J Food Sci Nutr
Vol 9, p 150~155 (2004)

J Food Science and Nutrition

Antioxidative Effect of *Rhus javanica* Linne Extract Against Hydrogen Peroxide or Menadione Induced Oxidative Stress and DNA Damage in $HepG_2$ Cells

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Abstract

The free radical scavenging activities and the protective effects of *Rhus javanica* extracts against oxidative damage induced by reactive oxygen species (ROS) were investigated. n-Hexane, ethyl acetate and water fractions were prepared from a methanol extract. DPPH radical, superoxide anion and hydroxyl radical scavenging activities were estimated. Intracellular ROS formation was quantified using fluorescent probes, 2', 7'-dichlorofluorescin diacetate (DCFH-DA) for hydroxyl radical and dihydroethidium (DHE) for superoxide anion. The oxidative DNA damage was investigated by the comet assay in $HepG_2$ cells exposed either to H_2O_2 or to menadione. The highest IC_{50} values for DPPH radical scavenging activity was found in the ethyl acetate fraction with a value of 5.38 μg/mL. Cells pretreated with ≥1 μg/mL of the ethyl acetate extract had significantly increased cell viability compared to control cells, which were not pretreated with the extract. Intracellular ROS formation and DNA damage in $HepG_2$ cells, which were pretreated with the various concentrations of *Rhus javanica* ethyl acetate extract and then incubated either with H_2O_2 or with menadione, reduced in a dose-dependent manner. These findings suggest that *Rhus javanica* might have biologically active components which have strong protective effects against ROS induced oxidative damages to the biomolecules, such as cell membranes and DNA.

Key words: *Rhus javanica* Linne, $HepG_2$ cells, reactive oxygen species (ROS), comet assay

INTRODUCTION

It is now well established that reactive oxygen species (ROS), such as superoxide anion ($O_2^{-\circ}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH°), are continuously produced *in vivo*. In consequence, organisms have evolved not only antioxidant defense systems to protect against them but also repair systems that prevent the accumulation of oxidatively damaged molecules (1, 2). But increased generation of ROS within cells often leads to damage to biological macromolecules leading to lipid peroxidation, protein oxidation, and DNA base modifications and strand breaks (3). Therefore, it is widely believed that oxidative stress has been related to aging and a variety of human diseases; including neurodegenerative diseases, cardiovascular diseases, lung diseases, UV-mediated skin diseases, and the age-related development of the major cancers, such as those of the colon, prostate, rectum, and breast (4,5). Thus, diets or therapeutic agents that decrease oxidative DNA damage may delay or prevent the onset of cancer and various age-related diseases. Recently, substantial efforts have been made to develop and identify both natural and synthetic antioxidants.

Rhus javanica Linne, a member of *Anacardiaceae* family, is widely distributed in Korea, Japan and China. Its aerial parts have been used as a folk medicine to treat diarrhea in Korea (6). Recently, in studies searching for natural antioxidants, ethyl acetate and chloroform extract of *R. javanica* were found to be very effective at retarding oxidation of palm oil and lard, and several non-cell studies have demonstrated that the extract of *R. javanica* was capable of reducing the lipid peroxidation caused by ROS (7-10). However, relatively little is known about the antioxidative activity of *R. javanica* on the oxidative damage induced by ROS in intact cells.

Highly reactive hydroxyl radicals frequently attack biologic molecules by abstracting hydrogen. Superoxide radical is much less reactive than hydroxyl radical, but a number of biologic targets are sensitive to it (2). We used the intracellular probes DHE and DCFH to study ROS generation in cultured cells. Specifically, oxidation

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of DCFH to fluorescent DCF was indicative of H_2O_2 or hydroxyl radical formation, while oxidation of DHE to fluorescent Eth-DNA provided a measure of superoxide formation (11). Intracellular oxidative stress was monitored by measuring changes in fluorescence resulting from intracellular probe oxidation. The single cell gel electrophoresis (SCGE) technique (also called the comet assay) developed by Singh et al (12) and can be used to evaluate low degrees of DNA damage in individual cells. The technique has been used mainly to quantitate single-strand breaks in DNA and the repair capacity of DNA subsequent to ultraviolet light, ionizing radiation, or oxidative damage in *in vitro* models.

