J. Toxicol. Pub. Health
Vol. 23, No. 1, pp. 25-31 (2007)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Evaluation of Protective Effects of *Houttuynia cordata* on H_2O_2-Induced Oxidative DNA Damage Using an Alkaline Comet Assay in Human HepG2 Cells

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Received February 8, 2007; Accepted March 7, 2007

To evaluate the protective effect of *Houttuynia cordata* on hydrogen peroxide-induced oxidative DNA damage in HepG2 cell line, we used an alkaline single-cell gel electrophoresis (SCGE; comet assay). The DNA damage was analyzed by tail moment (TM) and tail length (TL), which used markers of DNA strand breaks in SCGE. The 100 μg/ml of methanolic extract of *Houttuynia cordata* root showed significant protective effects ($p < 0.01$) against hydrogen peroxide-induced DNA damage in HepG2 cells and increased cell viability against hydrogen peroxide. The results of this study indicate that *Houttuynia cordota* root methanol extract acts as a potential antioxidant, and exhibits potential anticancer properties, which may provide a clue to find applications in new pharmaceuticals for oxidative stability.

Key words: DNA damage, Single cell gel electrophoresis (SCGE), Comet assay, *Houttuynia cordota*.

INTRODUCTION

Reactive oxygen species (ROS), including superoxide anion radicals, hydrogen peroxide, and hydroxy radicals are natural by-products generated by living organisms as a consequence of aerobic metabolism. These ROS produced under normal physiological condition are scavenged by antioxidant enzymes including superoxide dismutase (SOD), gluthathione peroxidase (GPx), and catalase (CAT). Accumulation of excess ROS in cells by endogenous and exogenous sources which are toxic to cells, can cause oxidative stress leading to damage to cellular organisms (Reilly *et al.*, 1991; Collins, 1999; Fang *et al.*, 2002). To protect against the toxic effects of oxygen-derived species, cells constitutively express enzymes that neutralize ROS and repair and replace the damage caused by ROS. If an excess of free radicals are formed, they attack cell membranes and cause lipid peroxidation and oxide liposome, deoxyribose, protein and 2'-deoxyguanosine, generating intermediates which can react with DNA and form adducts (Cadet, 1994; Alessandro *et al.*, 2001). When DNA replication occurred, 8-OHdG did not excise by glycosylase, causing the replacement of GC base to GG or AT base (Wilaons *et al.*, 1993). If DNA did not repair at replication, normal cell function was inhibited, causing various human chronic diseases such as cancer, alzheima's disease, and aging (Fraga *et al.*, 1990; Alpetite *et al.*, 1996; LePage *et al.*, 1998; Collins, 1999; Bartsch and Nair, 2004). DNA damage expressing as single or double strand break, abasic sites, DNA-DNA, and DNA protein crosslinks occur after treatment with radiation (Cadet, 1994; Nocentini, 1995; Kmuaravel and Jha, 2006). Because the level of oxidative DNA damage is much lower at the cellular level (Helbock *et al.*, 1998), gas chromatography (Dizdaroglu, 1991), high-performance liquid chromatography (Kasai, 1997; Roias *et al.*, 1999), and ionizing radiation methods were developed for detection of the level of oxidative DNA damage (Cadet *et al.*, 1997; Ravanat *et al.*, 1997). However, these methods were non-sensitive for detection of the level of oxidative DNA damage, the Comet assay,

