Research Article

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Thermostability of a marine polyphenolic antioxidant dieckol, derived from the brown seaweed *Ecklonia cava*

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The thermostability of antioxidant activity of dieckol, a phlorotannin isolated from brown seaweed *Ecklonia cava* was investigated. The thermostable antioxidant properties of dieckol were evaluated at 30, 60, and 90°C for 7 days using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radical scavenging activities, and comparing its performance to that of ascorbic acid. The intracellular reactive oxygen species (ROS) scavenging activity and apoptotic body formation were investigated using DCF-DA assay and nuclear staining with Hoechst 33342, propidium iodide and flow cytometry. Dieckol treated at different temperatures during 7 days showed stable scavenging activities on towards DPPH and hydroxyl radicals. In addition, dieckol showed a stable protective effect against H_2O_2-induced apoptotic body formation in Vero cells. On the other hand, the radical scavenging activities and intracellular ROS scavenging activities of ascorbic acid, used as a positive control, were significantly decreased at 60°C and 90°C from on the 4th day and 3rd days, respectively. In conclusion, the results indicated that food grade antioxidant extracts containing dieckol derived from *E. cava* remain a stable during the temperatures encountered during the processing of food and cosmetics.

Key Words: antioxidant; brown seaweed; dieckol; *Ecklonia cava*; phlorotannin; thermostable

INTRODUCTION

According to economic development and the increasing diversity in lifestyle choices, the customer awareness of food and food additives had been significantly increased, and the use of functional and safe and natural food additives is required for an ever increasing majority of products. Many food processing technologies have been developed, but during the processing and storage the functional components, color or flavor may be changed (Fritsch 1981, Yang et al. 2000, Ukeda et al. 2002). The lipid oxidation caused by several unfavorable environmental conditions such as oxygen, temperature, and light is a serious problem in the food industry (Park et al. 2005). Lipid oxidation produces unpleasant flavors in foods and can also produce reactive oxygen species (ROS) that may lead to harmful effects such as cancer, diabetes, mellitus, liver injury, skin damage, aging, and inflammation. Therefore, many attempts have been made to cope with lipid oxidation of food (Frölich and Riederer 1995).

Over the past few decades, considerable numbers of antioxidant studies have been conducted by many researchers and they have tried to find more effective lipid oxidation inhibitors which can be used as antioxidants for food or medicine. Currently, synthetic antioxidant such as butylatedhydroxyanisol and butylatedhydroxytoluene have been used as ingredients or additives to suppress oxidation in food. Synthetic antioxidants are suspected

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as being carcinogenic and responsible for a variety of unidentified health disorders (Sherwin 1990). Therefore, the development of natural antioxidants which can prevent oxidation of food or medicine in a more effective yet safe manner is increasingly important. Recently, many studies have been conducted on marine organisms, including algae and animals, in order to determine the biological activities of metabolites with potential functional values. Among the biological activities of seaweeds, antioxidant activity has attracted more attention (Ye et al. 2009). Seaweeds are rich in vitamins, polysaccharides, and various functional polyphenols (Lordan et al. 2011). Therefore, and not surprisingly, they have been identified as a rich source of bioactive compounds (Dykens et al. 1992, Sukenik et al. 1993, Matsukawa et al. 1997).

The marine brown seaweed, *Ecklonia cava* has been reported to contain polyphenol compounds such as dieckol, eckol, phloroglucinol, and phlorofucofuroeckol (Nagayama et al. 2002, Mayer and Hamann 2004, Athukorala and Jeon 2005, Kotake-Nara et al. 2005, Ahn et al. 2007, Heo and Jeon 2008, Lee et al. 2010, Park et al. 2011, Wijesinghe et al. 2011). These phlorotannins have been reported to possess various biological effects such as antioxidant, cytoprotective and anti-inflammatory effects (Heo et al. 2010, Devi et al. 2011). Especially, dieckol showed the excellent antioxidant and protective effects against oxidative stresses induced by exposure of UV and hydrogen peroxide (Kang et al. 2005, Ahn et al. 2007). It is well known that temperature plays an important role in the stability of antioxidant compounds. Hence, the use of antioxidant agents in food, and in other applications, is limited due to their sensitivity to heat treatments. However, the thermostability of dieckol has not yet been reported. Taken together, the objective of the present study is to evaluate the thermostable antioxidant properties of dieckol, a phlorotannin isolated from the brown seaweed *E. cava*.

