

Protective Effects of *Alpinia katsumadai* Extract Against Oxidative Stress

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In the present study, total methanol extracts prepared from *Alpinia katsumadai* showed significant protective effects against the oxidative stress induced by hydrogen peroxide, UV-C or γ -ray irradiation. These protective effects were substantially increased by treatment with 20~100 μ g/ml of the extract. The *A. katsumadai* total methanol preparation was further fractionated into n-hexane, dichloromethane, ethylacetate, n-butanol and water fractions. Among these five fractions, the ethylacetate and butanol fractions of *A. katsumadai* showed the strongest protective effects against oxidative stress induced by UV-C and γ -ray irradiation. These fractions also showed high DPPH radical scavenging and lipid peroxidation inhibitory activities. In addition, both fractions displayed cell proliferation activation effects, as evidenced by significant increases in colony formation. Our current data thus suggest that the mechanisms underlying the protective effects of *A. katsumadai* against oxidative damage may include radical scavenging, protection against cell membrane damage and stimulation of cell proliferation.

Key words: medicinal plant extract, protection, reactive oxygen species

Introduction

Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from the metabolism of oxygen. Moreover, ROS can cause extensive damage to cells and tissues, during infections and various degenerative disorders such as cardiovascular disease, aging, and neurodegenerative

diseases like Alzheimer's disease, mutations and cancer (Harman, 1994; Cox and Cohen, 1996; Finkel and Holbrook, 2000).

Mammalian cells possess elaborate defense mechanisms for radical detoxification. Key metabolic steps are the superoxide dismutase catalysis of the dismutation of superoxide to hydrogen peroxide and oxygen, and the conversion of H_2O_2 into water and oxygen by catalase and glutathione peroxidase, which destroys toxic peroxides. In addition to antioxidant enzymes, several small-molecule antioxidants play important roles in the antioxidant defense systems. These can be divided into compounds made *in vivo*, and compounds obtained from diet. Glutathione, bilirubin, and melatonin are examples of the former, and vitamins such as α -tocopherol, β -carotene, and ascorbic acid and micronutrient elements such as zinc and selenium examples of the latter (Halliwell and Gutteridge, 1998).

In recent years, there has been a worldwide trend towards the use of the natural phytochemicals present in berry crops, teas, herbs, oilseeds, beans, fruits and vegetables (Velioglu *et al.*, 1998; Deiana *et al.*, 1999; Kitts *et al.*, 2000; Lee and Shibamoto, 2000; Wang and Jiao, 2000; Lie and Xie, 2000).

Natural antioxidants have a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals, and alteration of intracellular redox potential (Finkel and Holbrook, 1991). One of our laboratory's interests is on the investigation of the mechanism of cellular response to various oxidative stress and other cell damaging agents. In addition, we are trying to develop a potential cellular damage-protective agent which can reduce cellular damages induced by various chemical and physical damages (Kim *et al.*, 1998; Lee *et al.* 2003; Han *et al.*, 2006).

The plant used in this study, *Alpinia katsumadai* (Zingiberaceae), was selected based on its traditional use in Chinese medicine and results reported earlier (Kim *et al.*, 1998, 2000). This plant has been used as traditional medicine to treat a variety of gastric disorders and emesis. Recent reports have shown antiviral activity against rotavirus and

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influenza virus (Kim *et al.*, 2010; Kwon *et al.*, 2010). It is also reported that neuro-protective effect of *A. katsumadai* after ischemic damage through anti-oxidative effect (Jeong *et al.*, 2007; Li *et al.*, 2011). Previous investigations of *A. katsumadai* have reported a variety of diarylheptanoids (Kuroyanagi *et al.*, 1983), chalcones and flavonoids (Yushiro *et al.*, 1968), monoterpenes, sesquiterpenoids (Saiki *et al.*, 1978; Lawrence *et al.*, 1972), stilbenes and labdanes (Brown *et al.*, 1998). Yang *et al.* (1999), also, isolated two novel diarylheptanoids from the seeds of *A. katsumadai* and their structures were determined by spectroscopic analysis. Although it is known that these components have antioxidant activity, very little research has been done on antioxidant or biological activity *in vivo* of *A. katsumadai*. In this study, we would like to report our findings of protective effect of *A. katsumadai* extract against cell damages caused by UV-C and γ -ray irradiation. In order to investigate the underlying mechanism of the protective effect, DPPH radical scavenging, the lipid peroxidation inhibitory and colony forming activities are tested and reported.