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which very rapidly detects DNA damage at the level of individual cells, was developed. The alkaline single cell gel electrophoresis assay (Comet assay) is considered to be a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells (Singh *et al.*, 1988). In addition, the assay also provides the opportunity to estimate DNA repair in cells following exposure to genotoxic agents (Singh *et al.*, 1990; Fairbairn *et al.*, 1995; Kumaravel and Jha, 2006). The main advantages of the Comet assay include collection of data at the level of the individual cell without DNA extraction, collection of analyses within one day, the need for only a small number of cells per sample, sensitivity for detecting DNA damage, and possible use of any eukaryote single cell population, including cells obtained from natural biota or aquatic organisms for eco-genotoxicological studies, and environmental monitoring (Singh *et al.*, 1988; Van Loon *et al.*, 1991; Fairbairn *et al.*, 1995; Hartmann *et al.*, 2003). Currently, several studies examining the protective effects of bioactive substances on DNA damage using Comet assay have come out with specialized measures of DNA damage. Alessandro *et al.* (2001) reported that *Resveratrol* have a protective effect on breast cancer, large intestinal cancer, and prostate cancer patients. Boyle et al (2000) reported that human dietary supplementation with onion meal has a significantly protective effect on DNA damage in lymphocyte and Kucuk *et al.* (2003) reported that human dietary supplementation with linoleic acid has a significantly protective effect on DNA damage in lymphocyte. As mentioned above, free radicals are closely associated with various human chronic diseases such as chronic inflammation reaction, arteriosclerosis, ischaemia-reperfusion injury, aging, cancer, and rheumatoid arthritis. The oxidative DNA damage in cancer development has become widely recognized (Ohshima, 2003; Klaunig and Kamendulis, 2004). There is need to discover new natural antioxidants which scavenge free radicals and protect against DNA damage. To test the availability and usefulness as a chemotherapy agent, we evaluated the protective effects of *Houttuynia cordata* on H_2O_2-induced oxidative DNA damage using an alkaline Comet assay in Human HepG2 cells.

MATERIALS AND METHODS

Materials. *Houttuynia cordata* were obtained from Institute of Traditional Medicinal Plants of Gyeongnam (Hamyang, Gyeongnam, Korea). *Houttuynia cordata* root were dried under dark light and cut into small pieces and stored at 4°C until used.

Extraction. *Houttuynia cordata* root (300 g) was extracted with 900 ml methanol in a shaking incubator at 80°C for 3 hr. The residue was re-extracted under the same condition 3 times. The extracts obtained were combined and filtered. The combined extract was evaporated to dryness in a vacuum rotary evaporator and weighed to the yield of soluble constituents.

Cell culture and treatment condition. The human HepG2 cells were kept in α-DMEM medium (Sigma) supplemented with 10% fetal bovine serum, 50 unit/ml penicillin and 50 μg/ml streptomycin. Cells were grown in a humidified incubator at 37°C, 48 hr under 5% CO_2/95% air. Cells were seed in a 25 ml^2 flask at a density to reach 1.5×10^5. At 48 h after seeding, cells were harvested, washed and used for assay.

Determination of cell viability evaluated as mitochondrial activity. Cell viability evaluated as mitochondrial activity was quantified by measuring dehydrogenase activity retained in the cultured cells, using an MTT assay. The assay is based on the ability of living cells to convert dissolved MTT into insoluble formazan; therefore, the amount of formazan produced is proportional to the number of living cells. The cells were seeded at a density to reach $1.5 \times 10^5/cm^2$ in 96 well plates. After methanolic extract treatments, the cells were incubated with 1 mg/ml MTT in DMEM for 4 h at 37°C. The MTT containing medium was removed and the intracellular formazan product was dissolved in DMSO for quantification at A_{560}.

Comet assay. Cells were diluted at an appropriate density to reach 200~300/0.1 μl. The Comet assay was performed according to the procedure described by Singh *et al.* (1988). Frosted microscope slides were cleaned and pre-coated with freshly prepared normal melting agarose (NMA, 0.8%), left at room temperature to allow the agarose to dry, and then kept overnight at 4°C. Diluted cells were mixed with the 100 μl of fresh low-melting-point agarose (LMA, 1%). The mixed sample was spread on each pre-coated slide and covered with a coverslip. After cooling for 5 min on ice, the coverslip was gently removed, and a third layer, consisting of 90 ul LMA, was added and allowed to solidify for 5 min on ice. After cooling for 5 min on ice, the coverslip was gently removed, and the positive control and samples were treated with 50 μM H_2O_2 at 4°C for 5 min except for the negative control. After the coverslips were gently removed. The slides were then immersed in freshly prepared ice-cold lysing solution (4°C, 2.5 M NaCl, 0.1 M Sodium EDTA, 10 mM Tris, and 1% Triton