MATERIALS AND METHODS

E. cava, a marine brown alga, was collected along the coast of Jeju Island, Korea between October 2009 and March 2010. To remove salt, sand, and epiphytes attached to the surface, the seaweeds were washed three times with tap water and maintained in a medical refrigerator at -20°C. The frozen samples were pulverized with a grinder prior to extraction. Then, the extract (extracted with 80% aqueous EtOH) was evaporated under a vacuum at 40°C to get the dry extract. The dry extract was dissolved in dimethyl sulfoxide (DMSO), and was then used for experiments, adjusting the final concentration of DMSO in culture medium to <0.1%. 1,1-diphenyl-2-picrylhy-drazyl (DPPH), ascorbic acid, Dulbecco's modified Eagle's medium (DMEM), medium, fetal bovine serum (FBS), penicillin-streptomycin and trypsine-EDTA were purchased from Gibco / BRL (Burlington, ON, Canada). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, dihydroethidium, propidium iodide (PI), DMSO and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

Isolation of dieckol from *Ecklonia cava*

The powdered *E. cava* was extracted with 80% aqueous EtOH, and was evaporated under vacuum. The EtOH extract was then partitioned with EtOAc. The EtOAc extract was fractionated via silica column chromatography with the stepwise evolution of a $CHCl_3$-MeOH mixture (100 : 1 to 1 : 1) to generate separated active fractions. The combined active fraction was then further subjected to a Sephadex LH-20 column (GE Healthcare, Piscataway, NJ, USA) saturated with 80% MeOH, and was finally purified via reverse-phase high performance liquid chromatography (HPLC; ThermoFisher Scientific, Rockford, IL, USA) using a Waters HPLC system equipped with a Waters 996 photodiode array detector and C18 column (150 × 20 mm, 4 μm, J'sphere ODS-H80; YMC Co., Kyoto, Japan) by stepwise elution with methanol-water gradient (UV range, 230 nm; flow rate, 0.8 mL min^{-1}). The purified compound, dieckol was confirmed by comparing their liquid chromatography-mass spectrometry (LC / MS), and ^1H NMR data to previously reported data (Li et al. 2009). Dieckol: LC / MS data (M^+, m/z: 742.0 Calcd. For $C_{36}H_{22}O_{18}$). ^1H NMR (400 MHz, DMSO-d_6) δ 9.71(1H, s, OH-9), 9.61 (1H, s, OH-9″), 9.51 (1H, s, OH-4″), 9.46 (1H, s, OH-4), 9.36 (2H, s, OH-3″, 5″), 9.28 (1H, s, OH-2″), 9.23 (1H, s, OH-2), 9.22 (1H, s, OH-7″), 9.15 (2H, s, OH-3′, 5′), 6.17 (1H, s, H-3″), 6.14 (1H, s, H-3), 6.02 (1H, d, J = 2.7 Hz, H-8), 5.98 (1H, d, J = 2.7 Hz, H-8″), 5.95 (1H, s, H-2′″, 6′″), 5.82 (1H, d, J = 2.7 Hz, H-6), 5.81 (1H, d, J = 2.7 Hz, H-6″), 5.80 (1H, t, J = 2.0 Hz, H-4′), 5.78 (2H, d, J = 2.0 Hz, H-2′, 6′).

The purity of dieckol (Fig. 1) was >95%, based on the peak area of all components absorbed at each specific wavelength in HPLC analysis. Dieckol was dissolved in DMSO and was used for experiments adjusting the final concentration of DMSO in the culture medium to <0.01%.

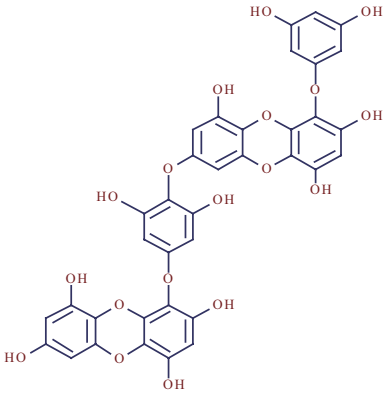

Fig. 1. Chemical structure of dieckol isolated from *Ecklonia cava*.

