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Full Length Research Paper

Antioxidative activity of taheebo (*Tabebuia impetiginosa* Martius ex DC.) extracts on the H_2O_2-induced NIH3T3 cells

Sung-Eun Lee[1], Byeoung-Soo Park[2] and Jong Hwa Yum[3]*

[1]School of Applied Biosciences, Kyungpook National University, Daegu 702-701, Republic of Korea.
[2]Research Station, Nanotoxtech Inc., Gyeonggi Technopark Technology Development Center, Ansan 426-901, Korea.
[3]Department of Clinical Laboratory Science, Dong-eui University, Busan 614-714, Republic of Korea.

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The antioxidant activities of *Tabebuia impetiginosa* methanolic extract (TIME) were examined with its serial solvent extracts using hexane, chloroform and ethyl acetate against hydrogen peroxide (H_2O_2)-induced oxidative stress in NIH3T3 cells. The three serial extracts were selected for the study of regeneration on antioxidant enzyme activities because butanol and water extracts significantly affected cell survival. Treatment of hydrogen peroxide on the cells showed a dramatic repression on superoxide dismutase (SOD) and cytosolic $NADPH^+$-dependent isocitrate dehydrogenase (IDPc) activities with the remaining activities of 56.1 and 37.5%, respectively. The three extracts significantly regenerated SOD activity with the range of 103 to 178% when compared to the control, and IDPc activity with the range of 34.4 to 42.2%. The three extracts also regenerated catalase and glucose-6-phosphate dehydrogenase activities with the range of 91.6 to 139% in comparison to the control. Hydrogen peroxide did not change intracellular glutathione content. The three serial extracts of TIME enhanced intracellular glutathione concentration, protected proteins from the oxidative attack by H_2O_2 and also decreased malonaldehyde formation in the cells. Taken together, the non-polar extracts of TIME protect NIH3T3 cells from the H_2O_2-induced oxidative stress.

Key words: *Tabebuia impetiginosa*, NIH3T3, antioxidant activity, hydrogen peroxide (H_2O_2), lipid peroxidation.

INTRODUCTION

For the living organisms, an imbalance of redox state between the production of reactive oxygen species (ROS) and removal of the ROS is referred to as oxidative stress and thus a biological system induces its ability to detoxify the reactive intermediates and to prevent or repair the resulting damage to cells. There is growing evidence that ROS are directly or indirectly involved with a variety of chronic diseases (Gaulton and Markmann, 1988). For instance, hydrogen peroxide (H_2O_2, a well-known oxidant) is generated *in vivo* by the dismutation of superoxide radicals via superoxide dismutase enzymes. On the other hand, hydrogen peroxide is primarily produced by a range of oxidases as well as via the peroxisomal pathway for beta-oxidation of fatty acids in living organisms. Interestingly, most biological systems have shown their peculiar adaptation to recover from a variety of oxidative stress with the development of an effective and complicated network for defense mechanisms, to efficiently handle the harmful oxidative environments (Ames et al., 1993; Fridovich, 1978; Lundberg and Weitzberg, 2010). Generally, these defense mechanisms include non-enzymatic and enzymatic defenses. The non-enzymatic systems include reduced glutathione (GSH), ascorbic acid, α-tocopherol, uric acid, and small peptide thioredoxin, while enzymatic defenses include superoxide dismutases (SOD), catalases (CAT), and peroxidases (Chance et al., 1979; McCord and Fridovich, 1969).

*Corresponding author. E-mail: auxotype@gmail.com.

Tabebuia spp. (Bignoniaceae) is native to tropical rain forests throughout Central and South America. The herbal products obtained from the bark of tabebuia trees are called "taheebo", "lapacho", "pau d'arco", and "ipe roxo" (Jones, 1995). Many studies on the biological and pharmacological effects of *Tabebuia* spp. extracts have been documented as anti-inflammatory effect (Byeon et al., 2008), anti-cancer activity (Rao and Kingston, 1982; Udea et al., 1994), anti-platelet activity and inhibitory effect on the proliferation of vascular smooth muscle cells (Son et al., 2006), antibacterial activity against *Helicobacter pylori* (Park et al., 2006), and selective growth-inhibiting effects on human intestinal bacteria (Park et al., 2005). In addition, beta-lapachone presented primarily in *Tabebuia* spp. extracts has been known as an anti-cancer compound on human prostate carcinoma and bladder cancer cells (Lee et al., 2006, 2005).

