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Determination of antioxidative potentials of *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem. in differentiated HL-60 cells

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Abstract

A compound with proven antioxidant activities, AE-7-CII, was isolated and purified from the ethanol extract of *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem. (Araliaceae), using a sequential procedure consisting of silica gel column chromatography, thin-layer chromatography and preparative HPLC. In the presence of AE-7-CII, profound inhibitory effects against the generation of 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced superoxide anion radicals ($O_2^{-\cdot}$) and the formation of hydrogen peroxide were observed in differentiated HL-60 cells. This suggests that the isolated compound functions as an effective $O_2^{-\cdot}$ generation inhibitor. The inhibitory effects of AE-7-CII were shown to occur in a concentration-dependent manner, with an IC_{50} value of 12.5 μg/ml. Also, when the radical-scavenging activity of AE-7-CII was compared to those of commercially available synthetic or natural antioxidants, AE-7-CII was shown to exert a more profound scavenging effect than did any of the natural antioxidants. Results indicated that the compound purified from *A. sessiliflorus* (Rupr. & Maxim.) Seem. possessed an antioxidative profile that identified it as both an $O_2^{-\cdot}$ inhibitor and scavenger.

Keywords: *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem.; Superoxide anion radical; TPA-induced $O_2^{-\cdot}$ generation; Radical scavenger; Antioxidant activity

1. Introduction

Acanthopanax sessiliflorus (Rupr. & Maxim.) Seem. (Araliaceae), which is commonly referred to as "Siberian Ginseng", is a perennial plant native to Siberia and Eastern Asia, and is similar to the *Panax* species. With regard to its pharmacological aspects, *A. sessiliflorus* (Rupr. & Maxim.) Seem. has been used in traditional medical protocols as a tonic, analgesic, antihypertensive, and antidiabetic agent (Deyama, Nishibe, & Nakazawa, 2001). However, only scanty data have been reported in relation to the medicinally relevant and biologically active compounds inherent to *A. sessiliflorus* (Rupr. & Maxim.) Seem.

In recent years, interest in reactive oxygen species (ROS), including superoxide anion radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and nitric oxide (NO), has substantially increased in direct relation to cellular abnormalities (Nakamura et al., 1998). The majority of the body's oxygen is generally reduced *via* the oxidative phosphorylation of the respiratory metabolic pathway, but some of the oxygen is transformed to ROS (Urso & Clarkson, 2003). These ROS can be scavenged by detoxifying proteins within the body, such as superoxide dismutase, glutathione peroxidase and catalase. However, quantities of ROS, which overwhelm the capacity of the body defence systems, may result in irreversible oxidative damage

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(Tseng, Kao, Chu, Lin-Wu, & Wang, 1997). Much of the available evidence indicates that excess ROS can result in deleterious changes to cells, including the disintegration of cell membrane, the oxidation of membrane protein, and the mutation of cellular DNA (Buettner, 1993; Mak, Misra, & Weglicki, 1983). In particular, the alteration or breakage of DNA templates and the interruption of cell signalling can initiate and/or propagate tumorigenesis (Halliwell, 1999). The $O_2^{-\cdot}$ has been specifically implicated in the promotion of lipid peroxidation, which is the primary relevant factor in a host of adult diseases, including arteriosclerosis (Halliwell, 1989), brain ischemia (Ginsberg, 2001), and cirrhosis of the liver (Abul, Mathew, Dashti, & Al-Bader, 2002).