X-100, adjusted to pH 10, final volume 500 ml) and left at 4°C, for 1 hr in the dark. The slides were then gently removed from the lysing solution and transferred to a horizontal electrophoresis tank containing freshly prepared alkaline buffer (0.3 M NaOH, 1 mM NaEDTA; pH > 13) and incubated for 30 min at 4°C in order to allow the DNA to unwind. Electrophoresis was carried out in the same buffer at 4°C, for 20 min by applying an electric field of 20 V and adjusting the current to 300 mA. Finally, the slides were gently washed three times in a neutralization buffer (0.4 M tris-HCl; pH 7.5) for 5 min before being dehydrated in ice-cold ethanol for 5 min. After dehydration, the slides were stained with 0.05 mM ethidium bromide solution (40 μl). Slide analysis was performed using a fluoromicroscope (Olympus, Japan) at 200 × magnification. Quantitative assessment of DNA damage in HepG2 cell nuclei was performed using Komet 5.5 image analysis software (Kinet Imaging, UK) by measuring tail extend moment (TEM; defined as the tail length weighted by the percentage of tail DNA), tail length (TL; length of the tail, distance between the head and the last DNA fragment), which used markers of DNA strand breaks in SCGE. The two slides were prepared per each treated group, and three replicates were performed per each treated group.

Statistical analysis. The results are expressed as mean ± standard deviation (S.D.). Differences between groups were assessed by one-way analysis of variance using the SAS software package for Windows. If in a one-way analysis of variance test a significant F-value of $p < 0.05$ was obtained, a Dunnett's multiple comparison test between the treated and control group was conducted. Differences between two groups were statistically evaluated by *t*-test.

RESULTS

Protective effects of Houttynia cordata root methnolic extract on H_2O_2 induced cell toxicity in HepG2 cells. The effect of H_2O_2 on cell viability was evaluated by determining the percentage of MTT reduction upon incubation of HepG2 cells with increasing H_2O_2 concentration in the range 0~70 μM. As shown in Fig. 1, H_2O_2 inhibited cells growth in a dose dependent manner.

For the protective effect of *Houttynia cordata* root methanolic extract on $HepG_2$ cells against H_2O_2, HepG2 cells were treated with 25, 50, and 100 μg/ml of *Houttynia cordata* root methanolic extract and incubated for 30 min. After incubation, 0~50 μM H_2O_2 was added and cell viability was determined by MTT test. The group treated with 100 μg/ml of *Houttynia cordata* root methnolic extract showed significant ($p < 0.01$) increase in cell growth (80~98%), unlike the group treated with H_2O_2 without *Houttynia cordata* root methanolic extract. The groups treated with 50 μg/ml and 25 μg/ml of *Houttynia cordata* root methanolic extract showed significant ($p < 0.05$) increase in cell growth 50~90% and 40~85% as apposed to the group treated with H_2O_2 without *Houttynia cordata* root methanolic extract (Fig. 2).

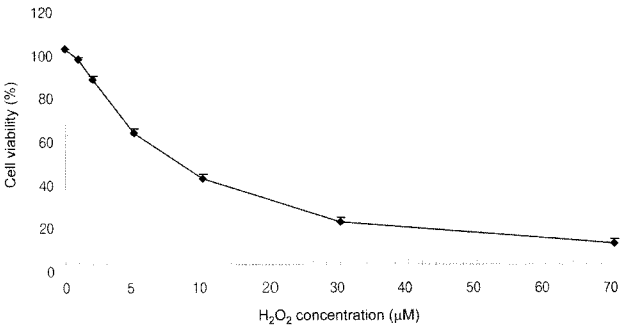

Fig. 1. The effect of H_2O_2 on cell viability in HepG2 cells. Cells were treated with each indicated concentration of H_2O_2 for 48 hr. Cell viability expressed as relative percentage.

