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Full Length Research Paper

Antioxidant and anticancer activity of fractions of the ethanol extract of *Naematoloma sublateritium*

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***Naematolma sublateritium* (Fr.) P. Karst (family: Strophariaceae) is known as a chestnut mushroom. Although *N. sublateritium* is currently a popular and edible fungus in the USA, Japan and Korea, its proximate composition and biological activities have been poorly studied. The present study was carried out to assess the antioxidant and anticancer potential of *N. sublateritium* and to identify the fractions that exhibit pharmaceutical activity among sequentially separated 5 fractions of *N. sublateritium*. *N. sublateritium* contained relatively higher amounts of protein (19.3%) than fat and ash. Ethyl acetate fraction of *N. sublateritium* (EFNS) showed more than 50% free radical scavenging activity and inhibition in lipid peroxidation at 200 µg/ml. In addition, exposure of human cancer cell lines to hexane fractions (HFNS) and a dichloromethane fraction of *N. sublateritium* resulted in the inhibition of cell proliferation and viability. Particularly, HFNS treatment in MDA-MB-231 cells induced apoptotic cell death, causing a condensation of nuclei, poly Adenosine diphosphate (ADP-ribose) polymerase cleavage and regulation of Bcl-2 and Bax protein expressions at an IC_{50} value of 200 µg/ml. These findings clearly demonstrate that *N. sublateritium* has antioxidative activity and anticancer effects, causing apoptotic cell death. In particular, EFNS and HFNS can be putative antioxidant or anticancer substances, respectively.**

Key words: *Naematoloma sublateritium*, antioxidant, anticancer, apoptotic cell death, hexane fraction of *N. sublateritium* (HFNS), dichloromethane fraction of *N. sublateritium* (DFNS), ethyl acetate fraction of *N. sublateritium* (EFNS), *n*-butanol fraction of *N. sublateritium* (BFNS).

INTRODUCTION

Natural products have received increasing attention in recent years due to the pursuit of the discovery of novel and effective biological agents. Mushrooms have traditionally been used to maintain health and their components have been used in the prevention and treatment of diseases in many countries. For example, *Cordyceps* spp. (Fr.) (family: Clavicipitaceae). Links are used as traditional folk medicine for the treatment of inflammatory diseases and stomach disorders in China, Korea, Japan and Thailand (Zhu et al., 1998; Xiao et al., 2004). *Naematoloma* (Syn. *Hypholoma*) *sublateritium* (Fr.) P. Karst (family: Strophariaceae) is known as a chestnut mushroom that can be found growing in tight clusters on hardwood stumps and logs.

Naematoloma sublateritium is rare and less well known than the other species of *Naematoloma* are. Currently, *N. sublateritium* is a popular edible fungus in the USA, Japan and Korea, although it is often considered inedible or even poisonous in Europe. Several studies have reported that the presence of a human Ras-farnesyl transferase inhibitor, clavaric acid, which is a triterpenoid compound, significantly contributes to the antitumor activity of *H. sublateritium* (Godio et al., 2004; 2007;

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Godio and Martin, 2009). However, the biological activities of *N. sublateritium* have been poorly studied. Therefore, the present study aimed to assess the antioxidant and anticancer activity of *N. sublateritium*. We tested the proximate composition and analyzed sequentially separated fractions of *N. sublateritium* with high-performance liquid chromatography (HPLC). A combination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and lipid peroxidation methods were used to evaluate the antioxidant activity of *N. sublateritium* fractions. We used 5 human cancer cell lines (cervical cancer (HeLa), ovarian cancer (SKOV3), colorectal (HT-29) carcinoma, human breast cancer MDA-MB-231 and MCF-7) to analyze the anticancer effect of *N. sublateritium*.

MATERIALS AND METHODS

Fruiting bodies of *N. sublateritium* were obtained and authenticated by Dr. Choi in the Gangwon Forest Research Institute (GFRI, Chuncheon, Korea). A voucher specimen (GFRI-NS-01) was deposited in the Entomopathogenic Fungal Culture Collection of the GFRI. The human cancer cell lines, HeLa, SKOV3 and HT-29 were obtained from the American Type Culture Collection (Rockville, MD) and human breast cancer cells MDA-MB-231 and MCF-7 were from the Korean Cell Line Bank. Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640, α-MEM, fetal bovine serum (FBS), L-glutamine and antibiotics were purchased from GIBCO BRL (Grand Island, NY). Phosphate buffered saline (PBS) and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO). Antibodies against poly (ADP-ribose) polymerase (PARP), Bax, and Bcl-2 were obtained from Cell Signaling Technology (Beverly, MA).

Preparation of *N. sublateritium* fractions

The fruiting bodies were dried at 50°C, crushed in a blender and the crude powder was extracted with ethanol at 70°C for 3 h according to a previous report, 14.6% yield (Kim et al., 2010). The extract was evaporated at 60°C under reduced pressure and resuspended in distilled water. The ethanol extract was sequentially fractionated with equal volumes of hexane, dichloromethane, *n*-butanol and ethyl acetate. Each fraction was concentrated on a rotary vacuum evaporator and passed through 0.2 µm filter paper to remove minor particles. The fractions were dried again by a freeze-drier and stored at -20°C.

