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일엽초 추출물의 항산화 및 항암 효과

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Antioxidant and Anticancer Properties of the Extracts from *Lepisorus thunbergianus* (Kaulf.) Ching

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ABSTRACT : *Lepisorus thunbergianus* (Kaulf.) Ching has been used in folk medicine in Korea. In this study, a *L. thunbergianus* methanol extract and its fractions were investigated for their antioxidant properties. The results showed that the ethyl acetate and butanol fractions of *L. thunbergianus* possess potent DPPH radical scavenging activities. Both fractions also possessed reducing power and inhibited reactive oxygen species formation. In addition, the cytotoxic activity of the *L. thunbergianus* n-hexane fraction (HF) was investigated. The results suggested that the HF remarkably suppressed proliferation of human breast, liver and colon cancer cells. These results demonstrate, for the first time, that *L. thunbergianus* extract induces apoptosis in SW620 cells, suggesting that *L. thunbergianus* may have potential as a therapeutic agent for colon cancer.

***Key Words* : *Lepisorus thunbergianus* (Kaulf.) Ching, Antioxidant, Anticancer, Apoptosis**

INTRODUCTION

Flavonoids are a large group of polyphenolic compounds with antioxidative activity and scavenging effects on reactive oxygen species (ROS) (Nijveldt *et al*., 2001). Highly reactive free radicals may cause cardiovascular disease (Sugamura and Keaney, 2011), neurodegenerative disease, diabetes (Jang and Seong, 2014), rheumatoid arthritis, cataracts (Seo and Jeong, 2014), and aging (Liochev, 2013). Superoxide is a ROS generated within the mitochondria that can result in the induction of additional ROS (Grivennikova and Vinogradov, 2006). Therefore, increasing the level of these ROS creates oxidative stress, which may lead to DNA damage and mutations (Hogan *et al*., 2010). Antioxidants function to prevent the adverse effects of oxygen by capturing free radicals and preventing chronic complications, in part, through their interactions with ROS (Seifried *et al*., 2007).

Cancer is the second leading cause of death in the Western world (Madhusudan and Middleton, 2005). According to the World Health Organization, more than 10 million new cancer cases are diagnosed each year worldwide, and statistical trends indicate 15 million new cases in 2020 (Mignogna *et al*., 2004). Different agents show a wide range of anticancer effects (Damm *et al*., 2001; Zou *et al*., 2006), but drugs often have unpleasant side effects and complications (Mittal *et al*., 2004). Thus, it is necessary to seek alternative medicines, preferably

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herbal therapies such as *Lepisorus thunbergianus*, which is an evergreen fern that grows on rocks and tree trunks throughout East and Southeast Asia and has been used in folk medicine for diuretic and hemostatic (Choi *et al*., 1999). Oral cavity cancer cells treated with a *L. thunbergianus* extract showed significant concentration-dependent growth inhibition (Chung *et al*., 1998). Previous studies have reported that isovitexin, orientin, isoorientin and chlorogenic acid were isolated from *L. thunbergianus* (Yang *et al*., 2015). However, no study has reported on the cytotoxic properties of *L. thunbergianus* on human breast, liver, and colon cancer cells. Thus, the objective of this study was to evaluate antioxidant activites and cytotoxic properties of the *L. thunbergianus* extracts by determining ROS production, reducing power, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability, and total phenolic and flavonoid contents.

MATERIALS AND METHODS

1. Preparation of the crude extract and fractionations

The whole plant of *L. thunbergianus* was obtained from Kangwons Medicinal Plant, Wonju city, Korea in July 2013. The plant was identified and authenticated by Dr. HeoKweon, a taxonomist in the department of Applied plant sciences of Kangwon National University. A voucher specimen is deposited in the herbarium of Department of Applied plant sciences, Kangwon National University.

The entire *L. thunbergianus* plant was dried in the shade at room temperature and then powdered. A 1.2 ㎏ of sample powder was extracted with 100% methanol. The methanol extract was filtered and three sample replicates were extracted under the same conditions with new solvent. The methanol extracts were filtered and evaporated under reduced pressure using a vacuum rotary evaporator (N-1110, Eyela, Tokyo, Japan) to produce a crude extract of 283.2 g. The crude extract was suspended in deionized water and partitioned sequentially with n-hexane, ethyl-acetate (EtOAc) and butanol (water saturated BuOH) fractions, which were evaporated using a vacuum rotary evaporator. The fractions were stored under refrigeration for further analysis.

