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Growth Period Effects on the Protective Properties of *Aloe vera* Against *t*-BHP-Induced Oxidative Stress in Chang Cells

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Aloe vera has been used in traditional medicine for the therapy of a variety of disorders, such as wounds and burns. However, few studies have examined the antioxidant capacities of *A. vera* plants during different growth periods. In order to investigate the effects of growth on antioxidant activity, *A. vera* was prepared from 2-, 4-, 6-, 8-, and 12-month-old aloe. The extracts from 6-month-old *A. vera* showed the highest contents of flavonoids (9.750 mg catechin equivalent/g extract) and polyphenols (23.375 mg gallic acid equivalent/g extract) and the highest ferric reducing antioxidant power (0.047 mM ferrous sulfate equivalent/mg extract). The extract from 6-month-old *A. vera* exhibited the highest free radical scavenging potential, and the lowest IC_{50} values were found for 2,2-diphenyl-1-picrylhydrazyl (0.26 mg/ml) and alkyl radicals (0.50 mg/ml). In addition, the extract from 6-month-old *A. vera* showed the greatest effects on cell viability in normal liver cells. Based on these findings, the extract from 6-month-old *A. vera* was examined further in order to determine its protective potential against *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative stress. The extract from 6-month-old *A. vera* at a concentration of 0.25 mg/ml showed the highest protective activity against *t*-BHP-induced reactive oxygen species production. These findings suggested that harvesting regimens were critical in the regulation of effects of the bioactive potential of *A. vera* on antioxidant activity.

Keywords: *Aloe vera*, growth period, antioxidant activity

Introduction

Free radicals are highly unstable atoms, molecules, and ions that have unpaired electrons and high reactivity. Free radicals that are derived from oxygen, nitrogen, and sulfur are referred to as reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species, respectively [5, 20]. ROS and other free radicals play an important role in many diseases, such as cancer, gastric ulcers, Alzheimer's, arthritis, and ischemic reperfusion [16].

Synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butylhydroquinone, and propyl gallate, are commercially available, but their use is limited and strictly regulated owing to their potential health hazards [31]. Therefore, the investigation for natural antioxidants that can be used as alternatives to synthetic ones is a big interest among researchers.

Aloe vera is a tropical or subtropical plant from North Africa, and it has turgid lace-shaped green leaves with jagged edges and sharp points [28]. It belongs to the Xanthorrhoeaceae family and Asphodeloideae subfamily. *A. vera*, which grows in arid climates, is widely distributed in Africa, India, and other arid areas. It is mostly cultivated in tropical or subtropical regions that do not experience freezing weather conditions [33].

The fresh gel, juice, or formulated products of *A. vera* have been used for cosmetic and medicinal purposes, including in traditional medicine for a number of curative

purposes, in the treatment of a variety of disorders, including wounds and burns [11], and for general health. In addition to its wound healing properties, it shows anti-inflammatory, antidiabetic, and hypoglycemic properties [2, 6, 42] and the curing of dermal damage in diabetic rats. Hart *et al*. [14] have isolated two chemically and functionally different immunomodulatory compounds in *A. vera* gel. However, most physical and biochemical studies on *A. vera* have focused on the polysaccharide components of the gel without characterizing the phenolic fraction and the effects of the age of the plants [12, 24, 32, 38]. In addition, most research on *A. vera* has concentrated on examining its gel, and few studies have examined the whole plant. Therefore, the objective of this investigation was to evaluate the *in vitro* antioxidant potential of the whole *A. vera* plant at various growth phases.

Materials and Methods

Materials

tert-Butylhydroperoxide (*t*-BHP), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), 2,2′-azo-*bis*(2-amidinopropane) hydrochloride (AAPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) as diammonium salt (ABTS), α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone, linoleic acid, ammonium thiocyanate, 3-(2-pyridyl)-5,6-*bis*(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), 2,6-di-*tert*-butyl-4-methylphenol, catechin, ferrous and ferric chloride, and potassium ferricyanide were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). In addition, the *A. vera* was obtained from Naturetech (Jincheon, Korea). All of the other reagents were of the highest commercial grade.

Preparation of the *Aloe* Extract

The *A. vera* plants were harvested at 2, 4 6, 8, or 12 months. After the plants were harvested, they were washed completely with distilled water and cut into small pieces. The pieces were then extracted with distilled water at 4°C in a shaking incubator for 3 days. The water extract was then filtered with Whatman filter paper No. 41 in order to remove any debris, and the filtrate was freeze-dried in order to obtain the *Aloe* extract.

