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Protective Effect of Some Medicinal Plants on *tert*-Butyl Hydroperoxide-Induced Oxidative Stress in Human Keratinocytes

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Abstract – It is well known that excessive production of reactive oxygen species (ROS) leads to oxidative stress, loss of cell function, and ultimately apoptosis or necrosis. To search for natural antioxidants able to modulate cellular oxidative stress, we investigated the protective effect of ethanol extracts of 17 medicinal plants selected from the preliminary antioxidant screening on *tert*-butyl hydroperoxide (*t*-BuOOH)-induced oxidative stress in human keratinocytes. The result showed that extracts of the four plants, *Distylium racemosum*, *Astilbe chinensis*, *Cercis chinensis* and *Sapium japonicum*, exhibited significant cytoprotective activity (over 50% protection) against *t*-BuOOH-induced cellular injury.
Keywords – oxidative stress, antioxidants, medicinal plant extracts, *tert*-butyl hydroperoxide, cytoprotective activity, human keratinocytes

Introduction

Reactive oxygen species (ROS) are so apt to react with biomolecules such as lipids, proteins, sugars, and DNA that they lead to oxidative stress in cells highly implicated in the pathogenesis of various degenerative diseases and cancer (Frei, 1994; Silva *et al*., 2005; Finkel and Holbrook, 2000). Accordingly, antioxidants capable of suppressing oxidative stress in cells are expected to treat or prevent various degenerative diseases and aging (Bandyopadhyay *et al*., 1999; Beal, 1995; Maxwell, 1995; Poulson *et al*., 1998). Although a great number of synthetic and natural antioxidants have been developed so far, a new antioxidant acting in biological system without toxicity still needs to be discovered.

tert-Butyl hydroperoxide (*t*-BuOOH), an organic hydroperoxide widely used as model compound to induce oxidative stress, can be metabolized to free radical intermediates by cytochrome P450 (hepatocytes) or hemoglobin (erythrocytes), which subsequently can initiate lipid peroxidation, mediate DNA damage, form covalent bonds with cellular molecules resulting in cell injury (Ruch *et al*., 1985). These phenomena are similar to the oxidative stress occurring in cells and tissues. In microsomal suspensions in the absence of NADPH, the *t*-BuOOH is transformed into a peroxyl radical (ROO•) through a one-electron oxidation process, whereas in the presence of NADPH it is transformed into an alkoxyl radical (RO•) through a one-electron reduction process (Davies, 1989). The metabolism of *t*-BuOOH to these radicals has also been demonstrated in isolated mitochondria and intact cells, in which the alkoxyl radical is transformed into a alkyl radical (•R) through a β-scission process (Kennedy *et al*., 1992; Timmins and Davies, 1993; O'Donnell and Burkitt, 1994). The radicals generated from *t*-BuOOH are analogous to those generated from fatty acids during the peroxidation of biological membranes, and are known to initiate the process in cells (Masaki *et al*., 1989).

In our previous antioxidant screening for plant extracts, we found out that several plants had anti-radical and anti-lipid peroxidation activities (Na *et al*., 2001 and 2003). To verify their antioxidant property in cellular level, in this study, we investigated the cytoprotective effect of 17 medicinal plants, selected from the preliminary study, using an oxidative stress model induced by *t*-BuOOH in human keratinocytes.

Experimental

Plant material – The list of plants studied is given in

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Table 1. The protective effect of ethanol extracts of plants on *t*-BuOOH-induced oxidative stress in human keratinocytes

