 doi:10.20944/preprints201807.0060.v1

Article

Sulforaphene Isolated from Radish (*Raphanus Sativus* L.) Seeds Inhibits Growth of Six Cancer Cell Lines and Induces Apoptosis of A549 Cells

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Abstract: Sulforaphene (SFE), a major isothiocyanate in radish seeds, is a close chemical relative of sulforaphane (SFA) isolated from broccoli seeds and florets. The anti-proliferative mechanisms of SFA against cancer cells have been well investigated, but little is known about the potential anti-proliferative effects of SFE. In this study, we showed that SFE purified from radish seeds inhibited the growth of six cancer cell lines (A549, CHO, HeLa, Hepa1c1c7, HT-29, and LnCaP), with relative

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half maximal inhibitory concentration (IC_{50}) values ranging from 1.37 to 3.31 μg/mL. Among the six cancer cell lines evaluated, SFE showed the greatest growth inhibition against A549 lung cancer cells. In A549 cells, SFE induced apoptosis via changes in the levels of poly (adenosine diphosphate ribose) polymerase and caspase-3, -8, and -9. Our results indicate that SFE from radish seeds may have significant anti-proliferative potency against a broad range of human cancer cells via induction of apoptosis.

Keywords: Anti-proliferation; *Brassica* vegetables; Human disease; Isothiocyanates; MTT assay

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1. Introduction

Glucosinolates (GSLs) are major secondary metabolites found in *Brassica* vegetables such as radish, broccoli, kale, and kimchi cabbage. Although GSLs are not bioactive, their hydrolytic breakdown products are thought to protect against cancer. GSLs are released from the vacuoles of myrosin cells in response to plant tissue damage and are hydrolyzed by cytosolic myrosinase, a thioglucoside glucohydrolase (EC 3.2.3.1) [**1,2**]. Isothiocyanates (ITCs) are produced by the hydrolytic breakdown of GSLs and are thought to play an important protective role in plants against fungi, bacteria, viruses, and insects [**3-6**].

ITCs have potential anticancer activities in animals and humans. Several ITCs have been shown to inhibit the growth of cancer cells *in vivo*. A previous study [**7**] showed that the growth of mouse or human prostate cancer cells inoculated subcutaneously into mice was slowed by treatment with allyl ITC (10 mM by intraperitoneal injection three times a week), phenethyl ITC (9–12 mM by oral intubation three times a week), and sulforaphane (SFA; 5.6 mM/kg diet daily). Moreover, oral administration of the phenethyl ITC-N-acetyl cysteine conjugate (8 mM/g diet daily) inhibited the proliferation of human prostate cancer cell xenografts in mice [**8**].

Although early studies focused mainly on the ability of ITCs to inhibit cancer cell formation, recent studies showed that ITCs also suppress the survival and proliferation of existing cancer cells. Investigations into the molecular bases of the anticarcinogenic and anticancer effects of ITCs have shown that ITCs target cancer cells via multiple mechanisms, including inhibition of carcinogen-activating enzymes, induction of carcinogen-detoxification enzymes, induction of apoptosis, and other mechanisms that are not yet well understood [**9–12**]. Among these mechanisms, apoptotic induction is of particular interest because it can be exploited for cancer

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prevention and treatment.

SFA obtained from broccoli is the most well-known example of a plant ITC with anticancer properties. SFA was isolated from broccoli extracts as a potent inducer of mammalian cytoprotective enzymes [**13**]. Broccoli seeds and sprouts are rich sources of glucoraphanin (GRA), which is the chemical precursor of SFA [**14–16**]. Consequently, broccoli has been used as a source of GRA or SFA in studies of human health. However, when fresh broccoli floret is consumed, the major degradation product of GRA is not SFA, but rather SFA nitrile, which has little anticancer activity [**17**].

Radish seeds and sprouts are rich in sulforaphene (SFE), a major ITC with a chemical structure similar to that of SFA. Unlike in broccoli, breakdown of glucoraphenin (GRE), the GSL corresponding to GRA in broccoli, results in the production of SFE, rather than SFE nitrile, in radish plant. This process occurs because radish seeds and sprouts lack the epithiospecifier protein (ESP), which promotes nitrile formation during the breakdown of GSLs by myrosinase [**18**]. The anticancer properties of SFA from broccoli have been well documented, but research on the potential biological activities of SFE from radish is limited to a few recent studies. In a previous study [**19**], a hexane extract containing a mixture of SFE and other ITCs from radish seeds showed anti-proliferative activity against cancer cells. Furthermore, it was found that SFE extracts have significant and specific anti-proliferative activity against HepG2 cells [**20**]. However, previous reports have not investigated the mechanism of action of SFE or its effect on apoptosis. In the present study, we investigated the biological activity of SFE obtained from radish seeds on six cancer cell lines in terms of apoptosis.