Determination of the Total Polyphenol Contents

The total phenolic contents of the extracts were determined with Folin–Ciocalteu assays [25]. Samples (0.1 ml) of the extract in distilled water (1 mg/ml) were mixed with 50 µl of the 50% Folin–Ciocalteu reagent, and 150 µl of 20% sodium carbonate (Na_2CO_3) was then added. The solution was incubated at room temperature for 30 min. The absorbances of the reaction mixtures were measured at 760 nm with a spectrophotometer (SECOMAM, Ales, France). Gallic acid was used as the standard, and the total polyphenol contents of the *Aloe* extracts were expressed in milligram gallic acid equivalents (mg GAE/g extract).

Determination of the Total Flavonoid Contents

The total flavonoid contents were estimated by the aluminum colorimetric method [30], and catechin was used as the standard. Test samples were dissolved in distilled water, and 150 µl of the sample solution was then blended with 150 µl of 2% aluminum chloride ($AlCl_3$). After incubating the mixture for 10 min at room temperature, the absorbance of the supernatant was measured at 510 nm with a spectrophotometer. The total flavonoid content was shown as catechin equivalents in milligrams per gram extract (mg CE/g extract).

Oxygen Radical Absorbance Capacity (ORAC)

ORAC assays were performed according to the method of Ou *et al*. [29] with some modification. Trolox was used as the standard. Samples (50 µl) of blank, Trolox, and extract solutions (in 75 mM of phosphate buffer, pH 7.4) were added to 50 µl of a fluorescein (7.8 µM) solution and incubated for 15 min at 37°C. After the addition of 25 µl of 221 mM AAPH, the fluorescence was measured every 5 min for about 120 min (excitation wavelength, 485 nm; emission wavelength, 535 nm) with a fluorescence microplate reader (SpectraMax M2/M2e; Molecular Devices LLC, Sunnyvale, CA, USA). The final ORAC values of the samples were shown as micromole Trolox equivalent (TE) per milligram extract (µmol TE/mg extract).

Ferric Reducing Antioxidant Power (FRAP) Assay

In order to determine antioxidant capacities, the FRAP method was conducted according to the methods described by Benzie and Strain [1]. A 3 ml aliquot of the FRAP reagent (mixture of 0.3 M acetate buffer, 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM hydrogen chloride, and 20 mM ferric chloride in 10:1:1 (v/v/v)) was mixed with 1 ml of the samples. The antioxidant capacities of the samples were calculated based on the standard curves of ferrous sulfate ($FeSO_4$; 0–10 mM). The antioxidant capacities were expressed as millimolar $FeSO_4$ equivalent per milligram extract (mM $FeSO_4$ eq./mg extract).

Free Radical Scavenging Activity

DPPH radical scavenging activity. The DPPH radical scavenging activity was measured with an electron spin resonance (ESR) spectrometer (JES-FA series ESR; JEOL Ltd., Tokyo, Japan) by using the method described by Kim *et al*. [18]. Samples (30 µl) and the control (ethanol) were mixed with 30 µl of DPPH (60 µM in ethanol). After 10 sec of vigorous mixing, the solutions were transferred to Teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adducts were determined by the ESR spectrometer exactly after 2 min.

Alkyl radical scavenging activity. Alkyl radicals were generated by AAPH. The reaction mixture containing 40 mM AAPH, 40 mM α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone, and the test samples

were mixed in phosphate-buffered solution (PBS; pH 7.4), incubated at 37°C in a water bath for 30 min, and then transferred to a 100 µl Teflon capillary tube where the spin adducts were measured.

Hydroxyl radical scavenging activity. Hydroxyl radicals were generated by the Fenton reaction and then reacted rapidly with a DMPO nitrone spin trap. The resultant DMPO-OH adducts were detected with an ESR spectrometer. The reaction mixtures containing 20 µl of 0.3 M DMPO, 20 µl of 10 mM $FeSO_4$, and 20 µl of 10 mM H_2O_2 were mixed with the test samples and then transferred to a Teflon capillary tube. The spin adducts were then measured.

ABTS radical scavenging activity. The total antioxidant activities of the extracts were measured by ABTS radical cation ($ABTS^{\cdot}$) decolorization assays involving the preformed $ABTS^{\cdot}$ [36]. The $ABTS^{\cdot}$ was produced by the 7 mM ABTS stock solution with 2.45 mM potassium persulfate ($K_2S_2O_8$), and the mixture was stored in the dark at room temperature for 14 h before use. In order to determine the scavenging activity, 0.9 ml of the ABTS reagent was mixed with 0.1 ml of the extracts, and the absorbance was measured at 734 nm after 6 min of reaction at room temperature. The antioxidant activities of the aloe extracts were expressed as the Trolox equivalents antioxidant capacity as mM TE /mg extract.

