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Comparative Analysis of the Antioxidant Activities of Green Peppers Cultivated under Conventional and Environmental-Friendly Farming conditions

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Environmental-friendly agriculture (EFA) is defined as the cultivation of crops with reduced amounts or without chemical-synthetic pesticides. Recently, the use of chemical pesticides has decreased significantly; therefore, we cultivated peppers following EFA- and conventional methods and compared their antioxidant activities. To accomplish this, the environmental-friendly cultivated peppers (EFPE) and conventionally cultivated peppers (CCPE) were extracted with 70% methanol and the effects of the extracts on the cell viability, intracellular ROS generation, lipid peroxidation and catalase activity of HepG2 cells were evaluated. EFPE showed a stronger protective effect against oxidative stress induced-cell death than that of CCPE. EFPE also reduced intracellular ROS generation (42.7% to 26.4%) following treatment with hydrogen peroxide more effectively than that of CCPE (24.2% to 6.3%). Furthermore, EFPE and CCPE showed protective effects against lipid peroxidation and induced catalase activity, although these effects were not statistically significant. Taken together, these results suggest that EFPE showed stronger antioxidant activities than CCPE, and thus represent evidence that EFA with biocontrol materials may improve the functional properties of crops and/or secondary metabolites with antioxidant activities when compared with conventional agricultural practices.

Key Words: Antioxidant, Catalase, Environmental-Friendly Agriculture, Intracellular ROS, Lipid Peroxidation, Pepper

INTRODUCTION

The growing demands for organic and sustainable agricultural plant foods can be attributed to concerns regarding the environment and food safety, as well as a broader interest in the relationship between diet and human health. The recent agricultural policy of Jeonnam Province in South Korea involves a reduction in the use of chemical pesticides, improvement of crop quality and decreased environmental contamination. Because the longevity and slow degradation rate of chemical pesticides create potential environmental and human health concerns, a variety of non-chemical pesticides have been developed in an attempt to reduce the use of such compounds (Simon *et al*, 2003). According to the agricultural policy of Jeonnam Province, environmental-friendly agriculture (EFA) is defined as the cultivation of crops with reduced amounts or without the aid of chemical-synthetic pesticides. Accordingly, EFA employs various biocontrol materials such as plant extracts and microbial cultures to control pathogens as alternatives for chemical-synthetic pesticides.

Many epidemiological studies have shown that increased consumption of fruits and vegetables is associated with the prevention of chronic diseases such as diabetes, heart disease and certain cancers (Block *et al.*, 1992; Kris-Etherton *et al.*, 2002). Additionally, phytochemicals (naturally occurring and synthetic) that are foreign to

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mammalian cells have been recognized as potent antioxidants (Kahl, 1984; Block, 1992), and phytochemicals derived from plants have attracted considerable attention because they do not show toxicity when metabolized in liver cells (Barclay *et al.*, 1990; Sato *et al.*, 1992). Indeed, it has been estimated that more than two-thirds of human cancers could be prevented by appropriate lifestyle modification. Phytochemicals can scavenge reactive oxygen species (ROS) and show antioxidant, anti-inflammatory and anticancer activities (Prior, 2003). Furthermore, evidence from in vitro laboratory studies, clinical trials and epidemiological investigations indicates that plant-based diets have protective effect against various cancers. Specifically, it has been suggested that about 7-31% of all cancer worldwide could be reduced by the consumption of diets high in fruits and vegetables (Surh, 2003).