Analysis of proximate composition

The proximate composition of *N. sublateritium* was determined by Association of Official Analytical Chemists (1990). Moisture and ash content were determined gravimetrically by desiccation at 105°C and incineration at 550°C for 6 h in an electric furnace, respectively. The crude protein content was obtained with the micro-Kjeldahl method. *N. sublateritium* lipids were extracted using a Soxhlet-extracor apparatus with petroleum ether for 16 h at a solvent condensation rate of 2 to 3 drops/s.

HPLC analysis of the *N. sublateritium* fractions

Each fraction was dissolved in 2 ml Dimethyl sulfoxide (DMSO) before HPLC analysis (WellChrom HPLC-pump K-1001, WellChrom fast scanning spectrophotometer K-2600, and 4-channel degasser K-500) was carried out. HPLC separations were performed on a Gemini 5 µm C_{18} 110A column (30 × 50 mm, Phenomenex, Inc., Torrance, CA). The HPLC mobile phase were H_2O with 0.1% CF_3CO_2H (v/v) for solvent A and CH_3CN with 0.08% CF_3CO_2H (v/v) for solvent B; the gradient condition was as follows: 0 to 5 min, 40% solvent B; 5 to 15 min, 40 to 60% solvent B; and 15 to 25 min, 60% solvent B. The flow rate was 1 ml/min and all samples were injected in volumes of 20 µl. An ultraviolet/visible (UV/VIS) detector was used for quantitation at 278, 335, 400 and 520 nm.

Cell culture

Human cancer cells were cultured in the appropriate medium supplemented with 10% (v/v) FBS, and antibiotics were maintained in an atmosphere of 95% air and 5% CO_2 at 37°C. Monolayer cultures of MCF-7 cells were maintained in α-MEM supplemented with 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, and 1.5 g/L sodium bicarbonate. HeLa, SKOV3, and MDA-MB-231 cultures and HT 29 cells were maintained in RPMI 1640 and DMEM culture medium, respectively.

Proliferation and viability assay

The effect of fractions of NS on cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method which measures the respiratory ability, metabolic reduction of tetrazolium to insoluble formazan by mitochondrial dehydrogenase. In brief, cell lines were plated in 96-well culture plates and allowed to adhere by overnight incubation. The cells were then treated with DMSO (control) or the desired concentration of NS fractions for 24. At the end of the incubation, 20 µl of MTT (5 mg/ml) was added for 4 h at 37°C. After incubation, the supernatant was removed carefully and 150 µl of DMSO was added to each well. After a 10 min incubation and vibration, the absorbance at 490 nm was read. Results are expressed as the ratio of the number of viable cells with *N. sublateritium* treatment fractions to that of the DMSO-treated control. The value of 50% inhibitory concentration (IC_{50}) was used as a parameter for cytotoxicity. Cell viability of the cultured cancer cells was also measured using an automatic cell counter (ADAM-MC, Digital Bio, Seoul, Korea) that analyzed propidium iodide (PI)-negative and PI-positive cells following the addition of PI. PI intercalates into double-stranded nucleic acids and is excluded by viable cells, but it can penetrate the cell membranes of dying or dead cells. The cell death induction by *N. sublateritium* fraction also assessed fluorescence microscopic analysis of cells with condensed nuclei following staining with 4',6-diamidino-2-phenylindole (DAPI) as described previously (Choi and Singh, 2005).

Antioxidant effects of NS: DPPH assay

The free radical scavenging activity of the fractions, based on the scavenging activity of the stable DPPH free radical was determined by the method described by Kuete et al. (2009). In brief, each *N. sublateritium* fraction was serially diluted to provide final concentrations of 100 to 400 µg/ml and was then added to 3 ml of 0.1 mM ethanol solution of DPPH. Absorbance at 517 nm was determined after incubation for 30 min and the percentage of inhibition activity was calculated with $[(A_C - A_{F(S)})/A_C] \times 100$, where A_C is the absorbance of the control and $A_{F(S)}$ is the absorbance of the fraction or standard. The absorbance values were converted into percentage of antioxidant activity. Ascorbic acid was used as a standard control.

Table 1. Proximate compositions of the *N. sublateritium.*

Proximate composition (%, w/w)			
Moisture	**Crude protein**	**Crude ash**	**Crude lipid**
6	19.3	12.4	1.8

Lipid peroxidation and thiobarbituric acid-reactive substances (TBARS)

Peroxidation was evaluated with the TBARS assay using an Oxltek kit from ZeptoMetrix according to the manufacturer's instructions. The assay measured malondialdehyde (MDA, end product of lipid peroxidation) oxidation products using the supplied MDA (0 to 100 nmol/ml) as a standard. Soybean oil and butylhydroxytoluene (BHT) (2.4 ml) or 10 mg/ml of each fraction (final conc. 0 to 400 µg /ml) were mixed in 90 × 20 mm Petri dishes and topped up to 10 ml with PBS. Sequentially, the mixture was incubated for 5 days in air in the dark at 60°C. TBA was added into the tubes containing 5 ml of oxidized mixture and the resulting mixture was then heated at 95°C for 60 min. After cooling, 5 ml of butanol was added to each tube, vortexed and centrifuged at 1200 × *g* for 10 min. The absorbance of the organic upper layer was measured at 532 nm using a spectrophotometer (Perkin-Elmer Lambda EZ 201, Rome, Italy). For correction of endogenous TBARS, fresh samples were boiled without oxidation and values were subtracted. The concentration of MDA (= TBARS) in a specimen was calculated by interpolation from the standard curve. Synthetic antioxidant BHT was used as a control for comparison.

