Saringosterol acetate from a popular edible seaweed, *Hizikia fusiformis* attenuated proliferation of human lung adenocarcinoma epithelial cells (A549 cells) via apoptosis signaling pathway

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Abstract

*Hizikia fusiformis* is a common, edible marine alga found in Asia. Although the anticancer activity of its extracts has previously been investigated, its active compounds have not been identified. In this study, saringosterol acetate (SA) was isolated from *H. fusiformis* extracts by centrifugal partition chromatography (CPC) system (two phase solvents condition: *n*-hexane:ethyl acetate/methanol:water = 5:3:7:1, v/v), exhibited anticancer effects in the human lung adenocarcinoma epithelial cell line, A549, by inducing apoptosis and sub-G1 phase cell cycle arrest. In addition, SA increased the expression of the pro-apoptotic proteins, Bax and cleaved caspase 3, and decreased that of the anti-apoptotic protein Bcl-xL. Although, SA did not affect the expression of p53, induces expression of Bid and caspase 8. In conclusion, we suggested that SA induces apoptosis against A549 cells via Bid and caspase 8 dependent pathway.

*Keywords*: Edible marine alga, Anticancer; *Hizikia fusiformis*; Saringosterol acetate; Apoptosis
1. Introduction

Cancer is the leading cause of death worldwide and results from abnormal cell growth [1]. Lung cancer is one of the most malignant types of cancer that occurs in humans and approximately 70% of patients die as a result of cancer metastasis. There has been a great interest in harnessing the anticancer properties of natural compounds as they are thought to be relatively non-toxic and have been used as natural remedies since ancient times [1,2].

Brown seaweeds are marine organisms that have a variety of biological activities including antioxidant, anticancer, anti-hypertension, antibacterial, and anti-inflammatory activities [3-7]. Their activities are attributable to the presence of a variety of bioactive secondary metabolites such as phlorotannins, fucoxanthin, fucoidan, and sterols. Metabolites such as sterols and polysaccharides can be extracted from *Hizikia fusiformis*, a popular, edible, marine brown alga, which is found in many tropical and sub-tropical regions of the world. The sterols have a variety of biological activities including antioxidant and anticancer [8,9].

Natural sterols such as fucosterol, phytosterol, cholesterol, stigmasterol, and β-sitosterol prevalent in marine algae have been reported to inhibit cancer cell migration and induce apoptosis (Bennani et al., 2007). Repetitive chromatography processes have commonly been used to isolate pure sterols from marine algae. Centrifugal partition chromatography (CPC) can also be used to isolate large quantities of compounds with a purity of >90% using a one-step process [10,11].

In this study, we applied CPC to isolate anti-cancer compounds from *H. fusiformis* and identified its signal pathway of apoptosis against human lung adenocarcinoma epithelial (A549) cells.
2. Materials and methods

2.1. Materials

*H. fusiformis* was obtained from Taerim, located in Jeju city, South Korea, in June 2011. The alga was ground and sifted through a 50 mesh standard testing sieve after being dried using a freeze-dryer. The dried *H. fusiformis* powder was stored at 4°C. All solvents and reagents used for the preparation of crude extract and CPC separation were of analytical grade (Daejung Chemicals & Metals Co., Seoul, Korea). The HPLC grade solvents used for the analysis of the extract and compound, were purchased from Burdick & Jackson (MI, USA). RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). M-MuLV reverse transcriptase was purchased from Promega (Madison, WI, USA). The protein assay kit and ECL detection reagent were purchased from Bio-Rad (Richmond, CA, USA) and Amersham Biosciences (Piscataway, NJ, USA), respectively. RNase A was purchased from Biosesang (Seoul, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dihydroethidium (DE), ethidium bromide (EtBr), dimethyl sulfoxide (DMSO), and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). DNA ladder size markers were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against Bax, Bcl-xL, p53, and GAPDH were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Antibodies against cleaved Bid, cleaved caspase-3, and cleaved caspase -8 were purchased from Cell Signaling Technology (Bedford, Massachusetts, USA). All other chemicals, reagents, and solvents were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of crude extracts from *H. fusiformis*

Dried *H. fusiformis* (600 g) was extracted three times for 3 h with 70% EtOH under
sonication at room temperature. The extract was then concentrated using a rotary vacuum evaporator and the concentrated extract was then stored in a refrigerator for CPC separation.

2. 3. CPC process

The CPC experiments were performed using a previously used protocol (Lee et al., 2014b) with some modifications. The two phases were composed of \( n \)-hexane:ethylacetate (EtOAc):methanol (MeOH):water = 5:5:7:1. The upper, organic phase was used as the mobile phase, whereas the lower aqueous phase was used as the stationary phase. The effluent from the CPC was monitored by TLC with a mobile phase composed of CHCl₃:MeOH=20:1.