Pepper fruits (*Capsicum annum* L.) are important vegetables used as foods and spices. Peppers are a good source of vitamin C and E as well as provitamin A and carotenoids (Krinsky, 2001; Matsufuji *et al.*, 1998). Additionally, hot pepper cultivars are rich in capsaicinoids-alkaloids that have pharmacological properties as well as impart unique tastes to the pepper fruit (Daood *et al.*, 1996; Wachtel, 1999). Phenolics, flavonoids and capsaicinoids from peppers show strong antioxidant activities and capsaicin and dihydrocapsaicin are the main compounds of capsaicinoids (Materska and Perucka, 2005; Kozukue *et al.*, 2005). Additionally, the presence of derivatives of cinnamic acid and flavonoids has been found in pepper fruits (Sukrasno and Yeoman, 1993). Previously, we reported the presence of monoamines and hydroxycinnamic acid amides (HCAA) of serotonin in pepper and other common vegetables (Ly *et al.*, 2008b). Specifically, peppers contain 17.9, 16.2 and 141.5 μg g^{-1} of dry weight of serotonin, tryptamine and tyramine, respectively, and these compounds all show strong antioxidant activities.

In the present study, peppers were cultivated according to an EFA manual in Nampung and Naju (Jonnam Province) and analyzed antioxidant activities such as protective effects against oxidative stress induced-cell death, intracellular ROS generation, lipid peroxidation and catalase activity in HepG2 cells. HepG2 cell, a hepatocellular carcinoma cell line, generally uses to evaluate the antioxidant activities and various biological activities of phytochemicals. To evaluate the quality of EFA peppers, comparative analysis was performed with conventionally cultivated peppers, which were cultivated in a neighboring greenhouse and analyzed the antioxidant activities of pepper extracts.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from PAA Laboratories (Pasching, Austria). Hydrogen peroxide (H_2O_2), DCFH-DA and TBA were purchased from Sigma-Aldrich (St. Louis, MO, USA). An XTT cell viability assay kit was purchased from WelGENE (Daegu, Korea).

Vegetable samples

The fruits of green pepper (*Capsicum annuum* var. Cheongyang) fruits were separately collected from two greenhouses (conventional and environmental-friendly) located in Nampung and Naju (Jonnam Province, Korea). All samples were harvested at 84, 92, 102, 112, 124 and 137 days after the first seedling was transplanted on February 28, 2008. For the conventional cultivation of peppers (CCP), several chemicals including Ridomil and Deomani (Dongbu Hitek Co., Seoul, Korea), Ongdalsam (Syngenta Co., Seoul, Korea), and Hint (Hankook Samgong Co., Suwon, Korea) were applied as fungicides, while A-pam, Bumelang, Mospilan, and Stonet (Syngenta Co.), Setis and Olgami (Dongbu Hitech Co.), and Conido (Byer Crop Science Co., Korea) were applied as insecticides, only. For the environmental-friendly cultivation of pepper (EFP), only environmental-friendly agents such as Top (GreenBiotech Co., Paju, Korea), Mai (Daeyu Co., Gyeongsan, Korea), BAC-1 (Chonnam National University, Gwangju, Korea), Eco (Shinyoung Agro, Goyang, Korea) and Fic (Bio-resource, Bucheon, Korea) were used for disease control, while Bugpam (Farmskorea Co., Yeoju, Korea), DipenseM (Dongbu Hitech Co.), Bestop and Bogum (Kyungnong Co., Seoul, Korea), and Olcatch (Nambo Co., Gyeongju, Korea). These agents are officially registered as biocontrol agents by the Rural Development and Administration, Suwon, Korea. To eliminate sampling bias and differences associated with the harvest time, peppers were harvested six times from May to July after seeding in February. During each sampling event, 100 g of fresh peppers were freeze dried, after which 1 g of the dried powder from each sample was mixed and extracted with 70% methanol. After extraction, we measured the dry

weight of the extracts using rotary evaporation.

Analyses of total phenolic compounds (TPC) and flavonoids contents (FC)

TPC and FC were extracted from one gram (dry weight) of each pepper as described previously (Ly *et al.* 2008a). The TPCs in the peppers were measured in accordance with the Folin-Ciocalteu procedure, and the results were expressed as gallic acid equivalents (GAE)/g of dry weight. The FC of the peppers was expressed as mg of rutin equivalents (RE)/g of dry weight. The data were expressed as the means ± SD for at least three replications.