2. Determination of total phenolic and flavonoid contents

Total phenolic content was measured by the method of Folin-Ciocalteu assay (Singleton and Rossi, 1965). Briefly, 0.1 ㎖ of sample at different concentrations was mixed with 0.05 ㎖ Folin-Ciocalteu reagent and mixed thoroughly. A 0.3 ㎖ aliquot of 20% sodium carbonate was added 3 min later, and the mixture was shaken intermittently. The reaction mixture was incubated for 15 min at 20℃. Finally, 1 ㎖ of distilled water was added and absorbance measured at 725 ㎚ in triplicate, and the data were expressed as gallic acid equivalent (GAE) per ㎎ of extract, based on the gallic acid calibration curve.

Total flavonoid contents of the sample were analyzed according to a colorimetric method reported previously (Park *et al*., 1997). In brief, 0.1 ㎖ of sample or quercetin (Sigma, St. Louis, MO, USA) standard was mixed with 0.9 ㎖ 80% EtOH. Each reaction mixture contained 0.5 ㎖ of standard or sample solution, 0.1 ㎖ 10% aluminum chloride (Sigma, St. Louis, MO, USA), 0.1 ㎖ 1 M potassium acetate and (Sigma, St. Louis, MO, USA) 4.3 ㎖ 80% ethanol. After a 40 min reaction at room temperature, absorbance of each reaction was measured at 415 ㎚. Results are expressed as g quercetin per ㎏ of the extract, based on the quercetin calibration curve.

3. DPPH radical scavenging activity

The DPPH radical scavenging activity of the extract was determined by the method of Blois (1958) with some modifications. Initially, 4 ㎖ of methanol solution containing 0.1 ㎖ of sample at different concentrations was mixed with 1 ㎖ 0.15 mM DPPH (Sigma, St. Louis, MO, USA) dissolved in methanol. The reaction mixture was incubated for 30 min at room temperature. The control contained all reagents without the sample, and methanol was used as the blank. Measurements were performed in triplicate. DPPH radical scavenging activity was determined by measuring absorbance at 517 ㎚ using a spectrophotometer (V530, JASCO Co., Tokyo, Japan). DPPH radical scavenging activity was expressed as the percentage of free radical inhibition by the sample and was calculated using the following formula, (%) inhibition rate = [absorbance of sample/ (1/2 absorbance of control)] × 100. The IC_{50} value (㎎/㎖) is the concentration at which scavenging activity is 50%.

4. Reducing power assay

Reducing power was measured according to a method reported by Oyaizu (1986) with some modifications. An

aliquot of each extract (0.1 ㎖) was mixed with 0.5 ㎖ of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 ㎖ 1% potassium ferricyanide (Sigma, St. Louis, MO, USA) followed by a 20 min incubation at 50℃. After 2.5 ㎖ of 10% trichloroacetic acid (*w/v*) was added, a 0.5 ㎖ aliquot of the upper layer was mixed with distilled water (0.5 ㎖). Ferric chloride (0.1 ㎖, 0.1%) was added and absorbance was measured at 700 ㎚ against a blank consisting of all reagents without the sample. A higher absorbance indicated higher reducing power. BHT and BHA were used for comparison.

5. Cell culture and sample treatment

RAW 264.7, $HepG_2$, Huh7, CoLo205, WiDr, HT-29, RKO, LoVo, HCT-116, MCF-7, and MDA-MD-231 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). $HepG_2$, Huh7, CoLo205, WiDr, HT-29, RKO, LoVo, HCT-116, MCF-7, and MDA-MD-231cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% antibiotics (penicillin and streptomycin, 100 U/㎖). RAW 264.7 cells were grown in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin, 100 U/㎖). The cells were incubated at 37℃ in a humidified chamber with a 5% CO_2 atmosphere.

6. Cell viability

Cell viability was determined by the MTT assay. Briefly, MTT (5 ㎎/㎖), dissolved in phosphate-buffered saline (PBS, Gibco, Carlsbad, CA, USA), was filtered through a 0.2 ㎛ filter and stored at 2℃ for routine use. This stock solution was diluted 10 fold with RPMI for each culture assayed, and the MTT (0.1 ㎎/㎖) solution was added to each well of a 96-well plate. The culture wells were washed once with 1 ㎖ of PBS to remove the remaining test sample after culture supernatants were collected and before the MTT solution was added. After an additional 4 h incubation for at 37℃ in a humidified chamber, the formazan crystals were dissolved by adding DMSO (100 ㎕). Absorbance was read at 540 ㎚ using an enzyme-linked immunosorbent assay reader multilabel counter (Wallac VICTOR^2, Tokyo, Japan).