To evaluate the antioxidant activity of *R. javanica*, we investigated the radical scavenging activity and the protective effect against cellular oxidative damage by intracellular ROS production in the $HepG_2$ cell line. We also examined the antigenotoxicity of the extract against oxidative DNA damage using the comet assay.

MATERIALS AND METHODS

Cell and chemicals

The human hepatoma cell line ($HepG_2$) was purchased from American Type Culture Collection. DPPH, NBT, H_2O_2, menadione, DCFH-DA, DHE, low melting and high melting point agarose, penicillin, streptomycin and ethidium bromide were all from Sigma (St. Louis, MO, USA). DMEM from GIBCO (Carlsbad, CA, USA), FBS from JRH Bioscience (Lenexa, USA) were used.

Preparation of methanol extract and fractionation

Dried stem barks and leaves of *R. javanica* were extracted with methanol 3 times under reflux. The extracts were concentrated to dryness in vacuo at 90°C to obtain the methanol extract. The MeOH extract was partitioned with hexane, water and ethyl acetate, successively.

Measurement of DPPH radical scavenging activity

DPPH radical scavenging activity was measured by the method of Hatano et al. (13). Various concentrations of test extracts were added to DPPH solution (200 μM). DMSO was used as solvent for lipid soluble extracts. After standing at room temperature for 30 min, the absorbance of the remaining DPPH was determined at 515 nm by microplate reader (Asys Hitachi, Expert 96). The radical scavenging activity of each sample was expressed as radical scavenging rate (%), and the concentration of 50% scavenging the radical (IC_{50}) was also calculated.

Measurement of ROS scavenging activity (*in vitro*)

The ability of plant extracts to scavenge reactive oxygen species was assessed using an *in vitro* system to generate specific ROS in the presence of the fluorescent probes (14). For superoxide anion radical, xanthine (0.4 mM)/xanthine oxidase (0.02 U/mL) were added to the cuvette containing DHE (100 μM) or DHE (100 μM) with the extract (5～50 μg/mL), and ethidium fluorescence was measured by the fluorospectrometer (ex 475 nm/em 610 nm) for 15 min. For hydroxyl radical formation, DCF fluorescence were detected for 15 min (ex 365 nm/em 435 nm) after the addition of H_2O_2 (1 μM)/$FeSO_4$ (50 mM) to the cuvette containing DCFH-DA (20 μM) or DCFH-DA (20 μM) with the extract (5～50 μg/mL).

Cell culture

$HepG_2$ cells were grown in a Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (w/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

Cytotoxicity

The protective effect of the extract on oxidant-induced cytotoxicity in $HepG_2$ cells was evaluated by the trypan blue exclusion test (15). After $HepG_2$ cells were pretreated with various concentrations (1.0～10.0 μg/mL) of the plant ethyl acetate extract for 1 hr at 37°C, they were exposed to oxidant, H_2O_2 (1 mM) or menadione (0.1 mM) in combination with DDC (0.5 mM), for 1 hr. Fifty μL of cell suspension was mixed with 50 μL of trypan blue isotonic solution (0.4%), and cell viability was determined on a haemocytometer under a microscope. Cell viability was expressed as a percentage of the viable cells.