Antioxidants are substances which delay or prevent the oxidation of inter- or intra-cellular oxidizable substrates arising from oxidative stress. Antioxidants exert their effects to prevent the generation of ROS, scavenge ROS or activate detoxifying proteins. Polyphenols, which are generally found in plants, exhibit a host of biological properties, including antioxidant activities (Dragsted, 2003). Antioxidants have also been implicated as possible chemopreventers against tumorigenesis (Manesh & Kuttan, 2003). Some antioxidants have been determined to protect cells from oxidative stress due to the tumor promoters, by preventing the formation of free radicals or by detoxifying free radicals, resulting in the prevention of a variety of pathophysiological processes (Mates & Sanchez-Jimenez, 2000). For instance, curcumin, a yellow pigment, which is found in tropical gingers, has been reported to function as both an oxygen-derived radical generation inhibitor under conditions of 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced oxidative stress and as a scavenger (Nakamura et al., 1998). As a result of our preliminary study, the ethanol extract of *A. sessiliflorus* (Rupr. & Maxim.) Seem. exhibited an appreciable inhibitory effect against TPA-induced $O_2^{-\cdot}$ generation, compelling us to attempt to determine the active compounds within the plant.

In the present study, we isolated the inhibitor, against TPA-induced $O_2^{-\cdot}$ generation, derived from the ethanol extract of *A. sessiliflorus* (Rupr. & Maxim.) Seem., using a differentiated HL-60 cell system. We then evaluated its inhibitory effects and cytotoxic potential. Furthermore, we conducted a comparison between its scavenging ability and those of commercially-available antioxidants.

2. Materials and methods

2.1. Sample and chemicals

A. sessiliflorus (Rupr. & Maxim.) Seem. was acquired from the Hanil Ginseng Co. (Seoul, Korea), and was authenticated by Dr. Sang In Shim, at Gyeongsang National University. The voucher specimen was deposited in the same institute. The human promyelocytic leukemia cell line, HL-60, was obtained from the American Type Culture Collection (Rockville, MD). RPMI 1640 medium, fetal bovine serum (FBS), and antibiotics were the products of Gibco BRL (Grand Island, NY). Cytochrome *c* (from horse heart), TPA, genistein, 2,2′-di-*p*-nitrophenyl -5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′diphenylene)-ditetrazolium chloride (NBT), 1,1-di-phenyl-2-picrylhydrazyl (DPPH), butylacetated hydroxyl toluene (BHT), butylated hydroxylanisole (BHA), curcumin, epigallocatechin gallate (EGCG), epicatechin, 2′,7′-dichlorofluoresceindiacetate (DCFH-DA) and *N*-acethyl-L-cystein (NAC) were all purchased from the Sigma Chemical Co. (St. Louis, MO). Silica gel 60 and Keiselgel 60 F254, used in the thin-layer choromatography (TLC), were from the Merck Co. (Darmstadt, Germany). All other chemicals were of analytical reagent-grade.

2.2. Cell culture

The human promyelocytic leukemia cell line, HL-60, was grown in RPMI 1640 medium supplemented with 10% FSB, penicillin (100 units/ml), and streptomycin (100 μg/ml). The stock cultures were maintained in T-25 flasks at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. When the cells reached >80% confluency, subculture was conducted at a cell density of 2×10^5 cells/flask. For cell differentiation, the HL-60 cells were treated with 1.25% dimethylsulfoxide (DMSO) for 4 days under an identical atmosphere to stimulate their differentiation into granulocyte-like cells.

2.3. Extraction and isolation

The dried materials of *A. sessiliflorus* (Rupr. & Maxim.) Seem. were extracted with four volumes of ethanol and filtered. The filtrate was dried with an evaporator under vacuum conditions. The crude extract was then resuspended in distilled water and mixed with an equal volume of chloroform, thereby yielding chloroform-solubles. The chloroform-solubles were applied to a column of silica gel 60 which had been equilibrated with *n*-hexane:ethylacetate = 4:1 (v/v), and eluted with the same mixed solvent. The fraction obtained from the silica gel chromatography was developed by preparative TLC (Keiselgel 60 F254 plate) with a mixture of *n*-hexane and ethylacetate (5:1, v/v) as a mobile phase. The spot with an *Rf* value of 0.41 was further purified *via* preparative HPLC on a μ-Bondapak C_{18} column (reversed phase type, 7.8 × 300 mm) with a gradient elution of 60–100% methanol as an eluent, at a flow rate of 2 ml/min, yielding a major active fraction.