7. Determination of ROS generation

The evolution of ROS was assessed by the 2', 7'-dichlorofluorescein diacetate (H_2DCFDA) assay (Schauss *et al.*, 2006). In brief, RAW 264.7 cells were plated in a 6-well dish in PBS buffer for 30 min at 37℃. The cells were washed twice with 1 ㎖/well PBS. ROS production was induced by lipopolysaccharide (LPS) (100 ng/㎖), which was added 30 min before. ROS production was analyzed immediately by fluorescence activated cell sorter (FACS, Becton Dickinson, San Jose, CA, USA).

8. Flow cytometry assay

The proportion of SW620 cancer cells undergoing apoptosis was measured using an Annexin V-FITC apoptosis detection kit (Enzo Life Scinece, New York, USA) according to the manufacturer's instructions. Cells (1×10^5) were seeded into 6-well plates for 24 h. Next, the culture medium was replaced with fresh medium, and the cells were treated with different concentrations of the hexane fraction (HF) for 24 h. Following digestion with 0.05% trypsin-EDTA for 3 min, the cells were collected and centrifuged at 2,000 rpm for 5 min. The pellets were then washed twice with PBS and re-suspended in a 1 × binding buffer at a concentration of 1×10^5 cells/㎖. Subsequently, 5 ㎕ of Annexin V-FITC conjugate and 5 ㎕ propidium iodide (PI) were added, and the cells were kept in the dark at room temperature for 10 min. The Annexin V-FITC-/PI-stained cells were analyzed using fluorescence activated cell sorter (Becton-Dickinson, Franklin Lakes, NJ, USA).

9. RNA preparation and reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA extraction, reverse transcription, and PCR analyses were performed as described previously (Lahti *et al.*, 2006) with some modifications. SW620 cells (1×10^5 cells) were incubated in a 6-well plate. After a 24 h incubation, cells were treated with sample for predetermined times and then the cells were harvested and washed twice with cold PBS. Total RNA was isolated with Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA (2 ㎍) containing oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). The primer and probe sequences and concentrations were optimized according to the manufacturer's instructions for Tag DNA Polymerase

part number P025A and were as follow; β-actin-F, 5'-TAC AGC TTC ACC ACC ACA GC-3', β-actin-R,5'-AAG GAA GGC TGG AAA GC-3', Survivin-F, 5'-ATT CGT CCG GTT GCG CTT TCC-3', Survivin-R, 5'-CAC GGC GCA CTT TCT TCG CAG-3'. Bax-F, 5'-TCC ACC AAG AAG CTG AGC GA-3'. Bax-R, 5'-GTC CAG CCC ATG ATG GTT CT-3'. Bcl-2-F, 5'-TGT GGC CTT CTT TGA GTT CG-3'. Bcl-2-R, 5'-TCA CTT GTG GCT CAG ATA GG-3'. p53-F, 5'-TGT GGA GTA TTT GGA TGA CA-3'. p53-R, 5'-GAA CAT GAG TTT TTT ATG GC-3'. Caspase-3-F, 5'-TCA CAG CAA AAG GAG CAG TTT-3'. Caspase-3-R, 5'-CGT CAA AGG AAA AGG ACT CAA-3'. After amplification, portions of the PCR reaction products were electrophoresed on 1% agarose gel, stained with ethidium bromide.