The purpose of this study was to assess the antioxidant effects of *Tabebuia impetiginosa* methanolic extract (TIME) as the regeneration of non-enzymatic and enzymatic defense systems on oxidative stress induced by hydrogen peroxide (H_2O_2) using NIH3T3 cells.

MATERIALS AND METHODS

Chemical

Hydrogen peroxide, menadione, NADPH, β-$NADP^+$, glutathione disulphide (GSSG), glutathione (GSH), pyrogallol, isocitrate, glucose-6-phosphate, glutathione reductase (GSR), *tert*-butyl hydroperoxide, 2-thiobarbituric acid, glucose-6-phosphate dehydrogenase (G6PD), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), pronase, RNase A, phenazine ethosulfate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide-thiazolyl blue (MTT), and 5-sulfosalicyclic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, ammonium persulfate, N,N,N',N'-tetramethylenediamide, riboflavin, and nitroblue tetrazolium (NBT) were from Bio-Rad (Hercules, CA). 2',7'-Dichlorofluoroscin diacetate (DCFHDA) was purchased from Molecular Probes (Eugene, OR). Other chemicals were of analytical grade.

Plant material extraction and fractionations

The dried inner bark of *T. impetiginosa* Mart. ex DC was purchased from Frontier (Norway, IA). The dried inner bark of *T. impetiginosa* Mart. ex DC (3.0 kg) was extracted two times with methanol (25 L) at room temperature for 2 days and filtered. The resultant extract was combined and concentrated under reduced pressure at 40°C to yield about 12.13% (based on the weight of the dried inner bark). The methanol (50 g) extract was sequentially partitioned into hexane (9.9 g), chloroform (4.6 g), ethyl acetate (6.4 g), butanol fractions (13.4 g), and water-soluble (15.7 g) portions for bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation at 40°C, while the water portion was freeze-dried.

Cell cultures and cytotoxicity assay

The NIH3T3 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT) and 10 µg/ml gentamycin at 37°C in an incubator at 5% carbon dioxide (CO_2). Cells were first grown on a 96-well plate at a density of 5×10^3 cells/well overnight before treatment. After overnight culture, the concentrations ranging from 0 to 2 mg/ml were used to study cytotoxic test of TIME and 0.5 mM of hydrogen peroxide was applied to the cells in 100 µl of DMEM/10%FBS, and cells were incubated for additional 48 h at 37°C. After 48 h of sample treatment to cells, 10 µL of water soluble tetrazolium salts (WST-1) was added and incubated for 2 h at 37°C. The absorbance of the resulting formazan product converted by the viable cells was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 450 nm with a 620 nm reference. Cell viability was expressed as a percentage of the absorbance seen in the untreated control cells (Mosmann, 1983).

Enzyme assays

Cells were collected at 10,000 g for 10 min at 4°C and were washed once with cold phosphate buffered saline (PBS). Briefly, cells were homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose, 10 mM Tris–HCl, pH 7.4). Cell homogenates were centrifuged at 1000 g for 5 min, and the supernatants were further centrifuged at 15,000 *g* for 30 min. The resulting supernatants were used as the cytosolic fractions. Protein concentration was determined by the method of Bradford using the reagents purchased from Bio-Rad. The activity of IDPc was measured by the production of NADPH at 340 nm (Loverde and Lehrer, 1973). The reaction mixture for IDPc activity contained 50 mM morpholinepropanesulfonic acid (MOPS), pH 7.2, 5 mM threo-ds-isocitrate, 35.5 mM triethanolamine, 2 mM $NADP^+$, 2 mM $MgCl_2$, and 1 µg/ml rotenone. One unit of IDPc activity is defined as the amount of enzyme catalyzing the production of 1 µmol of NADPH/min.

Catalase activity was measured with the decomposition of hydrogen peroxide, which was determined by the decrease in absorbance at 240 nm (Beers and Sizer, 1952). SOD activity in cell extracts was assayed spectrophotometrically using a pyrogallol assay (Marklund and Marklund, 1974), where one unit of activity is defined as the quantity of enzyme that reduces the superoxide-dependent color change by 50%. G6PD activity was measured by following the rate of $NADP^+$ reduction at 340 nm using the procedure described by Stanton and Seifter (1988).