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2. Results

2.1. Inhibition of cancer cell growth by SFE

Six cancer cell lines [A549 (lung cancer), CHO (ovarian cancer), HeLa (cervical cancer), Hepa1c1c7 (lung cancer), HT-29 (colon cancer), and LnCaP (prostate cancer)] of epithelial origin were used to evaluate cell growth inhibition by SFE using the 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide (MTT) assay (**Figure 1**). The dimethyl sulfoxide (DMSO) solvent control exerted no cytotoxicity against any of the six cancer cell lines. The cell viabilities of all cancer cell lines after SFE or SFA treatment were dependent on the SFE/SFA concentration, and ranged from 9.1% to 53.7% relative to the control cell density at high doses (**Figure 1**). Significant inhibitory activity was observed in A549, CHO, HeLa, Hepa1c1c7, and HT-29 cells, with strong inhibition at concentrations > 4.0 μg/mL (**Figures 1A–E**). However, growth inhibition by SFE in the LnCaP cell line was not significant (**Figure 1F**). The IC_{50} values of SFE and SFA are presented as relative values in **Supplementary Table S1**. The highest SFE's relative half maximal inhibitory concentration (IC_{50}) values, 1.82 and 1.50 μg/mL, were observed in the A549 and Hepa1c1c7 cell lines, respectively. The IC_{50} of SFE in HeLa cells was 1.37 mg/mL (**Figure 1C**); however, the cell viability at the highest SFE concentration evaluated (16 μg/mL) was still 30% of the control group viability. The IC_{50} values of SFE in CHO and HT-29 cell lines were respectively 3.31 and 2.50 μg/mL (**Supplementary Table S1**). In all cancer cell lines except HeLa and LnCaP, the inhibitory effect of SFE on cell growth was slightly greater than that of SFA (**Figure 1 and Supplementary Table S1**). Furthermore, the effects on inhibition of A549 and HT-29 cell growth were 1.25- and 1.31-fold greater, respectively, for SFE than for SFA.

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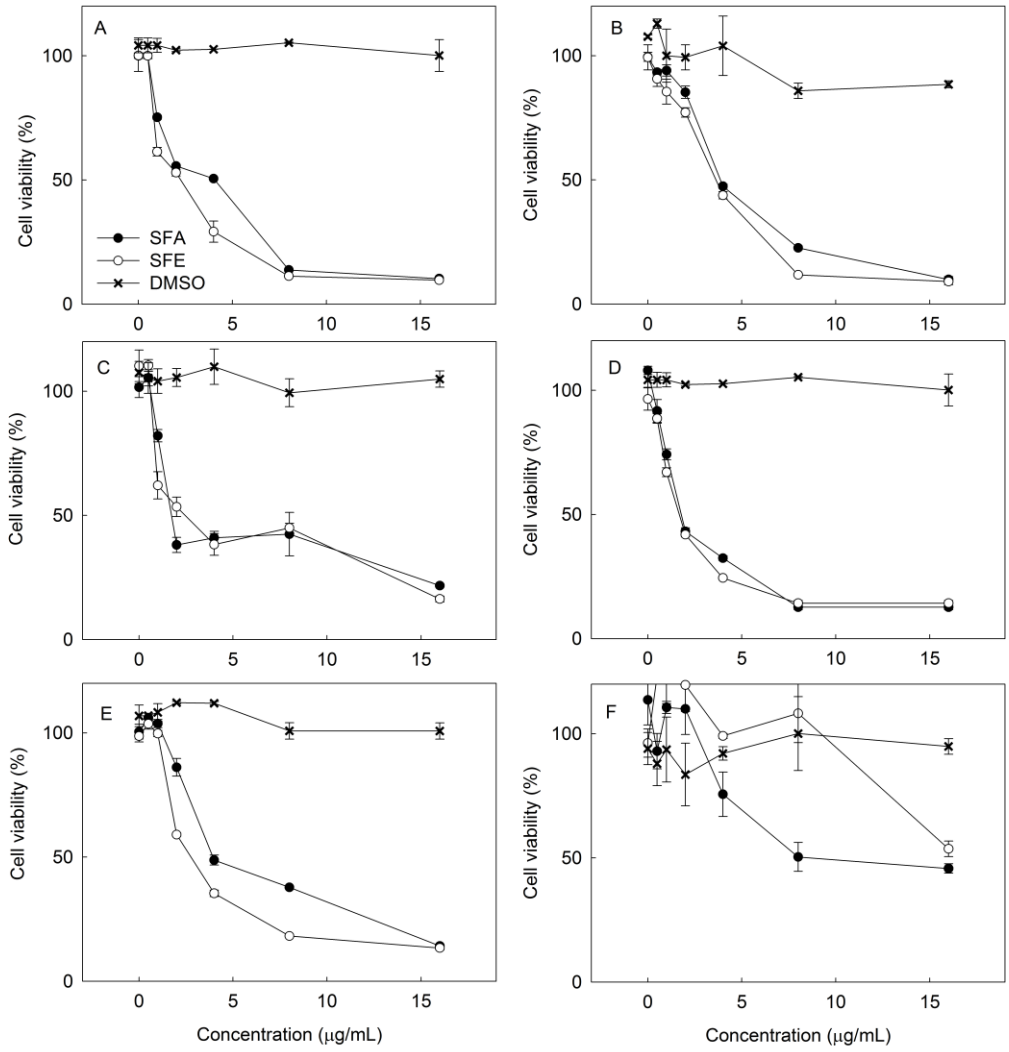