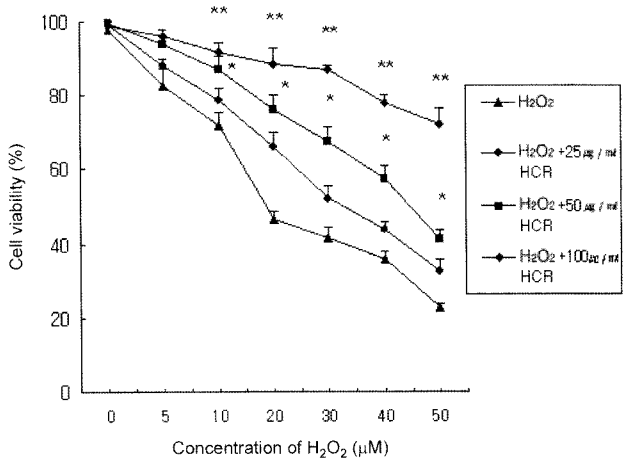

Fig. 2. The protective effect of various concentrations of *Houttynia cordata* root methanolic extract on H_2O_2 induced toxicity in HepG2 cell. Cytotoxicity was measured by MTT assay. The cells were preincubated in 96 well microplates for 30 min with 25, 50 and 100 μg/ml of *Houttynia cordata* methanolic extract, then incubated with H_2O_2 for 48 hr. The values are mean percentages of the control ± SD from 8 replicates. The percentage of cell growth in the control group was designated as 100%. *$p < 0.05$ vs. control, **$p < 0.01$ vs. control, HCR: *Houttynia cordata* root.

Protective effect of Houttynia cordata root methanolic extract on DNA damage induced by H_2O_2 in HepG2 cells. For the measurement of the protective effect of *Houttynia cordata* root methanolic extract on

Table 1. Inhibitory effect of *Houttuynia cordata* root methanolic extract on DNA damage induced by H_2O_2 in HepG2 cells

Experimental group	Tail moment (TM) Mean ± S.E.	Tail length Mean ± S.E.
PBS + DMSO	0.89 ± 0.16[a,1)]	12.97 ± 8.61[a]
HO + DMSO	5.19 ± 0.76[b]	29.81 ± 11.37[b]
H_2O + 25 μg/ml	8.96 ± 0.76[b]	31.52 ± 13.45[b]
H_2O + 50 μg/ml	4.22 ± 0.63[b]	26.42 ± 9.72[b]
H_2O + 100 μg/ml	1.48 ± 0.23[a]	18.50 ± 10.15[a]

[a,b]: Values with different superscript within the same column are significantly different ($p < 0.01$).
[1)]Mean ± S.E. (n = 100).

DNA damage induced by H_2O_2 in HepG2 cells, the Comet assay was performed. The method of Comet assay described in the methods and materials. We measured the inhibitory effect of *Houttynia cordata* root methanolic extract on DNA damage induced by H_2O_2 in HepG2 cell using Komet 5.5 image analysis software (Kinet Imaging, UK) by measuring tail extend moment (TEM; defined as the tail length weighted by the percentage of tail DNA), tail length (TL; length of the tail, distance between the head and the last DNA fragment), which used markers of DNA strand breaks in SCGE. Tail moment and Tail length of 100 μg/ml treated group were not different significantly from control group but those of 50 μg/ml and 25 μg/ml treated group were different significantly ($p < 0.01$) from control group. It

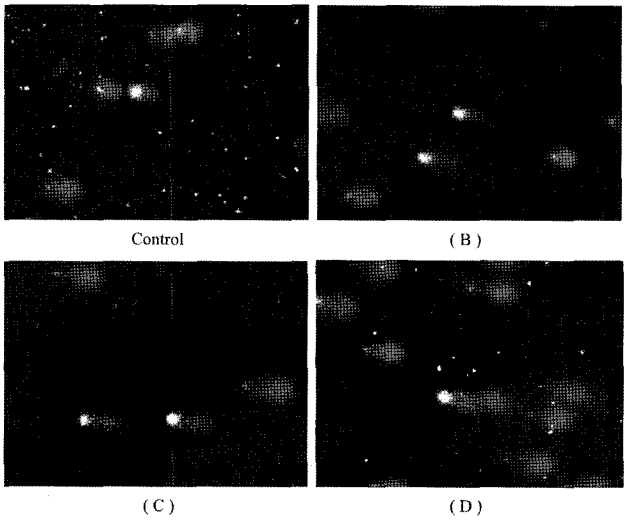

Fig. 3. Representative images of oxidative DNA damage in HepG2 cells. Cells were exposed to 100 μg/ml (B), 50 μg/ml (C), and 25 μg/ml (D) of *Houttynia cordata* root methanolic extract. After 30 min incubation, 50 μM H_2O_2 were added in B, C and D, and incubated for 5 min. The cells were analyzed by the Comet assay.