Heat treatment

To evaluate thermal stability, both dieckol and ascorbic acid were kept in the dark at 30, 60, and 90°C for 7 days. The antioxidant activities were determined via DPPH and hydroxyl radical scavenging activities. The intracellular ROS scavenging activity was investigated using a DCF-DA assay and the apoptotic bodies were investigated via nuclear staining with Hoechst 33342 and cell cycle.

DPPH radical scavenging assay

The DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). An ethanol solution of 60 μL each sample at the concentration of 62.5 μM and 125 μM (or ethanol itself as a control) was added to 60 μL of DPPH (60 μmol L^{-1}) in ethanol solution. After mixing vigorously for 10 s, the solutions were then transferred to a 100 μL Teflon capillary tube which was fitted to the cavity of the electron spin resonance spectrometer (JEOL, Tokyo, Japan). The spin adduct was measured on an electron spin resonance (ESR) spectrometer exactly 2 min later. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3 × 10^5, temperature 298 K.

Hydroxyl radical scavenging assay

Hydroxyl radicals were generated by Fenton reaction, and reacted rapidly with nitrone spin trap DMPO. The resultant DMPO-OH adducts were detectable with an ESR spectrometer. The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (pH 7.4) with 0.3 M DMPO 0.2 mL, 10 mM $FeSO_4$ 0.2 mL, and 10 mM H_2O_2 0.2 mL using a JES-FA electron spin resonance spectrometer (JEOL) set at the following conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 1 mW, gain 6.3 × 10^5, temperature 298 K.

Cell culture

The cells of an African green monkey kidney (Vero) were maintained at 37°C in an incubator, under a humidified atmosphere containing 5% CO_2. The cells were cultured in DMEM containing 10% heat-inactivated FBS, streptomycin (100 μg mL^{-1}), penicillin (100 unit mL^{-1}), and sodium pyruvate (110 mg L^{-1}).

H_2O_2 scavenging assay by DCF-DA

For the detection of intracellular H_2O_2, the Vero cells were seeded in 96-well plates at a concentration of 1.0 × 10^5 cells mL^{-1}. After 16 h, the cells were treated with dieckol samples (10 μL) and incubated at 37°C under a humidified atmosphere. After 30 min, H_2O_2 was added at a concentration of 1 mM, and then the cells were incubated for an additional 30 min at 37°C. Finally, DCF-DA (5 μg mL^{-1}) was introduced to the cells, and 2′,7′-dichlorodihydrofluorescein fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer (Perkin-Elmer, Waltham, MA, USA).

Nuclear staining with Hoechst 33342 and PI

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342 and PI. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and / or fragmentation was indicative of apoptosis (Gschwind and Huber 1995, Lizard et al.1995). The cells were placed in 24-well plates at a concentration of 1 × 10^5 cells mL^{-1} (950 μL). Sixteen hours after plating, the cells were treated with various concentrations of the compounds (50 μL), and were further incubated for 1 h prior to exposure to H_2O_2 (1 mM). After 24 h, 3 μL of Hoechst 33342 (stock 10 mg mL^{-1}) and PI were added to each well, a DNA-specific fluorescent dye, were added to each well, followed by 10 min of incubation at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera (Carsen Group, Markham, ON, Canada), in order to examine the degree of nuclear condensation.