Measurement of intracellular glutathione

GSSG was measured by the DTNB-GSSG reductase recycling assay after treating GSH with 2-vinylpyridine (Anderson, 1985). The total GSH level was measured in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mg NADPH, 30 µg DTNB, and 0.12 unit glutathione reductase. GSSG level was measured by the same method as the total GSH level, but after treatment of 1 µl of 2-vinylpyridine and 3 µL of triethanolamine for 1 h.

Measurement of intracellular ROS

Hydrogen peroxide oxidizes ferrous (Fe^{2+}) to ferric ion (Fe^{3+}) selectively in dilute acid and the resulting ferric ions can be determined using a ferric sensitive dye and xylenol orange as an indirect measure of intracellular hydrogen peroxide concentration. Cell extractions were added FOX solution (0.1 mM xylenol orange, 2.5 mM ammonium ferrous sulfate, 100 mM sorbitol and 25 mM H_2SO_4) and incubated in a room temperature for 30 min, and absorbance was measured at 560 nm. Hydrogen peroxide was used to draw standard curve as described Jiang et al. (1992).

Table 1. Effect of the various fractions obtained from methanol extract of the dried inner bark of *T. impetiginosa* on NIH3T3 cell viability after treatment of 0.5 mM H_2O_2.

Doses (mg/ml)	Cell survival (%)				
	Hexane ext.	Chloroform ext.	Ethyl acetate Ext.	Butanol ext.	Water ext.
0.125	3.0 ± 0.5	10.0 ± 1.5	18.4 ± 3.0	8.0 ± 0.6	2.1 ± 0.3
0.25	10.5 ± 1.4	62.1 ± 3.3	12.5 ± 1.8	1.1 ± 0.3	1.2 ± 0.1
0.5	33.2 ± 0.8	91.4 ± 6.2	93.6 ± 7.8	3.4 ± 0.2	3.6 ± 0.2
1.0	103.0 ± 4.5	18.3 ± 2.1	12.4 ± 2.8	12.8 ± 0.9	11.7 ± 0.8
2.0	42.2 ± 2.1	2.0 ± 0.1	21.4 ± 1.4	20.7 ± 2.1	14.6 ± 2.5

The cells (1×10^5) grown on 35 mm plates were treated with various concentrations of fraction samples and 0.5 mM H_2O_2. The treated cells were incubated for 24 h prior to the measurement of cell viability. Each value represents the mean ± SE from three independent experiments.

Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFHDA with fluorescent microscopy (Schwarz et al., 1994). Cells were grown at 3×10^4 cells per 8-well chamber slide glass and maintained in the growth medium for 24 h. Cells were treated with 10 µM DCFHDA for 15 min. Cells on the slide glass were washed with PBS and a cover glass was put on the slide glass. DCF fluorescence (excitation, 488 nm; emission, 520 nm) was imaged on a fluorescent microscope (Axioplan 2, Carl Zeiss, Thornwood, NY).

Lipid peroxidation

The production of MDA was measured by a spectrophotometric assay (Buege and Aust, 1976). Cells (2×10^6) were either untreated or treated with various concentrate of samples and 0.5 mM H_2O_2 for 24 h. For lipid peroxidation tests, different concentration of each sample was used as hexane extract (1 mg/ml), chloroform extract (0.5 mg/ml) and ethyl acetate extract (0.5 mg/ml). Then cell extracts (500 µL) were mixed with 1 ml of thiobarbituric acid-trichloroacetic acid-HCl solution (0.375% thiobarbituric acid, trichloroacetic acid in 0.25 N HCl, pH 2.0) and heated at 100°C for 15 min. The absorbance of thiobarbituric acid-reactive substance (TBARS) was determined at 535 nm.