Figure 1. The effects of SFA and SFE on the viability of six cancer cell lines: A549 (A), CHO (B), HeLa (C), Hepa1c1c7 (D), HT-29 (E), and LnCaP (F). Data represent the means ± standard deviations of six replicates.

2.2. Anti-proliferative effect of SFE in A549 cancer cells

Among the six cancer cell lines, we evaluated the anti-proliferative effect of SFE in A549 lung cancer cells and compared it with that of SFA as the control. At 24 h after treatment, SFE was removed from culture, and the cells were incubated for another 24, 30, or 36 h without SFE. Cell

viability was measured by MTT assay. The reduced cell viabilities induced by SFE were maintained even after SFE removal (**Figure 2**). In particular, the viability of A549 cells initially treated with 8.0 μg/mL SFA or SFE did not increase after the washout period. Thus, SFE effectively inhibited A549 cell proliferation.

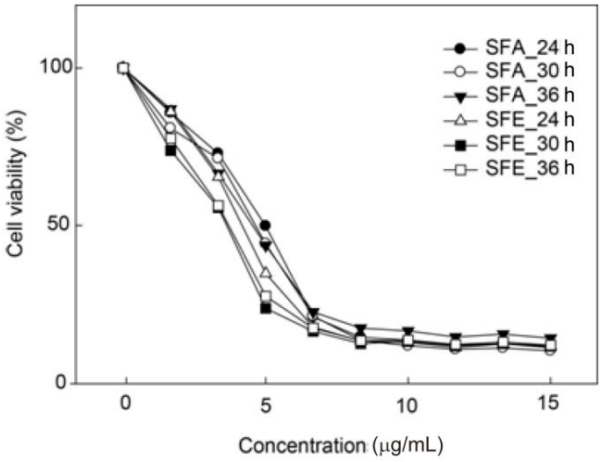

Figure 2. The anti-proliferative effect of SFE on A549 cells. Data represent the means of three replicates.

We observed changes in A549 cell morphology to determine whether cell cytotoxicity mediates SFE-induced cell growth inhibition (**Figure 3**). Morphological changes indicative of cell death were observed in SFE-treated cells compared with SFA-treated and control cells. Cells became detached from the plates at higher SFA and SFE concentrations. The numbers of detached, rounded, and condensed dead cells increased sharply at concentrations > 2.0 μg/mL SFA or SFE, with almost all cells exhibiting this phenotype at 8.0 μg/mL (**Figure 3**). The number of detached cells was greater after 2.0 μg/mL SFE than after 2.0 μg/mL SFA treatment.

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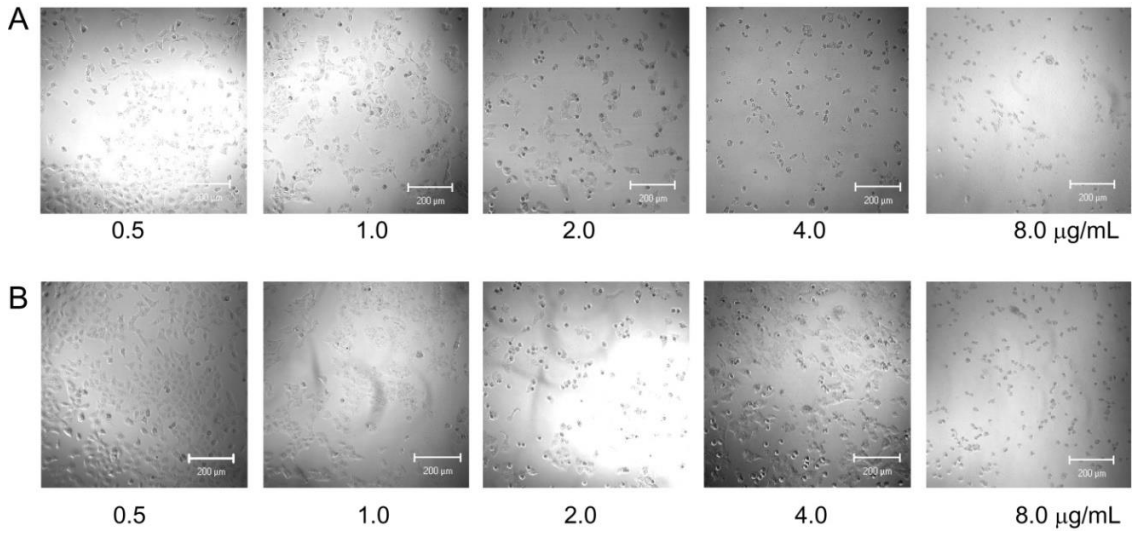