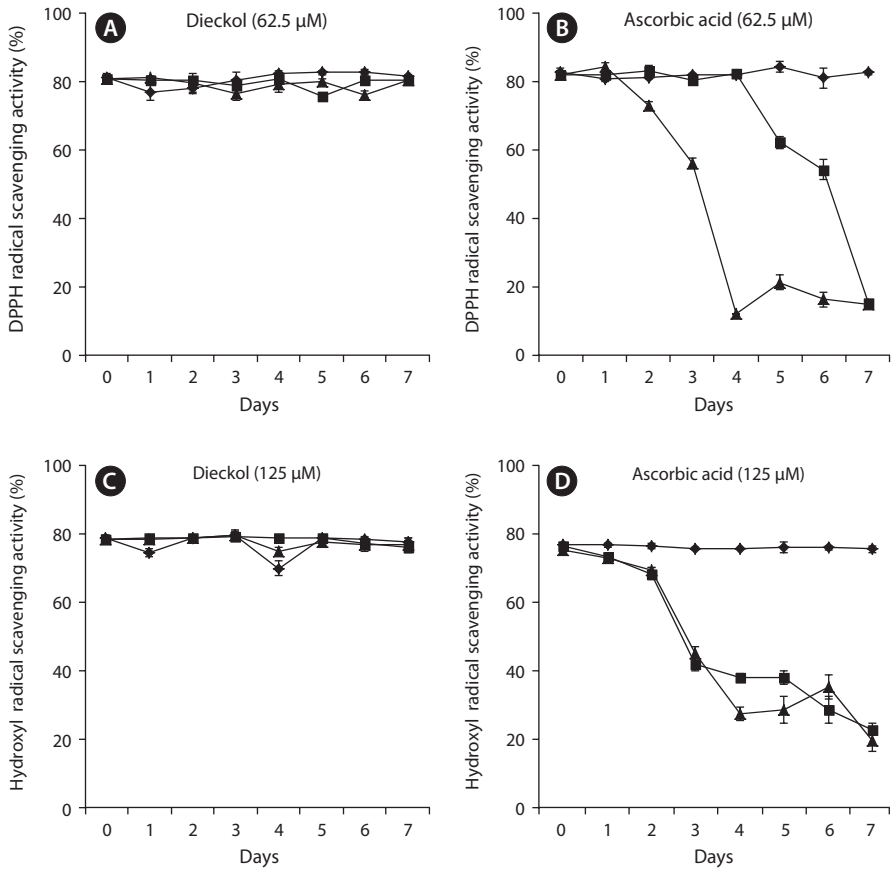

Fig. 2. Thermal stability of dieckol isolated from *Ecklonia cava* and ascorbic acid in DPPH, hydroxyl radical scavenging activity at the concentration of –◆– 30°C, –■– 60°C, and –▲– 90°C. (A) Dieckol 62.5 μM. (B) Ascorbic acid 62.5 μM. (C) Dieckol 125 μM. (D) Ascorbic acid 125 μM.

Cell cycle analysis

To check whether dieckol reduces the proportion of apoptotic sub-G1 hypodiploid cells increased by ethanol treatment, PI staining assay was used according to a method suggested by Nicoletti et al. (1991). The Vero cells were placed in a 6-well plate at a concentration of 4.0 × 10^5 cells mL^{-1}. The cells were treated with dieckol isolated from *E. cava* containing dieckol and further incubated for 1 h prior to exposure to H_2O_2 (1 mM). After 24 h, the cells were harvested at the indicated time, and were fixed in 1 mL of 70% ethanol for 30 min at 4°C. The cells were washed twice with phosphate buffered saline (PBS) and incubated in darkness in 500 μL of PBS containing 50 μg PI and 50 μg RNase A for 30 min at 37°C. Flow cytometric analysis was performed with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The effect on the cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by histograms generated by the computer program Cell Quest and Mod-Fit (Becton Dickinson, Mountain View, CA, USA).

RESULTS

Thermostability of dieckol on radical scavenging activities

In this study, the thermostability of dieckol derived from *E. cava* was assessed via radical scavenging activities against DPPH and hydroxyl radicals, and compared with a positive control, ascorbic acid. As shown in Fig. 2, dieckol exhibited a stable DPPH scavenging activity up to 90°C. No significant difference was observed during the experiment periods at 30, 60 and 90°C. The DPPH scavenging activity of dieckol was around 80% at a concentration of 62.5 μM at 90°C during the 7th day (Fig. 2A). However, the DPPH scavenging activity of ascorbic acid at the same concentration as dieckol was gradually

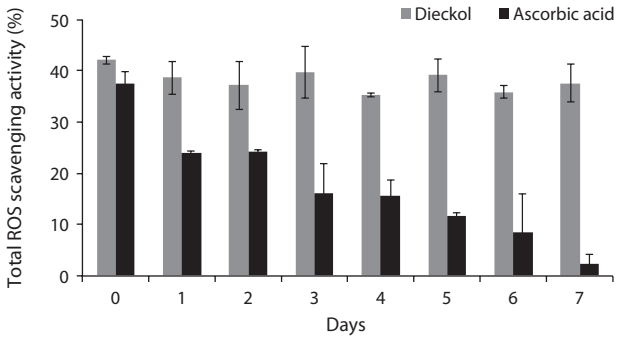

Fig. 3. Intracellular reactive oxygen species (ROS) scavenging activity of dieckol isolated from *Ecklonia cava* and ascorbic acid at 12.5 μM. The samples were treated a 60°C from day 0 to 7.