Immunoblotting

Cells were treated with fraction for the specified time interval and lysed as previously described (Choi and Singh, 2005). The cell lysate was cleared by centrifugation at 12,000 × *g* for 20 min and the supernatant was used for immunoblotting. Proteins were resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. Immunoblotting was performed using anti PARP, anti-Bax and anti Bcl-2 as previously described (Choi and Singh, 2005).

Statistical analysis

The statistical significance of the difference in measured variables between the control and each fraction of the *N. sublateritium*-treated groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's test for multiple comparisons. A difference was considered significant at $p<0.05$.

RESULTS

Proximate composition of *N. sublateritium*

The proximate compositions of *N. sublateritium* are displayed in Table 1. The crude protein content of *N. sublateritium* was high (19.3%) compared with that of fat (1.8%) and ash (12.4%), while the moisture content of *N. sublateritium* was approximately 6%. *N. sublateritium* contains higher amounts of protein and ash than other edible mushrooms do, but it contains lower moisture and lipid concentrations (Anonymous, 2006).

HPLC analysis of hexane and dichloromethane fractions of *N. sublateritium* extract

The HPLC analysis chromatogram for the hexane and dichloromethane fractions of the *N. sublateritium* extract is presented in Figure 1. As can be observed, the HFNS reveals major peaks at retention times of 101.3, 102. 1 and 103.5 min at a wavelength of 278 nm. The DFNS was also separated into several major peaks at retention times of 51.2, 66.7, 83.8, 101.9, and 103.3 min at a wavelength of 278 nm. The peak profiles of HFNS and DFNS displayed nonpolar characters at the late retention times.

DPPH free radical scavenging activity of *N. sublateritium*

Free radicals are known as a major cause of oxidative damage to biological molecules, causing coronary arterial disease, aging, and cancer (Siriwardhana et al., 2003). DPPH has been used to evaluate the free radical scavenging capacity of antioxidants. The reduction of DPPH in the presence of an antioxidant that donates a hydrogen atom or an electron is measured at 517 nm. Evaluation of the activity of each *N. sublateritium* fraction was compared with that of ascorbic acid, an antioxidant standard. A significant ($p<0.05$) decrease in the concentration of DPPH radicals was observed due to the scavenging ability of EFNS and BFNS and this decrease occurred in a concentration-dependent manner (Figure 2). Among the fractions, EFNS caused about 50% radical scavenging activity whereas ascorbic acid showed 75.3% inhibition at 200 µg/ml. Their IC_{50} values were 186.3 and of 85.7 µg/ml, respectively (Table2). BFNS had a lower inhibitory activity of approximately 20% at 200 µg/ml. On the other hand, the HFNS, or DFNS and H_2O fractions demonstrated little scavenging activity, indicating that they have no antioxidant activity.