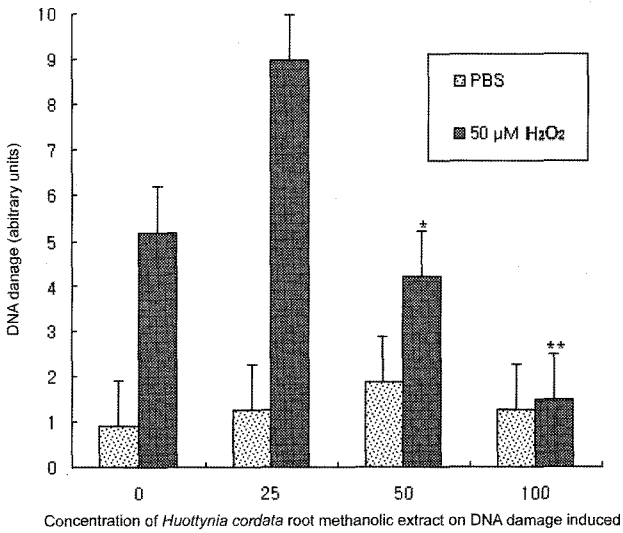

Fig. 4. Inhibitory effect of *Houttynia cordata* root methanolic extract on DNA damage induced by H_2O_2 (50 μM) in HepG2 cells. *, $p < 0.05$ vs. control; **, $p < 0.01$ vs. control.

means that *Houttynia cordata* root methanolic extract (100 μg/ml) showed significant protective effect on DNA damage induced by H_2O_2 (Table 1). As Fig. 3 was the results of image analysis of oxidative DNA damage in HepG2 cells, 100 μg/ml and 50 μg/ml of the *Houttynia cordata* root methanolic extract showed significant ($p < 0.05$) protective effect on DNA damage induced by H_2O_2 but 25 μg/ml did not show significant protective effect (Fig. 3, 4).

DISCUSSION

The *Houttuynia cordata* plant, like *Saururaceae* and its major constituents, are flavonoid and quercitrin. *Houttuynia cordata* are reported to possess anti-inflammatory, anti-bacterial , anti-viral and diuretic effects, but the antioxidant activities of *Houttynia cordata* methanolic extract have not been extensively examined by others *in vitro* and *in vivo*. We reported the antioxidant activities of *Houttynia cordata* root methanolic extract *in vitro* on lipid in our previous paper (Hah *et al.*, 2005). It also showed that it has a protective effect on hydrogen peroxide-induced chromosome damage in CHO cell line (data not report yet). From our results, we suggest that it may be useful for preventing oxidative stress-derived DNA damage. The oxidative DNA damage in cancer development has become widely recognized (Ohshima, 2003; Klaunig and Kamendulis, 2004). The Comet assay is used to detect various types of DNA damage such as single and double strand breaks, oxidative DNA base damage, DNA-DNA/DNA-protein/DNA-drug

crosslinks, and DNA repair in eukaryotic cells (Singh *et al.*, 1989; Tice *et al.*, 2000). Hydrogen peroxide is a reactive oxygen species causing DNA damage (Imlay and Linn, 1988), and generates hydroxyl radicals in the Fenton reaction. It can cause DNA damage, chromosomal aberration, and cancer (Collins *et al.*, 1995; Sweetman *et al.*, 1997). As shown in Fig. 1 and 2, cell viability in HepG2 cells was decreased with increasing concentrations of hydrogen peroxide. However, the cell viability of HepG2 cell was increased significantly by adding 100 μg/ml of *Houttynia cordata* root methnolic extract. This result corresponds with results of Basaran *et al.* (1996) who measured the protective effect of *Turkish* extracted from the plant on DNA damage induced by hydrogen peroxide and Duthie *et al.* (1997) who measured the protective effect of *Quercertin* and *Myricetin* on DNA damage induced by hydrogen peroxide and Ju *et al.* (2003) who measure the protective effect of *Betula var. japonica* on DNA damage induced by hydrogen peroxide.

