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Ethanol Eluted Extract of *Rhus verniciflua* Stokes Showed both Antioxidant and Cytotoxic Effects on Mouse Thymocytes Depending on the Dose and Time of the Treatment

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For a long time *Rhus verniciflua* Stokes (RVS) has traditionally been used as a herbal plant. It is known to contain various biological activities. Previously, a crude ethanol extract from RVS was reported to have antioxidant effects, and antiproliferative activities, on human cancer cell lines. In this report, we prepared a highly purified ethanol extract from RVS, which did not contain the urushiol derivatives, named REEE-1 (*Rhus* ethanol eluted extract-1), to investigate the mechanisms of the scavenging activity of hydroxyl radicals using mouse thymocytes. The results from the deoxyribose, DNA nicking, and glucose/glucose oxidase enzyme assays showed that REEE-1 contained a strong scavenging activity of oxygen free radicals, especially of hydroxyl radicals. However, interestingly, REEE-1 also showed cytotoxicity against the thymocytes, although the effect was variable, depending on the concentrations and times of treatment. The REEE-1-mediated cytotoxicity against thymocytes, which has been used as one of the well-characterized models for apoptosis studies, was verified to be apoptotic. This was proven by the following: the appearance of DNA laddering, increases in DNA fragmentation, low fluorescence intensity in the nuclei after propidium iodide staining, and positive Annexin V staining of the cells. These results suggested that REEE-1 had both antioxidative activity and cytotoxicity against the thymocytes, although the effect of the cytotoxicity was variable, depending on the dose and time of the treatment.

Keywords: Antioxidant, Apoptosis, Cytotoxicity, *Rhus verniciflua* Stokes

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Introduction

Oxidative stress is known to be implicated in the initiation and progression of many degenerative diseases, including atherosclerosis, diabetes, dysfunction of immune systems, and cancer (Wiseman and Halliwell, 1996; von Harsdorf *et al.*, 1999; Lee *et al.*, 1999c). Therefore, it is plausible that the alleviation of oxidative stress could be applied to decrease the incidence, or progression, of oxidation-associated diseases. Consequently, several approaches have been performed to diminish oxidative stress in order to treat oxidation-associated diseases, especially with the use of natural antioxidants. These approaches include the identification of the biological effects of some extracts from plants, fruits, and even vegetables, on the specific activity of the enzymes, such as superoxide dismutase, catalase, and glutathione transferase. Most of these enzymes are known as critical in protecting cells from endogenous and exogenous oxidative stresses (Thompson *et al.*, 1983; Slater and Orrenius, 1995; Sen and Packer, 1996; Lee *et al.*, 1999b; Miranda *et al.*, 1999; Chasi *et al.*, 2000; Fiander and Schneider, 2000; Park *et al.*, 2000).

Rhus verniciflua Stokes (RVS) is traditionally employed for both the preservation of antique furniture, as well as for herbal medicine in Korea (Jung, 1998; Hong *et al.*, 1999). Recently, the various biological activities of RVS were reported by a number of investigators. For example, RVS were characterized to contain an inhibitory effect on the proliferation of tumor cells (Kim *et al.*, 1997; Lee, 2000). Additionally, the crude ethanol extract from RVS was shown to have an antioxidant effect against hydroxyl radicals, the antiproliferative activity in human cancer cell lines, and stimulating activity for the activity of cell-associated detoxifying enzymes in hepatocytes. This occurred even though the direct uptake of RVS could possibly evoke cytotoxic activity, and/or extensive damage to the kidney, liver, and cells of the immune system (Lim and Shim, 1997; Lee *et al.*, 1999a; Lim *et al.*, 2000). Hence, a clear elucidation of the

function of ethanol extract from RVS on the scavenging activity of hydroxyl radicals, and on cytotoxicity, is considered to be an interesting research objective.

In this study, we prepared a highly purified ethanol extract from RVS, without the derivatives of urushiol. This is the most extensively studied chemical compound of *Rhus* for its toxic activity (Jung, 1998; Hong *et al.*, 1999). It is named REEE-1 (*Rhus* ethanol eluted extract-1). In addition, the scavenging ability of REEE-1 of hydroxyl radicals, and its cytotoxicity against mouse thymocytes, was investigated, since the thymocytes (prepared without the proteolytic enzyme treatment) were known as one of the most well-characterized model systems for apoptosis study (Oyama *et al.*, 1999). REEE-1 showed that there was a strong scavenging activity of oxygen free radicals. This was proven by the results from several assay systems for antioxidant activity. Also, REEE-1 showed the cytotoxicity against the thymocytes, although the effect was variable depending on the concentrations and times of treatment. The meaning of these results, and the mechanisms involved in REEE-1-mediated cytotoxicity against thymocytes, are discussed.

Materials and Methods

Chemicals and mice Unless otherwise specified, the chemicals and plastics used in this study were purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively. Four to six-weeks old inbred BALB/c mice were purchased from Damul Science (Yoosung, Korea).