decreased over the seven day period at 60°C and 90°C. Especially, on day 4 after heating at 90°C, the activity was decreased to 70.26%. On day 7, the activity was decreased to 66.81% and 67.61% at temperatures of 60°C and 90°C, respectively (Fig. 2B). This result demonstrated that dieckol is more stable under conditions of heating than the positive control, ascorbic acid.

The thermostable patterns of dieckol and ascorbic acid (125 μM) during hydroxyl radical scavenging were similar to those during DPPH scavenging (Fig. 2C & D).

Thermostability of dieckol in intracellular ROS measurement

The changes of intracellular ROS scavenging activity of dieckol and ascorbic acid during 7 days at 60°C were investigated and the results are exhibited in Fig. 3. In the case of dieckol, a slight change in ROS scavenging activity was observed, but there was no significant difference throughout the seven day experimental period. At day 0 the intracellular ROS scavenging activity of dieckol (12.5 μM) was 42.09% and at day 7 it was 37.5% However, the ROS scavenging activity of ascorbic acid at the same temperature dramatically decreased over time.

Protective effect of dieckol on H_2O_2 induced cell apoptosis

On the day 7 the changes of protective effects of dieckol and ascorbic acid against H_2O_2-induced cell apoptosis and necrosis were investigated via nuclear staining with Hoechst 33342 and PI. The microscopic photograph in Fig. 4A showed the uninjured nuclei and no necrosis. When the cells were treated with H_2O_2, a significant nuclear fragmentation and destruction which characteristics of