Voucher number	Plant name[a)]	Family name	Uses in traditional medicine (and/or reported activities)[e)]	Previously isolated classes of constituents[e)]	Part used[b)]	Cell viability (%)[c)]
	Blank					100
	t-BuOOH					11.2 ± 1.2
HK0055	*Areca catechu* L.	Palmae	anthelmintic, antidepressant effects	alkaloids, fatty acids, catechin	S	43.3 ± 3.6
CNU0251	*Platycarya strobilacea* Siebold & Zucc.	Juglandaceae	furuncle, distending pain in chest, abdominal pain, arthralgia, carbuncle, tinea and scabies	tannins, diarylheptanoids	L, St	49.6 ± 3.0
CNU0606	*Lindera obtusiloba* Blume	Lauraceae	fever, abdominal pain	lignans	L, St	22.9 ± 2.6
HK0094	*Paeonia lactiflora* Pall.	Paeoniaceae	sedative, antispasmodic, analgesic, anti-inflammatory, anti-allergic, immunomodulating effects, and tonic for blood	triterpenoids, flavonoids, monoterpene glycosides	R	21.7 ± 2.5
HK0037	*Paeonia suffruticosa* Andr.	Paeoniaceae	analgesic, sedative, antipyretic, anti-inflammatory effects	monoterpene glycosides	Rb	20.4 ± 1.9
HK0081	*Nelumbo nucifera* Gaertn.	Nymphaeaceae	diarrhea, gastritis, insomnia, nervous prostration	alkaloids, flavonoids	S	32.0 ± 2.9
CNU0855	*Distylium racemosum* Siebold & Zucc.	Hamamelidaceae	edema	Flavonoids	L, St	66.8 ± 4.6
CNU0880	*Astilbe koreana* Nakai	Saxifragaceae	headache, arthralgia, chronic bronchitis, stomachache	triterpenoids	L	22.9 ± 4.5
CNU0881	*Astilbe chinensis* (Maxim.) Franch. et Savat.	Saxifragaceae	headache, arthralgia, chronic bronchitis, stomachache	triterpenoids, bergenin	Rh	59.1 ± 4.0
HK0054	*Eriobotrya japonica* Lindl.	Rosaceae	skin disease, diabetes mellitus, cough, phlegm	triterpenoids, flavonoids	L	21.7 ± 2.5
CNU1122	*Cercis chinensis* Bunge	Leguminosae	dysmenorrhea, edema, bruising, injuries	flavonoids	L, St	50.5 ± 3.8
CNU1250	*Sapium japonicum* Pax & K.Hoffm.	Euphorbiaceae	tinea, scabies, furuncle, eczema	tannins	L, St	54.1 ± 4.0
CNU1334	*Euscaphis japonica* (Thunb.) Kanitz	Staphyleaceae	detumescence, invigorating the spleen, and analgesic effect	euscapholide	Ap	26.8 ± 3.3
CNU1787	*Callicarpa japonica* Thunb.	Verbenaceae	hemorrhage, anti-inflammatory, antiviral activity	flavonoids, essential oil	St	28.0 ± 2.8
HK0022	*Salvia miltiorrhiza* Bunge	Labiatae	coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, neuroasthenic insomnia	diterpenoid quinines, phenolic acids, flavonoids	R	29.3 ± 3.1
HK0101	*Lycium chinense* Mill.	Solanaceae	tonic, dizziness, headache, lumbago, diabetes	alkaloids	Rb	40.6 ± 3.0
HK0112	*Alpinia katsumadai* Hayata	Zingiberaceae	antiemetic, stomach disorders	stilbenes, diarylheptanoids, chalcones, monoterpenes, flavonoids	Fr	18.2 ± 2.1
	Quercetin[d)]					64.0 ± 4.2

[a)] Each plant extract was treated at 50 μg/ml as a final concentration.
[b)] Ap: aerial part, Fr: fruit, L: leaf, R: root, Rb: root bark, Rh: rhizome, S: seed, St: stem.
[c)] Values are expressed as mean ± SD of triplicate experiments.
[d)] Positive control.
[e)] References: Bae, 2000; Fenglin *et al*., 2004; Holdsworth *et al*., 1998; Ito *et al*., 2000; Kamiya *et al*., 1997; Kobaisy *et al*., 2002; Kwon *et al*., 1999; Lin *et al*., 1996; Lu and Foo, 2002; Namba, 1993; Ngo and Brown, 1998; Park, *et al*., 2003b; Salatino *et al*., 2000; Tanaka *et al*., 1998.

Table 1. Voucher number HK0022, HK0037, HK0054, HK0055, HK0081, HK0094, HK0101 and HK0112 were obtained from a pharmaceutical company of Korea, Han Kook Sin Yak Co., Ltd., and each voucher specimen was deposited in the Jakwang Research Institute of the Han Kook Sin Yak Co., Ltd., Nonsan, Korea. Other plant materials, CNU0251, CNU0606, CNU0855, CNU0880, CNU0881, CNU1122, CNU1250, CNU1334 and CNU 1787, were collected at Mt. Sulak or Jeju Island, Korea in July 2001. Each voucher specimen was identified by Prof. KiHwan Bae, College of Pharmacy, Chungnam National University and deposited in the herbarium of the College.