Evaluation of *N. sublateritium* antioxidant activities by TBARS assay

Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form complex compounds,

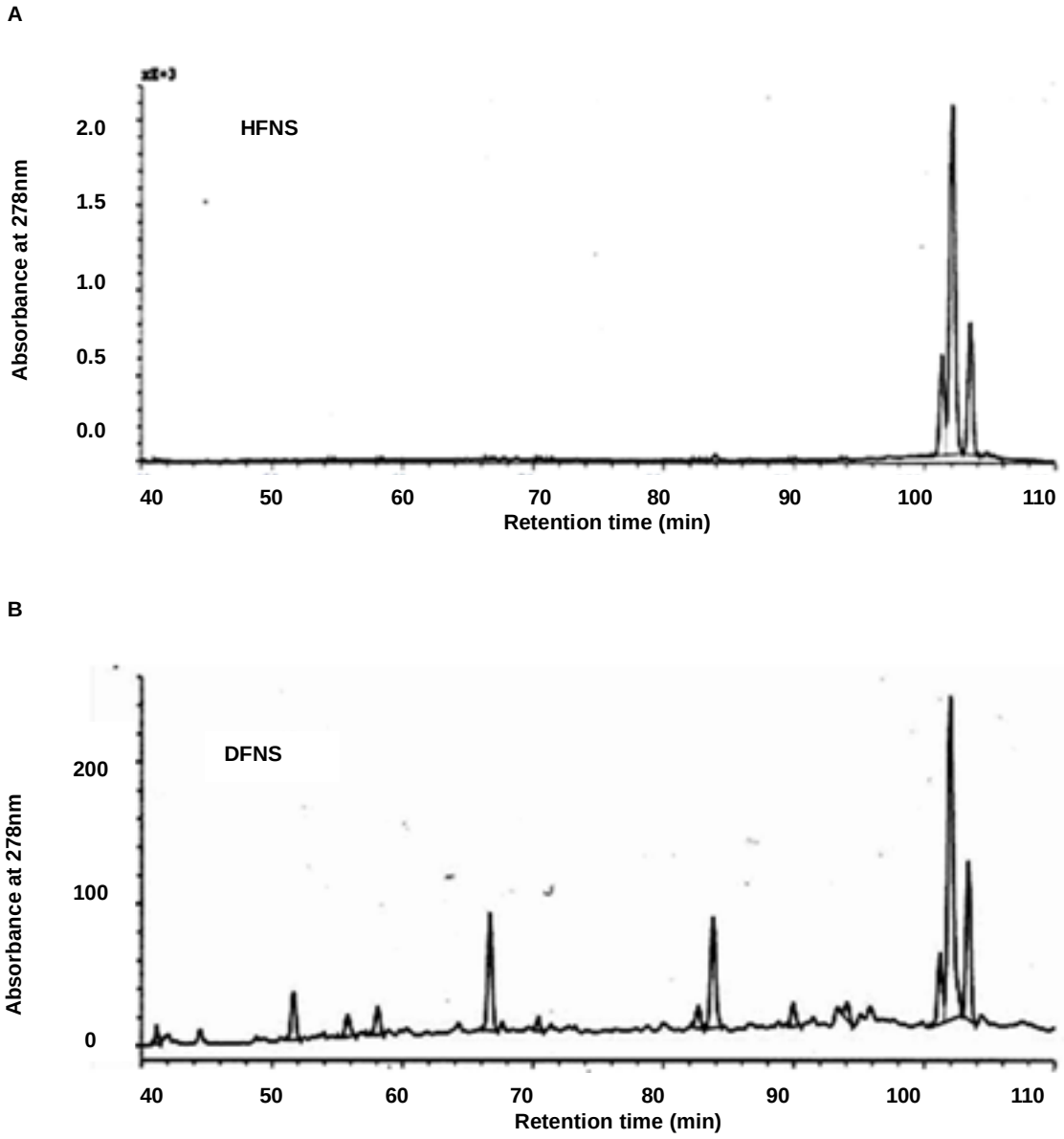

Figure 1. HPLC (278 nm) of the hexane and dichloromethane fractions of *N. sublateritium*. HFNS, A and DFNS, B fractions of *N. sublateritium* were analyzed with the KNAUER chromatography system. Elution solvents were distilled water and acetonitrile. The gradient step of the solvent was "water to acetonitrile 1%/min," and a Vydac C18 column was used.

including the reactive carbonyl group, TBARS. The most abundant product is MDA, which is used as a marker for oxidative stress-induced lipid peroxidation. Antioxidant activity is expressed as TBARS of each *N. sublateritium* fraction in comparison with the activity of BHT. The TBARS values were higher in all samples in which fractions were not added and differed depending on the concentration of each fraction. As shown in Figure 3, the EFNS and HFNS exhibited antioxidant activity with IC_{50} values of 162.4 and 317.2 µg/ml, respectively. While protective antioxidant activity of both EFNS and HFNS was observed in a concentration-dependent-manner, DFNS and H_2O fractions of *N. sublateritium* did not exhibit activity even at the highest concentration (400 µg/ml). These results indicate that EFNS and HFNS exhibited antioxidative potential by effectively removing the primary radical that initiates or propagates lipid peroxidation.

Effects of *N. sublateritium* extract fractions on proliferation and viability of cancer cells

Interestingly, some studies report that *H. sublateritium*

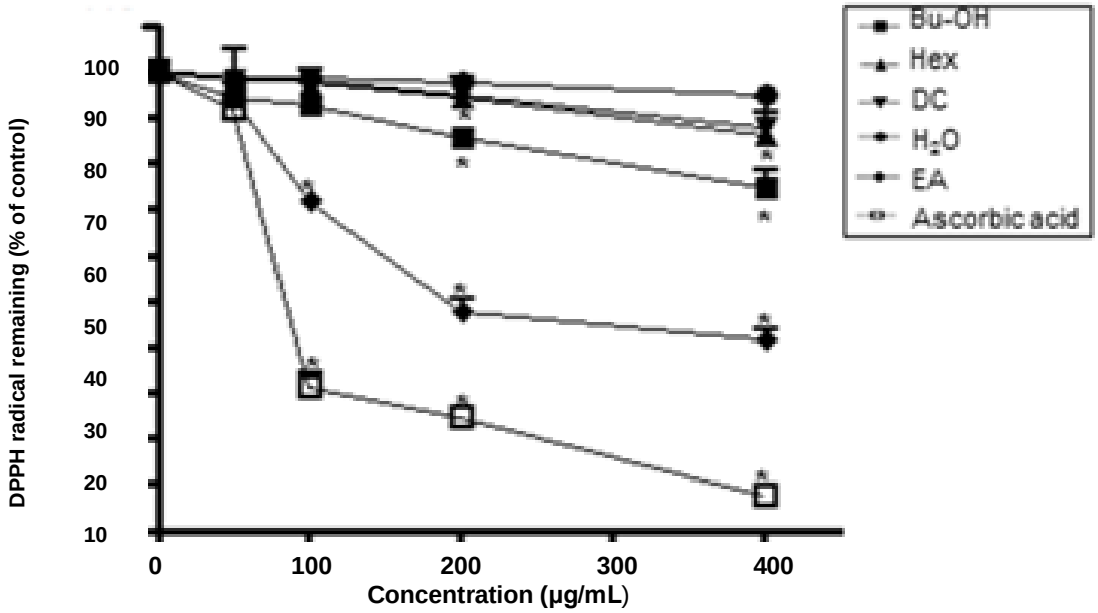

Figure 2. Free radical scavenging activity of fractions of the ethanolic extract of *N. sublateritium* on DPPH. The antioxidative effect of the *N. sublateritium* fractions was estimated by the DPPH assay. The desired concentration (0 to 400 µg/ml) of each fraction was incubated with DPPH solution and then the absorbance at 517 nm was measured. Points, mean (n= 4); bars, SE. *, $p<0.05$, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. Similar results were observed in three replicate experiments. Bu-OH; *n*-butanol fractions of NS, Hex; Hexane fractions of *N. sublateritium*, DC; dichloromethane fractions of *N. sublateritium*, EA; ethyl acetate fractions of *N. sublateritium*, H_2O; H_2O fractions of *N. sublateritium*.

