Protective Effect of *Caesalpinia sappan* L. on Hydrogen Peroxide

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In this study, we demonstrated the antioxidant effect of the *Caesalpinia sappan* L. extract through the scavenging effect against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and the protective effect on protein damage and PC12 cells against cupric ion/hydrogen peroxide. Its IC_{50} value of the scavenging effect against DPPH radical was 7.7 ㎍. Protection of its extract against oxidative bovine serum albumin (BSA)damage induced by hydrogen peroxide was more effective than that of vitamin C. The protective effect on PC12 cells by hydrogen peroxide was shown to be more potent in is extract than in vitamin C. DNA fragmentation analysis also supports this result.

Key words : *Caesalpinia sappan* L., 2,2-diphenyl-1-picrylhydrazyl, BSA degradation

Introdution

Caesalpinia sappan L.(C. sappan) has been used in Oriental medicine as an analgesic and anti-inflammatory agent, to cure emmeniopathy, sprains, and convulsions[1]. SL is well known to be used for a treatment medium of diabetic complications[2] and to promote blood circulation in rat thoracic aorta[3]. The extract of SL has also been reported to have some pharmacological activities such as sedative and depressing effects on the central nervous system[4], anticomplementary activity on the complement system[5], and vasorelaxation in rat aorta and human umbilical vein endothelial cells[6].

As reports stated above, *C. sappan* was composed of compounds extracted by methanol, such as brazilin, hematoxylin, sappanchalcone, etc. Brazilin, the major component of SL, is a natural red pigment and is usually used for histological staining[7]. Its effects show protection of hepatocytes from $BrCCl_3$-induced toxicity[8], inhibition of protein kinase C and insulin receptor serine kinase[9], immunological modulation and tolerance in mice[10], and vasorelaxation[3,6].

Especially, Moon et al.[8] reported that brazilin protects cultured rat hepatocytes from $BrCCl_4$-induced toxicity and suggested that brazilin is as an antioxidant and has protective effect on the toxicity of free radical. In this study, we demonstrated the antioxidant effects of SL on hydrogen peroxide were shown in in vitro and PC12 cells.

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Materials and Methods

1. Extraction of plants

To obtain the aqueous extract of SL, 200 g of dried SL was added to 1000 ㎖ of boiled distilled water and extraction was performed by heating at 80℃ for 2 h. Then the extract was filtered and lyophilized (Biocryos, Seoul, South Korea).

2. Scavenging assay against DPPH radical

The scavenging ability of extracts was examined in the presence of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma, St. Louis, MO, USA). The SL extract, vitamin C, and glutathione were diluted with distilled water to prepare sample solutions of dried samples per ㎖ solutions. 150 ㎕ of 0.004% DPPH solution was pipetted into each well of 96 well plate followed by 40 ㎕ of the samples. The mixture was incubated at 37℃ for 30 min. Absorbance was measured at 515 nm using a 96-well plate reader (Emax:Molecular Device, Menlo Park, CA, USA). The percentage (%) of radical scavenging activity was calculated from relative intensity compared with untreated samples. From the inhibition (%), the amount of the samples reducing the absorbance by 50% (IC_{50}) was determined.

3. Protection against oxidative bovine serum albumin (BSA) degradation

Bovine serum albumin (BSA)(Sigma, St. Louis, MO, USA) was dissolved in 150 mM phosphate buffer (pH 7.3) at a final

concentration of 0.5 ㎎/㎖. BSA was incubated with or without Cu^{2+} (100 mM) and H_2O_2 (2.5 mM) as previously described[11], for the durations indicated in the presence or absence of samples. Reactions were performed in a shaking water bath at 37℃. To identify BSA damage, BSA samples were loaded in 12% SDS-polyacrylamide gel and electrophoresed. After for running for 1 h, the gel wasstained with 0.15% Coomassie brilliant blue R-250 for 30 min, washed extensively and scanned.

4. Cell viability

Cell viability assay using crystal violet was performed as follows. PC12 cells were cultured with RPMI 1640medium (GibcoBRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (GibcoBRL, Gaithersburg, MD) at 37℃ in 5% CO_2, 95% air in each well of a 24-well plate (Corning Inc., Corning, NY) at a density of 2×10^5 cells per well. The cells were treated with various concentrations of H_2O_2 and with or without antioxidant pretreatment. After 16-20 h, the cells were washed and treated with crystal violet(0.5% crystal violet in 30% ethanol and 3% formaldehyde) for 10min at room temperature. Plates were washed four times with tap water. After drying, cells were lysed with 1% SDS solution, and dye uptake was measured at 550nm using plate reader. Cell viability was calculated from relative dye intensity compared with untreated samples.

5. DNA fragmentation

Cytosolic DNA was prepared as follows. Cell pellets were resuspended in 750 ㎕ of lysis buffer (20 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 8.0) and left on ice for 45 min with occasional shaking. DNA was extracted with phenol/chloroform and precipitated with alcohol. The precipitate was dried and resuspended in 100 ㎕ of 20 mM Tris-HCl, pH 8.0. After degradation of RNA with RNase (0.1 ㎎/㎕) at 37℃ for 1 h, samples (15 ㎕) were electrophoresed on a 1.2% agarose gel in 450 mM Tris borate-EDTA buffer (TBE, pH 8.0) and photographed under UV light.