Preparation of REEE-1 The preparation of the crude ethanol extract from RVS was performed as described previously (Lee *et al.*, 1999a). Briefly, 5 g of crude ethanol extract, obtained from RVS, was initially eluted through a distilled water-saturated silica gel column (2.5 × 60 cm, 22Å, 28-200 mesh) with distilled water and then with absolute ethanol. The ethanol-eluted extract was lyophilized, then further purified through HPLC (Waters model 2690 with Waters 996 photodiode array detector) that was equipped with a reverse-phase column (uBondapak™ C18 125Å 10 μm, 3.9 × 300 mm) using a gradient of acetonitrile in distilled water. The ethanol-eluted fractions were collected and lyophilized to a final amount of 100 mg, which was 2% of the initial amount, and named REEE-1. The quality and purity of the REEE-1 was evaluated using EI-MS and NMR spectra, which were serviced by the Korea Basic Science Institute. It showed that REEE-1 did not contain urushiols. Detailed information for the chemical composition of the REEE-1 will be reported elsewhere. The REEE-1 was dissolved in absolute ethanol for treatment of the cells. The final ethanol concentration did not exceed 0.5% (v/v) throughout the experiments.

Cell cultures Thymocytes were prepared from thymus glands, which were cut out and teased gently with a sterile rubber policeman in PBS (pH 7.4) that contained 5% FBS (HyClone, Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were then resuspended by pipetting with a RPMI 1640 medium that was supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% FBS, then collected at 1,000 × g for 10 min. One million cells were resuspended into either 2 ml, or 100 μl media for spreading onto either 35 mm culture dishes, or 96-well flat bottom plates, respectively. The culture media were changed with the RPMI 1640 that was supplemented with 0.5% FBS at 24 h before the REEE-1 treatment.

Antioxidative activity assay The antioxidative activity of the REEE-1 was assessed using deoxyribose, DNA nicking, and glucose/glucose oxidase (G/GO) assay systems. A deoxyribose assay was conducted following the methods of Halliwell *et al.* (1987). Briefly, varied concentrations of REEE-1 were mixed with a Haber-Weiss reaction buffer (100 μM $FeCl_3$, 104 μM EDTA, 1.5 mM H_2O_2, 2.5 mM deoxyribose, and 100 μM L-ascorbic acid with pH 7.4) to a final volume of 1.0 ml, then incubated for 1 h at 37°C. After the incubation, 1 ml of 0.5% 2-thiobarbituric acid in 0.025 M NaOH, and 1 ml of 2.8% trichloroacetic acid, were added into the mixture. The final mixture was further heated for 30 min at 80°C in a water bath. Finally, the mixture was cooled on ice, and the absorbance was measured at 532 nm using a spectrophotometer (Beckman, DU®530, Germany). The inhibitory effects on the activity of hydroxyl radicals were calculated as follows: inhibitory effect (%) = $[(OD_{control} - OD_{sample}) / OD_{control}] \times 100$.

To perform the DNA nicking assay, a supercoiled pBR322 plasmid DNA was initially prepared from the plasmid-harboring DH5a that was cultured in a LB medium. Extraction and purification of the plasmid DNA was performed using Wizard® Plus SV Minipreps (Promega, Madison, WI, USA). Plasmid DNA (0.5 μg) was added into the reaction mixture (30 mM H_2O_2, 50 μM ascorbic acid, and 80 μM $FeCl_3$) that contained the varied concentrations of REEE-1. The final volume of the mixture was brought up to 20 μl. The mixture was incubated for 30 min at 37°C. The DNA was analyzed using 1% agarose gel electrophoresis followed by ethidium bromide staining.

A G/GO assay was performed as described previously (Michikawa *et al.*, 1994). Briefly, thymocytes in 96-well plates were exposed to hydroxyl radicals that were generated by the G/GO enzyme system (0.5% D-glucose and 20 mU/ml glucose oxidase in RPMI 1640) for 4 h in the presence of REEE-1. After the incubation, 10 μl of the MTT solution (5 mg/ml in PBS) was added into each well. The cells were then further incubated for 4 h at 37°C. Finally, 70 μl of the acidic isopropanol was added into each well. The absorbance was measured at 560 nm using a SpectraCount™ ELISA reader (Packard Instrument Co., Downers Grove, IL, USA).

Measurements of DNA synthesis and cytotoxicity To measure the level of the DNA synthesis, thymocytes, which were pretreated with 5 μg/ml of concanavalin A for 24 h, were incubated with varied concentrations of REEE-1. The 1 μCi of [*methyl*-^{3}H] Thymidine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was added into each well, followed by further incubation for 4 h. Finally, the cells were collected with a cell harvester (Inotech Inc., Switzerland), and the tritium contents were measured with a liquid scintillation counter (Packard Instrument Co.).

REEE-1-mediated cytotoxicity was measured by a conventional MTT assay, as described previously. The cytotoxicity was

calculated as follows: cytotoxicity (%) = [($OD_{control}$ - OD_{sample}) / $OD_{control}$] × 100.

Apoptosis assay Influence of the REEE-1 treatment on the apoptosis of thymocytes was assessed using a DNA fragmentation assay, a flow cytometric analysis, and triple staining methods. For the DNA fragmentation assay, 2×10^6 thymocytes were incubated with 500 μl of a lysis buffer (1% NP-40, 1% SDS in 50 mM Tris-HCl, pH 8.0) for 1 h at 65°C. The DNA was extracted with phenol/chloroform/isoamyl alcohol. DNA fragmentation was analyzed using 2% agarose gel electrophoresis followed by ethidium bromide staining.