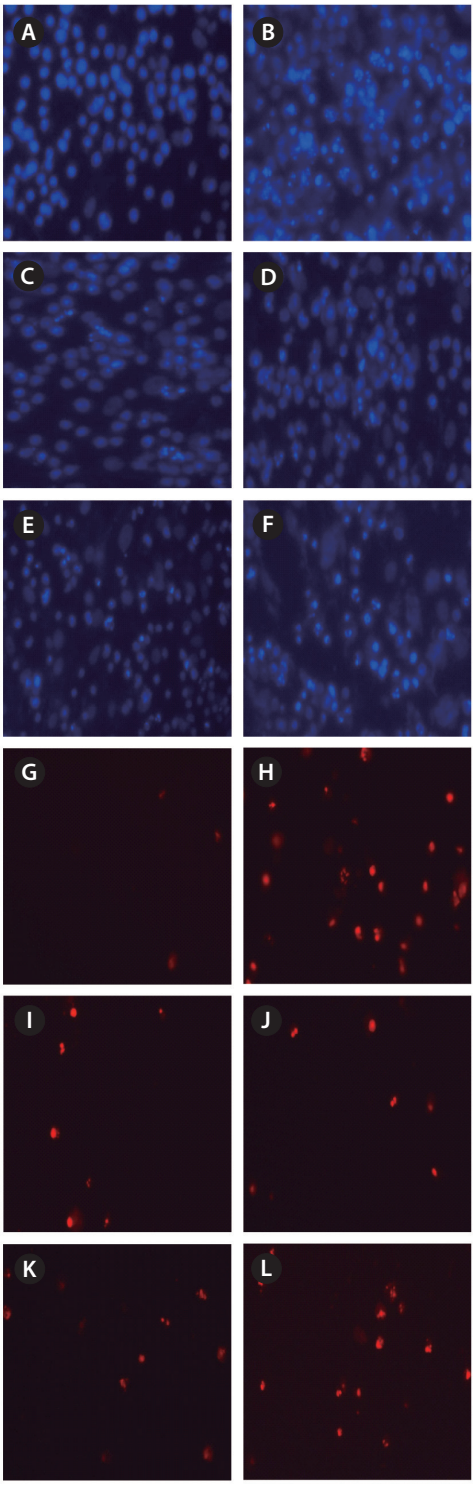

Fig. 4. Protective effect of dieckol isolated from *Ecklonia cava* and ascorbic acid (both 12.5 μM) on H_2O_2-induced cell damage in the Vero cell line. The samples were treated at 60°C from day 0 to day 7. Cellular morphological changes were observed under a fluorescence microscope after (A-F) Hoechst 33342 and (G-L) propidium iodide double staining. (A & G) Notx. (B & H) H_2O_2. (C & I) Dieckol at day 0. (D & J) Dieckol at day 7. (E & K) Ascorbic acid at day 0. (F & L) Ascorbic acid at day 7.

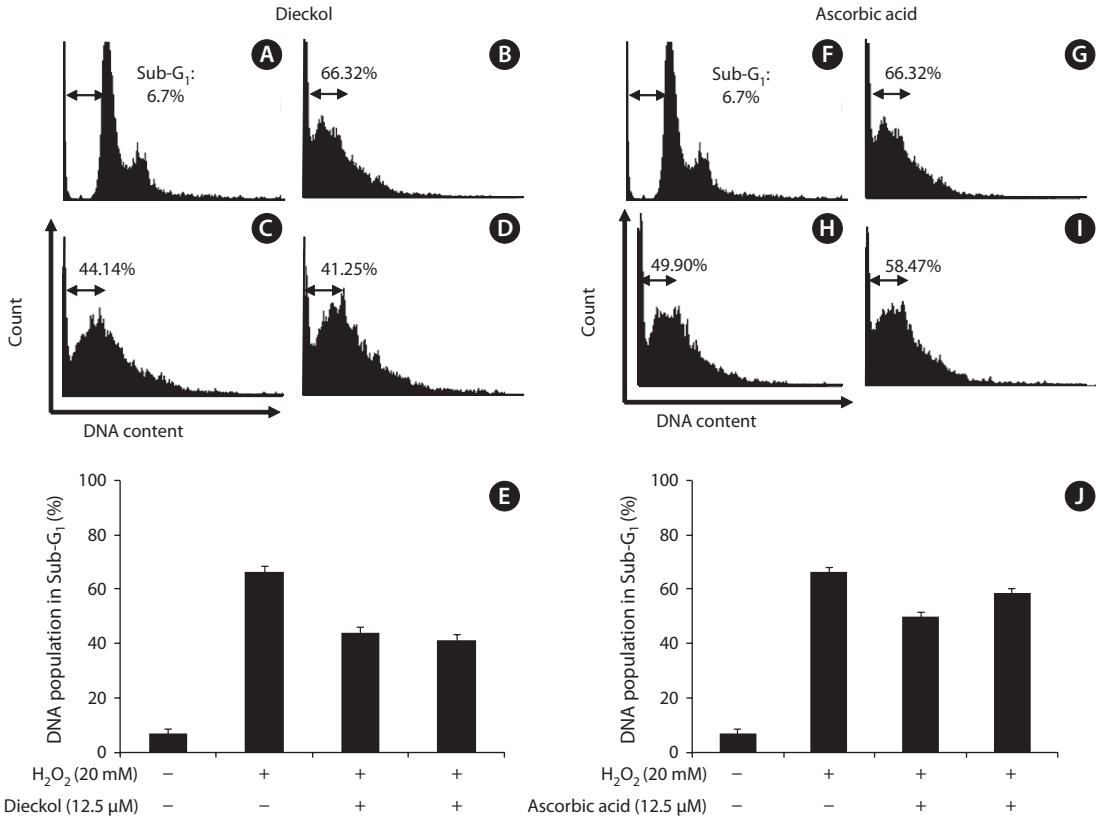

Fig. 5. Flow cytometry analysis of propidiumiodide stained Vero cell line treated with H_2O_2 alone, and in the presence of (A-E) dieckol and (F-J) ascorbic acid.

apoptosis (bright blue) and necrosis (red) were observed (Fig. 4B). However, cell damage, which was exhibited by both nuclear cleavage and necrosis, was significantly reduced for the cells treated with both dieckol and ascorbic acid at dat 0. On the 7th day, dieckol also showed a protective effect on the reduction of apoptosis and necrosis compared with H_2O_2-only treated cells. However, ascorbic acid showed no such effect after 7 days.