2.4. HPLC analysis

Analytical HPLC was conducted with a Waters M515 HPLC instrument which was equipped with an M486 UV detector and a Waters μ-Bondapak C_{18} column (reversed phase type, 3.9 × 150 mm). The gradient elution system, consisting of 60–100% methanol, was used at a flow rate

of 0.5 ml/min. The UV-absorbing substances were detected at 285 nm.

2.5. Cytotoxicity assay

The cytotoxicity of sample was assessed using the CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega Co., Madison, WI). In brief, the differentiated HL-60 cells were incubated with the sample at various concentrations, and then 100 μl of culture supernatant were transferred to a new 96-well microtitre plate. The assay quantitatively measured the amount of lactate dehydrogenase which was released upon cell lysis. The percentage of lysis was calculated according to the following formula: [(sample release − spontaneous release)/(maximum release − spontaneous release)] × 100.

2.6. Assay for inhibitory activity against TPA-induced $O_2^{-\cdot}$ generation

The differentiated HL-60 cells were harvested, washed with PBS, and resuspended at a density of 1×10^6 cells/ml. The $O_2^{-\cdot}$ production levels were measured according to the method of Murakami, Ohura, Nakamura, Koshimizu, and Ohigashi (1996) with some modifications. Prior to the addition of 5 μl of a TPA solution (20 μM), the cells were preincubated with the various concentrations of sample for 90 s at 37 °C and washed twice with phosphate-buffered solution to remove the extracellular test sample. Then, 50 μl of cytochrome *c* (20 mg/ml) were added and reincubated for 15 min. The reaction was stopped by the addition of 5 μl of a superoxide dismutase solution (15,000 U/ml). The cell suspension was then centrifuged and the absorbance of supernatant was measured at 550 nm. The percentage of inhibition against $O_2^{-\cdot}$ generation was calculated as $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$. The IC_{50} value was defined as the concentration (μg/ml) of sample required for a 50% inhibition of $O_2^{-\cdot}$ generation.

2.7. Assay for intracellular H_2O_2 formation

Hydrogen peroxide levels were determined by using DCFH-DA as an intracellular fluorescence probe (Bouhafs & Jarstrand, 2002). The HL-60 cells were differentiated as previously mentioned, and 5 μl of DCFH-DA solution (5 μM) were added to the cell suspension, followed by 15 min of further incubation at 37 °C. After incubation, 50 μl of the sample dissolved in DMSO (25 and 35 μg/ml) were added to the cell suspension, and the mixture was reincubated for a further 15 min. Ten minutes after 5 μl of a TPA solution (100 nM) were added, the reaction was stopped by adding 50 μl of an EDTA solution (10 mM). The cell suspension was subjected to centrifugation, and then resuspended with 700 μl of FACS buffer. The DCF fluorescence resulting from the reaction of DCFH with intracellular oxidative products was analyzed on an FACS flow cytometer (FACScalibur, Beckton Dickinson, San Jose, CA).

2.8. NBT reduction assay

The $O_2^{-\cdot}$ was generated by TPA in the differentiated HL-60 cells, and the scavenging activity on the $O_2^{-\cdot}$ was spectrophotometrically determined by an NBT reduction at 570 nm (Hanson, Berg, & Nilsen, 1989). In brief, 0.9 ml of the differentiated HL-60 cell suspension was mixed with 0.1 ml of NBT (5 mg/ml), after which a respiratory burst was initiated by adding 5 μl of TPA (20 μM) prior to the addition of 50 μl of sample dissolved in DMSO (25, 50, and 100 μg/ml). The cell mixture was then incubated for 15 min at 37 °C, and the reaction was stopped by the addition of 1 N HCl. The cells were lysed using DMSO, and absorbance was measured at 570 nm. DMSO without sample was employed as a control. The scavenging activity was calculated as follows; $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$.

