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Antioxidative Activity of the Extract from the Inner Shell of Chestnut

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Abstract – The ethanolic extract of chestnut (*Castanea crenata* S. et Z., Fagaceae) inner shell (CISE) and one of its components, ellagic acid (EA), were evaluated for their protective effects against 1,1-diphenyl-2-picryl hydrazine (DPPH) free radical generation and hydrogen peroxide-induced oxidative DNA damage in a mammalian cell line. CISE and EA were shown to possess the free radical scavenging effect against DPPH radical generation, significantly. They were also found to strongly inhibit hydrogen peroxide-induced DNA damage from Chinese hamster lung (CHL) cell, assessed by single cell gel electrophoresis assay and 8-hydroxy -2'-deoxy guanosine (8-OH-2'dG) assay. Furthermore, topical application of CISE [12.5%(w/w) cream] and ellagic acid [1.0%(w/w) cream] for 14 days potently inhibited malondialdehyde (MDA) formation of mouse dorsal skin (a marker of lipid peroxidation) induced by ultraviolet B exposure. Therefore, CISE and its component, ellagic acid, may be the useful natural antioxidants by scavenging free radicals, inhibition of lipid peroxidation and protecting oxidative DNA damage when topically applied.

Keywords □ *Castanea crenata*, ellagic acid, oxidative stress, antioxidant, free radical scavenging, single cell gel electrophoresis, 8-hydroxy -2'-guanosine (8-OH-2'dG), ultraviolet B

INTRODUCTION

An oxidative stress including reactive oxygen species (ROS)-generating chemicals, ultraviolet (UV) radiation and ionization radiation may cause damage to various cellular constituents such as DNA, protein, and lipid leading to many pathological conditions. For instance, DNA damage mediated by an oxidative stress plays a potentially important role in carcinogenesis and aging (Dizdaroglu M., 1992; Guyton *et al.*, 1993; Feig *et al.*, 1994; Kasai *et al.*, 1997; Beckman *et al.*, 1998; Lu *et al.*, 1999). The inner shell of chestnut (*Castanea crenata* S. et Z., Fagaceae) has been used as a cosmetic material for a long time in Korea. In one ancient literature, it is described as an anti-wrinkle and anti-aging agent mixed with honey [Huh, 1966]. And recent investigation has shown that this material inhibited the biosynthesis of melanin [Ahn *et al.*,1999] and enhanced the expression of adhesion molecules, fibronectin and vitronectin, of skin fibroblasts, possibly leading to skin-firming effect (Chi *et al.*, 2002).

Numerous plant constituents have proven to show free radical scavenging and/or antioxidative activities (Dragsted, 2003). Flavonoids and other phenolic compounds of plant origin have been reported as scavengers and inhibitiors of lipid peroxidation (Formica *et al.*, 1995). We found that the extract of chestnut inner shell contains relatively high amount of tannins, which are well-known polyphenolic antioxidants. Furthermore, no literature was available to describe antioxidative activities of this plant material so far. Therefore, it is worth to investigate antioxidative activities of chestnut inner shell. In the present study, the 70% ethanolic extract was evaluated for its free radical scavenging effect and the inhibitory activity against oxidative DNA damage in chinese hamster lung (CHL) cell. *In vivo* effect on lipid peroxidation was also examined using SKH-hairless mice.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), Dulbecco's modified eagles

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medium (DMEM), phosphate buffered saline (PBS), trypsin-EDTA, and antibiotics were obtained from Gibco BRL (Grand Island, NY). Normal melting point agarose (NMPA), low melting point agarose (LMPA), methyl linoleate, thiobarbituric acid (TBA), Triton X-100, dl-α-tocopherol (Vit-E), ellagic acid and ethidium bromide were purchased from Sigma Chem. (St. Louis, MO). The control cream base for topical application was provided by Coreana Cosmetic Co., LTD (Cheonan, South Korea).