Table 2. Antioxidant activity of *N. sublateritiumn* fractions, BHT and ascorbic acid determined with DPPH method and TBARS assay.

Sample	IC_{50} (µg/ml)	
	DPPH radical scavenging activity	TBARS
n-Butanol	> 400	333.3 ± 3.89
Hexane	> 400	317.2 ± 9.20
Dichloromethane	> 400	> 400
Ethyl acetate	186 ± 2.85[1]	162.4 ± 1.57
H_2O	> 400	> 400
Ascorbic acid	85.7 ± 0.59	ND
BHT	ND	126 ± 1.01

[1] Each value is the mean±SD of triplicate measurements; ND, not determined.

also contains an antitumor isoprenoid compound: the steroidal analogue clavaric acid (Godio et al., 2004, 2007). To investigate the potential of each *N. sublateritium* fraction for cancer cell growth inhibition, we examined their effects on cell proliferation and viability in 5 human cancer cell lines: HeLa, SKOV3, HT-29, MDA-MB-231, and MCF-7. Cancer cell proliferation decreased after 24 h exposure to each fraction in a concentration-dependent manner. For example, treatment with HFNS decreased the proliferation of MDA-MB-231 and SKOV3 cells at an IC_{50} of 200 µg/ml (Figure 4A), as judged by the MTT assay. DFNS also significantly inhibited proliferation of HT-29, SKOV3 and MDA-MB-231 cells (Figure 4B). Particularly, the proliferation of MDA-MB-231 cells was reduced by approximately 48 and 50% relative to DMSO-treated control cells following 24 h exposure to 200 µg/ml of HFNS and DFNS, respectively. BFNS and EFNS did not induce significant reductions even at the highest concentration of 400 µg/ml (data not shown).

We further confirmed the effect of HFNS and DFNS

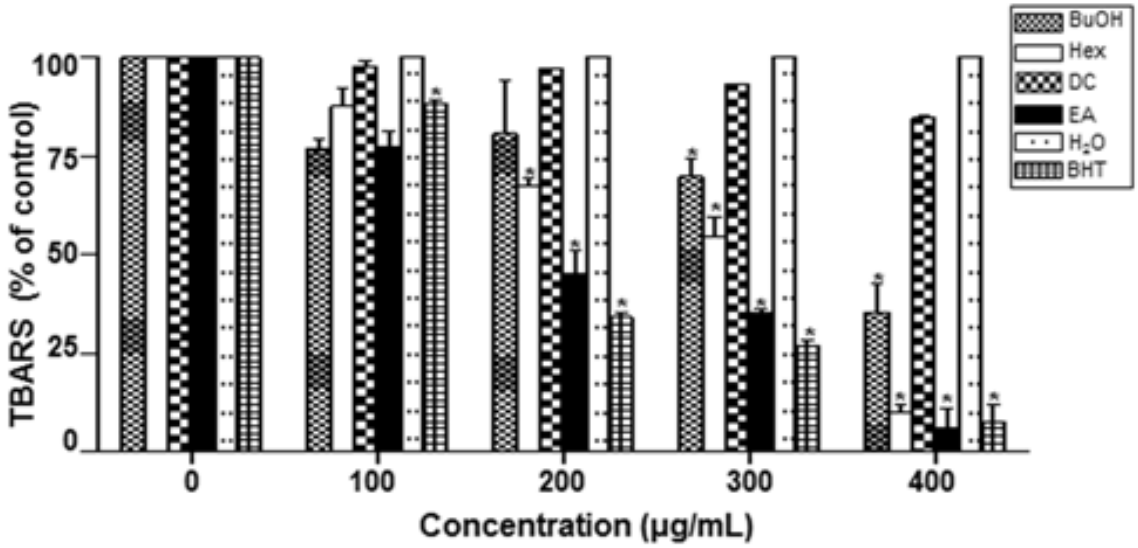

Figure 3. Anti-oxidative activity of fractions of the ethanolic extract of *N. sublateritium*. Anti-lipid peroxidative ability of fractions from the ethanolic extract of *N. sublateritium* was determined by TBARS assay. Soybean oil was incubated for 5 days with each fraction at the indicated concentration (0 to 400 μg/ml) or DMSO. The absorbance at 532 nm was measured. Columns, mean (n= 3); bars, SE. *, $p<0.05$, significantly different as compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. Similar results were obtained in duplicate experiments. Bu-OH; *n*-butanol fractions of *N. sublateritium*, Hex; Hexane fractions of *N. sublateritium*, DC; dichloromethane fractions of *N. sublateritium*, EA; ethyl acetate fractions of *N. sublateritium*, H_2O; H_2O fractions of *N. sublateritium*.