2.9. DPPH radical-scavenging activity

The free radical scavenging activities of the purified compound and other commercially-available compounds were measured by DPPH assay with some modifications (Bass et al., 1983). The 0.9 ml of 1.5×10^{-4} M DPPH radical solution in methanol was prepared, and then mixed with 0.1 ml of the sample dissolved in DMSO (0.7 mg/ml). The quantity of DPPH remaining in the mixed solution was measured at 517 nm for 30 min. The reduction in the absorbance of the DPPH solution indicated the degree to which radical-scavenging activity had increased. DMSO without the sample was employed as a control. The DPPH radical scavenging activity was calculated according to the following formula: $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$.

2.10. Statistical analysis

All experiments were repeated at least three times. Differences between groups were analyzed by the Duncan's multiple range test using SAS.

3. Results and discussion

3.1. Isolation of $O_2^{-\cdot}$ generation inhibitor from *A. sessiliflorus* (Rupr. & Maxim.) Seem

As shown in Table 1, the ethanol extract of *A. sessiliflorus* (Rupr. & Maxim.) Seem. displayed an appreciable inhibitory activity against TPA-induced $O_2^{-\cdot}$ generation ($IC_{50} = 45.0$ μg/ml) in the differentiated HL-60 cells. Therefore, the ethanol extract (10 g) was fractionated by the phase separation using chloroform. The resultant chloroform-solubles, AE-C, evidenced a profound inhibitory activity against the generation of $O_2^{-\cdot}$ ($IC_{50} = 26.0$ μg/ml), leading to the further purification by a sequential procedure involving silica gel column chromatography, TLC, and preparative HPLC. A relatively high degree of

Table 1
Inhibitory effects of fractions from *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem. against 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced superoxide anion radical generation and their yields in each purification step[A]

Purification step	IC_{50}[B] against $O_2^{-\cdot}$ generation (μg/ml)	Yield (%)
Ethanol extract (AE-0)	45.0 ± 3.8 a[C]	100
Solvent phase separation (AE-C)	26.0 ± 2.3 b	28.1
Silica gel 60 column (AE-7)	25.3 ± 2.7 b	3.10
Preparative TLC (AE-7-C)	17.5 ± 1.9 c	0.97
Preparative HPLC (AE-7-CII)	12.5 ± 1.1 d	0.65
Genistein	24.7 ± 2.1 b	–

[A] Data are expressed as the means ± S.D. of three experiments.
[B] IC_{50} = the concentration required for 50% inhibition.
[C] Values with different letters in a column are significantly different by Duncan's multiple range test ($P < 0.05$).

inhibitory activity was found in AE-7-CII (65 mg) among the three subfractions obtained as a result of preparative HPLC. The purified compound with the highest degree of $O_2^{-\cdot}$ generation inhibitory ability, AE-7-CII (UV max: 285 nm, IC_{50}: 12.5 μg/ml), showed a single peak on an analytical HPLC, indicating that it was highly purified (Fig. 1). The AE-7-CII reacted with Folin–Ciocalteu's reagent, H_2SO_4 and 1 N NaOH, while no reaction was observed with anthrone, Molisch, ninhydrin or ferric chloride. These results implied that the phenolic structure of the compound might be responsible for the inhibitory activity associated with *A. sessiliflorus* (Rupr. & Maxim.) Seem. against TPA-induced $O_2^{-\cdot}$ generation. The antioxidant activities of plant constituents have been found to be related to phenolic compounds (Kim, Lee, Shin, & Lee, 2004) as well as to caffeic acid derivatives and flavonoids (Namiki, 1990).

