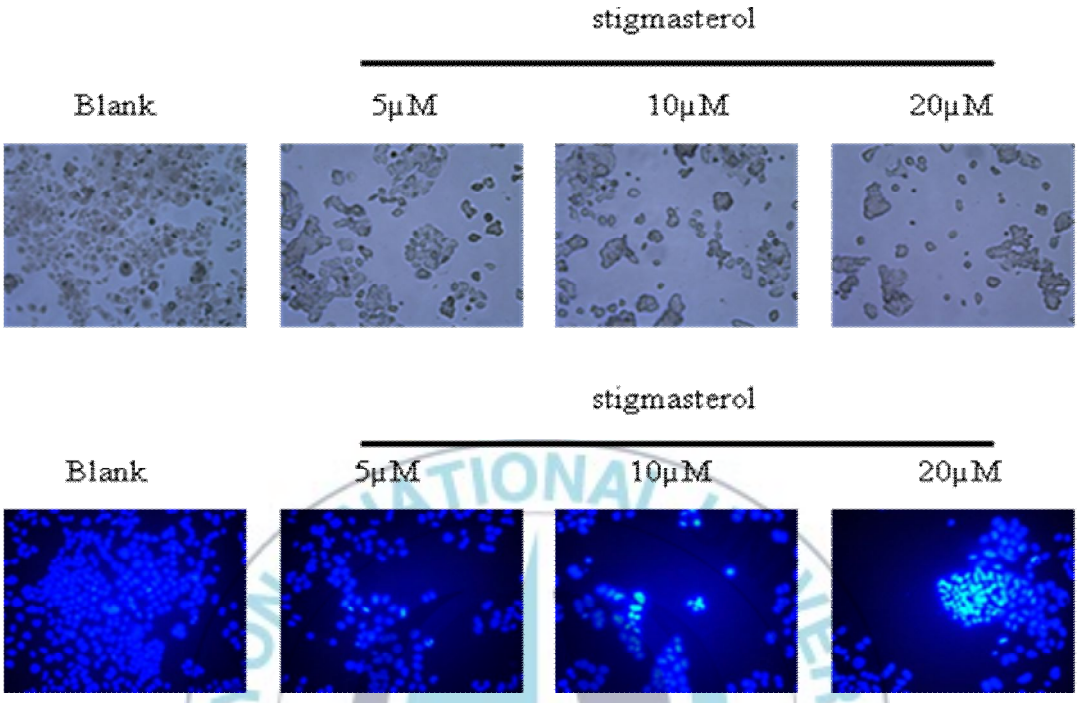


Figure 11. Morphological changes of stigmasterol treated HepG2 cells. For observation of morphological changes, HepG2 cells were grown and treated with stigmasterol for 24 h and morphological changes were detected under light microscope (viewed at magnification of 100 x). For Hoechst 33342 stained cells, treated with stigmasterol for 24 h, nuclear condition was detected under fluorescence microscope (viewed at magnification of 400 x)