Materials and Methods

Preparation of total methanol extract and fraction samples

A. katsumadai (100 g) was extracted at 80°C in 70% methanol for 3 hr. The extract was then filtered and the filtrate was concentrated under low pressure using a vacuum rotary evaporator (Eyela, Japan). The remaining residue was lyophilized in a freezing-dryer (Ilsin, Korea) and stored at -70°C. Approximately 10 g of powdered extract was recovered. The powder was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give final concentrations used in the experiments.

Fraction samples were prepared as the followings. Frozen-dried methanol extract sample was dissolved in d-H₂O and the equal volume of n-hexane was added and extracted twice. Then the equal volume of dichloromethane (CH₂Cl₂), ethylacetate (EtOAc) and n-butanol (BuOH) were added to the water fraction one by one and the extraction procedure was performed twice. Each fraction samples were dried in a vacuum rotary evaporator (Eyela, Japan) and the water fraction was frozen-dried using a freezing-drier (Ilsin, Korea).

Chemicals

The following chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA): dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH). Hydrogen peroxide was purchased from Fluka Chemical Co. (Buchs, Swiss). All other chemicals were of the highest analytical grade and purchased from common sources.

Cell culture, treatment and cell viability assay

Chinese hamster lung cell line V79-4 (ATCC CCL-93) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM glutamine, and antibiotics. Cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For hydrogen peroxide treatment, V79-4 cells were seeded in a 96-well plate and incubated for 16 hr. Then 100 μ M H₂O₂ was added to the culture and cells were incubated for 24 hr at 37°C. For γ -ray treatment, cells were irradiated with indicated doses of ⁶⁰Co γ -rays (Picker, USA) at a dose rate of 0.9 Gy/min at room temperature. For UV-irradiation, V79-4 cells were plated in a 96 well plate as described below and irradiated with 300 J/m² of UV-C (254 nm) using a UV cross-linker (Stratagene, USA).

Cell viability was estimated by the MTT assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells (Hansen *et al.*, 1989). V79-4 cells were treated with various concentrations of extract or fraction samples (4, 20, or 100 μ g/ml, respectively) for 1 hr where stated. Cells were incubated for an additional 24 hr at 37°C. During the last 4 hr, cells were incubated with 20 μ l of MTT stock solution (5 mg/ml) in 200 μ l medium at 37°C. Samples were then extracted with acidic isopropanol and the absorbance was measured with the ELISA reader (Bio-Rad, USA) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of formazan formed in control cells was taken as 100% of viability. The data are expressed as mean percentage of viable cells as compared to the respective control cultures.

DPPH free radical scavenging activity.

In order to measure antioxidant activity, the DPPH free radical scavenging assay was carried out as described elsewhere (Lee *et al.*, 2003).

Lipid peroxidation inhibitory activity.

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the method described elsewhere (Lee *et al.*, 2003).

Clonogenic assay

Cells were seeded in 90 mm plates, incubated for 7 days in a CO₂ incubator. The cells were fixed in methanol:acetic acid (3:1) and stained with trypan blue. Colonies of more than 50 cells were scored. Plating efficiencies were about 55–60% for the cell line used.

Statistics

All data represent means \pm S.E. Statistical analysis was performed using analysis of variance followed by the Student's *t*-test.

Results

Protective effect of total extract on various cellular damages

In order to study cell damage protective effect of a medicinal plant, *A. katsumadai* total methanol extract, we treated cells with chemical and physical stress which cause cellular damage. Cell viability after H₂O₂, UV-C and γ -ray treatment was reported in elsewhere (Ji and Kim 2009, Lee and Kim, 2008) and it was confirmed again in this study. First of all, cells were pre-treated with total extract before 50 μ M of H₂O₂ treatment at which cells showed approximately 50% of cell viability. At 4 μ g/ml concentration, there was no protective effect. However at higher concentration, the cell viability of H₂O₂ treated cells was increased as dose-dependent manner