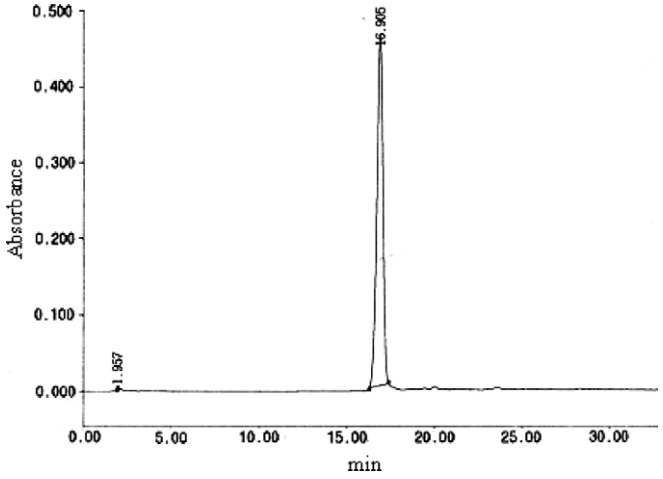

Fig. 1. HPLC profile of the antioxidant compound from *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem. HPLC analysis was conducted on a Waters M515 instrument, using a reversed-phase C-18 column (3.9 × 150 mm) and a Waters M486 UV detector at 285 nm. A methanol gradient (60–100%) was used as a mobile phase, with a flow rate of 0.5 ml/min.

Genistein is reported as the strong antioxidative and antitumorigenic isoflavone obtained from soybeans (Gerhauser et al., 2003; Lee et al., 2005). The IC_{50} of genistein (24.7 μg/ml) in the TPA-induced $O_2^{-\cdot}$ generation system was significantly higher than that of AE-7-CII (Table 1), indicating that the compound from *A. sessiliflorus* (Rupr. & Maxim.) Seem. was the potent radical inhibitor and antitumor promoter *via* inhibiting $O_2^{-\cdot}$ generation. A close relationship between the inhibition of $O_2^{-\cdot}$ generation and antitumor promotion has been observed in some natural chemopreventers (Murakami et al., 1996).

3.2. Inhibitory effect of AE-7-CII against TPA-induced $O_2^{-\cdot}$ generation

In the *in vitro* assay system, $O_2^{-\cdot}$ is generated in granulocyte-like HL-60 cells by stimulation with TPA, and is measured using the reduction of cytochrome *c*. Compounds which induce strong cytotoxic effects in the HL-60 cells may display profound inhibitory effects against the generation of $O_2^{-\cdot}$. This has been attributed to the low viability of these cells, which in turn, results in a lower level of $O_2^{-\cdot}$ generation, leading to only a small amount of reduction in the initial cytochrome *c* levels. Therefore, the actual inhibitory effects exerted by the compound against TPA-induced $O_2^{-\cdot}$ generation should be evaluated by considering its cytotoxicity. We measured the cytotoxicity and inhibitory activity of AE-7-CII concurrently at various concentrations, as shown in Fig. 2. Up to a concentration of 100 μg/ml, the reduction in cell viability did not occur by AE-7-CII, indicating no significant influence toward cytotoxicity at any tested concentrations. On the other hand, the treatment

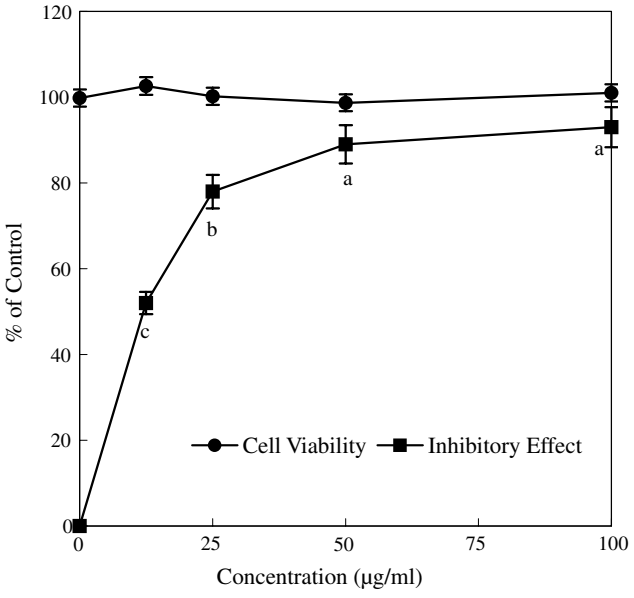

Fig. 2. Inhibitory effects and cytotoxicities of the antioxidant compound from *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem. on the 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced generation of the superoxide anion radical in the differentiated HL-60 cells. The values are expressed as means ± S.D. of three replicates. Different letters on the value are statistically different by Duncan's multiple range test ($P < 0.05$).