RESULTS

Cytotoxity

To evaluate the cytotoxicity against cell damage induced by H_2O_2, cells were exposed to H_2O_2 and then treated with the solvent serial fractions of TIME, whereas control cells were treated with H_2O_2 in the presence and the cells were incubated of vehicle only. The final concentration of DMSO was below 0.1% throughout this study, and DMSO had no cytotoxicity at the concentration. Relative cell survival was determined 24 h later by the WST assay. Among the treatment with the five fractions obtained from TIME, hexane, chloroform, and ethyl acetate fractions induced protective cell survivals in H_2O_2-treated cells. The cell damage protecting effects of hexane fraction at concentration of 0.25, 0.5, and 1 mg/ml increased to 10, 33, and 103%, respectively. In the chloroform fraction, cell survival effect at the dose of 0.125, 0.25, and 0.5 mg/ml increased in a dose-dependent fashion to 10, 62, and 91%, respectively. According to the result of treatment with ethyl acetate fraction, they have a significantly protecting effect only at the 0.5 mg/ml about 94%. However, the butanol and water fractions did not show any potent effects on the tested concentration. The overall description of cytotoxicity of the serial extracts of TIME is presented in Table 1.

Effect of the taheebo extracts on SOD, CAT, G6PD, and IDPc

Table 2 shows the antioxidant activities of various concentrations (0.25, 0.5, and 1 mg/ml) of three fractions on the following four enzymes activities; CAT, G6PDH, SOD, and IDPc on induced oxidative stress by 0.5 mM H_2O_2 in NIH3T3 cells. For the restoration of CAT activity, treatment with the hexane fraction at doses of 0.5 and 1 mg/ml induced 18 and 30%, respectively, when compared to the attacked CAT by H_2O_2. Chloroform and ethyl acetate fractions at 0.25 mg/ml improved 16 and 2% of CAT activity, respectively. At the concentration of 0.5 mg/ml, chloroform and ethyl acetate fractions improved 26 and 27% of in CAT levels, respectively. G6PDH activities showed a dose-dependent manner treated with three different solvent fractions at doses of 0.25, 0.5, and 1 mg/ml. At concentration of 0.5 and 1 mg/ml, the hexane fraction increased G6PDH activity by 18 and 56%, respectively, when compared to the attacked G6PDH. Treatment with chloroform and ethyl acetate fraction at 0.25 and 0.5 mg/ml enhanced G6PDH activity.

A dramatic regeneration was found in SOD activity as shown in Table 2. SOD was the enzyme strongly attacked by H_2O_2 during the study, as the remaining activity was only 56.1% in comparison to the control. The three serial extracts of TIME showed potent restoration activity on the SOD activity in the range of 103 to 178% in comparison to the control. IDPc activity strongly lost its activity by the oxidative attack and was not changed by the addition of TIME solvent fractions after H_2O_2

Table 2. Regeneration of antioxidant enzyme activities by the treatment of the three serial extracts of *Tabebuia impetiginosa* methanolic extract on H_2O_2- induced oxidative stress in NIH3T3 cells.

Treatments	Conc. (mg/ml)	Antioxidant enzymes*			
		CAT (%)	G6PDH	SOD	IDPc
Control	-	10.10 ± 0.27^{a} (100)**	0.063 ± 0.0016^{a} (100)	2.44 ± 0.023^{a} (100)	0.064 ± 0.001^{a} (100)
HP 0.5 mM	-	8.86 ± 0.16^{b} (87.7)	0.055 ± 0.0015^{b} (87.3)	1.37 ± 0.026^{b} (56.1)	0.024 ± 0.001^{b} (37.5)
HP / hexane extract	0.5	10.45 ± 0.20^{a} (103)	0.065 ± 0.0014^{a} (103)	3.06 ± 0.043^{c} (125)	-
	1	11.47 ± 0.20^{c} (114)	0.086 ± 0.0010^{c} (139)	3.84 ± 0.076^{d} (157)	0.027 ± 0.001^{b} (42.2)
HP / $CHCl_3$ extract	0.25	10.30 ± 0.24^{a} (102)	0.057 ± 0.0017^{b} (90.4)	4.10 ± 0.059^{e} (168)	-
	0.5	11.20 ± 0.30^{c} (111)	0.072 ± 0.0020^{d} (114)	4.35 ± 0.088^{f} (178)	0.022 ± 0.001^{b} (34.4)
HP / EtOAc extract	0.25	9.25 ± 0.10^{d} (91.6)	0.067 ± 0.0004^{a} (106)	3.90 ± 0.039^{d} (159)	-
	0.5	11.28 ± 0.11^{c} (112)	0.069 ± 0.0015^{a} (110)	2.51 ± 0.035^{g} (103)	0.024 ± 0.003^{b} (37.5)

*Enzyme activity represents units/mg protein. Results represent the means ± S.D calculated from three independent determinations. HP, H_2O_2; CAT, catalase; G6PDH, glucose 6-phosphate dehydrogenase; SOD, superoxide dismutase; IDPc, cytosolic $NADP^+$-dependent isocitrate dehydrogenase. Means in the column followed by the same letter are not significantly different (P<0.05). **Brackets indicate the remaining enzyme activity in comparison to the control.

treatment.