Figure 3. Morphological changes in A549 cells after treatment with SFA (A) and SFE (B).

To confirm and distinguish between apoptotic and necrotic cells induced by SFA and SFE, detached cells were stained with Hoechst and propidium iodide (PI) after SFA/SFE treatment (**Figure 4**). Treatment with both compounds resulted in nuclear changes in a concentration-dependent manner. Hoechst-positive and PI-negative cells with bright or condensed blue nuclei (**Figure 4**, indicated by arrows) displayed morphological features characteristic of apoptosis. Hoechst-negative (or weakly negative) and PI-positive cells, stained red, were necrotic. The SFA and SFE treatments produced detached A549 cells with highly condensed chromatin nuclei in a concentration-dependent manner. However, SFA treatment markedly induced necrosis in cells at 2.0–8.0 μg/mL (**Figure 4A**), whereas SFE treatment resulted in no or few necrotic cells at the same concentrations (**Figure 4B**). At a high concentration (8.0 μg/mL) of SFA or SFE, no apoptotic cells were observed.

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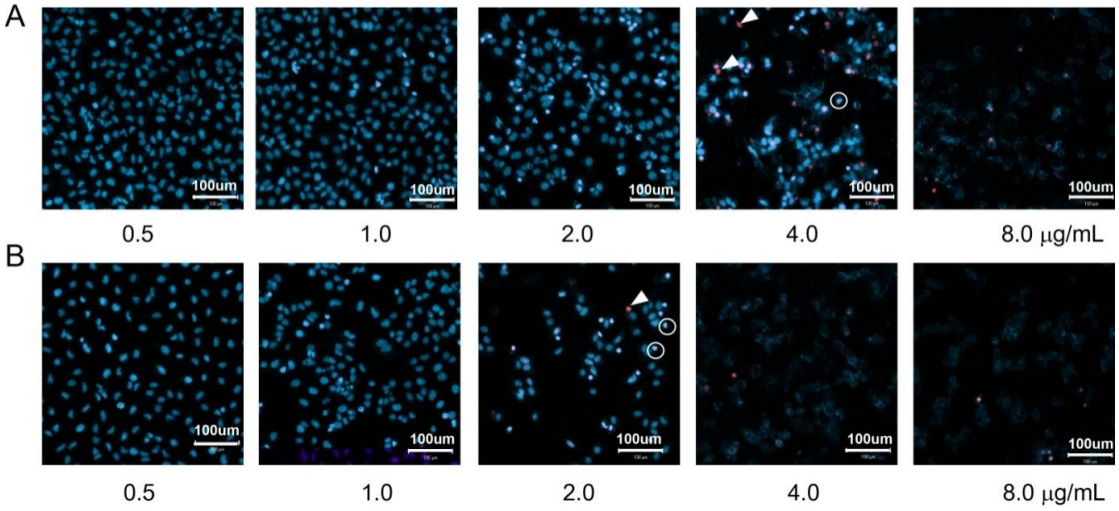

Figure 4. Necrosis and apoptosis induced by SFA (A) and SFE (B) in A549 cells.

The 2.0 μg/mL SFA group exhibited a higher proportion of cells with condensed chromatin among the detached cells (24%), indicating more apoptotic cells, compared with the 2.0 μg/mL SFE group (8%; **Figure 5**). At 4.0 μg/mL, SFE induced little necrosis in A549 cells, but SFA induced a significantly higher degree (55% of detached cells). At 8.0 μg/mL, SFE induced the death of most A549 cells, as indicated by the disappearance of normal blue-and red-stained cells (**Figure 5**).