of cells with 12.5 μg/ml of AE-7-CII inhibited the TPA-induced $O_2^{-\cdot}$ generation by nearly 50% (Fig. 2). The AE-7-CII exerted a concentration-dependent inhibitory activity towards $O_2^{-\cdot}$ generation. At a concentration of 25 μg/ml, $O_2^{-\cdot}$ generation was inhibited by approximately 78%, as compared to the control. The average inhibitory activities were 89% and 93% with the concentrations of 50 and 100 μg/ml, respectively. Based upon these results, we confirmed that the AE-7-CII isolated from *A. sessiliflorus* (Rupr. & Maxim.) Seem. very effectively inhibited TPA-induced $O_2^{-\cdot}$ generation. This pronounced degree of inhibition suggests that the purified compound from *A. sessiliflorus* (Rupr. & Maxim.) Seem. might be useful as a new natural antioxidant. It was reported that the antioxidant compound isolated from young green barley leaves showed a dose-dependent inhibitory activity toward superoxide generation and the phenolic moiety of the structure was presumably responsible for its ability (Arimoto, Ichinose, Yoshikawa, & Shibamoto, 2000). The inhibitory effect of AE-7-CII might be, at least in part, due to the inhibition of NADPH oxidase, since it is a key enzyme in the generation of $O_2^{-\cdot}$ by leukocytes during tumor promotion (Perchellet, Perchellet, Gali, & Gao, 1995). Note that, in the present study, we eliminated the possibility of disruption due to the scavenging effects of AE-7-CII by removing the compound before it was stimulated with TPA in the $O_2^{-\cdot}$ generation system.

3.3. Inhibitory effect of AE-7-CII against intracellular hydrogen peroxide formation

Nakamura et al. (1999) reported a positive correlation between the $O_2^{-\cdot}$ generation and intracellular H_2O_2 formation in granulocytes. Therefore, we attempted to investigate the inhibitory effect of AE-7-CII against H_2O_2 formation in the differentiated HL-60 cells, *via* FACS analyses of the cells using DCFH-DA as an intracellular fluorescent probe. Most of the cells produced H_2O_2 with the TPA stimulation alone (H_2O_2 positive percentage = 91.46%), as shown in Fig. 3. However, the H_2O_2 formation was remarkably reduced upon the introduction of AE-7-CII into the assay system. Its inhibitory effects (IE) against H_2O_2 formation were 26.7% and 68.7% at 25 μg/ml and 35 μg/ml, respectively. These data implicate AE-7-CII's ability to effectively suppress intracellular H_2O_2 formation, in a concentration-dependent manner. Combined with the results regarding the inhibitory effect of AE-7-CII against TPA-induced $O_2^{-\cdot}$ generation, we could confirm that the inhibitory effect of AE-7-CII against intracellular H_2O_2formation may be derived from its ability to inhibit $O_2^{-\cdot}$ generation, which is in general agreement with the observations of Lundqvist, Follin, Khalfan, and Dahlgren (1996).