treatment on MDA-MB-231 cell viability via PI staining (Figure 5A). Survival of MDA-MB-231 cells was significantly decreased in a concentration-dependent manner following 24 h exposure to HFNS with an IC_{50} of 200 μg/ml while DFNS elicited only 60% survival at a concentration of 400 μg/ml. HFNS-induced cell death was clearly observed by fluorescent microscopic analysis of cells with condensed nuclei following DAPI staining, which is another method for detecting apoptosis. Representative microscopic images for DAPI staining in MDA-MB-231 cells following 12 h exposure to DMSO (control) or 200 μg/ml HFNS are illustrated in Figure 5B. Consistent with the results of PI staining, apoptotic cells with condensed nuclei were visible in MDA-MB-231 cells cultured in the presence 200 μg/ml of HFNS. Collectively, these results indicate that HFNS treatment selectively decreases the viability of MDA-MB-231 breast cancer cells and does not exhibit nonspecific cytotoxicity.

Furthermore, HFNS-induced cell death was confirmed by cleavage of PARP, which is a deoxyribonucleic acid (DNA) repair enzyme and biochemical hallmark. Figure 5C shows that PARP cleavage was observed in HFNS-treated MDA-MB-231 cells. The Bcl-2 family proteins play critical roles in the regulation of apoptosis by functioning as either promoters or inhibitors of cell death (Choi and Singh, 2005). We also observed the effect of HFNS treatment on the expression levels of the Bcl-2 and Bax proteins. HFNS treatment resulted in a modest increase (1.4-1.9- fold increase over the control) in levels of the pro-apoptotic Bax protein, especially at concentrations of 100 to 200 μg/ml. HFNS-treated MDA-MB-231 cells exhibited a 37.5 to 43.8% decrease in the levels of anti-apoptotic protein Bcl-2 relative to the control (Figure 5C).

DISCUSSION

There has been an accumulating of evidence indicating that natural products not only offer protection against oxidative reaction but also suppress proliferation of cancer cells in culture as well as *in vivo* (Han et al., 1999; Siriwardhana et al., 2003; Li et al., 2006; Kuete et al., 2009). Furthermore, studies including those in our laboratory have indicated that inhibitory effects of natural constituents against the proliferation of cancer cells is attributable to cell cycle block as well as apoptosis induction (Choi and Singh, 2005; Wu et al., 2007; Kaur et al., 2008; Choi et al., 2009). In this study, we designed experiments to investigate the biological effects of NS using sequentially prepared fractions from an NS ethanol extract. We demonstrated for the first time that EFNS and HFNS have antioxidative activity and anticancer effects on cultured human cancer cells, with IC_{50} of 186 and 200 μg/ml, respectively.

To identify the biological properties of *N. sublateritium*, we extracted *N. sublateritium* with ethanol and the yield obtained was 14.6%. The ethanol extract was sequentially separated and fractions of hexane, dichloromethane,