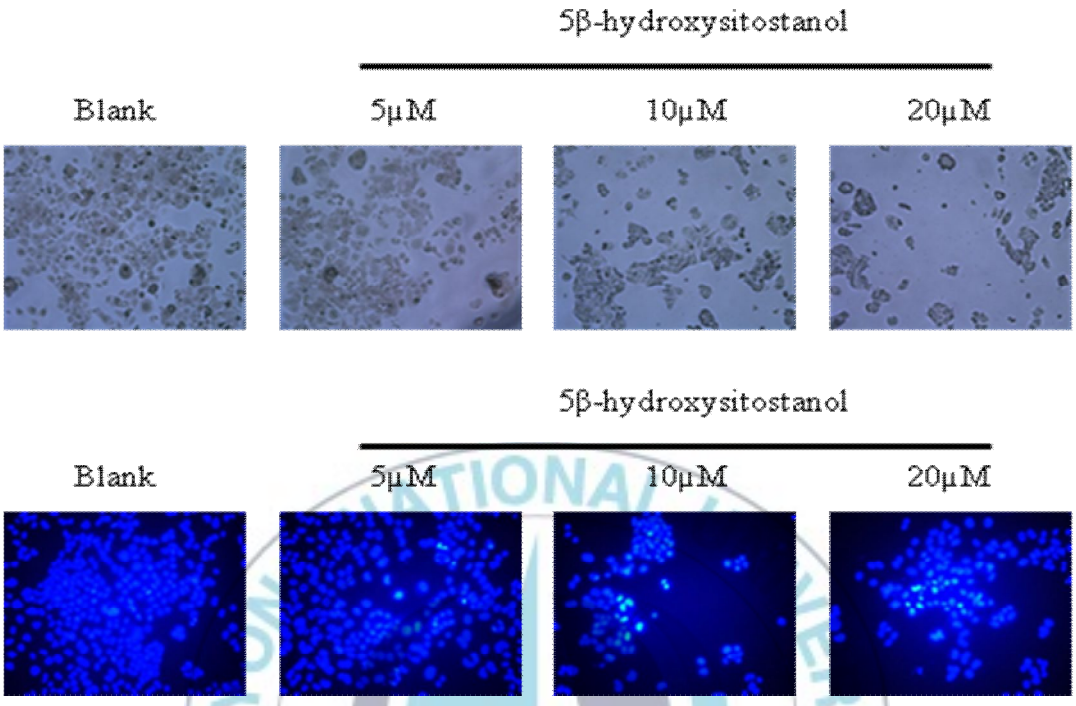


Figure 12. Morphological changes of 5β-hydroxysitostanol treated HepG2 cells. For observation of morphological changes, HepG2 cells were grown and treated with 5β-hydroxysitostanol for 24 h and morphological changes were detected under light microscope (viewed at magnification of 100 x). For Hoechst 33342 stained cells, treated with 5β-hydroxysitostanol for 24 h, nuclear condition was detected under fluorescence microscope (viewed at magnification of 400 x)

3.5. Flow cytometry analysis for the measurement of cell cycle arrest

In previous part, we observed morphological changes on the HepG2 cells treated with stigmasterol and 5β-hydroxysitostanol. When the apoptosis occurred, cell cycle is also changed. Considering the rounded cell morphology observed in HepG2 cells, cell cycle analysis was done to determine the effect of stigmasterol and 5β-hydroxysitostanol. HepG2 cells were cultured in 6 cm^2 dishes treated with different concentrations (5 µM, 10 µM, 20 µM) of stigmasterol and 5β-hydroxysitostanol. The cells were stained with PI, and the cell cycle was analysed in FACS. We observed that the G2/M pase of the cell cycle was arrested due to compound treatmet, and at the highest concentration it was corresponding to 26.87 % and 25.83% respectively. This confirms that the compounds arrest the cell cycle and thereby leading to cell death.

At key transitions during eukaryotic cell cycle progression, signaling pathways monitor the successful completion of upstream events prior to proceeding to the next phase. These regulatory pathways are commonly referred to as cell cycle checkpoints (Hartwell and Weinert, 1989). Cells can be arrested at cell cycle checkpoints temporarily to allow for: (i) cellular damage reparing of; (ii) the dissipation of an exogenous cellular stress signal; or (iii) availability of essential growth factors, hormones, or nutrients. Checkpoint signaling may also result in activation of pathways leading to programmed cell death if cellular damage cannot be properly

repaired. Defects in cell cycle checkpoints can result in gene mutations, chromosome damage, and aneuploidy, all of which can contribute to tumorigenesis.