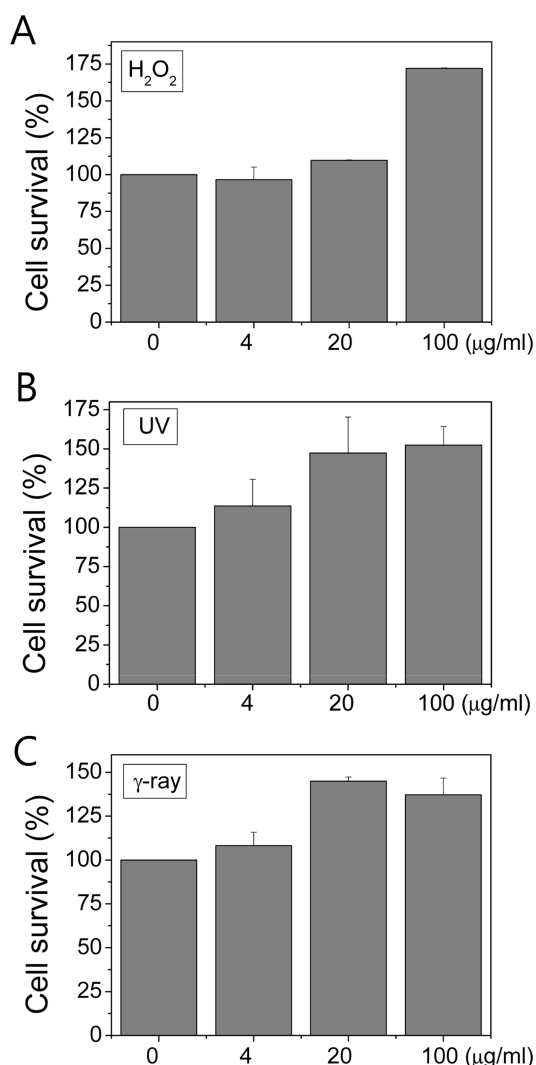


Fig. 1. Effect on cell viability of *A. katsumadai* total methanol extract against H₂O₂ (A) UV (B) and γ -ray (C) induced oxidative damages. Cells were treated with various concentrations of total extract 1 hr before H₂O₂ or UV or γ -ray treatment, then the relative cell viability was measured. Each experiment was performed at least 3 times and data are expressed as average percent change from control \pm S.D.

of total extract treated (Fig. 1A); 109.6% and 172.1% of cell viability at 20 and 100 μ g/ml of total extract, respectively.

In order to study UV-C protective effect of a medicinal plant, *A. katsumadai* total methanol extract, we pre-treated cells with the total extract 1 hr prior to the 300 J/m² of UV-irradiation. When cells were pre-incubated with total extract, cell viability was significantly increased (Fig. 1B). At 4, 20 and 100 μ g/ml of total extract treatment cell viability was increased to 111.1%, 147.3% and 152.4%, respectively.

From cell viability assay after γ -ray irradiation, we observed that approximately 65.9% of cells were survived after 10 Gy of γ -ray irradiation as shown in Fig. 1C. In order to investigate the effect of *A. katsumadai* total extract, cells were pretreated with total extract 1 hr prior to 10 Gy of γ -ray irradiation. The relative cell viability was measured and plotted as shown in Fig. 3. At 4, 20 and 100 μ g/ml of total extract treatment cell viability was increased to 108.2%, 144.9% and 137.2%, respectively.

Protective effect of fraction samples against UV and γ -ray-induced cell damages

In order to narrow down the fractions which contain the protective activity against cell damaging stress, we prepared

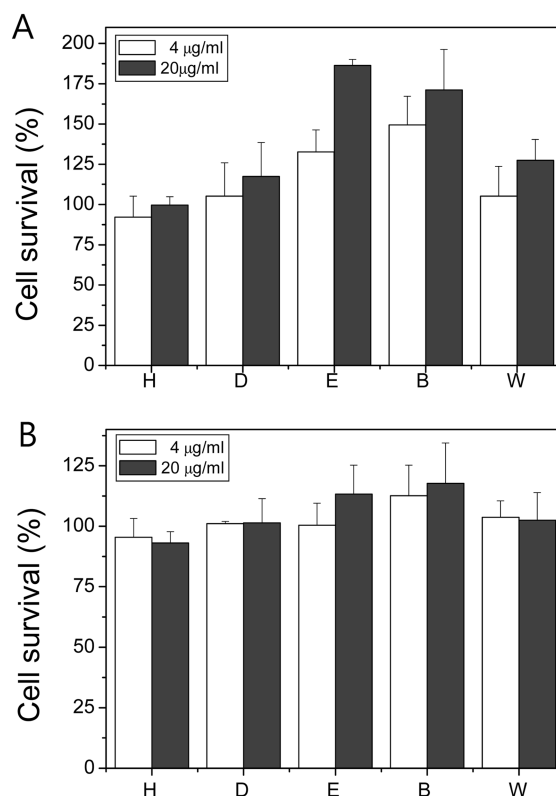


Fig. 2. Effect of fraction samples on the cell viability against UV (A) or γ -ray (B) induced oxidative stress. Cells were treated with fraction samples 1 hr prior to 300 J/m² of UV or 10 Gy of γ -ray irradiation. Each experiment was performed at least 3 times and data are expressed as average percent change from control \pm S.D. White and grey bars indicate 4 and 20 μ g/ml of samples, respectively.