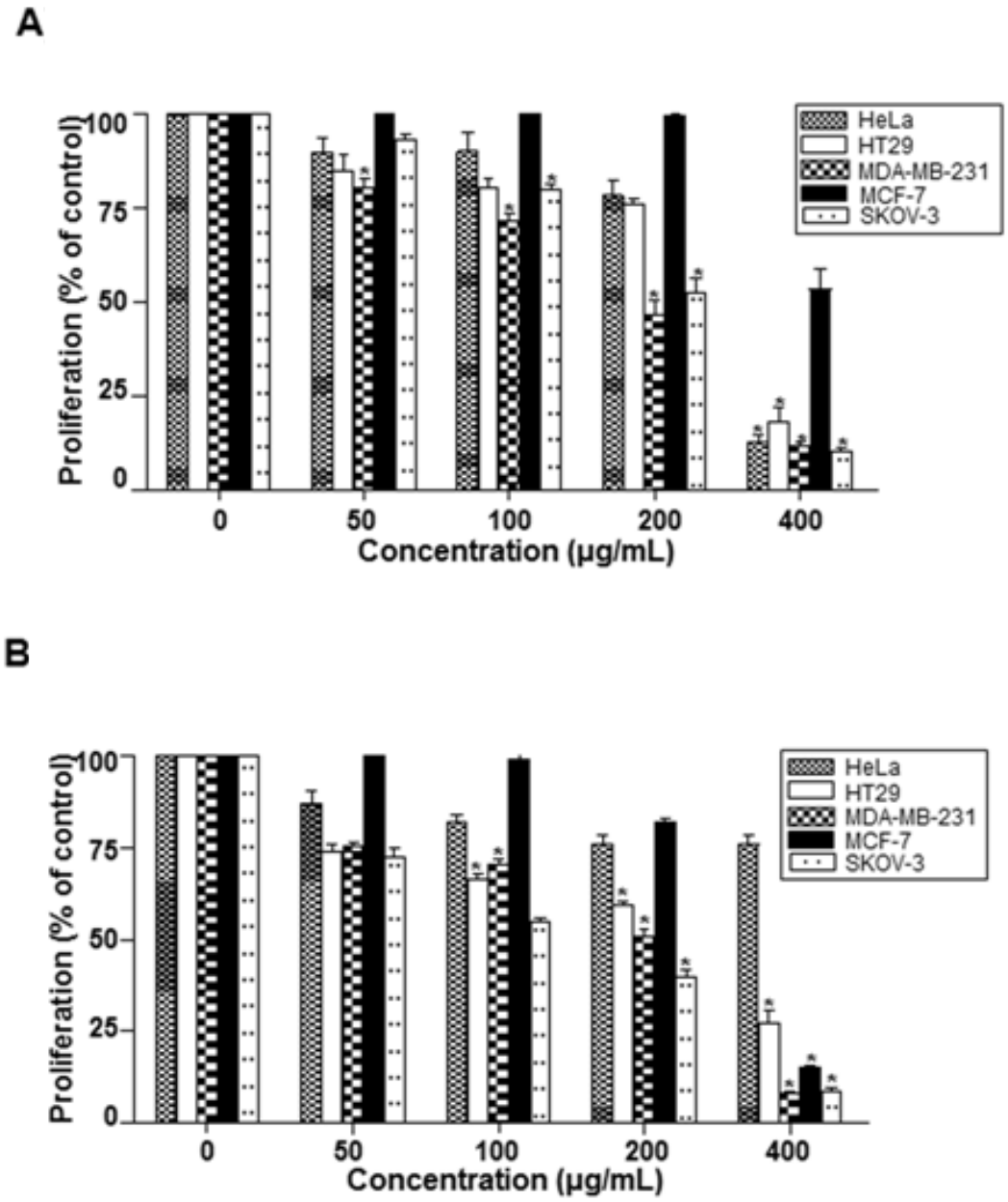

Figure 4. Treatment with the fractions of the ethanolic extract of *N. sublateritium* induced inhibition of cancer cell proliferation. The effects of HFNS; **A** and DFNS; **B** treatment on the proliferation of 5 human cancer cell lines were determined by MTT assay. Human cancer cell lines were exposed to DMSO (control) or the desired NS fraction concentrations for 24 h. Columns, mean (n = 3); bars, SE. *, $p<0.05$, significantly different as compared with control by one-way ANOVA followed by Dunnett's test. Similar results were observed in three independent replicate experiments.

n-butanol, and ethyl acetate generated yields of 0.56, 0.36, 0.15 and 1.65%, respectively. In addition, the amount of protein obtained from *N. sublateritium*, which was higher than that of other edible mushrooms, might be correlated with the antioxidative activity of *N. sublateritium*, due to the sulfate, thiol and amino groups it contains (Anonymous, 2006). Excessive production of free radicals results in oxidative stress, which leads to damage of macromolecules such as nucleic acids, proteins and lipids and may eventually lead to cancer (Sinclair et al., 1990; Dreher and Junod, 1996). Antioxidants interact with free radicals and stabilize them and may be used in cancer prevention and treatment to repair some of the damage caused by free radicals.

In this study, EFNS, BFNS and HFNS demonstrated antioxidant activity and EFNS exhibited statistically