Data are presented as mean ± S.E.M. of at least three separate experiments. Comparison between two values was analyzed using Student's t-test.

Results and Discussion

The scavenging effect of the C. sappan L. (SL) extract was investigated on DPPH compared with that of vitamin C (Vit C) and glutathione (GSH) (Table 1). As shown in Table 1, the scavenging action of antioxidants was observed in a dose-dependant manner. The efficiency of antioxidants was exhibited in the following order : Vit C > GSH > SL.

IC_{50} values for these antioxidants against DPPH radical were estimated : IC_{50} value of Vit C is 124 ㎍/㎖, GSH is 135 ㎍/㎖, and SL is 193 ㎍/㎖. This result suggests that the scavenging effect of Vit C against DPPH radical is more effective than those GSH and SL.

Table 1. Free radical scavenging effects on DPPH

Treatments	Concentration(㎍/ml)					IC_{50} (㎍/ml)
	0	39	78	156	312	
Vitamin C	100a	82.8	61.0	16.8	3.3	124
Glutathione	100	74.6	57.2	34.0	13.9	135
Caesalpinia sappan L.	100	90.3	78.2	57.2	19.8	197(7.7㎍)

a : % of the absorbance remained

To identify direct free radical scavenging effect of antioxidants through in vitro BSA degradation, antioxidants were incubated with BSA and hydrogen peroxide for 2 h and protein band was confirmed through 12% SDS-PAGE (Fig. 1).

As shown in Fig. 1, the scavenging effect of antioxidants against hydrogen peroxide was in the following order : GSH > SL > Vit C. This result suggests that the scavenging effects of GSH and SL against hydrogen peroxide are more potent than Vit C.

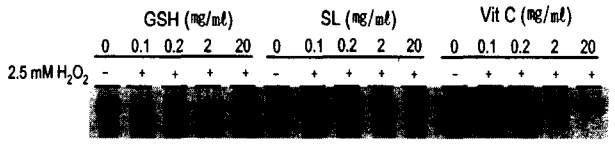

Fig. 1 . Protection effects of antioxidants on BSA degradation against H_2O_2. BSA was dissolved in 150 mM phosphate buffer (pH 7.3) at a final concentration of 0.5 ㎎/㎖. BSA was incubated with or without 100 mM Cu^{2+} and 2.5 mM H_2O_2 at 37℃. BSA degradation was identified in 12% SDS-PAGE. GSH, glutathione ; SL, Caesalpinia sappan L. ; Vit C, vitamin C.

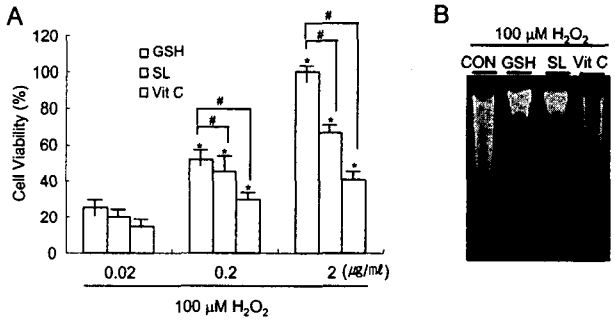

Fig. 2 Protective effects of antioxidants against H_2O_2-induced death of PC12 cells. PC12 cells were incubated in RPMI 1640 containing 10% FBS with or without antioxidants for 10 h before treatment with H_2O_2 for 16-24 h. For determination of cell viability, crystal violet assay was performed (A). DNA fragmentation was determined by gel electrophoresis (B). Values are represented as mean ± S.E.M. (bar) of three independent experiments. *P < 0.01 vs control, #P < 0.05 vs GSH. CON, control ; GSH, glutathione ; SL, Caesalpinia sappan L. ; Vit C, vitamin C.

To elucidate those protective actions against hydrogen peroxide, cell viability was assayed in PC12 cells. Their protective effects appeared according to a concentration-dependent action (Fig. 2A). Antioxidant functions against hydrogen peroxide were shown the same order as Fig. 1 : GSH > SL > Vit C. These data suggest that the actual antioxidant action of SL against hydrogen peroxide is more effective than Vit C (Fig. 1 and 2A). Also these results were supported by DNA fragmentation analysis (Fig. 2B).

Free radical is associated with a cause at pathological processes, some of which are related to aging, for example, atherosclerosis, arthritis, muscular dystrophy, pulmonary dysfunction, cancer, and several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis[12]. Lipid peroxidation and DNA and protein damage as a result of free radical attack has been widely studied. Protein damages are considered to contribute less prominently to total cellular damage.