Cell culture

HepG2 cells were maintained in DMEM containing 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37℃ in a humid atmosphere of 5% CO2. For all experiments, cells (1×10^5 cells/mL) were seeded in a culture dish and maintained in the tissue culture incubator.

Cell Proliferation Assay

Adherent cells were detached from culture plates with 0.05% trypsin-0.02% EDTA to prepare a cell suspension. Cells suspended in culture medium containing 10% FBS were divided and placed into a flat-bottomed 96-well plate. After the cells were attached the plate, various concentrations of pepper extracts (0.025 to 250 μg/mL) were applied to each well and the plates were then incubated at 37 ℃ for 24 h. Proliferation of HepG2 was determined by a colorimetric method based on an 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay. Briefly, 1 mL of XTT labeling reagent and 20 μL of electron coupling reagent phenazine methosulfate (PMS) were mixed together, after which 20 μL of this solution was applied to the 96-well plate via a pipette. The plate was then incubated at 37℃ for 3 h, and then the absorbance at 450 nm and 690 nm was measured using an ELISA reader and the values were derived from the absorbance as described below. Each experiment was repeated at least four times and the analyses were then conducted based on the mean values.

Cell proliferation (%) = Fluorescence of sample (450 nm - 690 nm)/ Fluorescence of control (450 nm - 690 nm) × 100

Measurement of intracellular reactive oxygen species (ROS)

Relative changes in the intracellular ROS of the HepG2 cells were monitored using a fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA diffuses through the cell membrane readily and is deacetylated by intracellular esterases to non-fluorescent DCFH, which is then rapidly oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS (LeBel *et al.*, 1992). The intensity of the DCF fluorescence is proportional to the amount of ROS formed intracellularly. HepG2 cells were seeded in a 96-well plate at 1×10^4 cells per well. At sixteen hours after plating, the cells were incubated in medium without FBS alone or with red pepper extracts for 24 h, and then they were incubated with 100 μM DCFH-DA in DMEM for 30 min at 37°C in the dark. The cells were then washed in HEPES-buffered saline (HBS) containing 135 mM NaCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES (pH 7.4) and 6.0 mM glucose, after which they were treated with 1 mM H_2O_2 for 1 h and the intensity of fluorescence was immediately read in a fluorescence spectrophotometer (Molecular Devices, IL, USA) at an excitation of 485 nm and an emission of 530 nm (Qiao *et al.*, 2004).

Increased ROS (%) = (1 h-0 h)/0 h × 100

Lipid Peroxidation

The cells were cultured with 1×10^5 cells per well in 24-well plate as described above. The HepG2 cells were then treated with the pepper extracts for 24 h, and then they were treated with 1mM H_2O_2 for 1 h. After incubation, the media were removed and the cells were washed with ice-cold PBS and then added to 640 μL of TBA mixture containing 6.25 mM Tris·Cl (pH 7.4), 0.78 mM EDTA, SDS 0.5% (w/v), acetic acid 9.4% (v/v) and TBA 0.375% (w/v) and boiled at 60°C for 1 h in the dark. The 24-well plates were then placed in an ice-bath for 10 min, after which 400 μL of n-butanol:pyridine (15:1, v/v) and 400 μL of the cell lysate were transferred into an eppendorf tube and shaken vigorously at room temperature for 10 min. The samples were then centrifuged for 10 min at 400 g at 4°C. Finally, the supernatant was transferred into a 96-well plate and the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured at an excitation of 515 nm and an emission of 553 nm.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD). Statistical differences were determined by ANOVA using SPSS 12.0K (Chicago, IL). P values <0.05 were considered significant.