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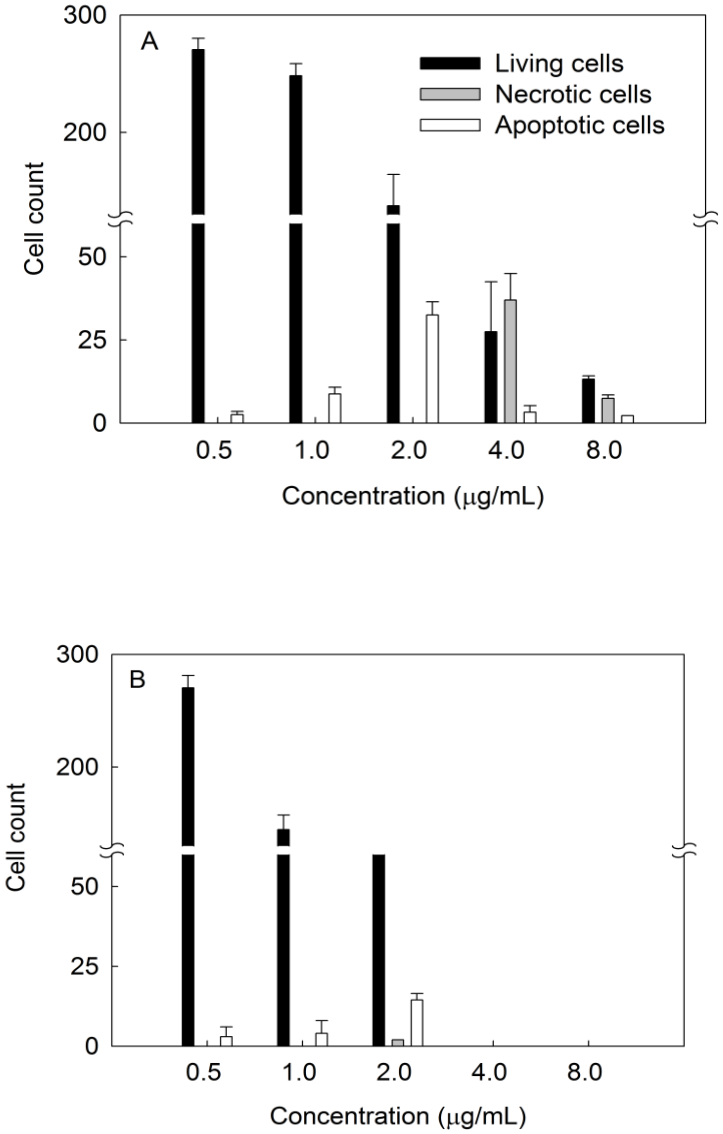

Figure 5. Necrotic and apoptotic cell counts among dead A549 cells after treatment with SFA (A) and SFE (B).

2.3. Induction of apoptosis by SFE in A549 cancer cells

Inactivated poly (adenosine diphosphate ribose) polymerase (PARP) and cleaved caspase-3, -8, and -9 are markers of activation of different apoptotic pathways. A549 cell lysates were prepared and subjected to western blot analysis using antibodies targeting inactivated PARP

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and cleaved caspase-3, -8, and -9. SFE and SFA elevated the levels of these proteins, consistent with apoptotic activation (**Figure 6**). At 8.0 μg/mL SFE, the PARP level was decreased, and the cleaved form of the protein was evident. Cleaved caspase-3 was up-regulated at lower SFE and SFA concentrations (2.0 and 4.0 μg/mL), whereas cleaved caspase-8 and caspase-9 were up-regulated at higher SFE and SFA concentrations (4.0 and 8.0 μg/mL). The levels of all three cleaved caspases were increased more by SFE than by SFA. At a high SFE and SFA concentration (16.0 μg/mL), most cells were necrotic (data not shown); thus, we did not evaluate these cells by western blot analysis.

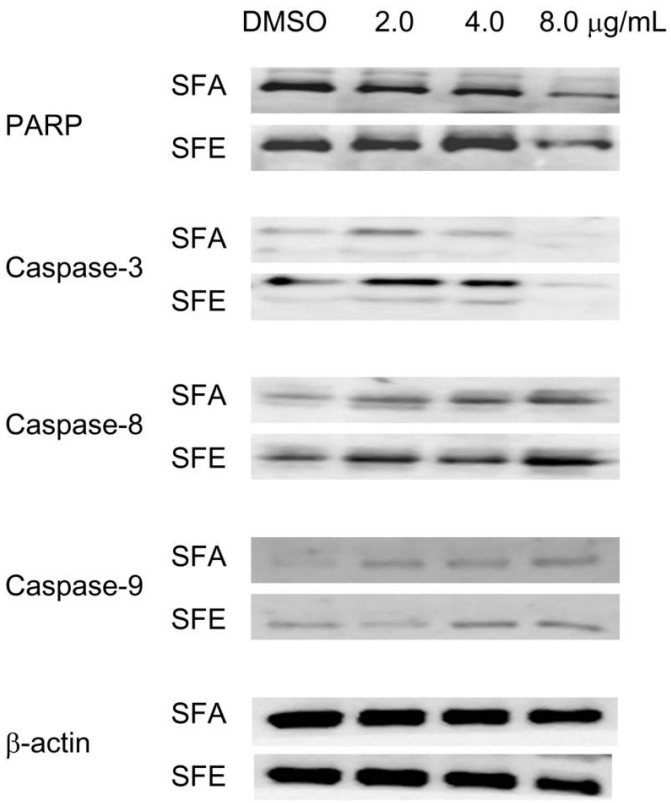

Figure 6. Western blot analysis of four apoptosis pathway proteins in A549 cells after treatment with SFA and SFE.