Measurement of intracellular glutathione

To examine intracellular GSH generation against oxidative stress, the cellular levels of GSH in the TIME solvent-spiked cells were determined (Figure 1). NADPH is required for GSH regeneration by glutathione reductase; therefore, it is essential in the cellular defense systems against oxidative damage. The ratio for cellular [GSSG]/[GSSG + GSH] in control cells was 0.0905 ± 0.006, whereas that of the cells on H_2O_2-induced oxidative damage was 0.0895 ± 0.006 (mean ± S.D., n = 3). As the cells were exposed to H_2O_2, the values of cellular [GSSG]/[GSSG + GSH] were significantly changed with the addition of the TIME solvent fractions. The hexane, chloroform, and ethyl acetate fractions showed values of cellular [GSSG]/[GSSG + GSH] as 0.0716 ± 0.001, 0.0782 ± 0.0006, and 0.0755 ± 0.002, respectively. This data indicates that GSSG might be reduced and GSH concentration might increase after the application of antioxidant fractions on the H_2O_2-treated cells. These results strongly suggest that the decrease in the efficiency of GSH recycling may be responsible for scavenging intracellular peroxides and the pronounced oxidative damage in cells in response to 0.5 mM H_2O_2 treatment.

Measurement of intracellular ROS

To investigate the role of samples in cellular defense against H_2O_2-induced cell damage, we determined the level of intracellular peroxide in cells after treatment with the three fractions obtained from TIME (Figure 2). In the cells treated with H_2O_2, the peroxide level increased by 42% compared with the control cell. However, it increased by 14, 10, and 7% with the addition of hexane, chloroform, and ethyl acetate fractions of TIME at the concentrations of 1, 0.5, and 0.5 mg/ml, respectively. The effect of the samples on ROS production was demonstrated further by the relative intensity of DCF (Bass et al., 1983). After treatment with 0.5 mM of H_2O_2, DCF fluorescence intensity was compared and it was stronger in the H_2O_2-induced cells than in the control cells. However, it was dramatically reduced in the three fractions obtained from TIME, leading to reduce the oxidative damage in cells after the treatment of 0.5mM H_2O_2.

Inhibitory effect on lipid peroxidation

We evaluated cellular antioxidant activity of three fractions obtained from TIME to inhibitory effects against TBARS production in H_2O_2-induced NIH3T3 cells (Figure 3). In the H_2O_2-treated cells, the TBARS level increased by 39% compared with that in control cells. However, hexane fraction at the concentration of 1 mg/ml significantly decreased the MDA level after H_2O_2 treatment. Chloroform fraction also showed a significant reduction of lipid peroxidation generation at the concentration of 0.5 mg/ml. The strongest inhibitory activity on lipid peroxidation mediated by H_2O_2 treatment was found on the ethyl acetate fraction with about 35% reduction of MDA generation.

DISCUSSION

Many studies on *Tabebuia* natural products has been focused on anti-inflammatory effects because novel compounds and the *Tabebuia* constituents showed potent