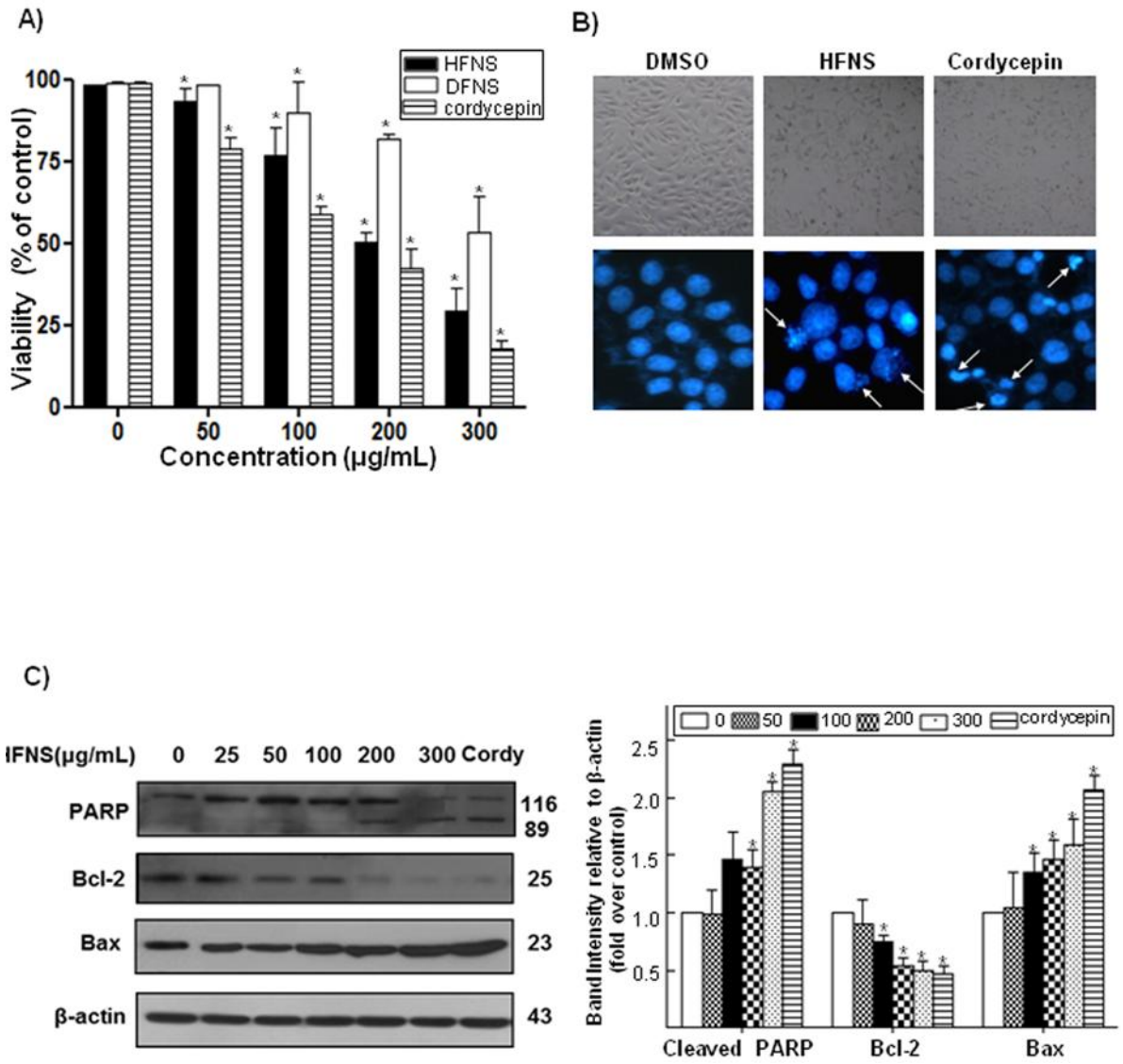

Figure 5. Both HFNS and DFNS treatment reduced viability in the human breast cancer cell, MDA-MB-231, causing apoptotic cell death. **A** Effects of HFNS and DFNS treatment on the viability of MDA-MB-231 cells were determined by PI staining and were compared with the DMSO-treated control. Corycepin (100 μM) was used as a positive control. Similar results were observed in three independent replicate experiments. **B** and **C**. MDA-MB-231 cells were exposed to DMSO (control) or 200 μg/ml HFNS for the indicated time. **B**. Upper panel: morphological changes of MDA-MB-231 cells were observed under phase-contrast microscopy following a 12 h exposure to solvent control (DMSO) or HFNS (Magnification, ×100). Lower panel: Representative DAPI staining for detection of apoptotic cells with condensed nuclei was performed. Arrows, apoptotic cells with condensed nuclei (magnification, ×400). **C**. Immunoblotting for PARP, Bcl-2, and Bax was performed using lysate from HFNS-treated cells (24 h). The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. Immunoblotting for each protein was done three times using independently prepared lysates, and the results were similar. Relative expression folds were calculated using quantified intensity followed by normalization to β-actin. Columns, mean (n = 3); bars, SE. *, $p<0.05$, significantly different as compared with control by one-way ANOVA followed by Dunnett's test.

significant antioxidant activity compared to the ascorbic acid and BHT controls. The BFNS and ascorbic acid did not display antioxidant activity in TBARS, although the BFNS demonstrated increased DPPH free radical scavenging activity. This could be explained by their polarity; nonpolar antioxidants in emulsions concentrate at the lipid/air surface and exhibit high protection of the emulsion itself (Koleva et al., 2000). The finding that the HPLC profile of the BFNS was more polar than that of other *N. sublateritium* fraction profiles supports this premise (data not shown). The BFNS, which contained compounds, such as water-soluble phenolics did not

generate the expected TBARS assay result. Cancer is a result of uncontrolled cell proliferation as well as the dysregulation of apoptotic cell death (Han et al., 2007). It has been suggested that induction of apoptosis or arrest of cell cycle progression by treatment of chemotherapeutic agents has become an indicator of tumor treatment response (Parker et al., 1998). We observed that the HFNS and DFNS containing nonpolar compounds, as shown in the HPLC histogram, caused growth-inhibitory effects against human cancer cells in MTT assay. It is still possible that the decrease of proliferation in MTT assay was attributed to uncontrolled event such as loss of cell homeostasis, cytostatic stress and cytotoxicity. However, the result of MTT assay in this study clearly showed a selective anti-proliferative activity of HFNS and DFNS against MDA-MB-231, SKOV3 and HT-29 cancer cells in a dose-dependent manner. The inhibitory effect of HFNS and DFNS on cancer cells was also confirmed by PI and DAPI staining, presenting condensed or fragmented nuclei. These results strongly indicate that HFNS and DFNS appear to be a potential source of broad-spectrum tumoricidal activities.

Indeed, HFNS treatment induced several features of apoptosis such as condensed nuclei, PARP cleavage and regulation of Bcl-2 and Bax protein expressions which was accompanied with an increase of concentration, while DFNS did not show significant features in MDA-MB-231 breast cancer cells. These data also demonstrate that the pharmacological activity of HFNS may have been brought about through the induction of apoptotic cell death in the cell lines examined. The results of the present study support the traditional use of mushrooms for the prevention and treatment of diverse diseases.

The American National Cancer Institute guidelines define the limit of activity for crude extracts at IC_{50} of proliferation as less than 30 µg/ml after 72 h exposure (Suffness and Pezzuto, 1990). Although the *N. sublateritium* fractions exhibited higher IC_{50} values than this threshold value at 24 h, they are still suggested as potential anticancer agents.

Conclusions

Collectively, our data provides evidence for the potential use of *N. sublateritium* fractions in the prevention, or even treatment of the oxidative process and human cancer cells. Further studies are needed to identify and characterize the bioactive substances that function as antioxidants as well as anticancer agents in these fractions.

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