10. Immunoblot analysis

SW620 cells (1×10^6 cells) were incubated in a 6-well plate. After a 24 h incubation, the cells were treated with the HF for 24 h and then harvested and washed twice with cold PBS. After centrifugation, the cells were lysed in 50 ㎕ lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglyco-tetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1% Triton X-100, 10% glycerol, 10 ㎍/㎖ aprotinin, 10 ㎍/㎖ pepstatin, 1 mM benzimide, and 2 mM hydrogen peroxide). The lysate was incubated on ice for 10 min followed by sonication for 5 sec. The lysate was then centrifuged at $12,000 \times g$ for 10 min at 4℃. The supernatant was collected and protein concentration was determined by the Bradford assay. Aliquots of the lysates (40 ㎍ of protein) were heated at 94℃ for 5 min and separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (Bio-Rad, Hercules, CA, USA) membranes. The membranes were blocked in blocking buffer (Tris-buffered saline containing 3% BSA, 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20) for 60 min at room temperature. The membrane was incubated for 60 min with appropriate primary antibody at room temperature, washed three times with the TBST buffer (Tris-buffered saline containing 20 mM NaF, 2 mM EDTA and 0.2% Tween 20), incubated for an additional 60 min with HRP-conjugated secondary antibody, and washed three times with TBST buffer. Bound antibodies were detected with an enhanced chemiluminescence system.

11. Data analysis

Data are expressed as means ± standard deviations. Statistical significance was determined by analysis of variance. Duncan's Multiple Range Test (DMRT) was used to detect differences between groups. A significant difference was considered at the level of $p < 0.05$.

Results and Discussion

1. Total phenolic and flavonoid contents

It is widely accepted that the antioxidant activity of plant extracts is related to total phenolic content (TPC) and total flavonoid content (TFC) (Zou *et al*., 2012), although solvents with different polarities have different effects on TPC and antioxidant activities (Xu and Chang, 2007). Therefore, we analyzed TPC and TFC from the methanol extract and different *L. thunbergianus* fractions. As shown in Table 1, TFC in the samples was EtOAc fraction (106.56 ± 2.86 ㎎ QE/g) > BuOH fraction (45.28 ± 1.22 ㎎ QE/g) > methanol extract (35.29 ± 0.64 ㎎ QE/g) > water fraction (12.76 ± 0.26 ㎎ QE/g) > n-hexane fraction (5.45 ± 2.27 ㎎ QE/g). TPC was 27.44 ± 8.77 ㎎ GAE/g in the water fraction. As expected, the EtOAc fraction (275.74 ± 1.67 GAE/g) showed higher TPC than that of the other fractions. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which play an important role adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, and decomposing peroxides (Zheng and

Table 1. Total phenolic and flavonoid contents and DPPH radical scavenging activity of the *L. thunbergianus* extract and fractions.

Fraction	DPPH radical Scavenging activity IC_{50} (㎍/㎖)	TPC[1)] (㎎ GAE/g)	TFC[2)] (㎎ QE/g)
E	11.69 ± 0.09^{a}*	275.74 ± 1.67^{a}	106.56 ± 2.86^{a}
B	13.30 ± 0.05^{b}	150.38 ± 1.92^{b}	45.28 ± 1.22^{b}
M	43.59 ± 0.35^{c}	106.27 ± 4.96^{c}	35.29 ± 0.64^{c}
W	157.28 ± 0.23^{d}	27.44 ± 8.77^{d}	12.76 ± 0.26^{d}
H	923.53 ± 0.28^{e}	18.76 ± 4.00^{e}	5.45 ± 2.27^{e}
BHA	14.73 ± 0.08^{d}		
BHT	132.99 ± 0.30^{d}		

[1)]Total phenolic contents, [2)]total flavonoid contents, M; methanol extract, H; n-hexane fraction, E; ethyl acetate fraction, B; butanol fraction, W; water fraction. *Mean values within the same column followed by different letters (a-e) are significantly different at $p < 0.05$ by DMRT.

Wang, 2001). Therefore, the higher TPC in the EtOAc fraction might account for its higher reducing power and DPPH radical scavenging effect.

2. DPPH radical scavenging activity

DPPH is an unstable purple free radical, but changes to a stable yellow compound upon reaction with an antioxidant. It is common to measure the concentration of antioxidant required to reduce or inhibit 50% of the DPPH radicals present (Ramrez-Mares *et al.*, 2010). The scavenging abilities of the extract and various fractions against the DPPH radical are illustrated in Table 1, and the results are normalized and expressed as IC_{50} values (㎍/㎖) for comparison. The results demonstrated that all extracts and fractions tested possessed radical-scavenging activity. The EtOAc fraction displayed a stronger ability to decrease DPPH radicals (IC_{50} = 11.69 ㎍/㎖) than that of the other fractions. In addition, the BuOH fraction (IC_{50} = 13.30 ㎍/㎖) showed higher radical scavenging activity than that of BHT (IC_{50} = 132.99 ㎍/㎖). Reynertson *et al.* (2005) reported a good linear correlation between total phenolic and DPPH antioxidant capacity. The conclusions were agree with Chirinos *et al.* (2010), which reported that the antioxidant potential of camu fruit, related to its phenolics content. From the above these results suggest that the antioxidant capacity is correlated with phenolic compounds. Thus, the EtOAc and BuOH fractions have a strong DPPH radical scavenging activities.