For the flow cytometric analysis, REEE-1-treated thymocytes were initially fixed overnight at 4°C with 80% ethanol. They were then stained overnight at 4°C with a propidium iodide (PI) staining mixture (250 μl of PBS, 250 μl of 1 mg/ml of RNase in 1.12% sodium citrate, and 500 μl of 50 μg/ml of PI in 1.12% sodium citrate). After the staining, the cells were analyzed with the FACS Vantage® system (Becton Dickinson, San Jose, CA, USA).

Apoptosis-mediated death of thymocytes was also examined through the triple staining method with H33258, Annexin V, and PI (Mesner, Jr. and Kaufmann, 1997). Initially, thymocytes (1×10^6 cells) were treated with 1 μg/ml of H33258 for 30 min and washed three times with ice-cold PBS. One microgram per ml of FITC-labeled Annexin V in a HEPES buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4) that contained 1 μg/ml of PI was added to the cells, and incubated for 30 min at RT. The cells were washed with PBS, cytocentrifuged at 500 rpm for 3 min, and observed with a fluorescence microscope (Carl Zeiss, Germany).

Statistical analysis All data were expressed as mean ± standard error. A one-way ANOVA using SPSS v 10.0 software was used for multiple comparisons. A value of $P<0.05$ was considered significant.

Results

Antioxidative activity of REEE-1 The antioxidative activity of REEE-1 was assessed through three different assay systems. Initially, the antioxidative activity of REEE-1 was measured by determining the level of the TBA-MDA adduct formation, as described in Materials and Methods (Table 1). As shown in the table, the hydroxyl radical-induced deoxyribose degradation was effectively inhibited by the addition of REEE-1. The level of inhibition was evaluated as 2.19% at 0.01 mg/ml, 22.62% at 0.1 mg/ml, 45.62% at 1 mg/ml, 55.29% at 2 mg/ml, and 62.23% at 3 mg/ml, respectively, compared to that of the control. These results indicated that REEE-1 contained antioxidative activity against hydroxyl radicals.

Table 1. Hydroxyl radical scavenging activities of REEE-1.

Amount of REEE-1 (mg/ml)	Optical density (A_{532}nm)	Inhibitory effect on hydroxyl radicals (%)
Control	0.548±0.046	0
0.01	0.536±0.024	2.19
0.1	0.424±0.020	22.62
1	0.298±0.014	45.62
2	0.245±0.012	55.29
3	0.207±0.019	62.23

Control contained the reaction buffer solution only. Values of the absorbance were expressed as a mean of the triplicates.

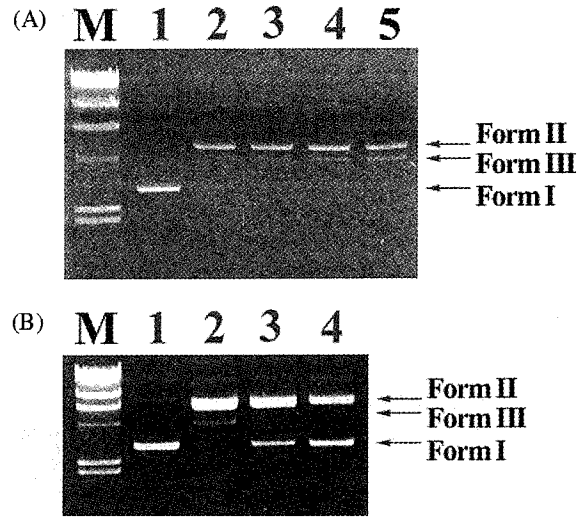

Fig. 1. Inhibitory effect of REEE-1 on DNA nicking caused by hydroxyl radicals. (A) The DNA nicking reaction was initiated by the addition of 0.5 μg of pBR322 plasmid DNA. The reaction mixture was then incubated at 37°C. Lanes 1 through 5 represent the results from the mixture incubated for 0, 5, 10, 20, and 30 min, respectively. M represents the λ/*Hin*d III DNA size marker. (B) REEE-1 was added to the reaction mixtures and incubated for 30 min at 37°C. Lanes 1 through 4 represent the results from the reaction mixtures containing 0, 5, 10, and 20 μg of REEE-1, respectively. M represents the λ/*Hin*d III DNA size marker.

In order to confirm the antioxidative activity of REEE-1, we investigated whether the REEE-1 treatment could reduce the hydroxyl radical-mediated DNA nicking by a DNA nicking assay. When the plasmid DNA was dissolved into a nicking reaction mixture, the time-dependent increases of a single-stranded nicked form of DNA, Form II, and of a double-stranded nicked and linear DNA, Form III, were observed (Fig. 1A). It was shown that hydroxyl radical-mediated DNA nicking clearly occurred within 30 min of the reaction (Fig. 1A, lanes 2-5). Interestingly, the addition of REEE-1 to the nicking reaction mixture decreased the hydroxyl radical-mediated appearance of Form II and III DNA (Fig. 1B). Especially, an addition of 20 μg of REEE-1 clearly increased the presence of Form I DNA, and mediated the complete loss of the hydroxyl radical-mediated Form III DNA formation (Fig. 1B, lane 4). However, the hydroxyl radical-mediated Form II DNA formation was not completely inhibited, but was reduced by the REEE-1 addition.