Preparation of the chestnut inner shell extract and identification of ellagic acid

The dried chestnut inner shell (1 kg) obtained from local market (Seoul, Korea) was soaked in 70% ethanol at room temperature for 7 days in order to extract the polyphenol compounds. After removing solid materials by filtration, the filtrate was evaporated in *vacuo* to dryness (260 g) and this dried powder (CISE) was used throughout the experiments. Tannin content in the extract was determined by Folin-Dennis method [Naczk et al., 1992]. In order to analyze ellagic acid content, HPLC procedure was employed. HPLC system (Shimadzu, Japan) comprised of a solvent delivery pump equipped with a UV detector (254 nm). A separation was carried out on a stainless steel ODS column (250 x 4.6 mm i.d., 5 μm spherical particle, Shimadzu, Japan), with 2% formic acid buffer in 24% acetonitrile as a mobile phase at 0.8 ml/min. Working solutions of CISE for *in vitro* and *in vivo* experiments were dissolved in DMSO.

Chinese hamster lung cell (CHL) culture

CHL cells obtained from American Type Culture Collection were grown as monolayers in MEM with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin at 37°C under 5% CO_2. The cells were plated at 5×10^4 cells/well in 24-well plates. One day after initial seeding, cells were incubated with H_2O_2 (10^{-3}M) / $FeCl_2$ (10^{-3}M) or H_2O_2 (10^{-3}M) / $FeCl_2$ (10^{-3}M) plus test compounds for 30 min. The medium was replaced by fresh complete medium. Two hours later, cells were harvested and subjected to single cell gel electrophoresis and measurement of 8-hydroxy-2'-deoxyguanosine (8-OH-2'dG). CISE, dl-a-tocopherol and ellagic acid were dissolved in 50% DMSO and then added to the cultures.

Animals

Female SKH-1 hairless mice (5 weeks old) obtained from Charles River Lab. (Wilmington, MA) were used. Animals were fed with lab chow (Purina Korea) and water *ad libitum*. They were acclimatized in a specific pathogen free facility under the conditions of 20-22°C, 40-60% relative humidity and 12/12 h (light/dark) cycle at least 7 days prior to experiment.

DPPH assay *in vitro*

The hydrogen-donating ability of the extract was examined in the presence of DPPH stable radical. The radical scavenging activity was routinely determined using 60 μM DPPH. Absorbance was measured at 520 nm after incubation of test sample at 37°C for 30 min according following the previous reported procedure of Fugita *et al.*, 1988.

Single cell gel electrophoresis in CHL cells

CHL cells were embedded in agarose on frosted microscopic slides according to the procedure of Sing *et al.*, 1988. First, 0.65% NMPA in PBS (100 μl) at 65 °C was dropped onto slides and they were covered with glass coverslip (18×18 mm, No.1). After leaving on ice for 10 min, the cover slip was removed. Cells were mixed with 200 μl of 0.5% LMPA and 50 μl of cell suspension was immediately pippeted onto the layer of agarose on the same slide. After covering with a coverslip, the slide was left on ice for 10 min. A final layer of agarose (100 μl of 0.5% LMPA) was applied in the same way. The slide without coverslip was immersed in ice-cold lysis solution (10 mM Tris, pH 10.0, containing 2.5 M NaCl, 100 mM EDTA, 10% DMSO and 1% Triton X-100) at 4°C for 1 hr. Electrophoresis was carried out in a tank containing 300 mM NaOH, 1 mM EDTA, pH 13.0 for 15 min under 25 V and 300 mA. Slides were then transferred to 0.4 M Tris buffer (pH 7.5), washed three times and gently dried. Ethidium bromide (2 μg/ml) was dropped onto the gel to stain DNA. Slides were examined at X400 magnification using a BH2 fluorescence microscope (Olympus, Japan) equipped with a 20BG-W2 dichromatic mirror (excitation filter: 515 nm, barrier filter: 590 nm). Image analysis was performed with the software Komet (version 3.0, Kinetic Imaging, Liverpool, UK) on 50 randomly selected cells. DNA damage was quantified by the increase of the tail moment, which was defined as a product of comet length and amount of DNA in the tail according to the original procedure of Olive *et al.*,1990.