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3. Discussion

The development of cancer therapeutics must take into account the anatomical location of the cancer cells to be targeted. To define the anticancer properties of natural products, researchers must evaluate inhibition of organ-specific cancer cell growth induced by a chemical carcinogen. Recently, the anticancer properties of ITCs have been demonstrated extensively in several cancer cell lines from different organs *in vitro*.

In this study, to define the general anticancer activities of SFE isolated from radish seeds, we evaluated six organ-specific cancer cell lines (**Figure 1 and Supplementary Table S1**). We demonstrated that SFE obtained from radish seeds inhibited the growth of A549, CHO, HeLa, Hepa1c1c7, and HT-29 cell lines. Moreover, SFE exhibited remarkably effective IC_{50} values (1.37–3.68 μg/mL = 7.8–18.9 μM). Based on our findings, we propose that SFE can be classified as a bioactive compound due to its low IC_{50} (below the defined upper limit of 20 μg/mL) [**21**]. Additionally, SFE isolated from radish seeds was slightly more effective against five cancer cell lines than was SFA obtained commercially. The lung cancer cell lines (A549 and Hepa1c1c7) exhibited greater cytotoxicity than did the other cancer cell lines derived from different organs. Strong anti-proliferative effects of SFE against ovarian and lung cancer cell lines have been reported [**22**]. LnCaP cells treated with SFA clearly exhibited anticancer mechanisms, such as cell cycle arrest [**23**]. However, the viability of LnCaP cells was not decreased by SFE treatment, even at high doses, but was decreased by SFA treatment at 8.0 μg/mL (**Figure 1F and Supplementary Table S1**).

From these results, we deduced that SFE exerts a different spectrum of anticancer properties on various cancer cell lines compared with SFA. The different structures of ITCs isolated from *Brassica* crops have been reported to be responsible for their differential anticancer effects [**24**].

Bioactive ITC concentrations of 5–50 μM, which are effective for the prevention of lung, bladder, and colon cancers, can be achieved by consuming 200–400 g cruciferous vegetables per day [**6**]. We analyzed GRE as a precursor of SFE in different plant organs of 'Taeback' radish plant (**Supplementary Figure S2)**. The highest concentration of GRE was 7.8 mM/g dry weight in mature seeds. Only a few radish seeds per day would be required to achieve the above concentrations.

The broad spectrum of anticancer effects demonstrated by ITCs derived from plant materials has been well investigated. According to the MTT assay results (**Figure 2**), SFE obtained from radish seeds elicited anti-proliferative effects in A549 cells, and the reduced cell viabilities were maintained after SFE washout. Our analysis of cell morphology (**Figure 3**) clearly indicated that SFE inhibits the spreading and elongation of cells, leading to a rounded shape and subsequent detachment from the culture plates. The detachment of cells from the plates is a typical indication of apoptosis. These results were supported by the inhibition of A549 cell proliferation observed after SFE treatment. Hoechst and PI staining demonstrated distinctive morphological features characteristic of apoptosis-related cell death in the dead cells produced after SFE treatment (**Figure 4**). Additionally, no necrosis was observed in the SFE-treated cancer cells at any concentration evaluated. The extent of apoptosis induced by SFE was similar to that induced by SFA (**Figure 5**). Previous reports found anti-proliferative effects of radish extracts on HeLa, hepatoma, and colon cancer cell lines by cell staining [**25–27].**

Some reports demonstrated anti-proliferative effects of SFE or radish extracts, based mainly on cell staining analyses [**19,28**]. We also evaluated apoptosis-related protein expression to show that SFE strongly induces apoptosis in A549 cells (**Figure 6**). PARP, which is responsible for repairing DNA damage, was found in its cleaved inactive form after SFE or SFA treatment.

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Inactivation of PARP implies that SFE induces programmed cell death in lung cancer cells via inhibition of cellular DNA repair systems. In general, the cleavage of caspase-3, -8, and -9 plays an important role in the execution phase of intrinsic and extrinsic apoptosis through inactivation of PARP [**29**].