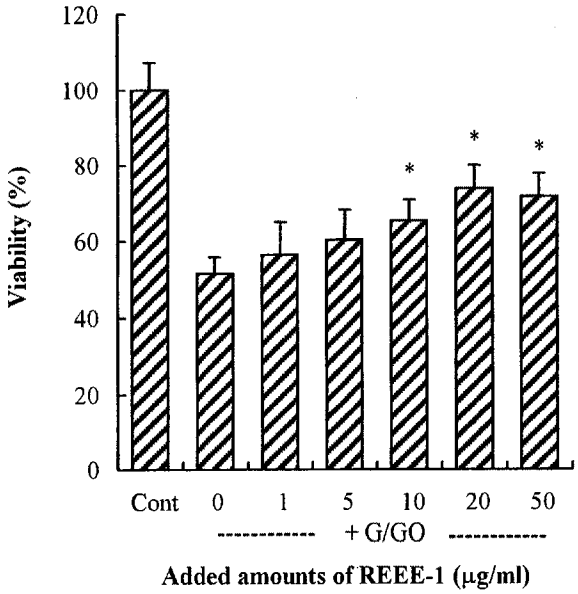

Fig. 2. Protective effect of REEE-1 on hydroxyl radical-induced cytotoxicity on thymocytes. Thymocytes were exposed to hydroxyl radicals generated from the G/GO enzyme for 4 h in the presence of REEE-1 as indicated. Each bar represents the mean ± SEM values of triplicates. The figure shows a representative result from the three separate experiments. *P values were less than 0.05, and represented the significant difference between the control and experimental treatment.

Finally, the REEE-1-mediated antioxidative activity was confirmed by measuring the protective activity of REEE-1 against hydroxyl radical-induced cell death through the G/GO assay system, as described in Materials and Methods (Fig. 2). As shown in Fig. 2, the addition of REEE-1 increased the viability of thymocytes. Especially, the addition of REEE-1 over 10 μg/ml increased significantly the viability of thymocytes (P<0.05) compared to that of the control. When the REEE-1 was added at 50 μg/ml, which was the highest REEE-1 concentration tested, the viability of the thymocytes reached up to 71.67%.

Cytotoxic effects of REEE-1 The effect of REEE-1 on DNA synthesis was determined by tritium incorporation into the DNA of thymocytes (Fig. 3). As shown in Fig. 3A, the addition of REEE-1 on thymocytes inhibited DNA synthesis in a dose-dependent manner. For example, when 5 μg/ml of REEE-1 was treated on thymocytes, which were pre-incubated with concanavalin A, the tritium uptake by the cells was 2,369±471 cpm. This represents about a 24.4% decrease from that of the untreated cells (3,135±442 cpm). Also, 50 μg/ml of the REEE-1 treatment dramatically inhibited the tritium uptake by thymocytes; the level was only 318±87 cpm. In addition, the REEE-1-mediated inhibition of DNA synthesis in thymocytes was time-dependent, as expected (Fig. 3B).

The REEE-1-mediated inhibition of DNA synthesis could be exerted in two possible ways; either by cytostatic, or the