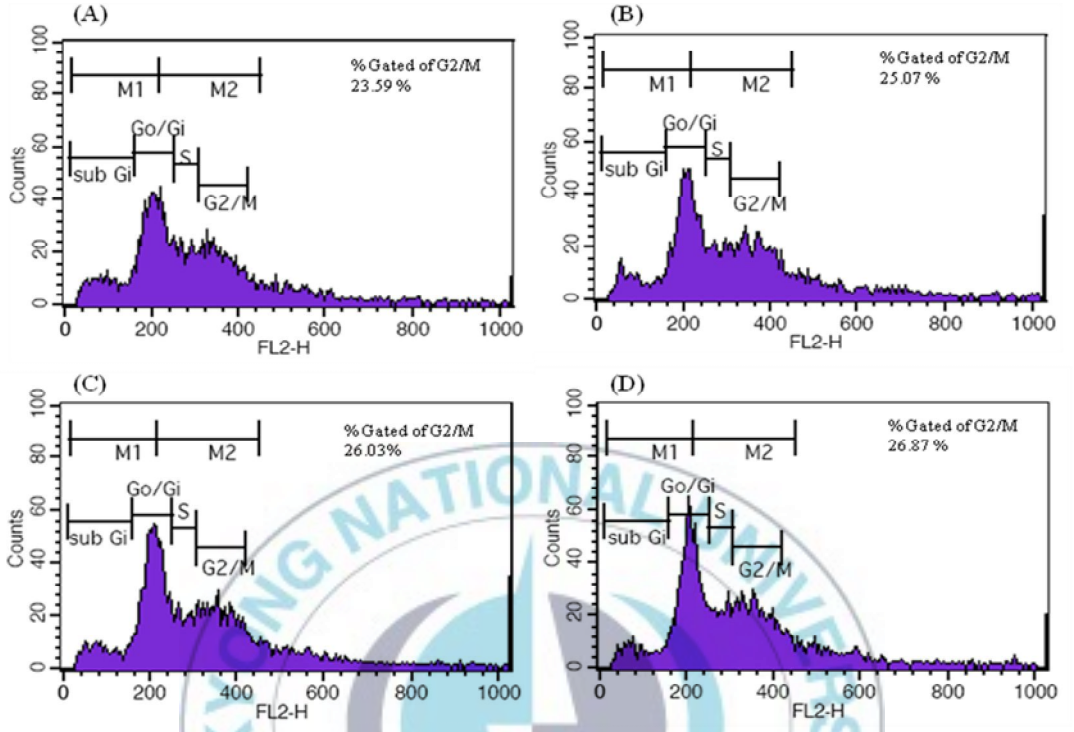


Figure 13. Detection of PI staining by FACS in stigmasterol treated HepG2 cells for 24 h. The percentage of cells in G2/M phase is indicated. (A):blank, (B): 5 μM, (C): 10 μM, (D): 20 μM of stigmasterol