Recently, however, the possibility that free radical damage to proteins might contribute to aging has also received attention[13]. Thus, it has been shown that oxidatively modified proteins accumulate during aging and in some pathological conditions[14]. Exposure of proteins to $OH^{\cdot}$ and /or $O_2^{\cdot -}$ leads to gross structural modifications. These oxidatively modified proteins may undergo spontaneous protein fragmentation and cross-linking or exhibit a substantial increase in proteolysis[13]. In this report, we demonstrated the antioxidant effect of SL against free radical, e.g. hydrogen peroxide, through the scavenging assay of DPPH radical, the BSA degradation, and the cell viability of PC12 cells.

The difference of antioxidant effects on DPPH radical and BSA degradation may be methodological consideration for potential antioxidant actions[11,15]. The study of antioxidant function against DPPH radical must be associated with methods used in evaluating antioxidant actions in vitro. There is need to agree on in vitro antioxidant methods based on an understanding of the mechanisms involved. Therefore, because DPPH assay are done in antioxidant effect concerned with scavenging DPPH radical, it is impossible to extrapolate the accurate results in vivo and in vitro. Recently the consensus of opinion is that a mix of tools should be used in assessing the antioxidant activities in vitro : the oxygen radical absorbance capacity, ferric reducing antioxidant power, total oxidant scavenging capacity, the deoxyribose assay, assays involving oxidative DNA damage, assays involving reactive nitrogen intermediates (e.g. $ONOO^-$), Trolox equivalent antioxidant capacity, and DPPH assay[15].

Among the changes caused by direct and indirect oxidative modification or degradation of proteins by free radical generation, one of the most common is the determination of carbonyl groups into the side chains[11]. Oxidative modification or degradation can be measured by monitoring the carbonyl content of a specific protein or analyzing through SDS-PAGE.

The results report that BSA is exposed to hydrogen peroxide, an increment in BSA damage is observed, strongly supporting the view that free radicals could be responsible for the oxidative modification or degradation[11] (Fig. 1).

In conclusion, the present report demonstrated the comparative study of scavenging effect between DPPH radical and BSA degradation on SL suggests that the actual antioxidant effect of SL is more effective than Vit C. This result elucidated that SL prevented significantly by hydrogen peroxide-induced cell death in PC12 cells, suggesting that SL may be useful antioxidant.

References

1. Baek, N.I., Jeon, S.G., Ahn, E.M., et al. Anticonvulsant compounds from the wood of Caesalpinia sappan L. Arch Pharm Res 23, 344-348, 2000.
2. Moon, C.K., Yun, Y.P., Lee, J.H., Wagner, H., Shin, Y.S.,Inhibition of lens-aldose reductase activity by brazilin and haematoxylin. Planta Med 51, 66-67, 1985.
3. Xie, Y.W., Ming, D.S., Xu, H.X., Dong, H., But, P.P., Vasorelaxing effects of Caesalpinia sappan involvement of endogenous nitric oxide. Life Sci 67, 1913-1918, 2000.
4. Nagai, M., Nagumo, S., Eguchi, I., Lee, S.M., Suzuki, T., Sappanchalcone from Caesalpinia sappan L., the proposed biosynthetic precursor of brazilin. Yakugaku Zasshi 104, 935-938, 1984.
5. Oh, S.R., Kim, D.S., Lee, I.S., Jung, K.Y., Lee, J.J., Lee, H.K.,Anticomplementary activity of constituents from the heartwood of Caesalpinia sappan. Planta Med 64, 456-458, 1998.
6. Hu, C.M., Kang, J.J., Lee, C.C., Li, C.H., Liao, J.W., Cheng, Y.W., Induction of vasorelaxation through activation of nitric oxide synthase in endothelial cells by brazilin. Eur J Pharmacol 468, 37-45, 2003.
7. Puchtler, H., Meloan, S.N., Waldrop, F.S.,Application of current chemical concepts to metal-hematein and -brazilein stains. Histochemistry 85, 353-364, 1986.
8. Moon, C.K., Park, K.S., Kim, S.G., Won, H.S., Chung, J.H., Brazilin protects cultured rat hepatocytes from BrCCl3-induced toxicity. Drug Chem Toxicol 15, 81-91, 1992.

9. Kim, S.G., Kim, Y.M., Khil, L.Y., et al. Brazilin inhibits activities of protein kinase C and insulin receptor serine kinase in rat liver. Arch Pharm Res 21, 140-146, 1998.
10. Choi, S.Y., Yang, K.M., Jeon, S.D., et al. Brazilin modulates immune function mainly by augmenting T cell activity in halothane administered mice. Planta Med 63, 405-408, 1997.
11. Mayo, J.C., Tan, D.X., Sainz, R.M., Natarajan, M., Lopez-Burillo, S., Reiter, R.J., Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: comparative effects of melatonin and other antioxidants. Biochim Biophys Acta 1620, 139-150, 2003.
12. Simonian, N.A., Coyle, J.T.,Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 36, 83-106, 1996.
13. Stadtman, E.R.,Protein oxidation in aging and age-related diseases. Ann N Y Acad Sci 928, 22-38, 2001.
14. Berlett, B.S., Stadtman, E.R.,Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 272, 20313-20316, 1997.
15. Aruoma, O.I., Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutat Res 523-524, 9-20, 2003.