Measurement of intracellular ROS generation

Intracellular oxidant stress was measured by the changes in fluorescence resulting from intracellular probe oxidation. Intracellular hydroxyl radical or superoxide anion production was measured using the oxidant-sensitive probe DCFH-DA (16) or DHE (17), respectively. Cells were pretreated with various concentrations of the ethyl acetate extract (1.0～10.0 μg/mL) for 1 hr, and then exposed to either H_2O_2 (1 mM) or menadione (0.1 mM) in combination with DDC (0.5 mM), which is an inhibitor of superoxide dismutase (18), for 1 hr. Then, DCFH-DA (20 μM) or DHE (5 μM) was added to the cells and incubated at 37°C for 30 min. After incubation, cells were washed twice with ice-cold PBS, collected using a cell scraper, and disrupted by three cycles of sonication for 10s at low output. After centrifugation, the DCF fluorescence (Ex 504 nm, Em 524 nm) for hydroxyl radical or the ethidium-DNA fluorescence (Ex 488 nm, Em 605 nm) for superoxide anion production of the supernatant was recorded using spectrofluorometer (Hitachi, F-4500).

Analysis of DNA damage (comet assay)

The comet assay was performed under alkaline con-

ditions by the procedure of Singh et al. (12). $HepG_2$ cells were pretreated with different concentrations (0.25~1.0 μg/mL) of the ethyl acetate extract for 1 hr, and then collected and suspended with PBS. Fully frosted slides were covered with 0.65% of normal agarose (NA) as the first layer, a mixture of cell suspension and 0.65% of low melting agarose (LMA) as the second layer, and finally with 0.65% of LMA (without cell) as the third layer. The slides were allowed to solidify at 4°C in the dark. DNA damage was induced by exposing the cells to an oxidant, such as hydrogen peroxide (25 μM) or menadione (0.1 mM), in combination with DDC (0.2 mM), for 5 min. The slides were placed in cold lysis buffer and kept at 4°C for 1 hr, and placed in a horizontal electrophoresis tank. The slides were left in the electrophoresis solution for 20 min to allow DNA unwinding and expression of alkali labile damage before electrophoresis. Electrophoresis was conducted at 4°C for 20 min using 25 V and 300 mA. After electrophoresis, the slides were neutralized in neutralization buffer, stained with ethidium bromide, and examined using a fluorescence microscope (200X, Leica). Images of 50 randomly selected cells from each slide and two slides per each treatment were analyzed with an image analysis system (Version 5.0, Kinetic Imaging, UK). The results were expressed in terms of tail extent moment (Tail length × % Tail DNA/100).

Statistical analysis

Data were presented as means ± SD and analyzed using one-way ANOVA with Duncan's multiple range test at α =0.05.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

The effects of *R. javanica* extracts on the reducing power of DPPH radical are shown in Fig. 1. Among the four extracts, the greatest DPPH radical scavenging activity was observed in the ethyl acetate extract, which exhibited stronger reducing activity than vitamin C which was used as a positive control at concentrations lower than 10 μg/mL. DPPH radical was scavenged about 76% by ethyl acetate extract and 63% by vitamin C at a concentration of 10 μg/mL. The scavenging effects decreased in the order of ethyl acetate > methanol > water and hexane extract. The concentration of the extracts for 50% scavenging the radical relative to a control (IC_{50}) was determined (Table 1). The IC_{50} of ethyl acetate extract and vitamin C were 5.38 and 5.61 μg/mL, respectively. There was no difference between ethyl acetate extract and vitamin C in DPPH radical scavenging power.

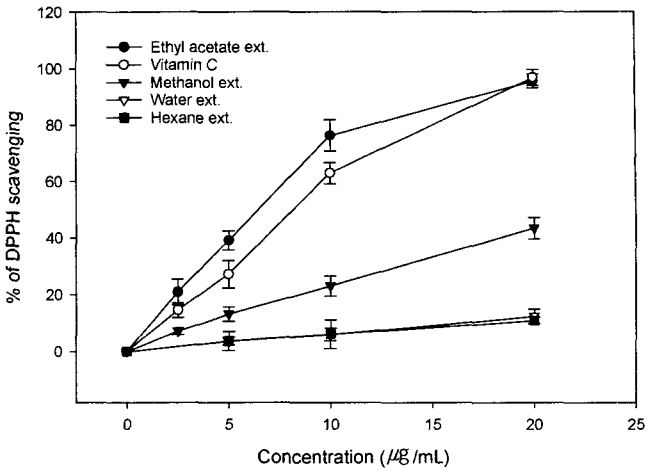

Fig. 1. The effects of various *R. javanica* extracts on the reducing power of DPPH radical. Data represent mean ± SD of the three different experiments.