3. Reducing power assay

Reducing power reflects antioxidant activity (Duan *et al.*, 2007). Thus, it is necessary to determine the reducing power of an extract to elucidate the relationship between their antioxidant effect and their reducing power. All fractions were measured by reading absorbance at 700 ㎚. A higher absorbance of the reaction mixture indicated greater reducing power. The extracts were tested at concentrations of 0.3, 0.5, and 1.0 ㎎/㎖, and all samples showed some degree of reducing power; however, all extracts showed increased reducing power with increasing extract concentration (Fig. 1). In addition, the higher absorbance values of the EtOAc extract were consistent with those of the other extracts. The water fractions appeared to be less effective. This result correlated well with the TPC results, showing that the EtOAc fraction

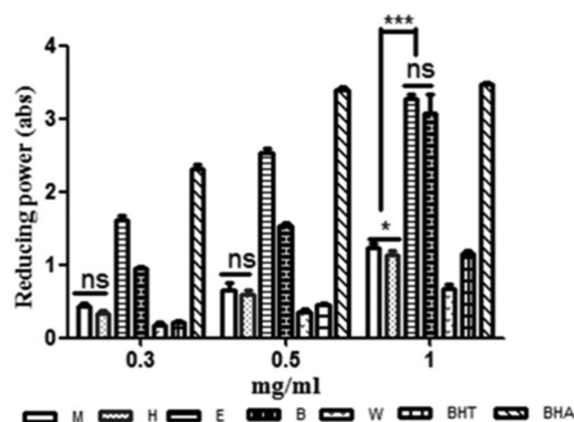

Fig. 1. Reducing power of the *L. thunbergianus* extract and fractions. M; methanol extract, H; n-hexane fraction, E; ethyl acetate fraction, B: butanol fraction, W; water fraction. Data are means ± SD of three experiments performed in triplicate (*$p < 0.05$, ***$p < 0.001$, ns; no significantly different).

possessed high phenolic content. Phenolic compounds have been proposed to donate an electron to free radicals, which terminate the radical chain reaction by converting free radicals to more stable products (Wang *et al*., 2007). Shimada *et al*. (1992) reported that the antioxidative effect is concomitant with the development of reductones. Therefore, the significant antioxidative activity of EtOAc fractions from *L. thunbergianus* may be related to their reducing power. These results agree with results from Odabasoglu *et al*. (2005), who showed that the highest reducing power was observed in the methanol extract of *Peltigera rufescens* (Weiss) Humb. A strong correlation was observed between reducing power and total antioxidant activity of the extracts.

4. ROS generation

ROS include superoxide radicals, hydroxyl radicals, singlet oxygen, and hydrogen peroxide, which are generated as byproducts of biological reactions or from exogenous factors. ROS are associated with many forms of apoptosis, and can also act as secondary messengers that activate production of other mediators involved in the inflammatory process (Maxwell, 1995), as well as in normal defense reactions. We investigated whether the EtOAc and BuOH fractions from *L. thunbergianus* could modulate ROS production in LPS-treated RAW 264.7 cells (Fig. 2). Untreated RAW 264.7 cells (no sample, no DCF-

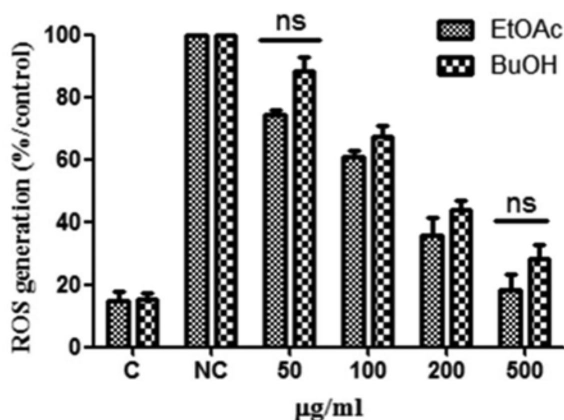

Fig. 2. Reactive oxygen species (ROS) generation in lipopolysaccharide-induced RAW 264.7 cells following treatment with *L. thunbergianus*. Data are means ± SD of three experiments performed in triplicate. EtOAc; ethyl acetate fraction, BuOH; butanol fraction, C; control, NC; negative control, ns; no significantly different.