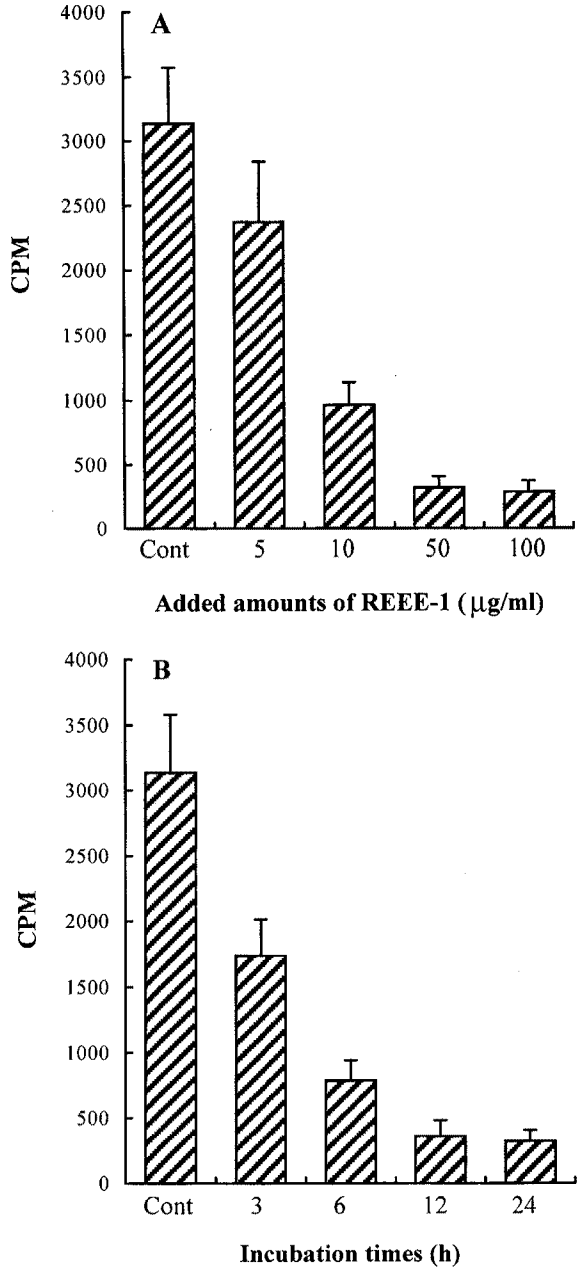

Fig. 3. Effects of REEE-1 treatment on DNA synthesis of thymocytes. (A) Thymocytes, which were preincubated with 5 μg/ml of concanavalin A for 24 h, were treated with the indicated concentrations of REEE-1 for 24 h in a low serum condition. They were then incubated for 4 h in the presence of [*methyl*-^{3}H] thymidine. (B) Thymocytes, stimulated with concanavalin A, were treated with 50 μg/ml of REEE-1 for the indicated times, then exposed to [*methyl*-^{3}H] thymidine for 4 h. Each bar represents the mean ± SEM values of triplicates and the figure shows a representative result from the three separate experiments.

cytotoxic activity of REEE-1 on thymocytes. To understand the nature of the inhibitory effect of REEE-1 on DNA synthesis by thymocytes, a MTT assay was performed, as

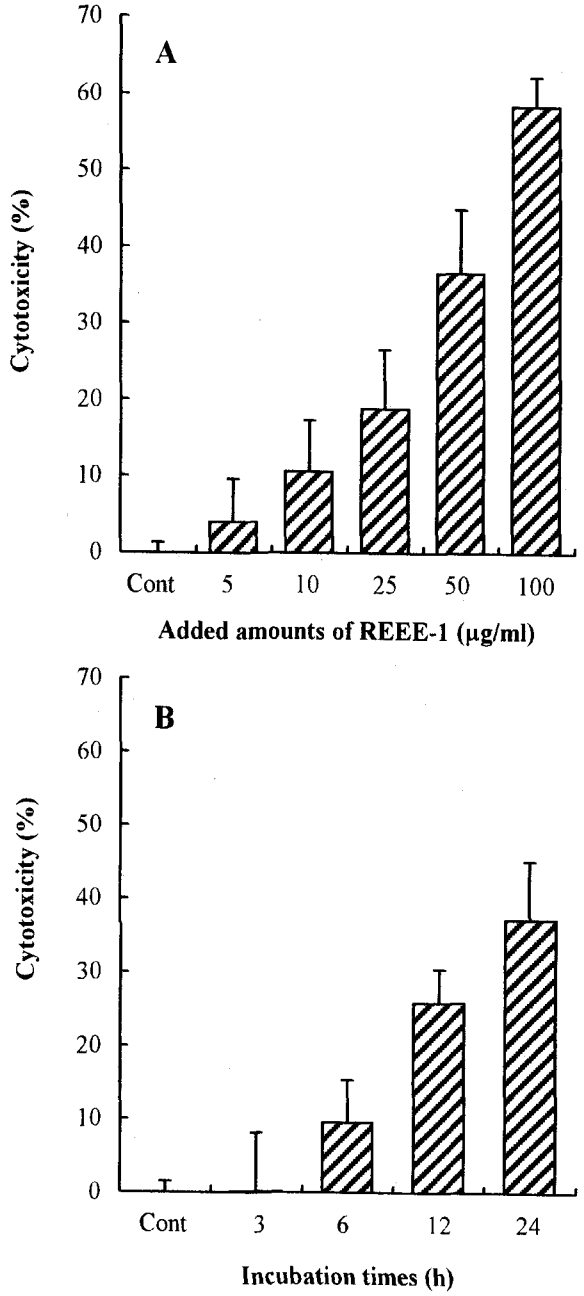

Fig. 4. Effects of REEE-1 treatment on cytotoxicity on thymocytes. (A) Thymocytes were treated with the indicated concentrations of REEE-1 for 24 h. (B) Thymocytes were incubated with 50 μg/ml of REEE-1 for the indicated times. Each bar represents the mean ± SEM values of triplicates. The figure shows a representative result from the three separate experiments.

described in Materials and Methods (Fig. 4). As shown in Fig. 4A, the addition of REEE-1 to the cultured thymocytes caused a cytotoxic effect in a dose-dependent manner. For example, REEE-1-mediated cytotoxicity on thymocytes was more than 60%, when 100 μg/ml of REEE-1 was used. Also, REEE-1-mediated cytotoxicity on thymocytes was time dependent, as expected, and a 24 h incubation with 50 μg/ml of REEE-1