Table 1. DPPH radical scavenging activity of *Rhus javanica* extracts

Sample	IC50 (μg/mL)
Methanol ext	20.29 ± 1.37[1)]
Ethyl acetate ext	5.38 ± 1.14
Hexane ext	87.03 ± 3.63
Water ext	148.72 ± 9.40
Vitamin C	5.61 ± 1.00

[1)]Values are mean ± SD (n=3).

ROS scavenging activity in *in vitro*

The ability of ethyl acetate extract of *R. javanica* to scavenge reactive oxygen species was assessed using an *in vitro* system to generate specific ROS in the presence of the fluorescent probes. As shown in Fig. 2, progressive increases in ethidium fluorescence due to the superoxide generation were seen in the cuvette containing xanthine/xanthine oxidase. However, addition of the plant ethyl acetate extract decreased the ethidium fluorescence in a dose-dependent manner. When compared to a positive control, such as vitamin C and E, the superoxide anion scavenging activity of the extract was similar to those two positive controls at the concentrations of 5 μg/mL (Fig. 2A) and 25 μg/mL (Fig. 2B). At 50 μg/mL (Fig. 2C), the superoxide scavenging activity of the extract was stronger than that of vitamin C and E. These results demonstrate that the ethyl acetate extract of *R. javanica* is a strong quencher of superoxide anion radical. As shown in Fig. 3, DCF fluorescence due to the hydroxyl radical generation increased in the cuvette containing H_2O_2 / $FeSO_4$. Addition of the ethyl acetate extract caused a concentration-dependent decrease of the DCF fluorescence. Vitamin E was not as good as vitamin C and the plant ethyl acetate extract in hydroxyl radical scavenging potency. At the concentration of 50 μg/mL (Fig. 3C), vitamin C and the ethyl acetate

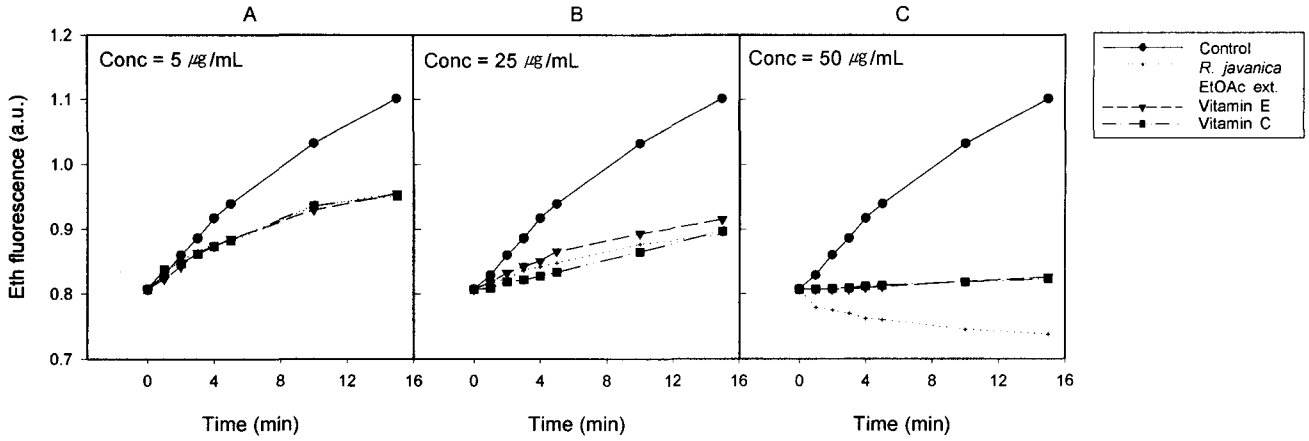

Fig. 2. The scavenging activity of *R. javanica* ethyl acetate extract on the superoxide generation by xanthine/xanthine oxidase system. Data represent mean±SD of the three different experiments.