Preparation of sample – Twenty gram of each dried plant material was extracted using 100 ml ethanol at room temperature for 2 weeks. The ethanol extract was filtered and dried in vacuo to give ethanol extract, which was then re-dissolved in DMSO or a medium to obtain a stock solution of 10 mg/ml. Appropriate dilutions were made before the experiments (final DMSO concentration never exceeded 1%, and control activity was not affected by this concentration).

Cell culture – The human epidermal keratinocytes-Neonatal/Foreskin (HEK-N/F) were purchased from Modern Tissue Technologies, Inc. (MC1312, Korea). The HEK-N/F cells were cultured in a type IV collagen coated plate with KGM® Bulletkit medium (CC-3111, Clonetics, San Diego, CA) in humidified atmosphere of 5% CO_2/95% air at 37 °C, and cultured to 90% confluence.

***t*-BuOOH induced oxidative stress** – The HEK-N/F cells (1×10^4 cells/100 μl) were seeded on a 96 well microplate and precultured for 24 h. The cells were then treated with 1 μl of the sample and 10 μl of *t*-BuOOH (1.5 mM) dissolved in Hank's balanced salt solution (HBSS) for 3 h in order to induce cellular peroxidation. The cell viability was measured using the methylthiazoletetrazolium (MTT) method. Briefly, the MTT (5 mg/ml) dissolved in phosphate buffered saline (PBS) was added to comprise less than 10% of the total volume. After 4 h incubation, the remaining medium was aspirated and 100 μl of DMSO was added to dissolve the formazan formed from the MTT. The absorbance was read at a wavelength of 570 nm. The inhibitory activity of lipid peroxidation was also determined using the thiobarbituric acid (TBA) method, as previously described (Park *et al*., 2003a). Briefly, the cell suspension was reacted with an equal volume of 10% trichloroacetic acid (TCA) and 1% TBA (in 50 mM NaOH). The mixture was heated in a boiling water bath for 5 min and then centrifuged at 10000 rpm for 10 min. The absorbance of supernatant was measured at 532 nm. A calibration curve was prepared using 1,1,3,3-tetraethoxypropane, a chemical releasing malondialdehyde in acidic conditions. Protein concentration was assayed using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA)

Statistical analysis – Results are expressed as the mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparison tests (GraphPad Prism program; GraphPad Software Inc., USA). *P* values of less than 0.05 were considered significant.

Results and Discussion

Oxidative stress is proposed to be involved in the etiology of degenerative diseases, as well as in the process of aging. Although a large number of antioxidants have been developed so far, due to the low bioavailability and the toxicity, a new antioxidant with potential action in biological system and no toxicity still needs to be developed. Plants produce various secondary metabolites capable of preventing themselves from oxidative stress, so that they are being considered as a good source for antioxidant drug discovery. Since the *t*-BuOOH has been widely applied for investigation of cell injury initiated by oxidative stress, we evaluated the protective effect of plant samples, which possess antioxidant activities against free radical formations and lipid peroxidation (Na *et al*., 2001 and 2003), on *t*-BuOOH-induced oxidative stress in human keratinocytes.

Of the 17 plant extracts tested, *D. racemosum, A. chinensis, C. chinensis*, and *S. japonicum* exhibited significant cytoprotective activity (over 50% protection) against *t*-BuOOH-induced cellular injury. In particular, ethanol extracts of *D. racemosum* and *A. chinensis*, with cell viability of 66.8 ± 4.6 and 59.1 ± 4.0%, showed potent cytoprotective activity comparable with that of quercetin (64.0 ± 4.2%), a positive control. Several phenolic compounds are known as constituents of the active plants, *D. racemosum, A. chinensis, C. chinensis*, and *S. japonicum*. Methyl gallate, kaempferol, quercetin and quercitrin have been isolated from an EtOAc soluble fraction of the leaves of *D. racemosum*, which have higher antioxidant activity compared with reference compounds, ascorbic acid (Park *et al*., 2003b). Bergenin, a constituent of *A. chinensis* exhibited encouraging antioxidant activity (Rana *et al*., 2005). Methyl gallate purified from *C. chinensis* leaves also showed free radical scavenging effect at low concentration (0.02 mM) and cell protective effect against H_2O_2-mediated oxidative stress (Whang *et al*., 2005). Seven compounds, gallic