Measurement of 8-hydroxy-2'-deoxyguanosine (8-OH-2'dG) in CHL cells

For the determination of 8-OH-2'dG concentration, DNA was isolated from CHL cells using phenol/chloroform/isoamyl alcohol (Kasai *et al.*, 1987). An equal amount of DNA was then

digested with nuclease P_1 and alkaline phosphatase, and the 8-OH-2'dG levels were analyzed by HPLC with an electrochemical detector (ESA, model LC-4C, West Lafayette, USA). A separation was carried out on a stainless steel ODS column (250 × 4.6 mm i.d., 5 μm spherical particle, Shimadzu, Japan), with 100 mM sodium acetate, pH 5.2 with phosphoric acid : methanol (95:5) as a mobile phase at 1.0 ml/min. Oxidative damage was expressed as the molar ratio of 8-OH-2'dG to 10^5 molecules of deoxyguanosine (2'dG), which was calculated from the absorbance at 254 nm (Shigenaga *et al.*, 1990).

Lipid peroxides assay in the dorsal skin hairless of mouse exposed to UV-B

Female SKH-1 hairless mice were divided into three groups. Six mice were used as negative control. Ten mice were treated with 120 mg of control cream base alone (positive control) and 10 mice with 120 mg of 12.5%(w/w) CISE in the control cream base or 1%(w/w) ellagic acid in the control cream base (treatment groups). Immediately after topical application on the dorsal skin, mice were irradiated with 180 J/cm^2 dose of UV-B (XX-15B, medium wavelength: 312nm, Spectroline, NY) for initial 3 consecutive days and 120 J/cm^2 dose of UV-B for following 11 days. Therefore, these treatments were continued daily for 14 days. The control cream base and test compound were topically smeared once in a day for 14 days. The distance of UV-B lamp from the skin was about 15 cm, and UV intensity was measured by UV-B meter (DIX series, Spectroline, NY). At the end of irradiation period, animals were sacrificed, and the full thickness dorsal skin (2 × 1 cm, L × W) was removed. After homogenization in PBS (pH 7.0), 1.0 ml of skin homogenate was mixed with 1.5 ml of 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid solution, successively. The mixture was heated at 95°C for 20 min. After cooling, 1.0 ml of distilled water and 5.0 ml of the mixture solution of *n*-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4,000 rpm for 10 min, the organic layer was obtained and absorbance was measured at 532 nm based on the procedure of Ohkawa *et al.*,1979. 1,1,3,3-Tetraethoxypropane was used as a standard, and the concentration of lipid peroxide was expressed as μmol of malondialdehyde (MDA) per the area of dorsal skin (6.3 cm^2) .

Statistical analysis

All results are expressed as arithmetic mean ± S.D. Statistical evaluation of the data was carried out using Student's *t*-test.

RESULTS

The inner shell of the chestnut is known to contain considerable amount of tannins. CISE revealed high content of tannic acids (2.5±0.5%, w/w, n=5). As one of antioxidant component in CISE, ellagic acid (a most simple form of tannin) was identified and its concentration was determined by HPLC analysis because this compound is also known to possess strong antioxidative activity. The actual content of ellagic acid in CISE was 0.09±0.02%, w/w (n=5). Although its content was not high, ellagic acid may be used as a good standard material for the identification and quality control of CISE.

Table I represented the free radical scavenging activity of CISE and ellagic acid. Both CISE and ellagic acid concentration-dependently showed potent free radical scavenging activity as expected. dl-α-Tocopherol used as a reference compound also showed free radical scavenging activity. The IC_{50} values were determined to be 1.9 μg/ml for ellagic acid, 7.5 μg/ml for CISE, and 7.6 μg/ml for dl-α-tocopherol by linear regression analysis. Ellagic acid was most potent, while the potency of CISE was similar to that of dl-α-tocopherol.

In order to find the protective effect against DNA oxidative damage, CHL cells were treated with $H_2O_2/FeCl_2$. As demonstrated in Table II, treatment of CISE as well as ellagic acid showed a significant reduction of the tail moment in single cell gel electrophoresis assay. dl-α-Tocopherol also showed a reduction of the tail moment concentration-dependently. The IC_{50} values found were dl-α-tocopherol (0.1 μg/ml), ellagic acid (0.8 μg/ml) and CISE (52.9 μg/ml).