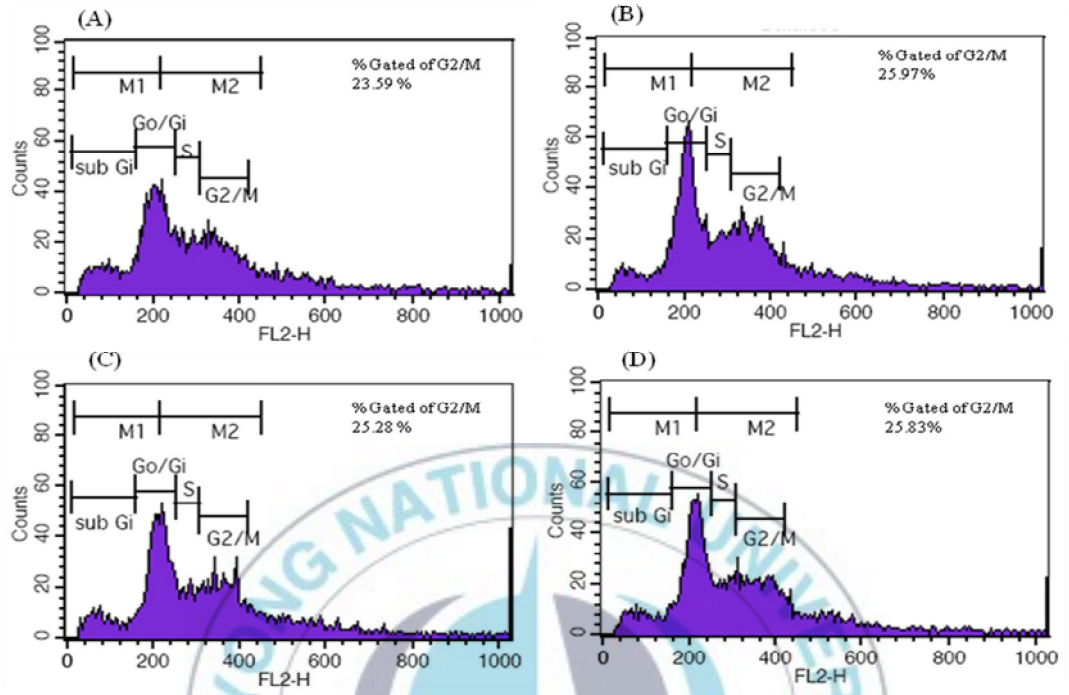


Figure 14. Detection of PI staining by FACS in 5β-hydroxysitostanol treated HepG2 cells for 24 h. The percentage of cells in G2/M phase is indicated. (A):blank, (B): 5 μM, (C): 10 μM, (D): 20 μM of 5β-hydroxysitostanol

3.6. Apoptotic effect in gene and protein expression levels

Balancing cell proliferation and cell death are involved in an important mechanism to maintain homeostasis in cells. Apoptosis is a programmed cell death (Hetz et al., 2005) and the ability to induce apoptosis has been known to be a promising strategy for cancer prevention. Bcl-2 family plays a vital role in mitochondrial apoptosis pathway (Antonsson, 2001; Zornig et al., 2001), which includes pro-apoptotic member, Bax. Caspase-9 in a member of the cysteine aspartic acid preotease or caspase family. The procaspase-9 is activated in apoptotic conditions and it is involved in the activation of the caspase cascade responsible for apoptosis execution and caspase-9 was shown to be downregulated in gastric cancer samples in comparison with normal mucosa tissues (Philchenkov et al., 2004). A tumor suppressor factor p53 plays a vital role in cancer cell death as well as apoptosis induction. The p53, in parallel with p21, can control the regulation of Bcl-2 family, which leads to disruption of mitochondrial membrane. Therefore, we characterized changing rates of caspase-9, Bax, p53 and p21 as an important regulators involved in apoptosis to examine the apoptotic issues by using RT-PCR and Western blot analysis. The results were given in figure 15-17.

According to the results of RT-PCR, stigmasterol induced the up-regulation for caspase-8, 9, Bax, p53, p21, FAS and FASL gene level in a dose-dependnet manner. This results comply with the results of western blotting assay. In western blot results,

caspase-8,9 and p53 also increasing in a dose-dependent manner. β-actin in the RT-PCR, and β-tubulin are internal standard mRNA and protein, respectively. Bcl-2 and XIAP gene are down-regulated in a dose-dependent manner by stigmasterol.

The role of p53 gene in apoptosis may be most important one among all apoptosis related genes, for contribution to the suppression of cellular proliferation, with potentially reversible cell cycle arrest (Linke et al, 1997). Also, induction of cyclin dependent kinase inhibitor p21 indicates apoptotic effect suggesting p53 mediated growth arrest (Hansen et al., 1997). Several studies have investigated anti-cancer effect of stigmasterol in breast cancer, prostate cancer cells, human colon cancer (HT-29) cells and human monocytic (U937) cells (Awad et al., 2000; Holtz et al. 1998; Raicht et al. 1980; O'Callaghan et al., 2010). In this study, stigmasterol showed inductive effect of apoptosis on HepG2 cells via up-regulation of p53 and p21 gene expression.

The caspases are a family of proteins those are one of the main executors of the apoptotic process. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive proforms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis (Budihardjo et al, 1999). In this research, caspase-8, 9, in gene expression were up-regulated in a dose-dependently and comply with wester blot results. Thus, stigmasterol significantly affect the caspase cascade via intrinsic pathway.