DA) served as a baseline, and RAW 264.7 cells treated with LPS in the absence of sample showed maximum ROS production. RAW 264.7 cells treated with sample produced fewer ROS than cells treated with LPS in the absence of sample. The inhibition of ROS formation was dose-dependent. The EtOAc and BuOH fractions inhibited LPS-induced intracellular production of ROS at 100, 200 and 500 ㎍/㎖ ($p < 0.05$). These results suggest that *L. thunbergianus* inhibits ROS generation.

5. Effect of the HF on cell viability

The effect of various concentrations of the resulting fractions (HF, EtOAc, BuOH and aqueous residue) on the growth of cancer cells was subsequently examined by an MTT-based assay. Among the extracts tested, the HF appeared to be most potent (Fig. 3). The liver (HepG2, and Huh7), breast (MCF-7, and MDA-MD-231) and colon (CoLo 205, WiDr, HT- 29, RKO, LoVo, HCT-116, and SW620) carcinoma cell lines were similarly susceptible to the cytotoxicity of the HF, resulting in < 40% cell viability. A slight, but significant, reduction in cell viability (15 - 20%) was observed at 200 ㎍/㎖ in SW620 cells treated with the HF (Fig. 4A). Both the 200 and 500 ㎍/㎖ concentrations resulted in similar cell viability. Khan and Mlungwana (1999) reported that the cytotoxicity of non-quinonoid fractions of the root wood of *Markhamia zanzibarica* and root bark of *Kigelia africana*

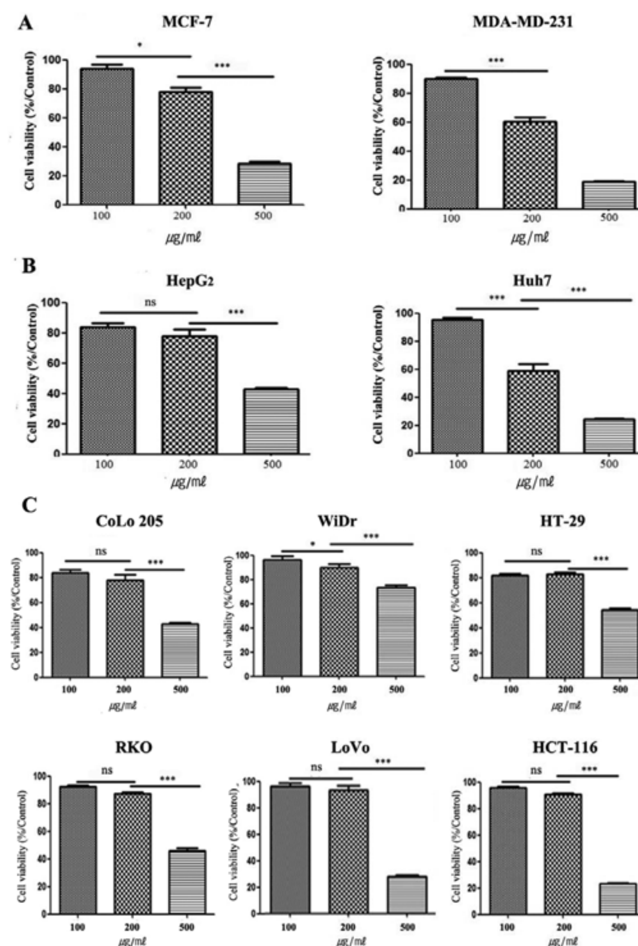

Fig. 3. Anti-proliferative effect of hexane fraction on human cancer cell lines. A; Inhibitory effect of hexane fraction on proliferation of MCF-7 and MDA-MD-231 (Human breast cancer cell), B; Inhibitory effect of hexane fraction on proliferation of HepG2 and Huh7 (Human liver cancer cell), C; Inhibitory effect of hexane fraction on proliferation of CoLo 205, WiDr, HT-29, RKO, LoVo and HCT-116 (Human colon cancer cell). Data are means ± SD of three experiments performed in triplicate (*$p < 0.05$, ***$p < 0.001$, ns; no significantly different).