Table I. DPPH radical scavenging activity of chestnut inner shell extract (CISE) and ellagic acid

Treatment	Concentration (μg/ml)	OD 520nm[a]	% Inhibition[b]
Control	-	0.539 ± 0.004	
CISE	5	0.329 ± 0.001*	38.9
	10	0.216 ± 0.003*	59.9
	20	0.154 ± 0.001*	71.4
Ellagic acid	1	0.405 ± 0.004*	25.0
	2	0.231 ± 0.019*	57.2
	5	0.110 ± 0.072*	79.6
dl-α-Tocopherol	5	0.361 ± 0.002*	33.0
	10	0.189 ± 0.001*	64.9
	20	0.114 ± 0.002*	78.8

[a]Values represent arithmetic mean ± S.D. (n=3), *p< 0.01, Significantly different from the positive control group.
[b]% Inhibition = [(OD control - OD treatment)/ OD control] × 100

Table II. Inhibition of chestnut inner shell extract (CISE) and ellagic acid on DNA damage by H_2O_2 / $FeCl_2$ in CHL cell

Treatment	Concentration (μg/ml)	Olive Tail Moment (OTM)[a]	% Inhibition[b]
Control	-	19.08 ± 0.55	
CISE	10	13.54 ± 2.91*	29.0
	50	10.65 ± 4.10*	44.1
	100	7.21 ± 1.07**	62.2
Ellagic acid	0.3	14.25 ± 0.10**	25.3
	3.0	13.76 ± 1.06*	27.8
	30.0	11.30 ± 0.73**	40.7
dl-α-Tocopherol	0.43	13.04 ± 2.35*	31.6
	4.30	7.45 ± 1.98**	60.9
	43.0	7.04 ± 0.96**	63.1

*p<0.01, **p<0.05, Significantly different from the positive control group.
[a]Values represent arithmetic mean ± S.D. (n=3),
[b]% Inhibition = [[OTM control - OTM treatment]/ OTM control] × 100

Table III represented that CISE inhibited H_2O_2/$FeCl_2$-induced 8-OH-2'dG formation, a biomarker of DNA oxidation (47.2% at 10 μg/ml, 79.3% at 50 μg/ml and 75.5% at 100 μg/ml). Ellagic acid showed strongest activity at low concentration (61.8% at 0.3 μg/ml), but not in a concentration-dependant manner. dl-α-Tocopherol also showed considerable reduction of 8-OH-2'dG formation concentration-dependently (24.9% at 0.43 μg/ml, 25.3% at 4.3 μg/ml and 38.7% at 43 μg/ml).

Finally, the inhibitory effect of lipid peorxidation was evaluated *in vivo*. Topical applications of 12.5%(w/w) CISE cream or 1.0%(w/w) ellagic acid cream for 14 days with UV-B exposure resulted in the potent reduction of lipid peroxidation expressed by MDA formation from the dorsal skin of hairless mouse (Table IV).

Table III. Inhibition of chestnut inner shell extract (CISE) and ellagic acid on the formation of 8-OH-2'dG by H_2O_2 (10^{-3}M) and $FeCl_2$ (10^{-3}M) in CHL cell.

Treatment	Concentration (mg/ml)	8-OH-2'dG / 10^5 2'dG [a]	
		Mean ± S.D.	% Inhibition
Control		173.63 ± 30.48	
CISE	10	91.62 ± 29.50**	47.2
	50	35.95 ± 6.32**	79.3
	100	42.56 ± 5.73**	75.5
Ellagic acid	0.3	66.29 ± 10.14**	61.8
	3.0	66.74 ± 7.70**	61.5
	30.0	62.84 ± 8.43**	63.8
dl-α-Tocopherol	0.43	130.34 ± 26.04	24.9
	4.3	129.64 ± 41.45	25.3
	43	106.38 ± 28.45*	38.7

*p<0.01, **p<0.05, Significantly different from the positive control group.
[a]Values represent arithmetic mean ± S.D. (n=3)
[b]% Inhibition = [(8-OH-2'dG / 10^5 2'dG)$_{control}$ − (8-OH-2'dG / 10^5 2'dG)$_{treatment}$] ÷ [(8-OH-2'dG / 10^5 2'dG)$_{control}$] × 100

Table IV. Inhibition of chestnut inner shell extract (CISE) and ellagic acid on lipid peroxidation induced by UV-B irradiation in the dorsal skin of mice

Treatment[a]	No. of mice	OD 532nm[b]	MDA μmol/6.3cm^2 of dorsal skin
Control	6	0.433 ± 0.088	1.05
UV-B	10	0.635 ± 0.119	1.55
CISE + UV-B	10	0.300 ± 0.082*	0.82
Ellagic acid + UV-B	8	0.333 ± 0.088*	0.88

[a]Female SKH-1 hairless mice were treated with 120 mg of control cream base alone (control and UV-B irradiated group), 12.5 %(w/w) CISE cream or 1%(w/w) ellagic acid cream (treatment groups). Immediately after topical application, mice were irradiated with UV-B for 14 days (UV-B, CISE and ellagic acid treated group) as described in materials and methods.
[b]Values represent arithmetic mean ± S.D.
*p<0.05, Significantly different from UV-B irradiated group.