2. 4. ESI/MS analysis of purified compounds

ESI/MS analyses were carried out using a Finnigan MAT LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a Finnigan electrospray source which is capable of analyzing ions. The negative ion mass spectra of the column eluate were recorded in the range m/z 100–2000. Operation program was Xcalibur software (Finnigan MAT).

2. 5. \(^1\)H-NMR and \(^{13}\)C-NMR analysis of purified compound

\(^1\)H-NMR spectra and \(^{13}\)C-NMR spectra were measured with a JEOL JNM-LA 300 spectrometer. Mass spectra (FAB-MS and EIMS) were recorded using a JEOL JMS 700 spectrometer.

2. 6. MTT assay

Measurements of the anti-cancer effects of the extract and compound were performed using the MTT assay described in Kotake-Nara et al. (2005). The human lung cancer cell line, A549,
was grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C with 5% CO₂. After a 24 h pre-incubation of A549 cells (1×10⁵ cells/ml) with various concentrations of the compound (0.5 ~ 50 µg/ml), MTT stock solution (50 µl; 2 mg/ml in PBS) was added to each of the wells in order to obtain a total reaction volume of 250 µl. Following a 4-h incubation, the plate was centrifuged at 2,000 rpm for 10 min and the supernatant was aspirated. The formazan crystals in each well were then dissolved in DMSO. The concentration of purple formazan was determined by measuring the absorbance at 540 nm.

2.7. Apoptotic body assay

The nuclear morphology of cells was analyzed using a cell-permeable DNA dye, Hoechst 33342. Cells displaying homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was interpreted as being indicative of apoptosis [11,12]. After treatment with the sterol, Hoechst 33342 dye was added into the culture medium at a final concentration of 10 µg/ml. The stained cells were then observed under a fluorescence microscope equipped with a CollSNAP-Pro color digital camera in order to examine the degree of nuclear condensation.

2.8. Cytometric (FACS) assay

Cell cycle analysis was performed to determine the proportion of apoptotic sub-G1 hypodiploid cells present [13]. The A549 cells (1×10⁵ cells/ml) were placed into the wells of a 5 cm of petri dishes. The cells were then treated with the sterol (1.5, 3, 6 and 12.5 µg/ml) for 24 h. After fixing the cells with 1 ml of 70% EtOH for 30 min at 4°C, the cells were then incubated with 100 µg/ml of PI/RNase A for 30 min. Flow cytometric analysis was performed
with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.9. Western blot analysis

A549 cells plated at $1 \times 10^5$ cells/ml were treated with the sterol (1.5, 3, 6 and 12.5 µg/ml) and harvested. The cell lysates were prepared with lysis buffer (50 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/l EDTA). The cell lysates were washed via centrifugation, and the protein concentrations in the lysates were then determined using a BCA™ protein assay kit. Lysates containing 30 µg of protein were subjected to electrophoresis on 10% or 12% sodium dodecyl sulfate–polyacrylamide gels. The gels were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were then incubated with primary antibodies against Bax, p53, cleaved capase 3, 8, Bcl-xl, cleaved Bid, and GAPDH in TTBS (25 mmol/l Tris–HCl, 137 mmol/l NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% non-fat dry milk for 1 h. The membranes were then washed with TTBS and incubated with secondary antibodies. Signals were developed using an ECL Western blotting detection kit and exposed onto X-ray film.

2.10. Statistical analysis

All measurements were made in triplicate and all values are represented as means ± standard error. The results were subjected to an analysis of the variance (ANOVA) using Tukey’s test to analyze the difference. A value of $p < 0.05$ was considered to indicate statistical significance.
3. Results and discussion

3.1. Separation of sterol by CPC and its structural identification

For optimization of two phases solvents, partition coefficients (K) were assessed to select a suitable two-phase solvent system [14]. In order to determine the most efficient system to separate target samples by preparative CPC, several two-phase solvent ratios were adopted using different compositions and volume ratios of two immiscible solvents such as n-hexane:EtOAc:MeOH:water (v/v). The K values of the samples were then determined using the size of bands obtained in TLC. The two-phase solvent system, which was composed of 5:3:7:1 (n-hexane:EtOAc:MeOH:water, v/v), exhibited a band size that indicates that this method is the most efficient for the separation of sterol from *H. fusiformis*. Preparative CPC was performed in descending mode (upper phase: stationary phase and lower phase: mobile phase), with 50% retention of the stationary phase in the coil and a pressure of 3.3 MPa at 360 min. TLC analysis of each fraction from preparative CPC is described in Fig. 1(a). The sterol in fraction 3 reacted with H2SO4, producing a purple color. After purification of the sterol in fraction 3 by preparative TLC, the chemical structure of the sterol was analyzed using HPLC-DAD-ESI/MS, 1H, 13C, and comparison with 2D NMR data and data from previous reports (shown in table 1) [15,16]. The sterol was confirmed as being saringosterol acetate (SA) with a molecular weight of m/z 469 (ESI-MS) (Fig. 1(b)). A total of 11 mg of pure sterol was isolated from 500 mg of the 70% EtOH extract with 2.2 % yield.