Cell Studies

Cell viability. Cell viability was estimated by MTT assays, which test the normal metabolic status of cells by assessing mitochondrial activities. Chang cells, which are normal liver cells that are widely used as a human normal hepatocyte model in various studies, were seeded in 96-well plates at a concentration of 4.0×10^5 cells/ml. After 20 h, the cells were incubated with different concentrations of the various extracts in a humidified incubator at 37°C for 1 h. Subsequently, 80 µM *t*-BHP was added and the cells were again incubated for 24 h. Thereafter, 100 µl of a MTT stock solution (0.5 mg/ml) was added and incubated for 4 h. The supernatants were then aspirated, and the formazan crystals in each well were dissolved in 150 µl of DMSO. Absorbance was measured with a spectrofluorometer (SpectraMax M2/M2e) at a wavelength of 540 nm. The optical density of the formazan that was formed in the control cells was set as 100%.

ROS generation in Chang cells. DCFH-DA was used to estimate the generation of ROS in oxidative stress. DCFH-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals (mainly hydrogen peroxide) and convert into its fluorescent product DCF, which is retained within the cells [19]. The cells were incubated with 5 µM DCFH-DA for 30 min at room temperature. The uptake of DCFH-DA fluorescence was measured by FACSCalibur (BD, Franklin Lakes, NJ, USA).

Cell cycle analysis by flow cytometry. For the flow cytometry analysis, the cells were collected and washed twice with PBS (pH 7.4). Subsequently, the cells were fixed overnight in 80% ethanol. They were then washed twice and resuspended in PBS containing 50 µg/ml propidium iodide and 5 µg/ml ribonuclease A for DNA staining. The cells were then analyzed by FACSCalibur by using the Cell Quest pro software. At least 10,000 events were evaluated.

Western blot analysis. In order to obtain the total cell lysate, 50 µl of radioimmunoprecipitation assay buffer was added to the Chang cells (3×10^5 cells/ml) that were cultured in 6-well plates. The cells were harvested, incubated for 10 min on ice, and centrifuged at 14,000 rpm for 10 min at 4°C. The protein concentration was determined with the DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and 20 g of whole cell lysate was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. Electrophoresis was performed, and the proteins were transferred to polyvinyl difluoride membranes with an electroblotting apparatus. The membranes were blocked for 1 h in Tris-buffered saline containing 0.1% Tween-20 and 5% dry skim milk and then incubated overnight with one of the following primary antibodies: Bcl-2 (1:5,000), Bax (1:5,000), or anti-β-actin (1:5,000). Subsequently, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:10,000). The optical densities of the antibody-specific bands were analyzed with a Luminescent Image Analyzer (LAS-3000; Fujifilm Corporation, Tokyo, Japan) [21, 22].

Statistical Analysis

The experimental data that are presented are summaries of data from at least three experiments. All of the data are presented as the mean ± standard deviation (SD). The statistical analyses were performed with SAS statistical software (SAS Institute, Inc., Cary, NC, USA). Paired *t*-tests were used to compare the data between the oxidative stress group and the *A. vera*-treated group. The treatment effects were analyzed with one-way analyses of variance, which were followed by Dunnett's multiple range tests. *P* values less than 0.05 indicated significance.

Results and Discussion

Total Polyphenol Contents

The nutraceutical properties of *A. vera* are mainly due to the presence of various phenolic compounds [38]. Polyphenols, which are one of the most abundant plant secondary metabolites, exhibit strong protective effects against cellular oxidative damage. Polyphenols have high antioxidant capacities, and they are therefore considered to be the most important dietary antioxidants [39].

The total polyphenolic contents of the samples are presented in Table 1. Extracts from 6-month-old *A. vera* possessed the highest amount of polyphenols (23.375 ± 0.065 mg), which was followed by 4-month-old extracts (20.157 ± 0.366) and then 2-month-old extracts (19.000 ± 0.030 mg GAE/g extract). The extracts from 8-month-old and 12-month-old *A. vera* had similar polyphenolic

Table 1. Concentrations of polyphenol and flavonoid in the *Aloe vera* extracts from plants from different growth periods.

Radicals \ Growth period	2 months	4 months	6 months	8 months	12 months
Polyphenol (mg GAE/g extract)	19.000 ± 0.030^{b}	20.157 ± 0.366^{ab}	23.375 ± 0.065^{a}	15.204 ± 0.098^{c}	15.110 ± 0.031^{c}
Flavonoid (mg CE/g extract)	4.750 ± 0.354^{cd}	8.000 ± 0.001^{b}	9.750 ± 1.061^{a}	3.950 ± 0.514^{d}	4.500 ± 0.001^{cd}

$^{a-d}$Means with different superscripts in a column differed significantly in the Dunnett's multiple range test ($p < 0.05$).

contents. Ray *et al*. [34] reported that the phenolic contents of *A. vera* gel extracts from various growth years ranged from 30.11 ± 1.89 μg GAE/mg to 34.37 ± 0.7 μg GAE/mg. Their results were higher than those of this study because their *A. vera* extracts were from different growth periods and extract parts: they harvested 2- to 4-year-old *A. vera* and used only *A. vera* gel.