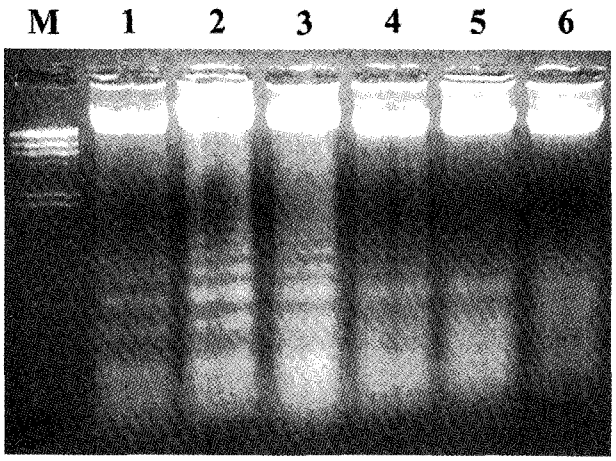

Fig. 5. DNA fragmentation in the thymocytes treated with REEE-1. Lanes 1 through 6 represent the results from the experiments treated with 0, 10, 25, 50, 100, and 200 μg/ml of REEE-1, respectively. M represents the λ/*Hin*d III DNA size marker.

represented 40% cytotoxicity (Fig. 4B). These results suggest that REEE-1 has a cytotoxic effect, rather than a cytostatic effect, on thymocytes.

Apoptotic death of thymocytes In order to characterize the nature of REEE-1-mediated cytotoxicity on thymocytes, we tested whether the REEE-1-mediated cell death was caused by either apoptosis, or necrosis, through a DNA fragmentation assay (Fig. 5), a flow cytometric analysis (Fig. 6), and H33258/Annexin V/PI triple staining (Fig. 7). As shown in Fig. 5, the REEE-1 treatment on the thymocytes produced a clear pattern of ladder formation that was created by the DNA fragmentation during the 24 h treatment (lanes 2-4). However, treatment of the high concentration (>100 μg/ml) of REEE-1 on thymocytes reduced the typical DNA ladder formation, and induced a gradual increase of the formation of DNA smear (lanes 5 and 6). These results suggest that the REEE-1-mediated cytotoxicity on thymocytes might be mediated through two different ways, depending on the concentration of the treated REEE-1. Namely, a low concentration of the REEE-1 treatment on thymocytes might exert the cytotoxicity through apoptosis compared to the necrosis, which might be induced by a high concentration of REEE-1.

In order to confirm the result that REEE-1-mediated cytotoxicity on thymocytes was exerted by apoptosis in a low REEE-1 concentration, the experiments focused on the characterization of the nuclear changes of the thymocytes through a flow cytometric analysis by permeabilizing and staining the cells with PI (Fig. 6). As shown in the figure, the majority of the permeabilized thymocytes were stained with PI. Only a few of the thymocytes appeared to contain low PI fluorescence, which represented the apoptotic cell population in the absence of REEE-1 treatment (Fig. 6A). However, when the thymocytes were incubated for 24 h in the presence

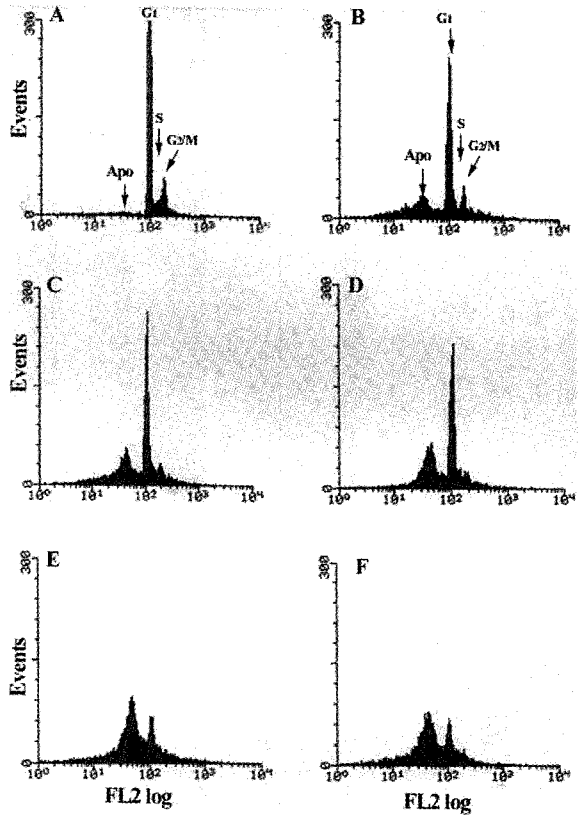

Fig. 6. Flow cytometric analyses of thymocytes after PI staining. Thymocytes, grown in a low serum condition, were incubated (A) without, or with, (B) 5, (C) 10, (D) 25, (E) 50, and (F) 100 μg/ml of REEE-1. The result shows a representative staining profile that was obtained from 10,000 cells for each experiment. The cells with fluorescence intensity that was less than 10^2 were defined as the apoptotic cell population.