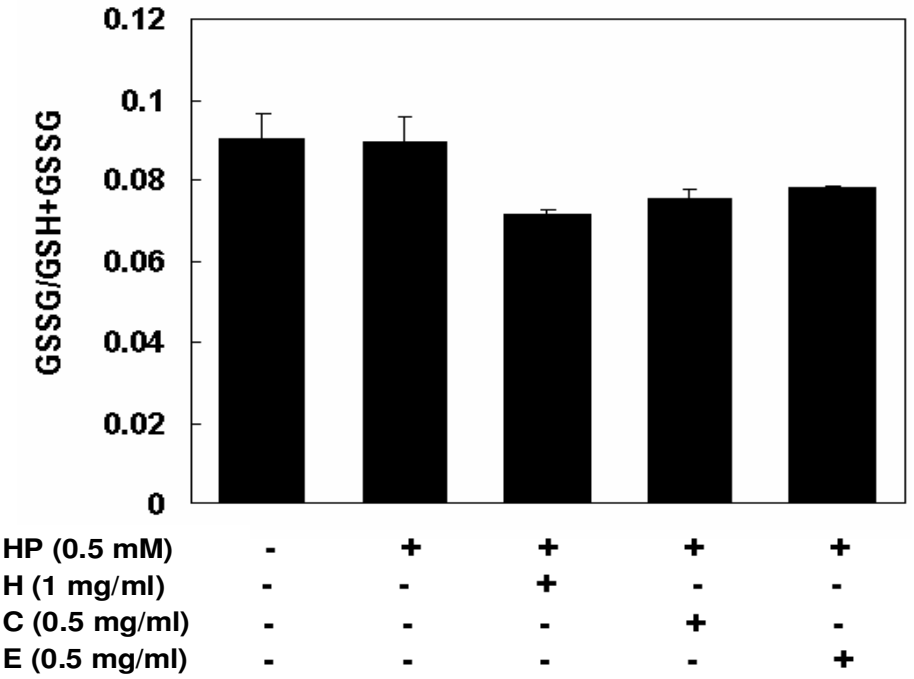

Figure 1. Effects of the three fractions obtained from methanol extract of the dried inner bark of *T. impetiginosa* on the level of intracellular glutathione. Each value represents the mean ± SE from three independent experiments. HP, Hydrogen peroxide; H, hexane extract; C, chloroform extract; E, ethyl acetate extract.

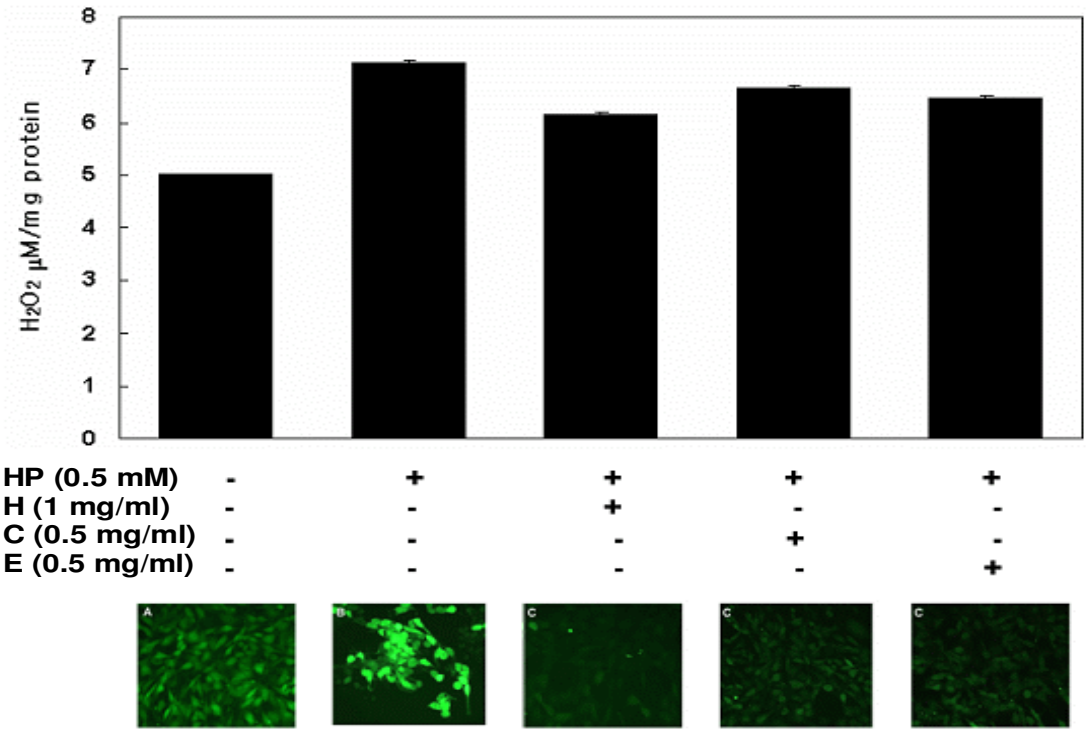

Figure 2. Measurement of *in vivo* molecular oxidation by DCF fluorescence. Subcultures of NIH3T3 cells were grown on poly-L-lysine coated glasses, loaded with H_2-DCF, and then exposed to either 0.5 mM H_2O_2 for 5 min. The pseudocolor images of DCF fluorescence by ROS are analyzed with the confocal microscopy. HP, Hydrogen peroxide; H, hexane extract; C, chloroform extract; E, ethyl acetate extract.