Total Flavonoid Contents

Flavonoids are a common group of polyphenols that are present in plants and that are known to provide powerful protection against many chronic diseases, such as diabetes, cardiovascular diseases, and cancer, possibly due to their antioxidant and anti-inflammatory activities [43].

The total flavonoid contents of the *A. vera* samples are shown in Table 1. Extracts from the 6-month-old *A. vera* plants showed the highest amount of flavonoids (9.750 ± 1.061), which was followed by 4-month-old (8.001 ± 0.001) *A. vera*. The extract from 2- and 8-month-old *A. vera* had similar flavonoid contents (4.750 ± 0.0354 mg CE/g extract). The 12-month-old *A. vera* possessed the least amount of flavonoids. The flavonoid contents of *A. vera* gel extract from various growth periods, 2 to 4 years, exhibited 11.00 ± 0.88 to 25.38 ± 1.30 μg RE/mg, and these contents were greater than those found in this study. Differences in the growth periods and extract parts may have contributed to the dissimilar results [34].

ORAC Assays

Currently, one of the methods that is most widely used for determining the antioxidant potential of a compound is an ORAC assay, in which the antioxidant capacity of a compound is reported by comparing it with an antioxidant standard, which is a water-soluble vitamin E analog known commercially as Trolox [9].

The ORAC values (μM TE) for the *A. vera* extracts are shown in Table 2. The adult 12-month-old *A. vera* extract showed the highest ORAC value, which was followed by the 8-month-old, 4-month-old, and 6-month-old *A. vera* extracts. The extracts from 2-month-old *A. vera* showed the smallest ORAC value. Nejatzadeh-Barandozi [26] has reported that the ethanol extract of *A. vera* gel possessed 136 ± 2.3 mol of TE. However, the growth period of the gel was not reported, and it was extracted with ethanol. Therefore, a direct comparison with the results of our study is not appropriate. Although the growth information for the gel was not reported, the results indicate that the ethanol extract contains much higher levels of polyphenols and flavonoids and antioxidant activities in assays such as ORAC and FRAP assays. In our investigation, whole *A. vera* was used and water extracted, which is the same process that is used by the Naturetech food company.

FRAP Assay

FRAP assays are used to evaluate the reducing power of antioxidants. In FRAP assays, the antioxidants that are contained in the test samples are treated as reductants in redox-linked colorimetric reactions. This method is used for the rapid evaluation of the total antioxidant capacities of various foods and beverages [7].

Table 2 lists the FRAP values of the *A. vera* extracts. Extracts from the 6-month-old (0.047 ± 0.002) *A. vera* showed the highest ferric reducing potential, which was followed by the 4-month-old (0.032 ± 0.001 mM $FeSO_4$ eq./mg

Table 2. Antioxidant activities of *A. vera* extracts from different growth periods.

Radicals \ Growth period	2 months	4 months	6 months	8 months	12 months
ABTS (mM Trolox eq./mg extract)	0.216 ± 0.003^{c}	0.238 ± 0.004^{b}	0.271 ± 0.006^{a}	0.187 ± 0.008^{d}	0.197 ± 0.006^{d}
FRAP (mM $FeSO_4$ eq./mg extract)	0.025 ± 0.003^{c}	0.032 ± 0.001^{b}	0.047 ± 0.002^{a}	0.022 ± 0.006^{c}	0.010 ± 0.003^{d}
ORAC (μM TE)	66.682 ± 0.201^{b}	67.574 ± 0.762^{ab}	67.093 ± 0.603^{ab}	68.432 ± 1.162^{ab}	68.851 ± 1.111^{ab}

$^{a-d}$Means with different superscripts in a column differed significantly in the Dunnett's multiple range test ($p < 0.05$).

extract) *A. vera*. The 2-month-old and 8-month-old *A. vera* extracts had nearly the same ferric reducing potential values. Nejatzadeh-Barandozi [26] reported higher concentrations of total polyphenols and total flavonoids, as well as higher antioxidant capacities, in the *A. vera* gel in the ORAC and FRAP analyses. However, in our study, there were no correlations of the results for the bioactive components and antioxidant activity.

Free Radical Scavenging Activity

ABTS assays are one of the methods that is most widely used to evaluate the antioxidant capacity of an extract. It involves the direct production of the blue/green $ABTS^{\cdot+}$ chromophore by reaction of ABTS with potassium persulfate. When antioxidants are added to this solution, they scavenge the $ABTS^{\cdot+}$ chromophores and reduce the radical back to ABTS, thereby decreasing its absorbance [36].

The ABTS radical scavenging activities of the *A. vera* extracts at different growth periods are shown in Table 2. The 6-month-old *A. vera* extract had the greatest scavenging potential, which was followed by the extract from the 4-month-old *A. vera*. The 12-month-old *A. vera* showed the least ABTS scavenging potential.