RESULTS AND DISCUSSION

Cellular oxidative injury has been implicated in aging and a wide array of clinical disorders including ischemia-reperfusion injury, neurodegenerative diseases, diabetes, inflammatory diseases, arthritis, hepatitis and drug-induced toxicity. Reactive oxygen and reactive nitrogen species can cause damage to all cellular macromolecules, including nucleic acids, proteins, carbohydrates and lipids (Hussain *et al.*, 2003; Squier, 2001). In this study, the antioxidant activities of EFA and CC peppers were compared. The extracted concentrations of EFPE and CCPE were 24.2 ± 2.0 μg/μL and 24.5 ± 1.8 μg/μL, respectively, and the extraction yields were 38.7 ± 3.2% and 39.2 ± 2.9%, respectively, which did not differ significantly. Diluted concentrations of EFPE and CCPE of 0.025 to 250 μg/mL were used in the following experiments.

Effects of pepper extracts on cell viability

To analyze the effects of pepper extracts on cell viability, HepG2 cells were treated with various concentrations of EFPE and CCPE ranging, after which the cell viability was measured by an XTT assay. Treatment with EFPE and CCPE at concentrations up to 25 μg/mL did not induce cell toxicity (Fig. 1). To evaluate the antioxidant activities of the pepper extracts, HepG2 cells were preincubated with various concentrations of the extracts and then treated with 1 mM H_2O_2 for 1 h (Fig. 2). H_2O_2 is converted to hydroxyl radical by making Fe^{2+} available for the Fenton reaction (Leonard *et al.*, 2004). The hydroxyl radical has a high reactivity, which enables it to attack DNA, proteins, carbohydrates and lipids in a destructive manner (Curtin *et al.*, 2002). Oxidative stress occurs when antioxidant systems are overwhelmed by ROS, and the resulting oxidative damage can lead to cell death. In the present study, the treatment with H_2O_2 induced cell death in almost 50%. However, pretreatment with EFPE and CCPE reduced the oxidative stress induced-cell death from 51% to 24.3% and from 41.4% to 8.3%, respectively. Additionally, the protective effects of EFPE were

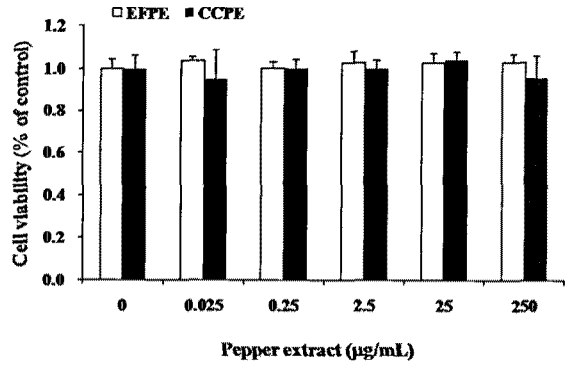

Fig. 1. Effects of EFPE and CCPE on cell viability. To evaluate the cell toxicity of EFPE and CCPE, HepG2 cells were incubated with various concentrations of extracts for 24 h and the cell toxicity was then determined using an XTT assay kit. Values represent the means ± S.D. of three determinations. *P < 0.05, **P < 0.01 (vs. 1 mM H_2O_2 treated control), $^{\dagger}P$ < 0.01 (EFPE vs. CCPE).