3.4. Scavenging effects of AE-7-CII on $O_2^{-\cdot}$

The use of synthetic antioxidants, such as BHT and BHA, has been restricted in the food industry due to their

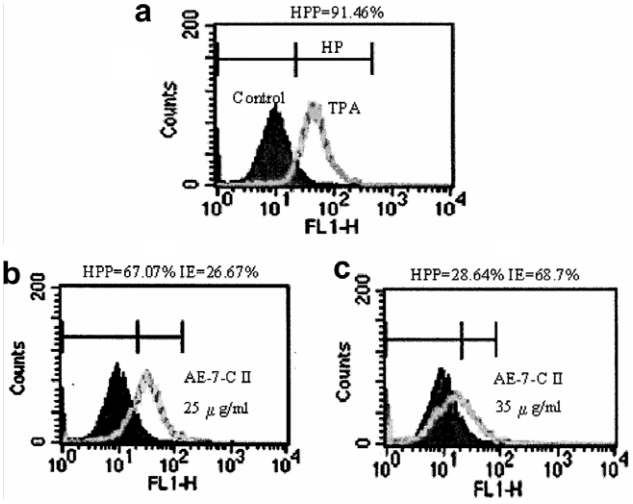

Fig. 3. Inhibitory effects of the antioxidant compound from *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem. on 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced intracellular hydrogen peroxide formation in the differentiated HL-60 cells. The DCF fluorescence was monitored on a flow cytometer with excitation and emission wavelengths at 488 nm and 525 nm, respectively. The control value was regarded as hydroperoxide positive (HP), and its percentage was expressed as the hydroperoxide positive percentage (HPP). IE represents the inhibitory effect (%). a; TPA alone, b; TPA + 25 μg/ml of sample, and c; TPA + 35 μg/ml of sample.

carcinogenesis. Subsequently, interest in the development of antioxidants with limited cytotoxicity has immensely increased in recent years. Ascorbic acid, α-tocopherol, curcumin, quercetin, epicatechin and EGCG have been revealed as effective antioxidants of natural origin (Frankel, 1996; Lee & Choe, 2003). Therefore, we assessed the scavenging activity of the purified compound from *A. sessiliflorus* (Rupr. & Maxim.) Seem., using an NBT reduction assay. The superoxide anion radical, which was observed in the mature granulocyte-like differentiated HL-60 cells, was reported to reduce the NBT, and to spur the formation of formazan in the cells (Collins, Bonder, Ting, & Gallo, 1980). In Fig. 4, the scavenging activity of AE-7-CII on $O_2^{-\cdot}$ increased in a concentration-dependent manner. The scavenging activity of AE-7-CII was 81% at 100 μg/ml, which was comparable to that of genistein ($P < 0.05$). These results indicated that the purified compound from *A. sessiliflorus* (Rupr. & Maxim.) Seem. had the ability to scavenge $O_2^{-\cdot}$ as well as to inhibit $O_2^{-\cdot}$ generation.

The DPPH radical is a lone highly-stable free radical, which appears violet at 517 nm. It is decolorized by the action of proton-radical scavengers; therefore, a compound which can lower the absorbance of the DPPH radical would be regarded as a radical scavenger (Bass et al., 1983). We compared the DPPH radical-scavenging activity of AE-7-CII, the purified compound from *A. sessiliflorus* (Rupr. & Maxim.) Seem. to a variety of commercially available antioxidants in a DPPH radical assay system (Fig. 5). The highest DPPH radical-scavenging activity was observed in *N*-acetylcysteine, a compound exhibiting antioxidant effects on malondialdehyde

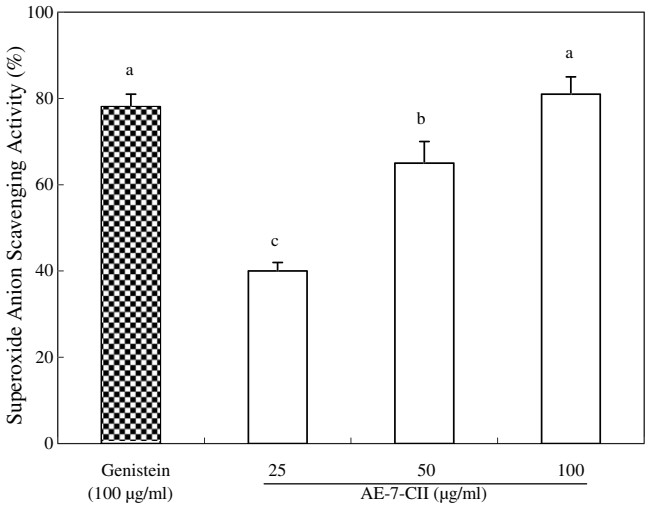

Fig. 4. Scavenging activities of the antioxidant compound from *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem. against the superoxide anion radical. The scavenging activity on 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced reactive oxygen species was determined in the differentiated HL-60 cells by NBT reduction assay. Genistein was employed as a positive control. Data are expressed as means ± S.D. of three experiments. Different letters above the bar are statistically different by Duncan's multiple range test ($P < 0.05$).