The chemical diversity of marine lipids, and particularly that of sterols such as cholesterol, sitosterol, and stigmasterol, has long been recognized as a valuable resource for the identification of biomarkers [17] for the chemotaxonomy of marine organisms [18]. Numerous researchers have isolated novel sterols from various harmful algal bloom species such as the marine sponge *Haliclona crassiloba*, marine red algae *Peyssonnelia* sp., and marine brown
algae [19-22]. Several of the sterols that have been derived from marine organisms have been reported to exhibit anti-bacterial, anti-inflammation, anti-diabetic, and anti-cancer effects. However, most of the sterols that exhibit anticancer effects were isolated from marine sponges and soft corals [23]. The isolation of sterols from marine algae and the evaluation of their anticancer properties are important areas of research for cancer drug development. In the current study, we successfully isolated pure SA from marine brown algae using a two-step process involving preparative CPC and TLC. This simple process can be used to purify sterol from marine algae and is an efficient alternative to conventional isolation processes such as repeated chromatography using normal and reverse phases [15,24]. A high yield of SA (2.2%) was obtained from the 70% EtOH extract. A mixture of 24R and 24S saringosterol acetate was obtained from *H. fusiformis* (Harvey) Okamura by Okano et al. [25]. However, its anticancer effects were not assessed in the current study.

### 3.2. Saringosterol acetate attenuates proliferation of A549 cells via apoptosis pathway

The relative cytotoxic activity of SA against tumor cells was measured by MTT assay using human lung cancer A549 cells. Fig. 2 (a) and (b) show the anticancer activity of SA against A549 cells and its cytotoxicity against monkey kidney cells (IC$_{50}$ value, 3 μg/ml, Fig. 2(a)). However, no cytotoxic effect was observed in Vero cells. The potential cytotoxic effect of SA on A549 cells was investigated by Hoechst 3334 assay and confirmed by flow cytometric assay. The apoptotic bodies in the cells treated with SA for 24 h are indicated using arrows in Fig. 3(A). Cells treated with SA exhibited a more heterogeneous morphology, compared with that of the control. The number of cells in each compartment of the cell cycle and sub-G1 phase was expressed as a percentage of the total number of cells. The data demonstrate that SA induced apoptosis (sub-G1 phase) in a dose-dependent manner (Fig. 3(B)) and that the
strongest effect was observed at a concentration of 12.5 μg/ml. The induction of apoptosis in cancer cells is one of the major strategies of cancer therapeutics. There are three distinct apoptosis pathways, extrinsic, caspase-dependent, and intrinsic mitochondria-dependent [26-28]. In this study, SA showed strong anticancer effects by inducing apoptosis. Previously, it was reported that various marine-derived sterols inhibit the proliferation of a variety of cancer cells via apoptosis [29].

3.3. Effect of SA on levels of apoptosis-associated proteins in A549 cells

In order to determine the mechanism of SA-induced apoptosis, we evaluated the expression of proteins such as Bax, Bcl-xL, cleaved caspase-3, cleaved caspase-8, p53, and cleaved Bid by western blot analysis. The expression level of Bcl-xL, an anti-apoptosis protein, was found to be downregulated, while the expression of pro-apoptosis proteins, including Bax and cleaved caspase-3, was found to be upregulated in A549 cells treated with SA. The expression levels of caspase-8 and Bid were also found to increase in a dose-dependent manner. However, levels of p53 did not change (Fig. 4). Caspase-dependent pathways activate caspase-8 and 9 and this leads to the activation of caspase-3 and apoptosis. DNA damage induced by external stress results in the expression of p53, which subsequently triggers apoptosis [30]. In the current study, SA induced up-regulation of caspase-3 and Bax whereas, the expression of p53 was not found to be affected. But, SA significantly increased the expression of caspase-8 and Bid. Bid has previously been reported to bind Bax and to regulate its bioactivity. Bid is thought to promote apoptosis via this mechanism. Caspase-8 is then activated, has the ability to cleave the amino portion of Bid to produce truncated Bid (t-Bid) which activates caspase-3 [31-33]. In this study, SA was found to upregulate both the expression of Bid and caspase-8, and thereby induce apoptosis in the human lung cancer cell line, A549. According to recent reports, baicalein, a
flavonoid, induced apoptosis in PC12 cells by suppressing the activation of the caspase-8- and Bid-dependent pathways [33]. This process may result in the activation of Bid, which interacts directly with Bax or Bak, a Bcl-2 homologous antagonist killer with pro-apoptotic action. Bid synergizes with Bax, leading to apoptosis [34,35]. These results suggest that SA induced Bax expression through the upregulation of Bid. Therefore, this study confirmed the possibility that apoptosis was induced through the bid and caspase 8 dependent pathway.