The *Houttynia cordata* root methnolic extract (100 μg/ml) showed a significant protective effect on DNA damage induced by H_2O_2 (Figs. 3, 4, and Table 1) in HepG2 cells. This result corresponds with results of Anderson *et al.* (1994) and Chen *et al.* (1996) who measured the protective effect of natural antioxidants and ascorbic acid, tocopherol, and butylated hydroxytoluene (BHT) on hydrogen peroxide-induced DNA damage of lymphocytes in patients, and Alessandro *et al.* (2001) who measured the protective effect of *Resveratrol* on hydrogen peroxide-induced DNA damage of breast epithelial cells of rats, but disagrees with the results of Kucuk *et al.* (2003) showing that linoleic acid did not inhibit the hydrogen peroxide-induced DNA damage of lymphocytes and plasma of patients. This discrepancy may be due to a difference of sample, While linoleic acid is an unsaturated fatty acid and does not has antioxidant effects, it accelerates oxidation of lymphocytes and plasma, making organs targets of free radicals. Therefore, we suggest that this methanolic extract has a more powerful protective effect on hydrogen peroxide induced DNA damage in HepG2 cells. Generation of oxygen radicals *in vivo* is thought to be relevant to carcinogenesis, and 8-OH-2'dG formation in DNA may be related to tumorgenesis. In addition, 8-OHdG as biomarker of oxidative DNA damage was produced by glycosylase enzyme excisable activity after 8-hydroxylation of guanine in DNA (Van Loon *et al.*, 1991). When DNA reproduction occurred, 8-OHdG was not excise by glycosylase, causing the replacement of GC to GG or AT (Wilson *et al.*, 1993). If damaged DNA did not repair at reproduction, it inhibited normal cell function and cause various human chronic diseases such as cancer, Alzheimer's disease, and aging *et al.* (Fraga *et al.*, 1990; Kucuk *et al.*, 2002). DNA damage expressed as single or double strand breaks, a basic sites, DNA-DNA and DNA protein crosslinks occur after treatment with radiation (Cadet *et al.*, 1997). Expression of genotoxicity such as mutation, chromosomal aberration and micronuecli formation was caused by ROS. Recently, medicinal plants containing antioxidants that scavenge ROS were examined as potential preventive and treatment strategies for initiation, promotion and progression of cancers (Makino *et al.*, 2006). We expect that these medicinal plants exert their chemopreventive effects by scavenging ROS and detoxifying potent genotoxic carcinogens without side effects, instead of synthetic anticancer drugs and radiation therapy which have many side effects. From the our results and these expectations of medicinal plants, we expect that the *Houttynia cordata* root could be used to treat and prevent ROS induced chronic diseases such as cancer. The mechanism of the protective effects by which methanolic extract protects against oxidative DNA damage may involve reactive oxygen scavenging activity.

In conclusion, these results show that methanolic extracts were effective in the protection of hydrogen peroxide-induced DNA damage in HepG2 cells. This might have some commercial application in the remedy and protection of oxidative DNA damage diseases. Further protective effects of methanolic extract in various cells and tissue *in vivo* and isolation of the antioxidative components in *Houttynia cordata* plants are under investigation.

ACKNOWLEDGEMENTS

This project was supported by grants from Gyeongsang National University.

REFERENCE

Alessandro, S., Raffaele, A., Beatrice, F., Alma, B., Federical, W. and Achille, C. (2001). *Resveratrol*, a natural phenlic compound, inhibites cell proliferation and prevents oxidative DNA damage. *Mutat. Res.*, **496**, 171-180.

Alpetite, C., wachter, T., Sage, E. and Moustacchi, E. (1996). Use of the alkaline comet assay to detect DNA repair deficiencies in human fibroblasts exposed to UVC, UVB, UVA and r-rays. *Inter. J. Rad. Bio.*, **59**, 359-369.