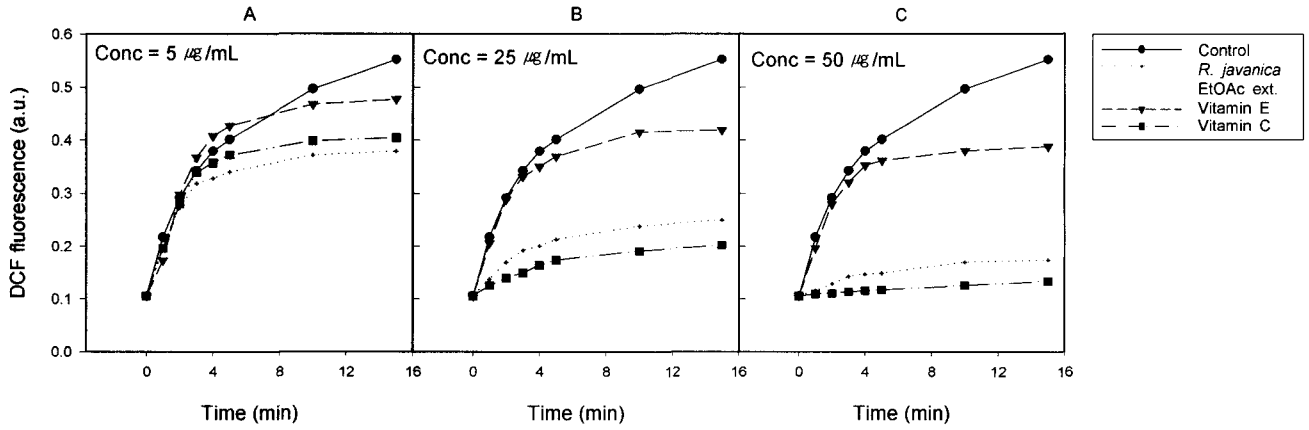

Fig. 3. The scavenging activity of *R. javanica* ethyl acetate extract on the hydroxyl radical or H_2O_2. Data represent mean±SD of the three different experiments.

extract scavenged hydroxyl radical almost completely over 15 min.

It has been reported that the addition of *R. javanica* ethyl acetate or chloroform extract to palm oil or lard greatly improves the shelf life of those lipids by lowering TBA and peroxide value (7-10). We cannot evaluate the strength of the chloroform extract of *R. javanica* in our system, but it is reasonable to assume that the chloroform extract might also have antioxidative activity as the ethyl acetate extract based on those previous reports (7-10).

Cell viability

As shown in Fig. 4, when cells were exposed to H_2O_2 (1 mM) or menadione (0.1 mM) in combination with DDC (0.5 mM) for 1 hr, cell viability was 75.9% or 50.0%, respectively, as measured by trypan blue exclusion test. However, the cells pretreated with the ethyl acetate extract of *R. javanica* for 1 hr showed resistance to the oxidative stress. Cells pretreated with ≥1 μg/mL of the ethyl acetate extract significantly increased the cell viability compared to control cells, which were not

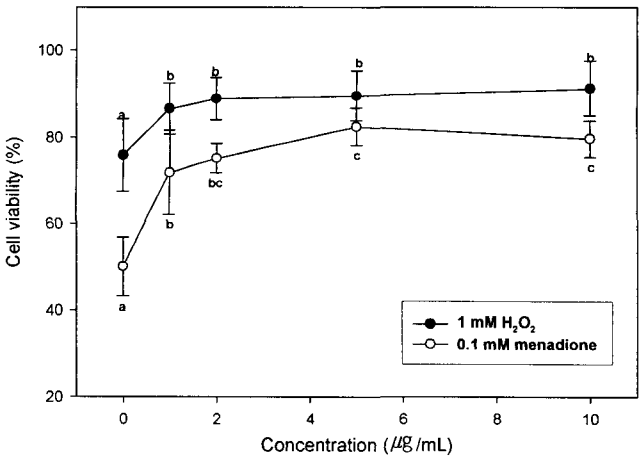

Fig. 4. Cell viability of $HepG_2$ cells pretreated with the *R. javanica* ethyl acetate extract for 1 hr and then exposed to either 1 mM H_2O_2 or 0.1 mM menadione with 0.5 mM DDC for 1 hr. Different letters in each graph indicate significant differences from one another ($p<0.05$).