acid, ellagic acid, 3,3'-di-*O*-methylellagic acid, 4-*O*-(β-D-xylopyranosyl)-3,3'-di-*O*-methylellagic acid, 4-*O*-(α-D-arabinofuranosyl)-3,3'-di-*O*-methylellagic acid, isoquercitrin, and geraniin have been reported to be constituents of *S. japoninum* (Kang, *et al*., 2006), and they have been demonstrated to have significant free radical scavenging capacities with IC_{50} values ranging from 0.011 to 0.032 mM, which were much more active than trolox, the positive control, with IC_{50} values of 0.026 mM (Zhang, *et al*., 2009). Considering the above results, the protective effect of *D. racemosum, A. chinensis, C. chinensis*, and *S. japonicum* on the oxidative stress induced by *t*-BuOOH might be associated with the phenolic constituents of the plants. However, their antioxidant properties in cellular level or animal models are not clear. Thus, further investigations on the active constituents of the species as well as on the protection mechanism in cells are required, which makes them useful in preventing the deleterious consequences of oxidative stress.

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References

Bae, K.H., The Medicinal Plants of Korea. Kyo-Hak Publishing Co., Ltd, Seoul, pp. 204, 245, 427, 452, 2000.

Bandyopadhyay, U., Das, D., and Banerjee, R.K., Reactive oxygen species: oxidative damage and pathogenesis. *Curr. Sci.* **77**, 658-666 (1999).

Beal, M.F., Aging, energy and oxidative stress in neurodegenerative diseases. *Ann. Neurol.* **38**, 357-366 (1995).

Davies, M.J., Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with rat liver microsomal fractions. *Biochem. J.* **257**, 603-606 (1989).

Fenglin, H., Ruili, L., Bao, H., and Liang, M., Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medical plants. *Fitoterapia* **75**, 14-23 (2004).

Finkel, T. and Holbrook, N.J., Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247 (2000).

Frei, B., Reactive oxygen species and antioxidant vitamins: mechanisms of action. *Am. J. Med.* **97**, 5S-13S (1994).

Holdsworth, D.K., Jones, R.A., and Self, R., Volatile alkaloids from *Areca catechu*. *Phytochemistry* **48**, 581-582 (1998).

Ito, H., Kobayashi, E., Takamatsu, Y., Li, S.H., Hatano, T., Sakagami, H., Kusama, K., Satoh, K., Sugita, D., Shimura, S., Itoh, Y., and Yoshida, T., Polyphenols from *Eriobotrya japonica* and their cytotoxicity against human oral tumor cell lines. *Chem. Pharm. Bull.* **48**, 687-693 (2000).

Kamiya, K., Yoshioka K., Saiki Y., Ikuta A., and Satake T., Triterpenoids and flavonoids from *Paeonia lactiflora*. *Phytochemistry* **44**, 141-144 (1997).

Kang, S.J., Hong, S.S., Hwang, B.Y., Ro, J.S., Lee, K.S., and Towers, G.H.N, Phenolic compounds from the stems of *Sapium japonicum*. *Nat. Prod. Sci.* **12**, 125-128 (2006).

Kennedy, C.H., Church, D.F., Winston, G.W., and Pryor, W.A., *tert*-Butyl hydroperoxide-induced radical production in rat liver mitochondria. *Free Radic. Biol. Med.* **12**, 381-387 (1992).

Kobaisy, M., Tellez, M.R., Dayan, F.E., and Duke, S.O., Phytotoxicity and volatile constituents from leaves of *Callicarpa japonica* Thunb. *Phytochemistry* **61**, 37-40 (2002).

Kwon, H.C., Choi, S.U., Lee, J.O., Bae, K.H., Zee, O.P., and Lee, K.R., Two new lignans from *Lindera obtusiloba* Blume. *Arch. Pharm., Res.*, **22**, 417-422 (1999).

Lin, H.C., Ding, H.Y., Wu, T.S., and Wu, P.L., Monoterpene glycosides from *Paeonia suffruticosa*. *Phytochemistry* **41,** 237-242 (1996).

Lu, Y. and Foo, L.Y., Polyphenolics of Salvia - A review. *Phytochemistry* **59**, 117-1140 (2002).

Masaki, N., Kyle, M.E., and Farber, J.L., *tert*-Butyl hydroperoxide kills cultured hepatocytes by peroxidizing membrane lipids. *Arch. Biochem. Biophys.* **269**, 390-399 (1989).