We also found that SFE on A549 cells induced the cleavage of caspase-3, -8, and -9 involved in apoptosis mechanism (**Figure 6**). The cleaved caspase protein levels varied depending on the SFE/SFA concentration. Additionally, apoptotic activation following caspase-3 protein cleavage resulted in inactivation of PARP, which repairs damaged DNA. The induction of caspase-8 and -9 by 4.0 μg/mL SFE suggests that SFE up-regulates the intrinsic and extrinsic apoptosis pathways in A549 cells. The elevated levels of these apoptotic marker proteins indicate that multiple apoptosis pathways are involved in the cell death elicited by SFE.

Most cruciferous vegetables, such as broccoli, kale, and cabbage, are ranked among the top 10 fruits and vegetables with cancer-fighting abilities. Compared with broccoli, kale, and other cruciferous vegetables, the radish is a better source of the ITC SFE because it lacks ESP, which inhibits the formation of ITCs. When fresh broccoli containing active ESP is consumed, the major hydrolysis product of GRA is predominantly SFA nitrile, which is an inactive form of SFA [**18**]. The major ITCs in the radish plant are SFE and raphasatin (RH), which are abundant in the seeds and sprouts, respectively. Previous studies have demonstrated the potential anticancer properties of SFE and RH [**30,31**]. However, given its rapid degradation, RH has only a weak effect in fighting cancer cells. SFE can remain stable for 1 day, whereas RH is degraded within 28 min after exposure to ambient temperatures [**20**]. Thus, we predict that SFE has greater potential as an anticancer agent than does RH, although we have not compared the anticancer activities of the two ITCs.

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4. Materials and Methods

4.1. Chemical reagents

'Taeback' radish seeds were obtained from Monsanto Korea (Anseong, Korea). All chemicals, antibiotics, and broths were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Becton Dickinson (San Jose, CA, USA). All solvents used for high-performance liquid chromatography (HPLC) analysis were purchased from J.T. Baker (Center Valley, PA, USA). SFE and SFA standards were obtained from LKT Laboratories Inc. (St. Paul, MN, USA). The A549 (lung cancer), CHO (ovarian cancer), HeLa (cervical cancer), Hepa1c1c7 (lung cancer), HT-29 (colon cancer), and LnCaP (prostate cancer) cell lines were a generous donation from the Korean Cell Line Bank (Seoul, Korea; http://cellbank.snu.ac.kr/index.php).

4.2. Extraction and qualitative identification of SFE from radish seeds

The procedure for SFE extraction from radish seeds was adopted from a previous report [**32**]. Radish seeds (100 g) were combined with 1 L water and homogenized for 3 min at 20,000 rpm on ice. The homogenates were incubated for 3 h at 37°C to allow hydrolysis of GRE by endogenous myrosinase. The hydrolyzed seed meals were filtered through five layers of cheesecloth and subjected to centrifugation for 20 min at 8,000 rpm. The supernatant was subjected to three extractions using 1 L dichloromethane (MC). The resulting MC extracts were passed through anhydrous sodium sulfate to remove any water. The MC layer was evaporated to 30 mL using a rotary evaporator (N-1001 series; Eyela Co., Tokyo, Japan) at 35°C. To remove any contaminants, the MC extracts were run through a Sep-Pak silica cartridge (Waters, Milford, MA, USA)

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equilibrated with MC. The cartridge was washed with ethyl acetate, and SFE was eluted with DMSO.

SFE purification was performed using a recycling preparative HPLC system (LC-9104; JAI, Tokyo, Japan) equipped with a Bondapak C18 HPLC column (500 × 3.9 mm, 10 μm particle size; Waters) fitted with a C18 guard column. The mobile phase consisted of an 8:1:1 (v:v:v) chloroform/methanol/water mixture. The flow rate was set at 3.0 mL/min, and the absorbance was monitored at 254 nm. After three rounds of recycling HPLC, crude SFE was partitioned into three fractions. The presence of SFE in all fractions was confirmed by HPLC analysis.

Purified SFE was identified by gas chromatography–mass spectrometry using a modified procedure [**33**]. The HP Model 5890 II system (Hewlett Packard Co., Palo Alto, CA, USA) coupled with the HP 5970 mass selective detector and a fused silica capillary column (DB-5, 0.25 × 30 mm; Agilent Co., Santa Clara, CA, USA) was used. The oven temperature was programmed to increase from 50°C to 250°C at a rate of 10°C/min. The injector temperature was maintained at 250°C. The flow rate of the helium carrier gas was 0.8 mL/min. Mass spectra were obtained by electron ionization at 70 eV over a mass range of 40–500 *m*/*z*. The mass spectrum of SFE isolated from radish seeds was compared with that of a commercial SFE standard (**Supplementary Figure S1**).