five fraction samples as described in materials and methods; hexane, dichloromethane, ethylacetate, butanol and water fractions. Fraction samples were tested for the protective effect against UV-induced cell damage. Among 5 fraction samples, ethylacetate, and butanol fractions revealed significant protective effect against UV-induced cellular damage (Fig. 2A). At 20 $\mu\text{g/ml}$, ethylacetate fraction revealed 186.4% cell viability compared to no-treatment control group. Butanol fraction showed 171.2% cell viability at the same concentration as that of ethylacetate fraction.

Fraction samples were tested for the protective effect against γ -ray irradiation. Among 5 fraction samples, ethylacetate and butanol fractions showed significant increased the relative cell viability of γ -ray irradiated cells. At a concentration of 20 $\mu\text{g/ml}$ of ethylacetate and butanol fraction samples, the relative cell viability was similar, 113.3% and 117.7%, respectively (Fig. 2B).

DPPH radical scavenging and Lipid peroxidation inhibition activity

In order to investigate the possible mechanism involved in the protection activity against UV-induced cellular damage, DPPH radical scavenging and lipid peroxidation inhibition activity of the fraction samples were measured. Among fraction samples, ethylacetate and butanol fractions which showed significant UV and γ -ray induced cellular damage protective effect in previous experiment were tested for DPPH radical scavenging activity. Both fractions revealed similar scavenging activities and the response was dose-dependent (Fig. 3); for ethylacetate fraction, 69% at 4 $\mu\text{g/ml}$ and 90% at 20 $\mu\text{g/ml}$; for butanol fraction, 69% at 4 $\mu\text{g/ml}$ and 88% at 20 $\mu\text{g/ml}$.

We further tested the lipid peroxidation inhibition activity of ethylacetate and butanol fraction samples. As shown in Fig. 4, Ethylacetate fraction showed slightly higher lipid peroxidation inhibitory effect; at concentration of 4 $\mu\text{g/ml}$, approximately 73.5% and at 20 $\mu\text{g/ml}$, approximately 84.5% of inhibition

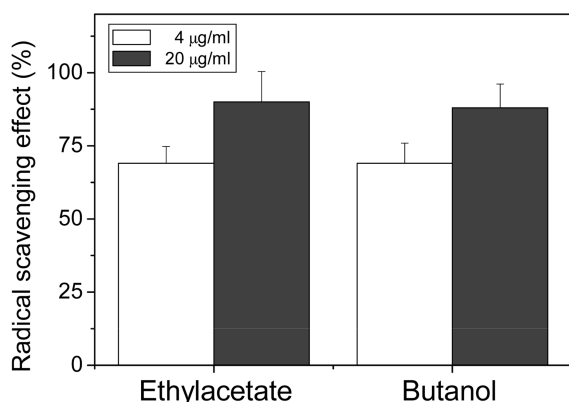


Fig. 3. DPPH radical scavenging activity of fraction samples. Fraction samples were added to DPPH solution and radical scavenging activity was measured at 520 nm. Each experiment was performed at least 3 times and data are expressed as average percent change from control \pm S.D. White and gray bars indicate 4 and 20 $\mu\text{g/ml}$ of samples, respectively.

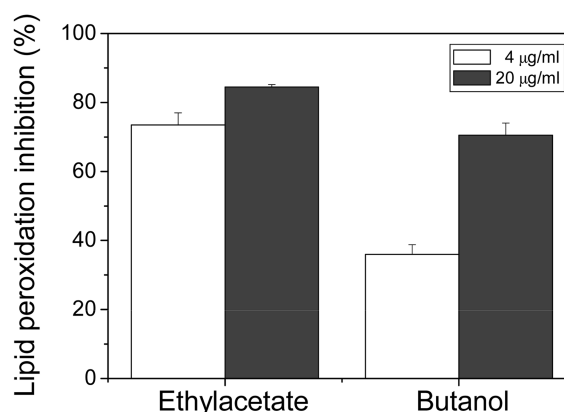


Fig. 4. Lipid peroxidation inhibition activity of ethylacetate and butanol fraction samples. Each experiment was performed at least 3 times and data are expressed as average percent change from control \pm S.D.