be attributed to the presence of γ-sitosterol, the 24S-epimer of β-sitosterol. β-sitosterol has been identified as a potential candidate for cancer chemotherapy (Moon *et al*., 2008). Non-polar and low polar solvent fractions (n-hexane fraction and chloroform fraction) were significantly cytotoxic to six cancer cell lines (MCF-7, MDA-MB-435S, HeLa, Bel-7402, HepG2, and ACHN), compared with that of polar solvent fractions (EtOAc fraction and 1-butanol) (Yu *et al*., 2007). This result is in agreement with ours. In our study, the HF inhibited SW620 cell proliferation the most; thus, we wondered which of the active

constituents contributed to this activity. Therefore, SW620 cells were used in further experiments.

6. Induction of apoptosis by the HF

To elucidate whether the HF-induced decrease in viability was attributable to apoptosis, we used Annexin V-FITC and PI staining (Fig. 4B). The percentage of apoptotic SW620 cells induced by the HF (100, 200, 300 ㎍/㎖) was 5.43 - 27.84%. A concentration-dependent increase in apoptotic cells was observed. In conclusion, our study shows that the HF inhibited proliferation of SW620 cells by inducing apoptosis. Apoptosis is an important homeostatic mechanism that balances cell division and cell death and maintains the appropriate number of cells in the body. A balance between cell proliferation and apoptosis controls normal organ development. Uncontrolled proliferation and suppressed apoptosis occur during cancer development (Evan and Vousden, 2001; Hanahan and Weinberg, 2000). The induction of apoptosis in tumor cells is considered a valuable way to treat cancer (Dixon *et al*., 1997). Agents that suppress the proliferation of cancer cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy. While many anticancer agents have been developed, unfavourable side effects and resistance are serious problems (Panchal, 1998). Thus, there is growing interest in the use of plant materials for the treatment of various cancers and the development of safer and more effective therapeutic agents (Ramos, 2007). Thus, we plan to explore the potential of HF as a therapeutic agent for the prevention of cancer in future studies.

7. Effect of the HF on expression of caspase-3, p53, survivin, Bax, and Bcl-2

mRNA expression of apoptosis-related genes in response to different levels of the HF was assessed. Bcl-2 and survivin expression decreased slightly in SW620 cells exposed to the HF (200 ㎍/㎖). A low dose (100 ㎍/㎖) had no apparent effect on Bcl-2, whereas the HF (300 ㎍/㎖) remarkably upregulated the expression of Bax, p53, and caspase-3 genes (Fig. 4C). Apoptosis is a key process in cancer development and progression. The ability of cancer cells to avoid apoptosis and continue to propagate is one of the basic characteristics of cancer and is a major target of cancer-therapy development