The DPPH and alkyl radical scavenging activities and the IC_{50} values for the extracts of *A. vera* are shown in Figs. 1A and 1B. The DPPH radical is widely used to measure the efficacy of antioxidants. This colorimetric assay is currently popular owing to the relative stability of the DPPH radical, its sensitivity, and its technical simplicity [15].

The extract from the 6-month-old *A. vera* showed the highest DPPH radical scavenging activity with an IC_{50} value of 0.26 mg/ml, which was followed by the 4-month-old (0.37 mg/ml of IC_{50}) *A. vera*. The 2-month-old and 8-month-old *A. vera* extracts had similar DPPH radical scavenging potentials (0.42 and 0.48 mg/ml of IC_{50}, respectively). However, the extracts from 12-month-old *A. vera* showed the least scavenging of the DPPH radical.

The alkyl radical scavenging activities and the IC_{50} values for the *A. vera* extracts are shown in Fig. 1B. The extract from the 6-month-old *A. vera* showed the highest alkyl radical scavenging activity with an IC_{50} value of 0.50 mg/ml. The extracts from the 4-month-old (0.67 mg/ml of IC_{50}) and 12-month-old *A. vera* (0.64 mg/ml of IC_{50}) had similar alkyl radical inhibitor activities. The extract from 2-month-old (0.85 mg/ml of IC_{50}) *A. vera* had the least alkyl radical scavenging activity.

Hydroxyl radicals are generated in the Fe^{2+}/H_2O_2 system. These radicals are then trapped by a DMPO-forming spin adduct, which were detected by an ESR spectrometer [8]. The hydroxyl and superoxide scavenging activities of the *A. vera* extracts are shown in Figs. 1C and 1D. The extracts were tested at a concentration of 2 mg/ml. As can be seen in the figures, the extract from 4-month-old *A. vera* showed the greatest hydroxyl scavenging activity, whereas the extract from 6-month-old *A. vera* had the greater superoxide scavenging activity.

Interest in polyphenols has been increasing, mainly because of their antioxidant properties, and their roles in the prevention of various diseases, including cardiovascular disease, cancer, neurodegeneration [27], and diabetes [17]. However, the concentration of each polyphenol antioxidant is not the only factor that influences antioxidant activity; the structural disposition (positions and numbers of the aromatic rings, double bonds, and hydroxyl groups) of these compounds also play a role [23]. Therefore, further investigations on the other bioactive components, including indoles, alkaloids, ketones, and sterols, which are well known for their health benefits, are needed [26].

Cell Culture Studies

Cell viability. The effects of the *A. vera* extracts on the viability of Chang cells were evaluated with MTT assays. MTT is reduced to insoluble purple MTT formazan crystals with succinate by the pyridine nucleotide cofactors, NADH and NADPH [3], and this results in a quantifiable color change from yellow to blue. MTT production is therefore inversely related to cell death.

Based on the results from the MTT assays, the extract from 6-month-old *A. vera* was selected for further study, and different concentrations (0.25, 0.5, 1, and 2 mg/ml) were tested for their effects on cell viability in Chang cells. The results are shown in Fig. 2A. The *A. vera* extract at a concentration of 2 mg/ml showed the highest cell viability, which was followed by the 1, 0.5, and 0.25 mg/ml concentrations. The extract from 6-month-old *A. vera* was further tested for its protective effects by evaluating its effects on cell viability against *t*-BHP-induced damage, and the results are shown in Fig. 2B. Treatment with *t*-BHP reduced the cell viability of Chang liver cells, but, as can be seen in Fig. 2B, cell viability was increased in the range of 0.25–2.0 mg/ml in the group treated with the extract from 6-month-old *A. vera*.

ROS generation in Chang cells. ROS, which are produced during cellular respiration, damage cells by oxidizing critical cellular macromolecules, including nucleic acids, proteins, and membrane lipids [10]. ROS have been implicated in a number of disease states, including the processes of cancer, aging, inflammation, diabetes mellitus,