Hence, 5β-hydroxysitostanol has not affected to cell viability. In RT-PCR and western blotting assay, caspase-8, Bax, and FASL gene and protein levels were slightly increased in a dose-dependent manner. Subsequently, 5β-hydroxysitostanol showed moderate inductive effect of apoptosis in the HepG2 cells, but its effect on cells were much lower tha that of stigmasterol.

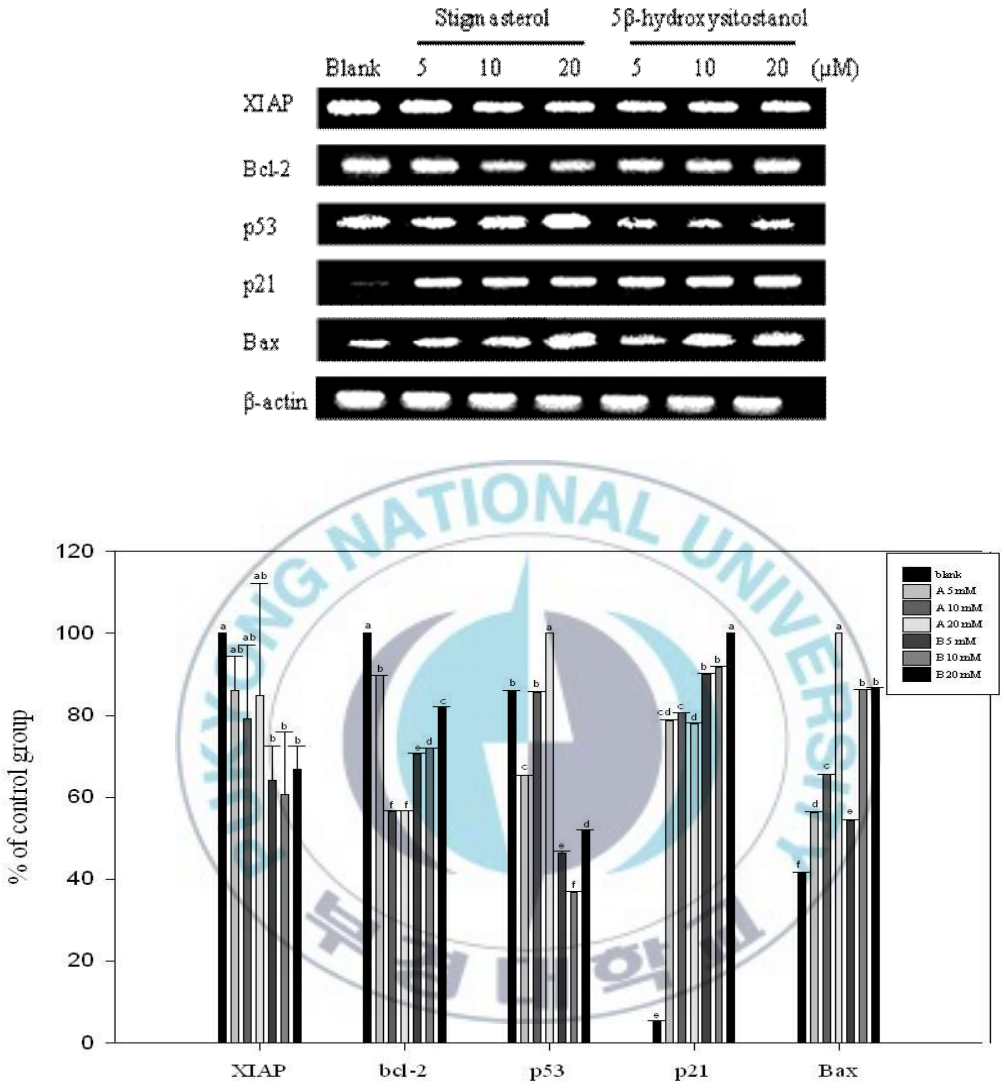


Figure 15. RT-PCR of XIAP, Bcl-2, p53, p21 and Bax for stigmasterol and 5β-hydroxysitostanol treated HepG2 cells. Cells were incubated with stigmasterol and 5β-hydroxysitostanol for 24 h and the expression levels of XIAP, Bcl-2, p53, p21 and Bax mRNA were derected using RT-PCR. β-actin was used as an internal standard