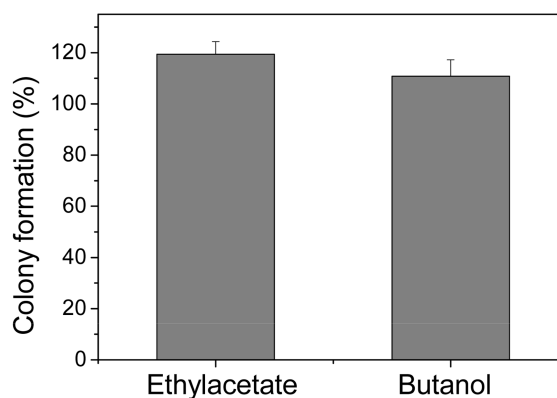


Fig. 5. Effect of ethylacetate and butanol fraction samples to clonogenic activity of Chinese hamster lung (V79-4) cells. Cells were treated with 20 $\mu\text{g/ml}$ of fraction samples and incubated for 7 days. The relative number of colonies formed were observed and plotted.

was observed. For butanol fraction 36% (at 4 $\mu\text{g/ml}$) and 70.5% (at 20 $\mu\text{g/ml}$) of inhibitory activity were observed.

Colony formation of ethylacetate and butanol fraction samples

The cytotoxicity of the ethylacetate and butanol fraction samples was measured using clonogenic assay (Fig. 5). Cells were seeded in multiple culture dishes incubated with 20 $\mu\text{g/ml}$ of fraction samples for 7 days. After 7 days of incubation, colonies with more than 50 cells were counted and the relative colony formation was plotted. As shown in Fig. 5, colony formation was slightly increased compared to no-treatment control; for ethylacetate fraction, 119.4% and for butanol fraction 110.8%.

Discussion

In recent years, there has been a worldwide trend towards the use of the phytochemicals present in natural plants.

Efforts to find oxidative cell damage attenuators including radioprotectors among plants products are also made (Arora *et al.*, 2005). Earlier studies showed that extracts from *Areca catechu* var. *dulcissima* showed strong scavenging activity against the superoxide anion radical (Ohsugi *et al.*, 1999). A cyclic phenylacetamide of *Solvia miltiorrhiza* was found to be a scavenger of the DPPH radical (Choi *et al.*, 2001). Methyl gallate from *Toona sienensis* showed protective against H₂O₂-induced stress (Hsieh *et al.*, 2004). The active fraction of *Pilea microphylla* (L.) ethanol extract showed anti-oxidant and radioprotective effect (Prabhakar *et al.*, 2007). The radioprotective effect of *Coleus aromaticus* on Chinese hamster fibroblast cells was reported (Rao *et al.*, 2006).

In addition, the protective activity of sulforaphane-containing broccoli sprout extract against UV-B-induced skin cancer was reported (Dinkova-Kostova *et al.*, 2006). The animal received high dose of extract showed 50% reduction in tumor burden, incidence and multiplicity. Treatment of Green tea polyphenol (GTP) resulted in inhibition of UV-B-induced protein oxidation *in vitro* and *in vivo*. GTP also inhibited UV-induced expression of matrix metalloproteinases (Vayalil *et al.*, 2004). The protective effect of soybean oil and its methanolic extract against UV-C irradiation by using comet assay was reported. It was concluded that the potential antioxidants found in the extract may be responsible for the protective effect (Wang *et al.*, 2003).

In this study we reported that the protective effect of *A. katsumadai* extract against various oxidative damage including H₂O₂ treatment, UV-C and γ -ray irradiation. Methanolic total extract showed significantly increased cell viability against oxidative cell damaging treatment used in this study. Among fraction samples, ethylacetate, and butanol fractions increased the cell viability significantly. Among two fractions ethylacetate fraction showed the highest relative cell viability ratio even though both samples showed significant increase in cell viability. In order to investigate the mechanism behind the cell protection activities, we measured DPPH radical scavenging activity and lipid peroxidation inhibitory activity. Among fraction samples, ethylacetate fraction revealed the highest DPPH radical scavenging and lipid membrane peroxidation inhibition activity. Fraction samples showed higher protective activity also revealed higher colony forming activity measured by clonogenic assay. Taken together, it seems that the mechanism behind the protective effect against oxidative cellular damaging treatment is free radical scavenging and cell membrane protection against peroxidation. In addition, increase in cell proliferation is involved in the protection mechanism. Further studies are needed to be confirmed by *in vivo* study and to identify the active protective molecule in fraction sample.

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