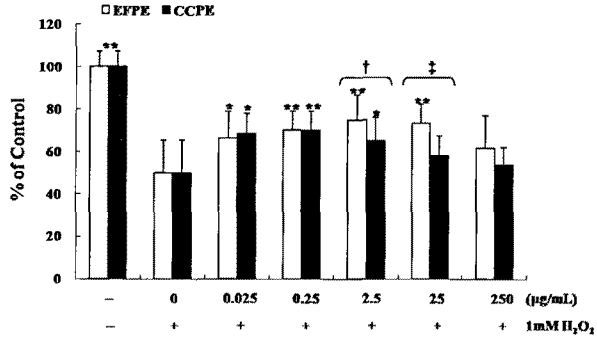

Fig. 2. Effects of EFPE and CCPE on oxidative stress induced cell death. To analyze the antioxidant activity of EFPE and CCPE, HepG2 cells were pre-incubated with various concentrations of both extracts for 24 h and then treated with 1 mM H_2O_2 for 1 h. The cell viability was measured using an XTT assay kit. Values represent the means ± S.D. of three determinations. *P < 0.05, **P < 0.01 (vs. 1 mM H_2OS treated control), $^{\dagger}P$ < 0.05, $^{\dagger}P$ < 0.01 (EFPE vs. CCPE). +/ - means treatment with or without 1 mM H_2O_2.

stronger than those of CCPE at concentrations ranging from 2.5 to 25 μg/mL.

Effects of pepper extracts on intracellular ROS generation

As pepper extracts showed the protective effects against oxidative stress induced-cell death, the effects of pepper extracts on intracellular ROS generation were analyzed. The relative changes in intracellular ROS in HepG2 cells were monitored using the fluorescent probe, DCF-DA. The DCF fluorescence intensity

is proportional to the amount of ROS formed intracellularly (LeBel *et al.*, 1992). When HepG2 cells were treated with EFPE and CCPE for 24 h, EFPE reduced the intracellular ROS generation by approximately 20-30% when compared to the controls, whereas CCPE increased the ROS by approximately 34% at a concentration 250 μg/mL (Fig. 3A). Because CCPE itself induced the production of intracellular ROS, we suspected the contamination of residual pesticides. Thus, the presence of 102 types of residual pesticides was analyzed by gas chromatography and HPLC analysis through the Environmental-Friendly Agricultural Product Certification Service (data not shown). However, no residual pesticides were detected in the EFA or CC peppers. These findings suggest that the effects of CCPE on cell viability and other antioxidant activities were not related to the presence of residual chemical pesticides; however, they did not exclude the possibility of side effects from chemical compounds. When HepG2 cells were treated with H_2O_2, the effects of EFPE and CCPE on intracellular ROS generation were analyzed (Fig. 3B). Treatment with H_2O_2 induced an approximately 1.9 fold increase in intracellular ROS generation when compared to the untreated-control (Fig. 3B). Additionally, intracellular ROS generation was reduced from 42.7% to 26.4% and 24.2% to 6.3% in response to treatment with 0.025 to 25 μg/mL EFPE and CCPE, respectively, when compared with the H_2O_2-treated control. However, treatment with CCPE at 250 μg/mL increased ROS by approximately 21.6%, and this compound may have acted as a pro-oxidant at concentrations of higher than 25 μg/mL. Based on a comparative analysis of antioxidant activities between EFPE and CCPE, treatment with EFPE at 0.25 to 250 μg/mL more effectively reduced intracellular ROS generation than treatment with CCPE at the same concentrations.