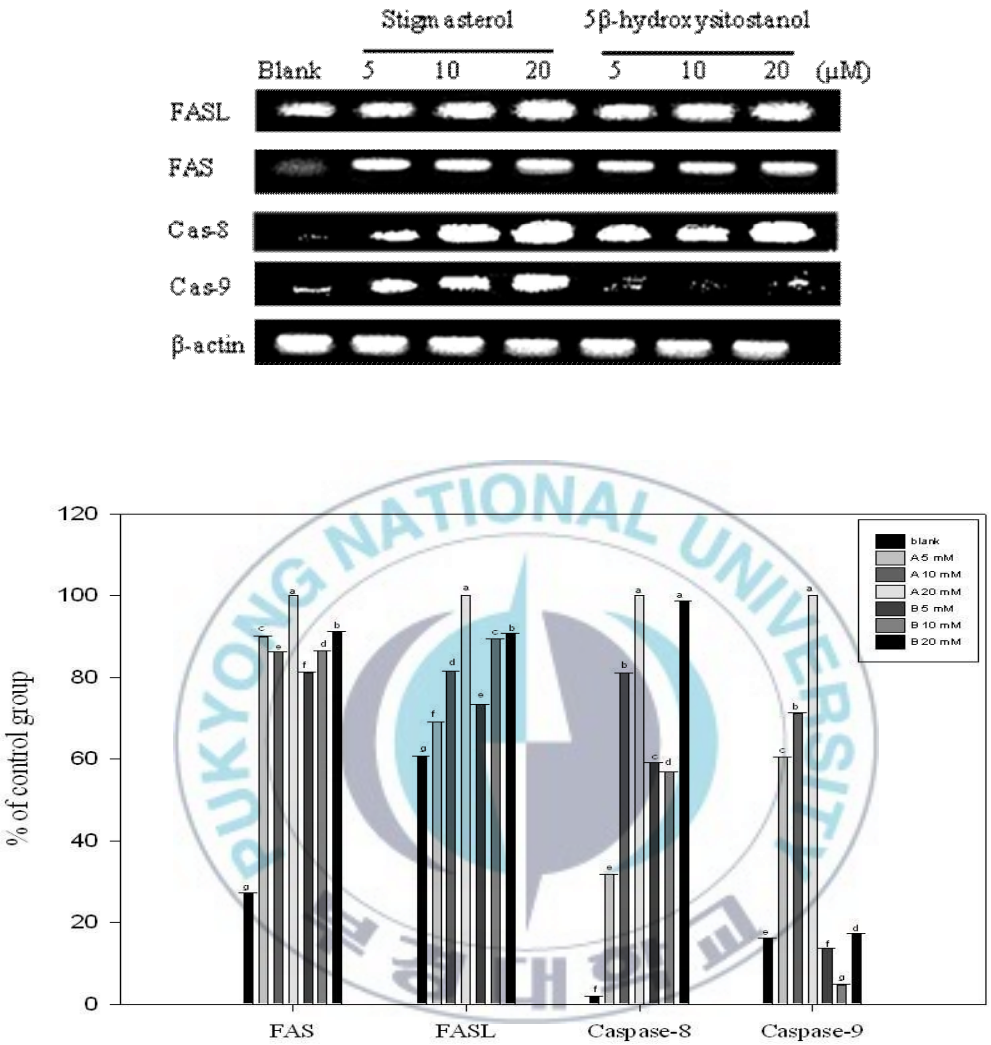


Figure 16. RT-PCR of FASL, FAS, caspase-8 and 9 for stigmasterol and 5β-hydroxysitostanol treated HepG2 cells. Cells were incubated with stigmasterol and 5β-hydroxysitostanol for 24 h and the expression levels of FASL, FAS, caspase-8 and 9 mRNA were derected using RT-PCR. β-actin was used as an internal standard