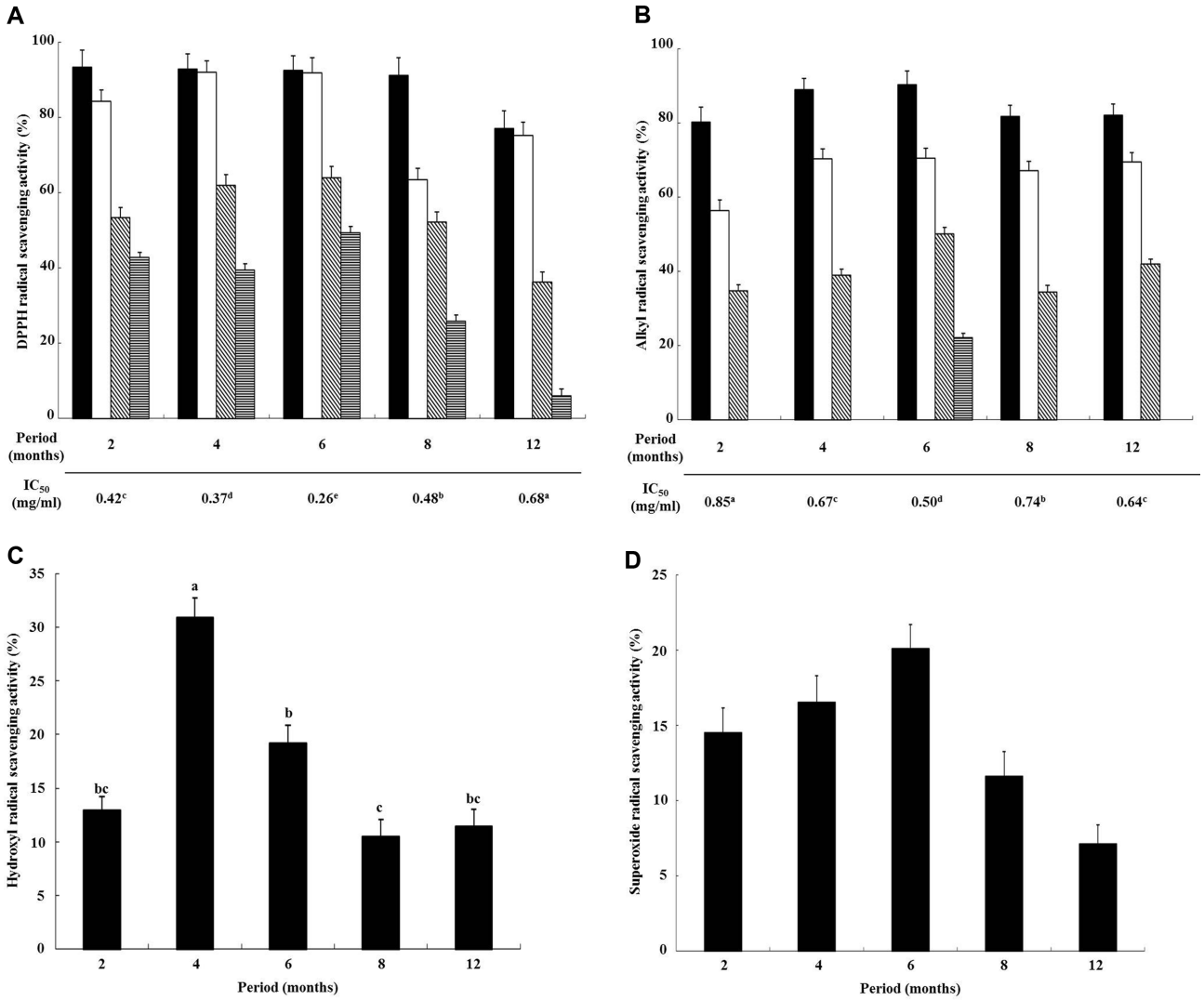

Fig. 1. Free radical scavenging activities of the extracts from 2-, 4-, 6-, 8-, and 12-month-old *Aloe vera*.

(**A**) 2,2-Diphenyl-1-picrylhydrazyl (DPPH), (**B**) alkyl, (**C**) hydroxyl, and (**D**) superoxide radical scavenging activities. [a-d]Means with different superscripts in a column differed significantly in Dunnett's multiple range test ($p < 0.05$). ■, 2.0 mg/ml; □, 1.0 mg/ml; ▨, 0.5 mg/ml; ▤, 0.25 mg/ml.

neurological disorders, and coronary heart, as well as Alzheimer's disease [4].

t-BHP, which is the short-chain analog of lipid hydroperoxides, is used to investigate oxidative stress-induced cell damage, and it can be metabolized to initiate lipid peroxidation, damage cell integrity, and cause a liver inflammatory reaction [13].

The protective effects of the extract from 6-month-old *A. vera* against *t*-BHP-induced oxidative damage was tested in Chang cells with DCFH-DA. The results are shown in Fig. 3. ROS production was markedly elevated by *t*-BHP treatment compared with non-tBHP treatment. However, ROS production was reduced by treatment of *A. vera* in the range of 0.25–2.0 mg/ml in a dose-dependent pattern. In addition, treatment with 2.0 mg/ml of *A. vera* resulted in a significant decrease ($p < 0.05$). Fig. 2B shows that cell viability was significantly increased with treatment with extracts in the concentration range of 0.5–2.0 mg/ml. However, ROS production was significantly inhibited by the 2.0 mg/ml concentration. We assumed that this difference was probably a result of the mechanisms of cell viability and ROS generation. Therefore, further investigations are needed in order to elucidate the precise mechanisms.