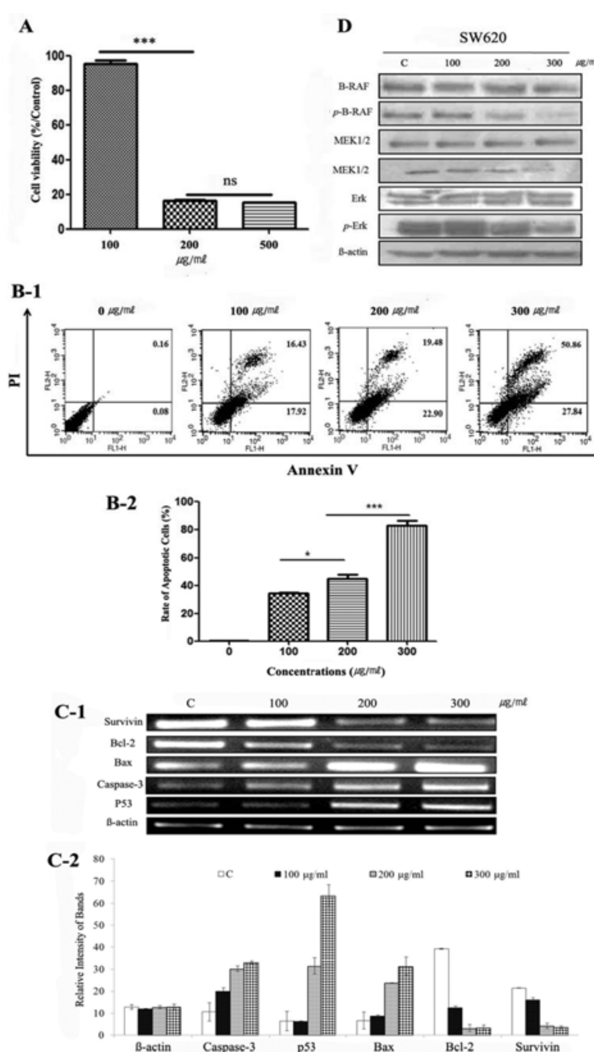

Fig. 4. Effect of hexane fraction on anti-proliferative activity in SW620 cells (human colon cancer cell). A; Inhibitory effect of the hexane fraction on proliferation of SW620. Data are means ± SD of three experiments performed in triplicate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns; no significantly different). B; Induction of apoptosis in SW620 cells. After being treated with a different concentration of hexane fraction for 24 h, the cells were processed for flow cytometry (B-1). The percentage of apoptotic cells was expressed as the percentage of apoptotic cells doubly positive for propidium iodide and Annexin V (B-2). Data are means ± SD of three experiments performed in triplicate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.). C; Hexane fraction treatment of SW620 cells inhibited genes expression. Gel electrophoresis of the amplified products of β-actin, caspase-3, p53, bax, bcl-2, and survivin (C-1) and quantitative data of panel C-1 (C-2). D; Effect of the hexane fraction on expression of the indicated proteins in SW620 cells.

(Ashkenazi, 2002). We provided evidence that HF induced apoptosis in SW620 cells. RT-PCR experiment showed that a clear high expression on caspase-3 mRNAs in

SW620 cells. HF downregulated the antiapoptotic gene survivin. Investigations have shown that there is a strong association between decreased survivin levels and induction of apoptosis in cancer cells. Antiapoptosis function of surviving also seems to be related to the ability to disturb the function of caspases (Zaffaroni *et al*., 2007). To support this finding our results also show that HF inhibit the expression of survivin. Furthermore, Bax, one of the bcl-2 gene family proteins, acts as a cell death promoter. The antiapoptotic bcl-2 gene was detected in our cells. bax was present predominantly in the cytosol, which partially translocates to the mitochondria during the induction of apoptosis. p53-dependent apoptosis is activated by Bax. Bcl-2 and Bax expression are regulated by p53 both in vitro and in vivo, and Bax is a direct target of p53 transcriptional activation (Wang *et al*., 2004). The result demonstrate, for the first time, that the HF have the potential to induce apoptotic cell death.

8. Effect of the HF on Raf, MEK, and ERK protein expression

Raf, MEK, ERK, and mitogen activated protein kinase (MAPK) are conserved proteins that control cellular responses to the environment by regulating gene expression, cell proliferation, division, and apoptosis (Johnson and Lapadat, 2002). Aberrant activation of the Raf/MEK/ERK pathway is involved in various cancers (Wong, 2009). Thus, the development of anticancer drugs has focused on inhibitors capable of blocking the Raf/MEK/ERK pathway. To study their role in HF-treated SW620 tumor cells, we monitored Raf, MEK, and ERK protein levels. The results showed that the levels of Raf, MEK and ERK decreased remarkably after treatment with 100 and 200 ㎍/㎖ HF (Fig. 4D). The protein levels were measured by quantitative Western blot analysis after being normalized to β-actin content. These data show that the HF inhibited Raf, MEK, and ERK activities. The results suggest that HF-induced cell apoptosis might be due to activation of the Raf/MEK/ERK pathway. Growth factors and mitogens use the Ras/Raf/MEK/ERK signaling cascade to transmit signals from their receptors to regulate gene expression. Importantly, this increased expression is associated with a poor prognosis. The Ras/Raf/MEK/ERK pathways to regulate growth and in some cases tumorigenesis. Mutations in Ras and Raf are reported in a wide variety of human tumors (Holderfield *et al*., 2013). Ras-activated signaling pathways may also be triggered by other means, such as by the action of growth factors working through their cognate receptors. Oncogenic transformation of cells by Ras and Raf leads to dramatic changes in cell morphology, adhesion, motility and intracellular architecture (Sun *et al*., 2014). This pathway has diverse effects which can regulate cell cycle progression, apoptosis or differentiation. Thus in treatment of cancer, it may be beneficial to inhibit Raf/ MEK/ERK expression to promote cell cycle arrest.

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