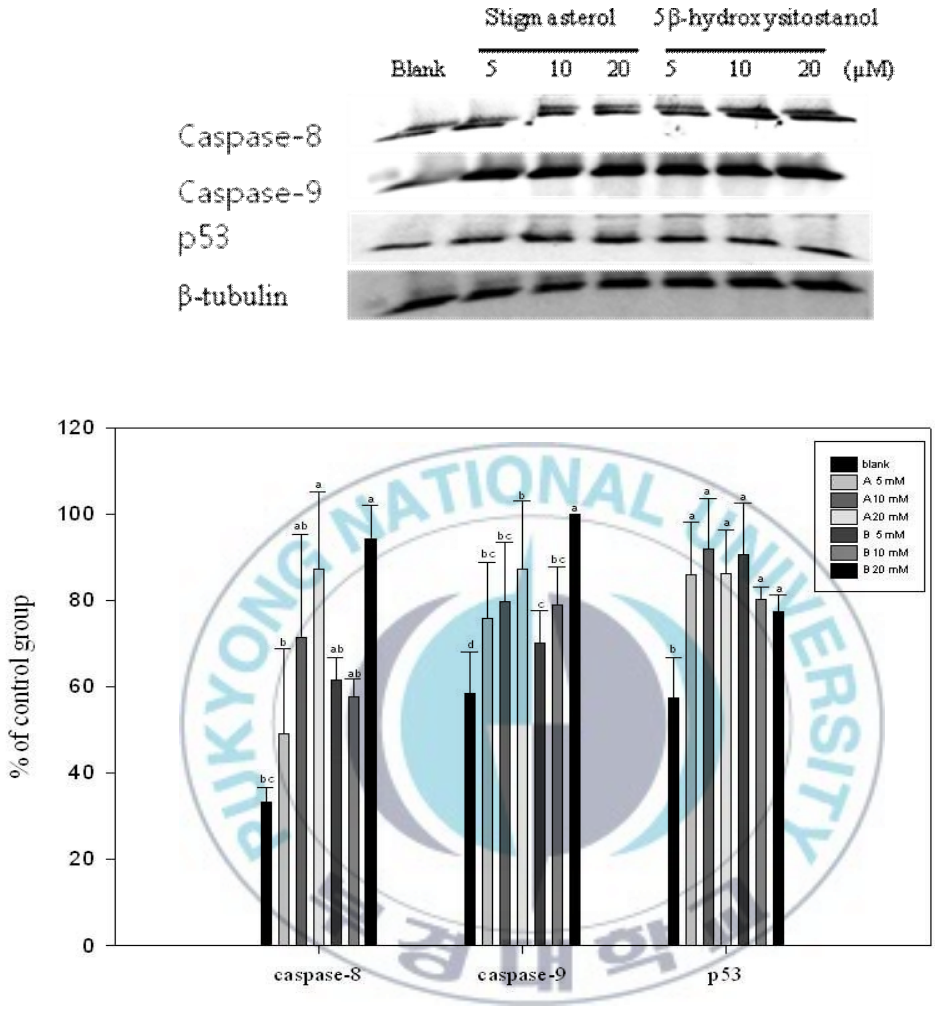


Figure 17. Western blot of caspase-8, 9 and p53 for stigmasterol and 5β-hydroxysitostanol treated HepG2 cells. Cells were incubated with stigmasterol and 5β-hydroxysitostanol for 24 h and the expression levels of caspase-8, 9 and p53 proteins were derected using western blot analysis. β-tubulin was used as an internal standard (A: stigmasterol, B: 5β-hydroxysitostanol)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4. Conclusion