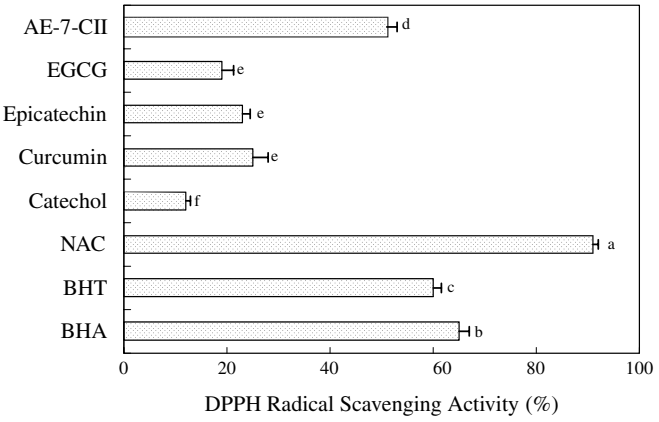

Fig. 5. DPPH radical-scavenging activities of the antioxidant compound from *Acanthopanax sessiliflorus* (Rupr. & Maxim.) Seem. and the commercial antioxidants. BHT: butylated hydroxytoluene, BHA: butylated hydroxyanisole, NAC: *N*-acetylcysteine, EGCG: epigallocatechin gallate. The sample was dissolved in DMSO (0.7 mg/ml). Data are expressed as means ± S.D. of three experiments. Different letters next to the bar are statistically different by Duncan's multiple range test ($P < 0.05$).

(Cotgreave, 1997), followed by the synthetic antioxidants, BHT and BHA. The AE-7-CII had a relatively low DPPH radical-scavenging ability compared to the synthetic antioxidants, but evidenced a higher degree of scavenging activity than did the natural antioxidants. The activity of AE-7-CII was at least twice as high as the activity levels registered by EGCG, epicatechin, curcumin and catechol ($P < 0.05$). Based upon these results, AE-7-CII was implicated as a potentially useful radical-scavenger for a host of radicals.

4. Conclusions

Aerobic organisms produce ROS as the result of oxygen metabolism. Although ROS are generated in small amounts, their highly reactive properties readily initiate sequential reactions, propagate ROS generation and inflict damage upon cells, including cell dysfunction and, ultimately, cell death. Therefore, the elimination of ROS can successfully protect cells against such damage. Small molecules with antioxidant properties are the new tools for the prevention of such oxidative stress-related diseases. In the present study, we isolated a compound from *A. sessiliflorus* (Rupr. & Maxim.) Seem., AE-7-CII, which exhibited profound antioxidant effects. The high inhibitory ability of AE-7-CII against TPA-induced $O_2^{-\cdot}$ generation might exert a beneficial effect against pathophysiological alterations caused by the presence of $O_2^{-\cdot}$. Interestingly, AE-7-CII was also shown to manifest scavenging activity against $O_2^{-\cdot}$, thereby implicating the compound as a relatively effective radical-scavenger. Considering the results, AE-7-CII might be used both as an inhibitor of TPA-induced $O_2^{-\cdot}$ generation and as a radical scavenger. Research is currently underway on the structural identification of the radical-generation inhibitor inherent to *A. sessiliflorus* (Rupr. & Maxim.) Seem., and the evaluation of its *in vivo* effects both as an antioxidant and as an antitumor-promoter.

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