Sub-G1 DNA content

The changes of protective effects of dieckol and ascorbic acid on the influence of the cell population for 1 day were also measured by flow cytometry. In the control, the sub-G1 DNA content was 6.7% (Fig. 5A). When the cells were exposed to H_2O_2, the sub-G1 DNA content was increased to 66.32% (Fig. 5B). At 0 day, dieckol and ascorbic acid at the concentration of 12.5 μM reduced the sub-G1 DNA content to 44.1% and 49.9% (Fig. 5C & H). This indicated that both dieckol and ascorbic acid have the protective effect via the influence of the cell population distribution. At day 7, dieckol can reduce sub-G1 content to 41.25% in H_2O_2-treated cells (Fig. 5D). While cells treated with ascorbic acid for 7-days showed a slight reduction in the sub-G1 content (Fig. 5I).

DISCUSSION

Oxidation of food which occurs during storage of raw materials, food processing heat treatments, and the storage of the final products is one of the most common causes of food spoilage (Lehtinen et al. 2003). Oxidation by ROS such as superoxide anions, hydroxyl radicals, and hydrogen peroxide causes the decay of food, destruction of fat and vitamins and development of toxicants and off-flavors. In particular, the heating of food caused lipid oxidation (Laguerre et al. 2007). Ascorbic acid is widely used in the food industry as an antioxidant, and many researchers have reported the profound antioxidant effect of ascorbic acid over past decades (Cordenunsi et al. 2002). Ascorbic acid is an important water-soluble anti-

oxidant (Frei et al. 1989). However, the antioxidant properties of ascorbic acid will be affected by thermal processing. In addition, ascorbic acid is also sensitive to light and oxygen (Gupta et al. 2008). Therefore, thermostable antioxidants are useful in various applications such as food processing industry. In addition, the use of thermostable natural antioxidants may have beneficial health implications (Amarowicz et al. 2000).

A wide variety of bioactive compounds and antioxidant properties have been reported for the brown seaweed *E. cava* (Kang et al. 2005, Ahn et al. 2007, Wijesinghe et al. 2011). In contrast, numerous *in vitro* studies reported the antioxidant properties of phlorotannins isolated from *E. cava* (Ahn et al. 2007, Li et al. 2009). The purpose of this study was to evaluate the antioxidative activity and the thermostability of dieckol, a phlorotannin isolated from *E. cava*. Thus, the research was performed in order to compare the antioxidant properties of dieckol at different temperatures.

Dieckol exhibited a more stable scavenging effect when compared to ascorbic acid. From the results, it is clear that dieckol has a higher degree of thermostability at the tested temperatures. In addition, the phlorotannin compound effectively scavenged the intracellular ROS generation in Vero cells in response to H_2O_2 induced stress at 60°C up to 7 days. Furthermore, the results of the current study confirmed that the dieckol isolated from *E. cava* has the ability to protect Vero cells from oxidative stress-related cell apoptosis in a thermostable manner. In contrast, the thermostability of dieckol antioxidant properties after heat treatment remained comparatively higher than the commercially available antioxidant ascorbic acid. During food processing and storage, ascorbic acid can be degraded by heat and light (Nagy and Smoot 1977, Esteve et al. 1995, Alwazeer et al. 2003). The effects of heat treatments and processing conditions on the antioxidant properties of plant extracts and isolated compounds were previously reported (Choi et al. 2006, Spigno et al. 2007, Im et al. 2011). Based on the previous reports, the brown seaweed *E. cava* can be considered as an excellent source of natural antioxidants (Kang et al. 2005, Heo et al. 2010). In addition, according to the findings of the present study, dieckol derived from the marine brown alga *E. cava* showed a high degree of stability un to 90°C. The thermostability of dieckol indicates that this may be a very useful compound, as an additive in food processing industries.

In conclusion, dieckol derived from *E. cava* showed a higher thermostability than ascorbic acid which is widely used as a food additive or an antioxidant agent in the food and pharmaceutical fields. Based on our results, it could be suggested that dieckol, a phlorotannin derived from *E. cava*, is a useful functional ingredient for application as a natural antioxidant in food, and is more applicable than ascorbic acid to food processes where higher temperatures are used.

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