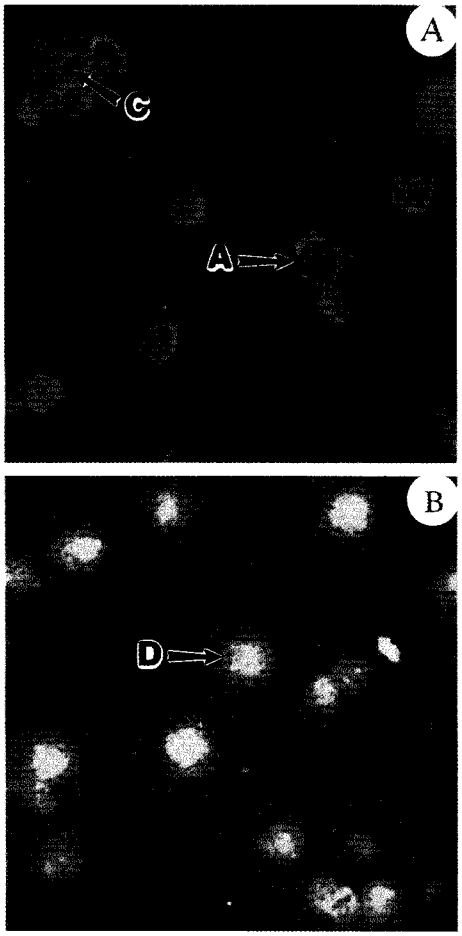

Fig. 7. H33258/Annexin V/PI triple staining of thymocytes. Thymocytes were treated with either (A) 25, or (B) 50 μg/ml, of REEE-1 for 24 h and cytocentrifuged after triple staining, as described in the Materials and Methods. C, A, and D represent the normal, apoptotic, and necrotic cells, respectively. The original magnification was ×400.

of up to 100 μg/ml of REEE-1, the apoptotic cell population increased in a dose-dependent manner (Figs. 6B-F). These results confirmed that the REEE-1-mediated cytotoxicity on thymocytes was mediated by the apoptotic process in the presence of a low concentration of REEE-1.

Finally, the observations that a low concentration of REEE-1 induced an apoptosis-mediated cytotoxicity on thymocytes, and a high concentration of REEE-1 induced a necrosis-mediated cytotoxicity, was confirmed by H33258/Annexin V/PI triple staining (Fig. 7). When the thymocytes were treated with 25 μg/ml of REEE-1, the proportion of the non-apoptotic viable cells, which were highly positive for chromatin staining with H33258 and negative with both Annexin V and PI ($H33258^{++}$/annexin V^-/PI^-, green color in intracellular), was very low (Fig. 7A). In addition, there were a few cells that showed as highly positive with the Annexin V staining, and slightly positive with H33258, but almost negative for PI uptake ($H33258^+$/Annexin V^{++}/PI^-, mainly green color in plasma membrane). In contrast, the cells were extensively stained with either fluorescence-conjugated Annexin V and PI, or PI alone and negatively stained with H33258 ($H33258^-$/Annexin V^+/PI^{++}, green and dark yellow color) when the thymocytes were treated with 50 μg/ml of REEE-1 (Fig. 7B). These results finally confirmed that the cytotoxicity that was exerted by REEE-1 was mediated differentially, depending on the concentrations of the REEE-1 used. A low concentration of REEE-1 exerted the cytotoxicity on thymocytes through apoptosis, compared to the necrosis, which was induced by a high concentration of REEE-1.

Discussion

The reaction mechanism of the hydroxyl radical with biomolecules has been a subject of particular interest, due to its diverse action on biological systems (Ames and Shigenaga, 1993). The importance of the iron-mediated hydroxyl radical formation, both *in vivo* (particularly under oxidative stress) and *in vitro*, has already been highlighted (Mello-Filho and Meneghini, 1991). Especially, the hydroxyl radical reactions

of the iron-mediated system, such as deoxyribose and DNA nicking assays, have frequently been used in screening radical scavengers that are derived from a number of natural materials, including foods (Halliwell *et al.*, 1987; Schneider *et al.*, 1989; Joseph and Aravindakumar, 2000). In this study, the results from deoxyribose, and DNA nicking assays, showed that REEE-1 contained strong scavenging activity of the hydroxyl radical (Table 1 and Fig. 1). REEE-1 acted as a scavenger of hydroxyl radicals, and the increases of hydroxyl radical-induced deoxyribose degradation were decreased by the REEE-1 treatment in a dose-dependent manner (Table 1). In addition, the DNA nicking assay demonstrated that the nicking potential, caused by the hydroxyl radical produced in the ascorbic acid/Fe(III)/H_2O_2 system, was inhibited dose-dependently by the addition of REEE-1 (Fig. 1B). Finally, the scavenging activity of REEE-1 of hydroxyl radical was confirmed by the glucose/glucose oxidase (G/GO) assay system in thymocytes primary cultures (Fig. 2). For example, hydroxyl radical-mediated cytotoxicity in thymocytes was significantly reduced by the addition of REEE-1 into the culture ($P<0.05$). Therefore, the addition of 10 μg/ml REEE-1 increased the viability of the thymocytes by 1.26-fold. Also, the addition of 20 μg/ml of REEE-1 increased the viability of thymocytes by 1.43-fold, compared to that of the control GO alone treated cells. This was in agreement with the previous observation that the cell viability of thymocytes, incubated with the G/GO system for 4 h in the presence of 100 μg/ml of crude ethanol extract of RVS, was increased about 1.45-fold (78.42%) (Lee, 2000). In addition, the thymocytes, which were incubated with more than 50 μg/ml of REEE-1 for 4 h without the G/GO enzyme system, were completely undamaged by the REEE-1 (data not shown). These results suggest that REEE-1 contained a protective effect on hydroxyl radical-mediated cytotoxicity in cultured thymocytes, and that the activity was higher than that of the crude ethanol extract of RVS. Collectively, these results showed that REEE-1 could be considered a strong antioxidant on hydroxyl radical-induced cell damage.