4. Conclusion

In this study, we confirm that the sarigosterol acetate isolated from *H. fusiformis* by CPC system exhibits strong anti-cancer activity through apoptosis pathway inducing expressions of Bid and caspase 8.

Acknowledgment
References


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<sup>a</sup> Multiplicity determined from HSQC-DEPT experiments
Figure legends

Figure 1. TLC of each fraction collected from 70% EtOH extract of *H. fusiformis* by CPC (a), and structure and ESI-MS data of isolated saringosterol acetate (b). CPC producer: Stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 2 mL/min; rotation speed: 1000 rpm; sample: 500 mg dissolved in 6 mL mixture of lower phase and upper phase (1:1, v/v) of the solvent system.

Figure 2. Cytotoxicity on A549 (A) and Vero cells (B) of saringosterol acetate from *H. fusiformis*. Cells were treated to indicate concentrations of saringosterol acetate and measured by MTT assay. *Significantly different from no-treated A549 cell (P < 0.05); **Significantly different from no-treated A549 cell (P < 0.01); ***Significantly different from no-treated A549 cell (P < 0.001).

Figure 3. Effect of saringosterol acetate on the induction of apoptotic bodies (A) and DNA contents of sub G1 phase (B) in A549 cells. Cells were treated to indicate concentrations of DTCP and stained with PI to determine DNA fluorescence and cell cycle phase distribution. The sub-G1 fraction of the cell cycle indicative of DNA damage was analyzed from the hypodiploid fraction of DNA cell cycle analysis. Data are representative one of three similar experiments. *Significantly different from no-treated A549 cell (P < 0.05); **Significantly different from no-treated A549 cell (P < 0.01)

Figure 4. Effect of saringosterol acetate on apoptosis-related proteins in A549 cells. Cells were treated with saringosterol acetate at the indicated concentration for 24 hr. Whole cell lysates were subjected to Western blot analysis of anti-Bax, -Bel-xL, -cleaved caspase-3, -cleaved caspase-8 and -bid monoclonal antibodies. GAPDH was used as internal control. Each experiment was repeated three times with similar results. *Significantly different from no-treated A549 cell (P < 0.05); **Significantly different from no-treated A549 cell (P < 0.01);
***Significantly different from no-treated A549 cell (P < 0.001).
Fig. 1. 

Saringosterol acetate (11 mg) 

Diagram: 

Fraction numbers 

Si TLC - CHCl₃ : M = 20 : 1 

TOC5371F1 #4409 RT: 45.25 AV:1 NL: 2.07E2 
F: ITMS + c ESI Full ms [100.00-2000.00] 

[M-H]=469.58 

Saringosterol acetate
**Fig. 2.**

(a) Saringosterol acetate concentration (µg/ml) vs. Cell viability (%). The IC₅₀ value is 3 µg/ml.

(b) Saringosterol acetate concentration (µg/ml) vs. Cell viability (%).
Fig. 3.
Fig. 4.

(A)

Saringosterol acetate (µg/ml)

- 1.5  3  6  12.5

Bax  

![Image of Bax protein levels with various concentrations of Saringosterol acetate](image)

Bcl-xL  

![Image of Bcl-xL protein levels with various concentrations of Saringosterol acetate](image)

Cleaved Caspase 3  

![Image of Cleaved Caspase 3 protein levels with various concentrations of Saringosterol acetate](image)

p53  

![Image of p53 protein levels with various concentrations of Saringosterol acetate](image)

Cleaved Caspase 8  

![Image of Cleaved Caspase 8 protein levels with various concentrations of Saringosterol acetate](image)

Cleaved Bid  

![Image of Cleaved Bid protein levels with various concentrations of Saringosterol acetate](image)

GAPDH  

![Image of GAPDH protein levels with various concentrations of Saringosterol acetate](image)

(B)

- **Bax**

![Bar graph of Bax protein levels with various concentrations of Saringosterol acetate](image)

- **Cleaved caspase 3**

![Bar graph of Cleaved caspase 3 protein levels with various concentrations of Saringosterol acetate](image)

- **Bcl-xL**

![Bar graph of Bcl-xL protein levels with various concentrations of Saringosterol acetate](image)

- **p53**

![Bar graph of p53 protein levels with various concentrations of Saringosterol acetate](image)

- **Cleaved caspase 8**

![Bar graph of Cleaved caspase 8 protein levels with various concentrations of Saringosterol acetate](image)

- **Cleaved Bid**

![Bar graph of Cleaved Bid protein levels with various concentrations of Saringosterol acetate](image)