Cell cycle analysis by flow cytometry. The results on the inhibitory effects of the extract from 6-month-old *A. vera* on *t*-BHP-induced apoptosis in Chang liver cells are presented

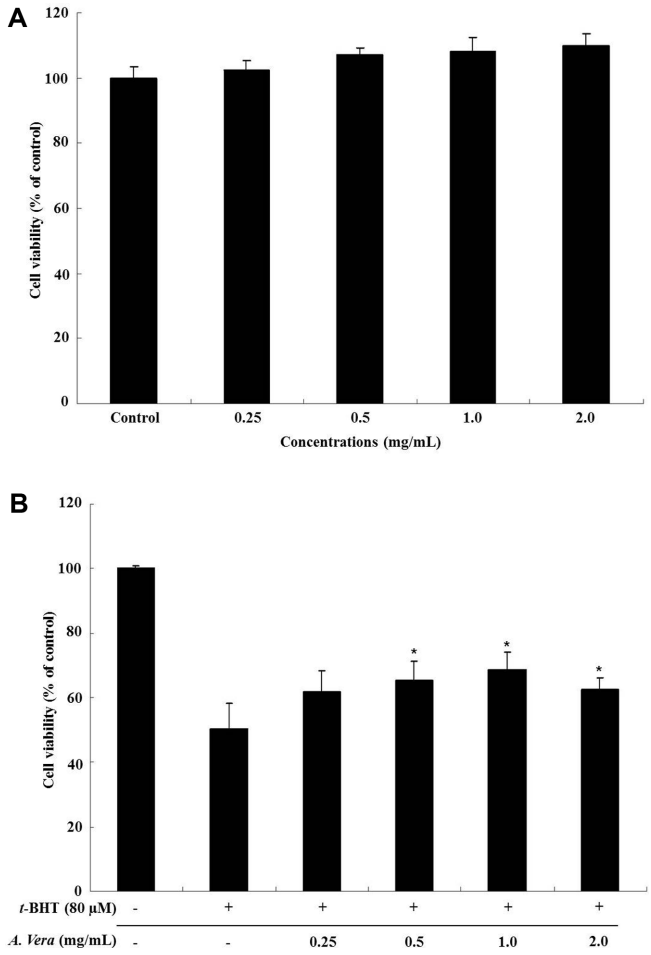

Fig. 2. Effect of *Aloe* extract on cell viability.
(**A**) Cell viability studies in Chang liver cells of the effects of the extracts from 6-month-old *A. vera*. (**B**) The protective effects in Chang liver cells of the extracts from 6-month-old *A. vera* on *t*-BHP-induced oxidative stress. *($p < 0.05$), indicating a significant difference in the paired *t*-test comparing the oxidative stress group with the *A. vera*-treated group.

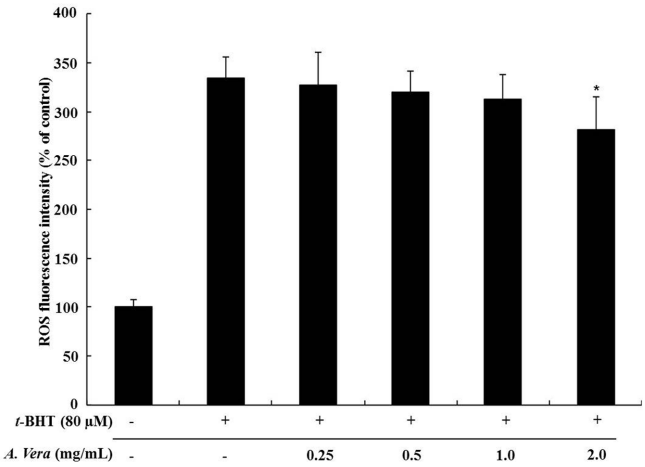

Fig. 3. Inhibition of reactive oxygen species (ROS) activity in Chang liver cells by the effects of the extracts from 6-month-old *A. vera* against *t*-BHP- induced oxidative stress.
*($p < 0.05$), indicating a significant difference in the paired *t*-test comparing the oxidative stress group with the *A. vera*-treated group.