In this study we purified and isolated stigmasterol and 5β-hydroxysitostanol from *Navicula incerta*. Further, we demonstrated that treatment of HepG2 cells with stigmasterol and 5β-hydroxysitostanol can induce apoptotic body formation, microalgae. Stigmasterol significantly induced apoptosis gene expression in a dose-dependently. The apoptosis inductive effect of apoptosis was mediated by extrinsic and intrinsic pathways via FAS, FASL and Bcl-2 family. Thus, we conclude that stigmasterol possess potential apoptosis inductive effect and beneficial characteristics for the treatment of liver cancer.

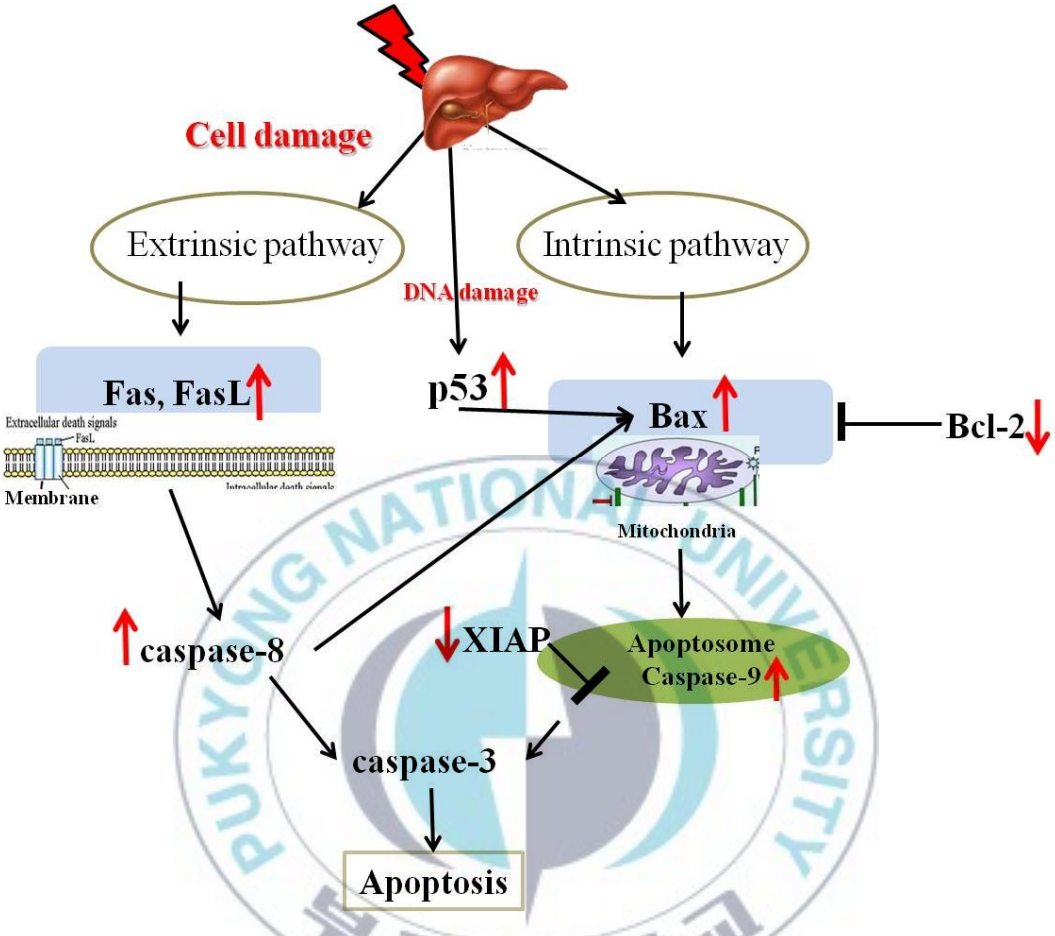

Figure 18. Scheme of apoptosis pathways activated by stigmasterol

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