4.3. Cell culture conditions and strains

Six cancer cell lines, A549 (lung cancer), CHO (ovarian cancer), HeLa (cervical cancer), Hepa1c1c7 (lung cancer), HT-29 (colon cancer), and LnCaP (prostate cancer), were evaluated for their sensitivities to SFE. SFA from broccoli was used as a positive control. Cell lines seeded at 2 ×

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10^5/mL were maintained under 5% CO_2, 100% relative humidity, and 37°C. The cancer cell lines were cultured as monolayers in Roswell park memorial institute 1640 medium (Sigma-Aldrich Co.) supplemented with 10% fetal bovine serum (Sigma-Aldrich Co.) and 1 mM antibiotic at 37°C in humidified air containing 5% CO_2.

4.4. MTT assay

Anticancer activity was evaluated using a modified MTT cell proliferation assay [**34**]. Cells were seeded at 1 × 10^5/mL in a 96-well plate and incubated for 24 h in 5% CO_2 and 100% humidity at 37°C. Solutions of 0.5–16 μg/mL SFE or SFA were added to each well and gently mixed with the MTT reagent using a multi-channel pipette. DMSO was used as the control. The mixtures were incubated for 24 h until a purple precipitate was visible. The MTT reaction was stopped by the addition of detergent (100 μL). The mixtures were incubated at room temperature in the dark for 2 h. The number of living cells in the wells was determined by measuring the absorbance at 570 nm using a microplate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA, USA). Viability was expressed as IC_{50} and presented as stationary graphs according to concentration. Each experiment was conducted in triplicate, and the results are expressed as means and standard deviations. SFE was removed from culture, and proliferation was assessed again by MTT assay after SFE washout periods of 24, 30, and 36 h.

4.5. Hoechst and PI staining

Phase-contrast microscopy was used to observe morphological changes in cells treated with SFE. Cells were cultured in six-well plates with various concentrations (0.5–8.0 μg/mL) of

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semi-purified SFE for 24 h at 37°C. At 24 h after treatment, SFE was removed from culture, and treated cells were incubated an additional 24 h without SFE. After incubation, photomicrographs were taken using a phase-contrast microscope. PI staining was used to distinguish between apoptotic and necrotic cells. After incubation, cells were washed with phosphate-buffered saline (PBS) solution, fixed in ethanol for 30 min at 4°C, rehydrated with PBS, and incubated with 100 μL PI at 37°C for 5 min. Photomicrographs were taken using a fluorescence microscope (BX51; Olympus Co., Tokyo, Japan).

4.6. Western blot analysis of apoptotic proteins

A549 cells grown on culture plates were treated with SFE or SFA. After treatment, detached cells were collected and washed twice with cold PBS. Proteins were extracted from the cells using radioimmune precipitation assay buffer (50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1.0% IGEPAL CA-630/NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease and phosphatase inhibitors). Protein concentrations were determined using the Bradford protein assay. Extracted proteins (100 μg) were loaded onto a 10% polyacrylamide gel, subjected to electrophoresis, and transferred to a polyvinylidene fluoride membrane. Electrophoresis and blotting were performed using the PowerPac200 Electrophoresis System (Bio-Rad, Hercules, CA, USA). After blocking with 5% nonfat milk for 1 h, membranes were incubated overnight at 48°C with primary antibodies and subsequently probed with horseradish peroxidase–conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG antibodies for 1 h. Protein bands were detected using the Kodak *In Vivo* Imaging System (FX; Kodak Co., New Haven, CT, USA).

5. Conclusions

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In conclusion, previous studies have shown that ITCs derived from radish sprouts and seeds possess antioxidant and anticancer activities using colorimetric methods such as the 2, 2-diphenyl-1-picrylhydrazyl and MTT assays, respectively. Hence, in this study we determined the anti-proliferative properties of SFE obtained from radish seeds by evaluating biochemical markers of apoptosis and performing MTT assays. Our results contribute to knowledge of the anti-proliferative properties of SFE and demonstrate that it is a powerful stimulator of apoptosis. Finally, the results of the present research suggest that the consumption of radish seeds is associated with the prevention of cancer. In the future, biotechnological and agricultural departments will focus on breeding radish seeds or their edible parts to produce optimal SFE contents for cancer prevention.

Supplementary Materials: The following are available online.

Supplementary Table S1. Effects of SFE and SFA on the viability of six cancer cell lines.

Supplementary Figure S1. GC-MS spectra of SFE. (A) A commercially obtained SFE standard and (B) SFE isolated from radish seeds.

Supplementary Figure S2. GRE concentrations in different organs of 'Taeback' radish plant.

Author Contributions: S.L. And J-C.A. And J.K. conceived and designed the experiments. S.L. And E.J.L. analyzed data and wrote the manuscript. S.L. performed the experiments. The authors read and approve the final manuscript.

Acknowledgments: Sooyeon Lim was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2015R1C1A2A01055027).

Conflicts of Interest: The authors declare no conflict of interest.

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