Maxwell, S.R.J., Prospects for the use of antioxidant therapies. *Drugs* **45**, 345-361 (1995).

Na, M., An, R.B., Lee, S.M., Hong, N.D., Yoo, J.K., Lee, C.B., Kim, J.P., and Bae, K.H., Screening of crude drugs for antioxidative activity. *Kor. J. Pharmacogn.* **32**, 108-115 (2001).

Na, M., An, R.B., Jin, W.Y., Min, B.S., Yoo, J.K., Kim, Y.H., and Bae, K.H., Antioxidant effects of plant extracts on free radicals and lipid peroxidation. *Nat. Prod. Sci.* **9**, 226-231 (2003).

Namba, T., The Encyclopedia of Wakan-Yaku (Traditional Sino-Japanese Medicines with Color Pictures Vol. I and II.. Hoikusha Publishing Co., Ltd, Osaka, pp. 300 (Vol. I), 102 (Vol. I), 132 (Vol. II), 216 (Vol. I), 80 (Vol. II), 239 (Vol. I), 1993.

Ngo, K.S. and Brown, G.D., Stilbenes, monoterpenes, diarylheptanoids, labdanes and chalcones from *Alpinia katsumadai*. *Phytochemistry* **47**, 1117-1123 (1998).

O'Donnell, V. and Burkitt, M.J., Mitochondrial metabolism of a hydroperoxide to free radicals in human endothelial cells: an electron spin resonance spin-trapping investigation. *Biochem. J.* **304**, 707-713 (1994).

Park, E.J., Zhao, Y.Z., Na, M., Bae, K.H., Kim, Y.H., Lee, B.H., and Sohn, D.H., Protective effects of honokiol and magnolol on tertiary butyl hydroperoxide- or D-galactosamine-induced toxicity in rat primary hepatocytes. *Planta Med.* **69**, 33-37 (2003a).

Park, Y., Lee, W.Y., Ahn, J.K., Lee, H.J., Chin, H.S., and Kwon, Y.J., Antioxidant compounds from *Distylium racemosum* leaves. *Mokchae Konghak* **31**, 6-72 (2003b)

Poulson, H.E., Preime, H., and Loft, S., Role of oxidative DNA damage in cancer initiation and promotion. *Eur. J. Cancer Prev.* **7**, 9-16 (1998).

Rana, V.S., Rawat, M.S.M., Pant, G., and Nagatsu, A., Chemical constituents and antioxidant activity of *Mallotus roxburghianus* leaves. *Chem. Biodivers.* **2**, 792-798 (2005).

Ruch, G.F., Gorski, J.R., Ripple, M.G., Sowinski, J., Bugelski, P., and Hewitt, W.R., Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. *Toxicol. App. Pharmacol.* **78**, 473-483 (1985).

Salatino, A., Salatino, M.L.F., and Giannasi, D.E., Flavonoids and the taxonomy of Cercis. *Biochem. Syst. Ecol.* **28**, 545-550 (2000).

Silva, C.G., Herdeiro, R.S., Mathias, C.J., Panek, A.D., Silveira, C.S., Rodrigues, V.P., Renno, M.N., Falcao, D.Q., Cerqueira, D.M., and Minto, A.B.M., Evaluation of antioxidant activity of Brazilian plants. *Pharm. Res.* **52**, 229-233 (2005).

Tanaka, T., Jiang, Z.H., and Kouno, I., Distribution of ellagic acid derivatives and a diarylheptanoid in wood of *Platycarya strobilacea*. *Phytochemistry* **47**, 851-854 (1998).

Timmins, G.S. and Davies, M.J., Free radical formation in isolated murine keratinocytes treated with organic peroxides and its modulation by antioxidants. *Carcinogenesis* **14**, 1615-1620 (1993).

Whang, W.K., Park, H.S., Ham, I.H., Oh, M., Namkoong, H., Kim, H.K., Hwang, D.W., Hur, S.Y., Kim, T.E., Park, Y.G., Kim, J.R., and Kim, J.W., Methyl gallate and chemicals structurally related to methyl gallate protect human umbilical vein endothelial cells from oxidative stress. *Exp. Mol. Med*. **37**, 343-352 (2005).

Zhang, Z., Liao, L., Moore, J., Wu, T., and Wang, Z., Antioxidant phenolic compounds from walnut kernels (*Juglans regia* L.). *Food Chem*. **113**, 160-165 (2009).

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