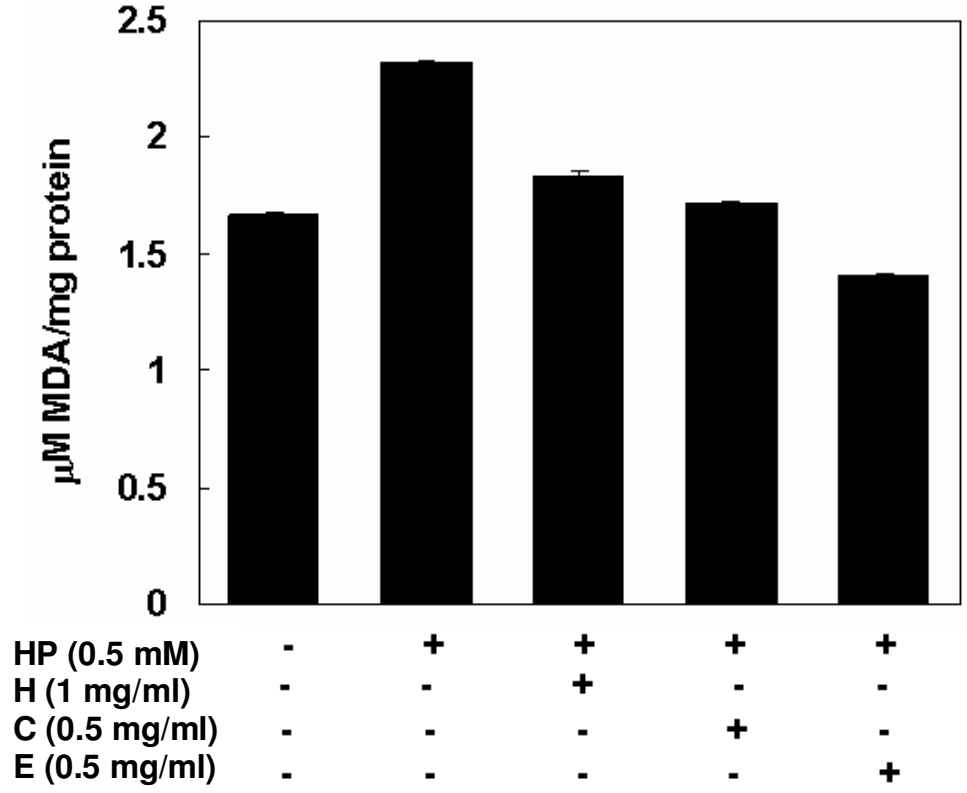

Figure 3. The production of TBARS in cells determined in triplicates. The level of TBARS accumulated in the cells treated with 0.5 mM H_2O_2, respectively. Each value represents the mean ± SD from three separate experiments. HP, Hydrogen peroxide; H, hexane extract; C, chloroform extract; E, ethyl acetate extract.

inhibitory effect on the production of nitric oxide and prostaglandin E2 (Awale et al., 2005; Byeon et al., 2008). Additionally, other pharmacological properties including anti-ulcerogenic activity and anticarcinogenic activity have been reported (Moon et al., 2010; Twardowschy et al., 2008). However, there are only a few studies concerning on the antioxidant properties of *Tabebuia* constituents. In relation to antioxidant activity of *T. impetiginosa*, our previous study showed that volatiles isolated from the dried inner bark of *T. impetiginosa* using steam distillation exhibited a potent inhibitory effect on the formation of conjugated diene hydroperoxides (from methyl linoleate) at a concentration of 1000 μg/ml. The extract also inhibited the oxidation of hexanal for 40 days at the level of 5 μg/ml. The major volatile constituents of *T. impetiginosa* were 4-methoxybenzaldehyde (52.84 μg/g), 4-methoxyphenol (38.91 μg/g), 5-allyl-1,2,3-trimethoxybenzene (elemicin; 34.15 μg/g), 1-methoxy-4-(1E)-1-propenylbenzene (trans-anethole; 33.75 μg/g), and 4-methoxybenzyl alcohol (30.29 μg/g) (Park et al., 2003).