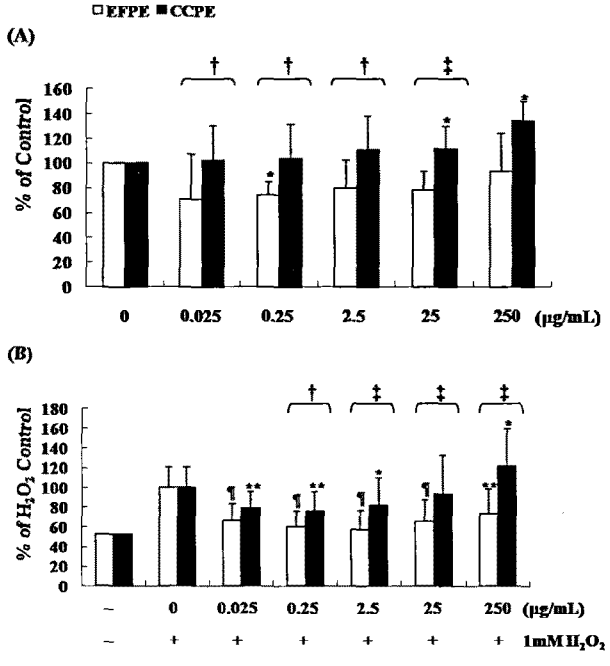

Fig. 3. Effects of EFPE and CCPE on intracellular ROS generation. To analyze the effects of EFPE and CCPE on intracellular ROS generation, HepG2 cells were incubated with various concentrations of both extracts for 24 h (A) or followed by treatment with 1mM H_2O_2 for 1 h (B), after which the intracellular ROS generation was analyzed using the fluorescent dye, DCF-DA. Values represent the means ± S.D. of three determinations. *$P < 0.05$ compared to the control, †$P < 0.05$, ‡$P < 0.01$ (EFLE vs. CCLE). +/ - means treatment with or without 1 mM H_2O_2.

Effects of pepper extracts on lipid peroxidation

Lipid peroxidation is initiated through one-electron redox reactions in which an initiating radical species is combined with an unsaturated lipid and then with oxygen to form a lipid peroxyl radical (Niki *et al.*, 2005). Lipid peroxidation induces the disturbance of fine structures, as well as alteration of the integrity, fluidity, and permeability of biomembranes and their functional loss. In addition, lipid peroxidation modifies the low density lipoprotein to proatherogenic and proinflammatory forms and generates potentially toxic products (Niki, 2009). When HepG2 cells were treated with 1 mM H_2O_2, lipid peroxidation was increased by approximately 34.5% (Fig. 4). However, pretreatment with

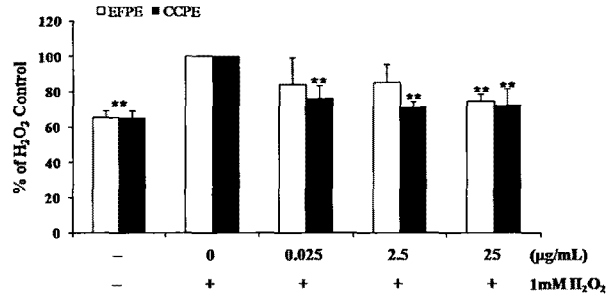

Fig. 4. Effects of EFPE and CCPE on lipid peroxidation by hydrogen peroxide. HepG2 cells were pre-incubated with different concentrations of both extracts for 24 h and then treated with 1 mM H_2O_2 for 1 h. Lipid peroxidation was assayed by determining the rate of MDA production. Values represent the means ± S.D. of three determinations. **$P < 0.01$ when compared to the 1 mM H_2O_2 treated control. +/ - means treatment with or without 1 mM H_2O_2.

EFPE and CCPE reduced the lipid peroxidation from 25.7% to 14.9% and 27.8 to 23.9%, respectively. According to previous study, capsaicin and phytochemicals from hot pepper prevent lipid peroxidation induced by oxidative stress (Luqman and Rizvi, 2006). Thus, active compounds including capsaicin from EFPE and CCPE showed protective effects on lipid peroxidation induced by hydrogen peroxide.

Effects of pepper extracts on CAT enzyme activity

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms. Defense mechanisms against oxidative stress involve preventive mechanisms, repair mechanisms, physical defenses and antioxidant defenses (Valko *et al.* 2007). Enzymatic antioxidant defenses include superoxide dismutase, glutathione peroxidase and catalase. To evaluate the effects of pepper extracts on catalase activity, HepG2 cells was measured catalase activity following treatment with pepper extracts under oxidative stress (Fig. 5). H_2O_2 reduced the catalase activity by approximately 16.5%, while pretreatment with EFPE and CCPE increased the enzyme activity from 10% to 21% and 23.3% to 39.2%, respectively. However, the effects of pepper extracts on catalase activity were not statistically significant. Superoxide dismutase (SOD) activity was also measured following treatment with H_2O_2 and pepper extracts; however, the effects of pepper extracts on SOD activity were very weak (data not shown). Although antioxidant enzyme activities were not affected by the treatment with pepper extracts in this study,