Ames, B.N., Shigenaga, M.K. and Hagn, T.M. (1993). Oxidants, antioxidants and degenerative disease of aging. *Proceeddings of the National Science of the United States of America.*, **90**, 7915-7922.

Anderson, D., Yu, T.W., Phillips, B.J. and Schmezer, P.

(1994). The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in Comet assay. *Mutat. Res.*, **307**, 261-271.

Barsaran, A.A., Yu, T.W., Plewa, M.J. and Anderson, D. (1996). An investigation of some Turkish herbal medicines in *Salmonella typhimurium* and in the Comet assay in human lymphocytes. *Terato. Carcino. Mutat.*, **16**, 125-138.

Bartsch, H. and Nair, J. (2004). Oxidative stress and lipid peroxidation-derived DNA-lesions in inflammation driven carcinogenesis. *Cancer Detec. Preven.*, **28**, 385-391.

Boyle, S.P., Dobson, V.L., Duthie, S.J., Kyle, J.A. and Collins, A.R. (2000). Absorption and DNA protective effects of flavonoid glycosides from an onion meal. *Eur. J. Nutr.*, **39**, 213-223.

Cadet, J. (1994). DNA damage caused by oxidation, deamination, ultraviolet radiation and photoexcitated psoralens. In : DNA adduct : identification and biological significance, edited by Hemminki, K.A., Dipple, D.E., Shuker,, F.F., Kadulbar, D., Segerback, D. and Bartsch, H. pp. 245-276.

Cadet, J., Berger, M., Douki, T. and Ravanat, J. (1997). Oxidative damage to DNA: formation, measurement and biological significance. *Review Physi. Bioch. Pharm.*, **31**, 1-87.

Chen, C., Pearson, A.M. and Gray, J.I. (1996). Effect of synthetic antioxidants (BHA, BHT and PG) on the mutagenecity of IQ-like compounds. *Food Chem.*, **43**, 177-183.

Collins, A.R. (1999). Oxidative DNA damage, antioxidants, and cancer. *BioEssays*, **21**, 238-246.

Dizdaroglu, M. (1991). Chemical determination of free radical-induced damage to DNA. *Free Radical Biol. Med.*, **10**, 225-242.

Duthie, S.J., Collins, A.R., Duthie, G.G. and Dobson, V.L. (1997). *Quercertin* and *myricetin* protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes. *Mutat. Res.*, **393**, 223-231.

Fairbairn, D.W., Oliver, K.L. and O'Neill. (1995). The Comet assay : a comprehensive review. *Mutat. Res.*, **339**, 37-59.

Fang, Y.Z., Yang, S. and Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutr.*, **18**, 872-879.

Floyd, R.A. (1990). The role of 8-hydroxylguanine in carcinogenesis. *Carcino.*, **11**, 1447-1450.

Fraga, C.G., Shigenaga, M.K., Park, J.W., Degan, P. and Ames, B.N. (1990). Oxidative damage to DNA during aging:8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci., USA*, **87**, 4533-4537.

Hah, D.S., Kim, C.H., Kim, G.S., Kim, E.K. and Kim, J.S. (2005). Antioxidative effects of traditional medicinal plants on lipid peroxidation. *Korean J. Vet. Res.*, **45**, 341-350.

Hartmann, A., Plappert, U., Poetter, F. and Suter, W. (2003). Comparative study with the alkaline comet assay and the chromosome aberration test. *Mutat. Res.*, **536**, 27-38.

Helbock, H.J., Beckman, B.K., Shigenage, M.K. and Walter, B.N. (1998). DNA oxidation matters : the HPLC-electochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guannine. *Proc. Natl. Sci., USA*, **95**, 288-293.

Imlay, J.A. and Linn, S. (1988). DNA damage and oxygen radical toxicity. *Sci.*, **240**, 1302-1309.

Ju, E.M., Lee, S.E., Hwang, H.J. and Kim, J.H. (2003). Antioxidant and anticancer activity of extract from *Betula platpylla* var. *japonica*. *Life Sci.*, **87**, 14-27.