As shown in Table 2, four solvent extraction of TIME was conducted and the butanol and water extracts significantly affected the cell survival. Therefore, those two serial extracts were not considered in this study. In the present study the three serial extracts of TIME showed potent regeneration activity on superoxide dismutase (SOD) (Table 1). Among the antioxidant enzyme activities determined in this study, SOD with the remaining activity of 56.1% when compared to the control, was the enzyme strongly depressed by H_2O_2 with its oxidation behavior. cytosolic NADPH+-dependent isocitrate dehydrogenase (IDPc) was another enzyme that lost its activity by H_2O_2 attack. Interestingly, the addition of the three serial extracts of TIME contributed to the regeneration of SOD activity and the enhancement of the SOD activity was significant as shown in Table 1. However, the findings were not applied to the IDPc enzyme. Therefore, the non-polar extraction of TIME may contain constituents having a specific ability to protect SOD from H_2O_2 attack. The three serial extracts also possessed the regeneration activity on catalase and glucose-6-phosphate dehydrogenase as shown in Table 1. However, the level of regeneration for the two enzymes was not as much as SOD case.

Many researchers have reported that antioxidants induce expression of antioxidant enzyme referring on elevated enzyme activity. Recently, a study with butin to verify its antioxidant property for elucidating cyto-protective effect against H_2O_2-induced cell damage

showed intracellular reactive oxygen species (ROS) scavenging, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, inhibition of lipid peroxidation, and DNA damage. In addition, butin restored the activity and protein expression of SOD and CAT in H_2O_2-treated cells (Zhang et al., 2008). Similarly, salvianolic acid B (SalB) isolated from *Radix Salviae miltiorrhizae* showed neuroprotective effects against H_2O_2-induced rat pheochromocytoma line PC12 injury. Following exposure of cells to H_2O_2 at the concentration of 150 µM, a dramatic decrease in cell survival and activities of SOD, CAT and glutathione peroxidase (GSH-Px), as well as increased levels of MDA production and lactate dehydrogenase (LDH) release were observed. However, addition of SalB into the H_2O_2-induced rat pheochromocytoma line PC12 cells blocked these H_2O_2-induced cellular events noticeably (Kang et al., 2007).

In addition to these results, the protective effect of *Castanopsis cuspidata* on H_2O_2-induced cell damage has been reported. The ethanol extract of *C. cuspidate* was found to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and reduce intracellular ROS generation, and thus prevent lipid peroxidation and cellular DNA damage induced by H_2O_2. Castanopsis extract has been also found to increase CAT activity and its protein expression (Kang et al., 2007). Therefore, our findings as regards the regeneration of SOD activity with the addition of the serial extracts of TIME in the H_2O_2-treated NIH3T3 cells were similar to the previous results and may protect the cells from the cytotoxicity mediated by H_2O_2. Figure 1 shows the recovery of intracellular glutathione concentration in the cells as observed in this study. As shown in the figure, the intracellular glutathione was not dramatically changed by the H_2O_2-induced oxidative attack. However, the three serial extracts of TIME contributed to the change in intracellular glutathione concentration in the cells. Furthermore, the effect of the formation of ROS on proteins is shown in Figure 2. DCF fluorescence intensity showed that H_2O_2 changed the aggregation of proteins. However, the addition of the three serial extracts of TIME enhanced protein distribution as found in the control group. It is likely that the three serial extracts of TIME may contribute to the regeneration or protection of oxidized proteins by H_2O_2 treatment in the cells. Another evidence of antioxidative activities of the three serial extracts was the decrease of malonaldehyde production in the cells as shown in Figure 3. It is observed from the figure that the extracts protected the cells from the lipid peroxidation by the peroxide. Therefore, these antioxidant properties of TIME prevented lipid peroxidation and protein oxidation by reduction of MDA and ROS production.

Conclusively, the non-polar extracts of taheebo possess antioxidant activities with the regeneration of SOD activity and the reduction of ROS and MDA production in NIH3T3 cells. Further studies may be focused on the isolation and structural determination of the active constituents from the extracts.

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