In the [*methyl*-^{3}H] Thymidine incorporation assay, REEE-1 showed an inhibitory effect on DNA synthesis (Fig. 3). Especially, the 24 h treatment of 10 μg/ml of REEE-1 inhibited the growth of thymocytes, but did not cause cell death. This result indicated that REEE-1 did not seem to contain mutagenic activity. The inhibitory effect of REEE-1 on DNA synthesis could be exerted through either cytostatic, or the cytotoxic effect of REEE-1. We assume that the REEE-1-mediated inhibition of DNA synthesis might be exerted through cytotoxicity, rather than the cytostatic effect that is based on the observation that the absorbance from the MTT assay was decreased, rather than maintained, as the treatment time continued (Fig. 4). However, additional studies on the signaling molecules that are involved in the cell cycle and apoptosis are required in order to reach a clear conclusion on this phenomenon. This is because the antiproliferative activity, caused by the 12 h treatment of 50 μg/ml of REEE-1, could not be completely attributed to the cytotoxic effect (Wong and McLean, 1999).

In this study, we used several methods to detect the process of apoptosis to characterize the nature of REEE-1-mediated cytotoxicity on thymocytes. Results from the experiments demonstrated that REEE-1 could elicit both apoptosis and necrosis in thymocytes, depending on the dose and time of the REEE-1 treatment (Figs. 5-7). For example, REEE-1 caused apoptosis in thymocytes at the concentration of 10 μg/ml, while more than 50 μg/ml of REEE-1 caused necrotic death in thymocytes (Fig. 5). Similarly, the results from flow cytometric analyses showed that REEE-1, with a concentration of less than 50 μg/ml, caused the apoptosis of thymocytes after a 24 h treatment. However, more than 50 μg/ml of REEE-1 caused apoptotic cell death, although the possibility of necrotic cell death could not be excluded in this experiment (Fig. 6). This finding was further demonstrated by monitoring the triple staining of the cells, which could discriminate the difference between apoptotic death and the necrotic cell death of thymocytes. For example, the early stage of apoptosis might be implicated in the loss of membrane phospholipid asymmetry and the appearance of phosphatidyl serine on the outer surface of the cells, which should be found in the inner leaflet of the plasma membrane in normal cells, and highly stained with a Ca^{2+}-dependent phospholipid-binding protein Annexin V. The phosphatidyl serine translocation was believed to be the hallmark of apoptosis. The detection of the phosphatidyl serine externalization might be a more sensitive method than the measurement of histone-associated DNA fragmentation to indicate the occurrence of apoptosis (Koopman *et al.*, 1994). In addition, the results from the H33258/PI staining, which could be utilized as a method to identify between viable and necrotic cell deaths (Vermes *et al.*, 1995; Mesner, Jr. and Kaufman, 1997), suggested that the REEE-1 induced apoptosis in thymocytes, when more than 10 μg/ml of REEE-1 was treated for 24 h (Fig. 7A). It also provoked necrotic cell death rather than apoptosis in thymocytes when more than 50 μg/ml of REEE-1 was treated (Fig. 7B). Although the exact intracellular mechanisms of the REEE-1-mediated initiation and amplification of thymocyte death was not clearly elucidated in this study, some explanations could be found from the reports that flavonoids had an inhibitory effect on PI3-kinase, protein kinase C, protein tyrosine kinase, and some transcriptional factors; thereby, arresting cell growth and inducing cell death in several carcinoma cell lines (Yang *et al.*, 1998; Gamet-Payrastre *et al.*, 1999).

In summary, the results of this study pointed out that REEE-1, prepared from the crude ethanol extract of RVS, could play a beneficial role in scavenging hydroxyl radicals that are generated from ascorbic acid/Fe(III)/H_2O_2, or G/GO enzyme systems. On the contrary, a high concentration of REEE-1 (50 μg/ml) was believed to be involved in apoptosis, and lead the thymocytes to necrotic cell death. These two aspects of REEE-1, demonstrating an antioxidative and

inductive activity of apoptotic cell death, lead us to assume that REEE-1 was possibly involved in the direct signaling pathway, such as the fas ligand pathway in cytoplasm. This is because several of the defined antioxidants showed the functions to scavenge the hydroxyl radical and transmit the signal (Rao *et al.*, 1993; Sen and Packer, 1996; Wong and McLean, 1999). Another possible assumption could be that REEE-1 worked through another signaling pathway, so that more than 50 μg/ml of REEE-1 could stimulate the fas/fas ligand-associated signaling pathway. However, less than 50 μg/ml of REEE-1 could run the signaling pathway, as compared to other antioxidants. This assumption could explain why REEE-1 acted as an antioxidant at a low concentration. It, however, worked as a stimulator of the toxic signaling pathway, such as the fas signaling pathway, at a high concentration. In order to clearly unravel the mechanisms involved in REEE-1-mediated cell signaling, many questions regarding the cell membrane receptor and signaling pathway should be addressed.

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