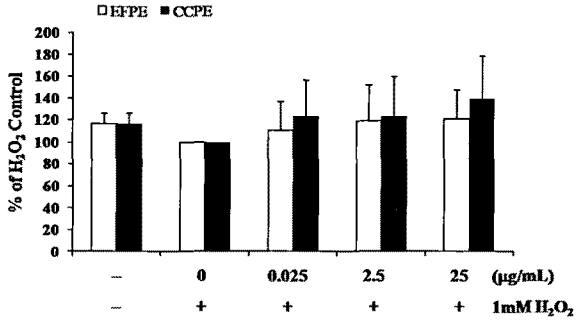

Fig. 5. Effects of EPPE and CCPE on CAT activity. HepG2 cells were pre-incubated with various concentrations of both extracts for 24 h, after which they were treated with 1 mM H_2O_2 for 1 h. The CAT activity was assayed by monitoring the decrease in absorbance of H_2O_2 at 240 nm. Values represent the means ± S.D. of three determinations. +/ - means treatment with or without 1 mM H_2O_2.

there are reports about the activities of pepper extracts and capsaicin on activation of antioxidative defense system including catalase and superoxide dismutase (Anandakumar *et al.*, 2008). Thus, more delicate experiment to evaluate the effects of pepper extracts on enzyme activity will be necessary.

Many factors such as resource availability, soil quality, climate and herbivory pressures from insects and animals are known to affect the levels of nutrients in plants. However, there is very little information available regarding the impact that various cultural practices have on the production of secondary phenolic metabolites in plants (Brandt and Mølgaard, 2001). Given that there is the increasing evidence that plant phenolics play a role in human health, further studies should be conducted to investigate the relationships between cultural practices and the level of phenolics in crops. Many studies have reported that organically cultivated crops have high antioxidant activities, total phenolic contents and secondary metabolites (Asami *et al.*, 2003; Olsson *et al.*, 2006; Wang *et al.*, 2008). According to the study for the effects of conventional, integrated, and organic farming, grown in a controlled greenhouse, on color, minerals, and carotinoids of sweet pepper, organic farming provided peppers with the highest intensities of red and yellow colors, contents of minerals, and total carotenoids and integrated fruits presented intermediate values (Pérez-López *et al.*, 2007). Recently, we reported that EFA crops exhibited higher flavonoids contents (FC), biogenic monoamines and high radical scavenging activities than CC crops (Ly *et al.*, 2008a). In this study, the total phenolic compounds (TPC) and FC of EFA and CC peppers were determined as shown in Table 1. The TPC levels of EFA and CC peppers were 468.1 ± 138.4 and 483.7 ± 137.7 mg GAE/g of dry weight, respectively, while the FC levels of EFA and CC peppers were 55.7 ± 17.9 and 67.7 ± 4.2 mg RE/g of dry weight, respectively. Although the levels of TPC and FC were higher in CC pepper than EFA pepper, this difference was not significant ($P > 0.05$).

Based on the results of this study, EFPE generally exhibited higher antioxidant activities than CCPE. There was also a correlation between oxidative stress induced-cell death and intracellular ROS generation. The higher antioxidant activities of EFPE suggest that the content of secondary metabolites with antioxidant properties was higher in the EFA peppers even

Table 1. Total phenolic compounds and flavonoid contents of peppers

Compounds	EFA	CCA	P
Total phenolic compounds (mg GAE/g of dry weight)	468.1±138.4	483.7±137.7	0.85
Flavonoid contents (mg RE/g of dry weight)	55.7±17.9	67.7±4.2	0.33

though the levels of TPC and FC were not. To better understand the physiological effects of pepper extracts on human cells, further studies are needed to investigate molecular markers defining the changes in antioxidant activities in pepper plants subjected to different cultivation conditions and practices.

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