DISCUSSION

The present investigation clearly demonstrates that CISE and its component, ellagic acid possess significant antioxidative activity in several experiments of *in vitro* and *in vivo*. There are particular interests on ellagic acid, a dimeric derivative of gallic acid, in vegetables, fruits and beverages because of the increasing evidence of its antioxdant effects (Nepka *et al.*, 1999; Thiem *et al.*, 2003). Previously, ellagic acid was reported to possess radical scavenging activity (Su *et al.*, 1988) and inhibit lipid peroxidation (Priyadarsini *et al.*, 2002). Furthermore, ellagic acid was shown to reduce DNA damage in comet assay (Festa *et al.*, 2001) and to protect formation of 8-OH-2'dG in mice (Sai-Kato *et al.*, 1995). Our results were well correlated with these previous findings that ellagic acid showed antioxidative properties.

In the beginning of experiments, the radical scavenging assay using DPPH was employed in order to evaluate the antioxidative activity *in vitro*. This assay evidently offers a convenient and accurate method for titrating the oxidazable groups of natural or synthetic antioxidant (Cao *et al.*, 1997). In this assay, the inhibitory potencies of CISE and ellagic acid were comparable to that of an antioxidant, dl-α-tocopherol. Previously, ellagic acid was reported to possess much stronger radical scavenging activity compared to dl-α-tocopherol (Festa *et al.*, 2001) and to inhibit gamma-radiation (hydroxyl radical)

induced lipid peroxidation in rat liver microsomes using the DPPH radical assay (Priyadarsini *et al.*, 2002).

In addition to the free radical scavenging activity, CISE and ellagic acid also showed the protective activity against oxidative DNA damage in CHL cells measured by comet assay and 8-OH-dG determination. The cellular mechanism to reduce DNA damage is not clear at present. But, it is reasonably thought that CISE and ellagic acid as antioxidants may protect DNA strand break induced by oxygen free radicals (Cozzi *et al.*, 1995; Masuda *et al.*, 1999; Rojas *et al.*, 1999), since comet assay detects DNA breaks in a single cell. Previous study also reported that a marked reduction of H_2O_2- induced DNA damage in comet assay exerted by ellagic acid (Festa *et al.*, 2001). Furthermore, ellagic acid was shown to protect formation of 8-OH-2'dG in male B6C3F1 mice treated with pentachlorophenol (Sai-Kato *et al.*, 1995). Our results on the inhibition of oxidative DNA damage in comet assay were confirmed by the reduction of CISE and ellagic acid against 8-OH-2'dG formation.

Moreover, CISE and ellagic acid were shown to greatly reduce the *in vivo* formation of lipid peroxidation products on the dorsal skin irradiated with UV-B when topically applied. These substances did not serve simply as a physical barrier for UV exposure because the treatment and control groups used same cream base, and the treatment groups showed higher inhibition compared with that of control group. Although ellagic acid was found to be effective in decreasing the microsomal lipid peroxidation in the lung and liver of mice (Khanduja *et al.*, 1999), there was no report concerning the dorsal skin of mice. Exposure of UV-B on the skin provokes lipid peroxidation partly through formation of hydroxyl radicals. Wei *et al.*, 2002 demonstrated that subacute exposure to UV-B substantially increased the level of H_2O_2, lipid peroxides (MDA), and 8-OH-2'dG in skin of hairless mice. Reactive oxygen species (ROS) are thought to associate with UV-B mediated cutaneous damage mainly due to lipid peroxidation (Black *et al.*, 1987).

In conclusion, our results clearly demonstrated *in vitro* and *in vivo* antioxidative activity of CISE, which is likely contributed, at least in part, by its component, ellagic acid. This investigation also offers one of scientific rationales of using chestnut inner shell as an antioxidant by topical application.

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