Kasai, H. (1997). Analysis of a form of oxidative DNA damage, 8-hydroxyl-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat. Res.*, **387**, 147-163.

Kasai, H., Tanooka, H. and Nishimura, S. (1984). Formation of 8-hydroxyguanine residues in DNA by X-irradiation. *Gann.*, **75**, 1037-1039.

Klaunig, J.E. and Kamendulis, L.M. (2004). The role of oxidative stress in carcinogenesis. *Annu. Rev. Pharma. Toxi.*, **44**, 239-267.

Kucuk, C.M., Rhijnsburger, E., Haenen, G.R. and Kleinjans, J.C. (2002). New opportunities in chemoprevention research. *Cancer Inve.*, **20**, 237-245.

Kucuk, C.M., Zwingman, I., Moonen, E.J., Schilderman, E.L., Rhijnsburger, E., Haenen, G.R. and Kleinjans, J.C. (2003). Analysis of oxidative DNA damage after human dietary supplementation with linoleic acid. *Food Chem. Toxi.*, **41**, 351-358.

Kmuaravel, T.S. and Jha, A.N. (2006). Reliable Comet assay measurements for detecting DNA damage induced by ionizing radiation and chemicals. *Mutat. Res.*, **605**, 7-16.

LePage, F., Guy, A., Cade, J., Sarasin, A. and Geftil, A. (1998). Repair and mutagenic potency of 8-oxoG:A and 8-oxo G:C base pairs in mammalian cells. *Nucleic Acids Res.*, **26**, 1276-1281.

Makino, T., Tsubouchi, R., Murakami, K., Haneda, M. and Yoshino, M. (2006). Generation of reactive oxygen species and induction of apoptosis of HL60 cells by ingredients of traditional herbal medicine. *Pharma. Toxi.*, **98**, 401-405.

Nocentini, S. (1995). Comet assay analysis of repair of DNA strand break in normal and deficient human cells exposed to radiations and chemicals. Evidence for a repair pathway specificity of DNA ligation. *Rad. Res.*, **144**, 170-180.

Ohshima, H. (2003). Genetic and epigenetic damage induced by reactive nitrogen species: implications in carcinogenesis. *Toxi. Lett.*, **140-141**, 99-104.

Reilly, P.M., Schiller, H.J. and Bulkley, G.B. (1991). Pharmacologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites. *Am. J. Surgery*, **161**, 488-503.

Ravanat, J.L., Douki, T., Turesky, R.J. and Cadet, J. (1997). Measurement of oxidized base in DNA. Comparison between HPLC-EC and GC-MS assays. *J. Chem. Phys.*, **94**, 306-312.

Roias, E. and Lopez, M. (1999). Single cell gel electrophoresis assay: Methodology and application. *J. Chromato.*, **31**, 722-225.

Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L. (1988). A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184-191.

Singh, N.P., Danner, D.B., Tice, R.R., McCoy, M.T., Collins, G.D. and Schneider, E.L. (1990). DNA damage and repair

with age in individual human lymphocytes. *Mutat. Res.*, **237**, 123-130.

Sweetman, S.F., Strain, J.J. and McKelvey, V.J. (1978). Effect of antioxidant vitamin supplementation on DNA damage and repair in human lymphoblastoid cells. *Nutr. Cancer*, **27**, 122-130.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F. (2000). Single cell gel/comet assay : guidelines for *in vitro* and *in vivio* genetic toxicology testing. *Envir. Mutat.*, **35**, 206-221.

Van-Loon, A.A., Groenendijk, R.H., Vander, G.P., Lohman, P.H. and Nbann, R.A. (1991). Detection of base damage in DNA in human blood exposed to ionizing radiation at biologically relevant doses. *Int. J. Radi. Biol.*, **59**, 651-660.

Wilson, V., Taffe, B.G., Shields, P.G., Povey, A.C. and Harris, C.C. (1993). Detection and quantification of 8-hydroxydeoxyguanosine adducts in peripheral blood of people exposed to ionizing radiation. *Enviro. Heal. Persp.*, **99**, 261-263.