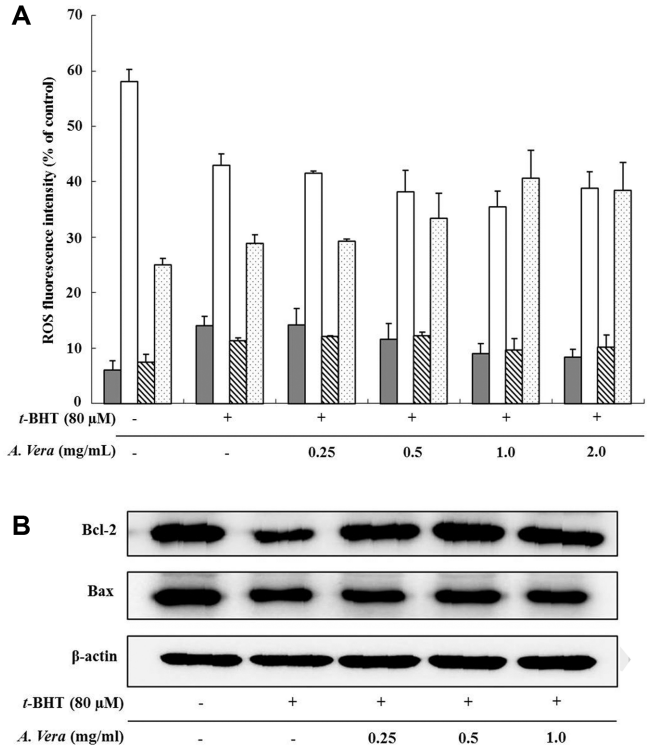

Fig. 4. Inhibitory effects of 6-month-old *Aloe vera* extracts on *t*-BHP-induced apoptosis.
(**A**) Inhibition of oxidative stress-induced apoptosis in Chang liver cells by extracts from 6-month-old *A. vera*. *($p < 0.05$), indicating a significant difference in the paired *t*-test comparing apoptosis in the oxidative stress group with that in the *A. vera*-treated group. (**B**) Effects of extracts from 6-month-old *A. vera* on the expression of Bcl-2 and Bax in Chang liver cells.

in Fig. 4A. When the cells were subjected to *t*-BHP, an increase in the percentage (14.01%) of apoptotic cells (sub-G1 DNA content) was observed, thus indicating cell damage. In contrast, treatment with the 0.5, 1, and 2 mg/ml concentrations of the Aloe extracts decreased the percentages of apoptotic cells by 11.63%, 9.01%, and 8.32%, respectively.

Western blot analysis. Mitochondria play a key role during apoptosis. During early apoptosis, permeabilization of the mitochondrial outer membrane occurs, which causes the release of pro-apoptotic factors, such as cytochrome *c*, into the cytosol. Cytochrome *c* release then amplifies apoptosis. However, this event is regulated through interactions between proteins of the Bcl-2 family. In this

family, effector proteins, such as BAX, are essential for permeabilization of the mitochondrial outer membrane. In contrast, anti-apoptotic members, such as Bcl2, inhibit this process by directly binding to the pro-apoptotic effector proteins [41]. The results for the western blots of the whole-cell lysate that were performed to determine the levels of expression of Bcl-2 and BAX after treatment with *Aloe* extracts are presented in Fig. 4B. Actin was used as the internal standard.

As shown in Fig. 4B, the western blot clearly exhibits a decrease in Bcl-2 concentration when the cells were subjected to *t*-BHP treatment. However, the extract from the 6-month-old *Aloe* extract reversed this effect at each concentration. The concentration of Bax, however, did not change significantly in the cells that were treated with the extract from 6-month-old *A. vera* compared with the *t*-BHP cells.

Antioxidant therapy has been suggested as a unique medical strategy to attenuate free radicals in the human body [35, 37]. Based on this perspective, different antioxidative assays can provide valuable information on herbal medicine product-process optimization. The yields of different phenolic extraction techniques and various extraction solvents, such as absolute ethanol, methanol, aqueous ethanol, and aqueous methanol, on the total phenol content of *A. vera* have been investigated by Sultana *et al*. [40]. Among the different solvents, higher extract yields with increased phenol contents were obtained with aqueous organic solvents, especially the 80% (v/v) methanolic extraction. However, the effects of the ages of plants remain uninvestigated. Qualitative and quantitative modifications in the component compounds of *A. vera* have been shown to alter its potential physicochemical properties and bioactivities [12, 25]. Most of the physical and biochemical studies on *A. vera* have focused on the polysaccharide components of the gel without prior characterization of the phenolic fraction and the effects of the age of plants [11, 24, 33, 38]. Hence, information on the biochemical characterization of the phenolic-rich extract of the AG that were obtained from different periods of plant growth, with reference to the antioxidant potential that was obtained, would be helpul for optimizing the components of the value chain of *A. vera* processing. Therefore, this study evaluated the antioxidant potentials of extracts that were obtained from *A. vera* from different growth periods. The results suggested that the extract from 6-month-old *A. vera* has potent antioxidant activity and can be used commercially.

In conclusion, in the present study, we found that the extract from 6-month-old *A. vera* exhibited the greatest amounts of total polyphenols and flavonoids, and it showed the highest free radical scavenging ($ABTS^{\cdot+}$, DPPH, alkyl, and superoxide) and ferric reducing potential. In addition, we confirmed that the *A. vera* extract had protective effects against *t*-BHP-induced oxidative damage by improving cell viability and cell cycle distribution and inhibiting ROS production. However, further studies are necessary in order to isolate and identify the active constituent that is responsible for these antioxidant effects.

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