pretreated with the extract, suggesting that the preincubation of cells with increasing concentrations of the

R. javanica ethyl acetate extract reversed the cell damage induced by H_2O_2 or menadione, providing significant protection of the extract against cell death caused by hydroxyl or superoxide radical, respectively.

Intracellular ROS generation

DCFH-DA diffuses through the cell membrane readily and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent DCFH, which is trapped inside the cell and rapidly oxidized to highly fluorescent DCF in the presence of ROS, particularly hydrogen peroxide (H_2O_2) and hydroxyl radical. Thus, the DCF fluorescence intensity is believed to parallel the amount of hydrogen peroxide (H_2O_2) and hydroxyl radical formed intracellularly. As shown in Fig. 5, dose-dependent decreases in intracellular DCF fluorescence by ethyl acetate extract of the plant were observed. Fluorescence in cells exposed only to 1 mM H_2O_2 (110.8 ± 3.5) increased by more than 10 times of the control (8.5 ± 1.2), which was not treated with the oxidant. In the presence of the ethyl acetate extract, DCF fluorescence intensity dropped significantly and dose-dependently from 85.3 ± 11.8 at the concentration of 1 μg/mL to 27.4 ± 14.7 at 10 μg/mL.

Menadione is a quinone compound that undergoes redox cycling. One electron transfer, mainly from the mitochondrial respiratory chain, forms semiquinone radicals that can rapidly reduce O_2, thereby generating superoxide radical intracellularly and regenerating the quinone (11). DHE enters the cell and can be oxidized by superoxide radical to yield fluorescent ethidium (Eth). Eth binds to DNA (Eth-DNA), further amplifying its fluorescence. Thus, increases in DHE oxidation to Eth-DNA (i.e. increases in Eth-DNA fluorescence) are suggestive of superoxide generation. However, when SOD is active, superoxide generated by menadione would be expected to yield H_2O_2. Therefore, diethyldithiocarbamic acid (DDC) was used to inhibit SOD activity (19). Menadione with DDC would favor accumulation of superoxide by inhibiting its removal via SOD. Addition of ethyl acetate extract of *R. javanica* decreased Eth fluorescence, suggesting that a concentration-dependent quenching of superoxide generated from menadione had occurred (Fig. 5). Eth-fluorescence increased from 12.3 ± 4.3 in control cells, which were not treated with menadione, to 80.2 ± 15.5 in cells treated menadione only. However, the fluorescence intensity was progressively decreased to values between 54.6 ± 1.5 and 34.8 ± 8.4 by the pretreatment of the ethyl acetate extract at concentrations of 2 ~ 10 μg/mL. Superoxide radical generation, as measured by Eth-fluorescence, was inhibited by 56% by the addition of 10 μg/mL of *R. javanica* extract to the medium, compared to the cells treated menadione only.

DNA damage

The comet assay can be a valid tool for analyzing the effect of natural antioxidants on a reliable biomarker of oxidative stress such as the status of cellular DNA. After 1 hr preincubation with various concentrations of the ethyl acetate extract (0.25 ~ 1.0 μg/mL), cells were exposed to the oxidants for 5 min and quantified for DNA damage by the comet assay, as a measure of tail extent moment (TEM) (Fig. 6). The oxidants, H_2O_2 (25 μM) or menadione (0.1 mM) with DDC (0.2 mM), caused a significant increase in TEM, indicating extensive DNA damage in these cells, compared with that of the unexposed controls. Ethyl acetate extract of the plant sig-

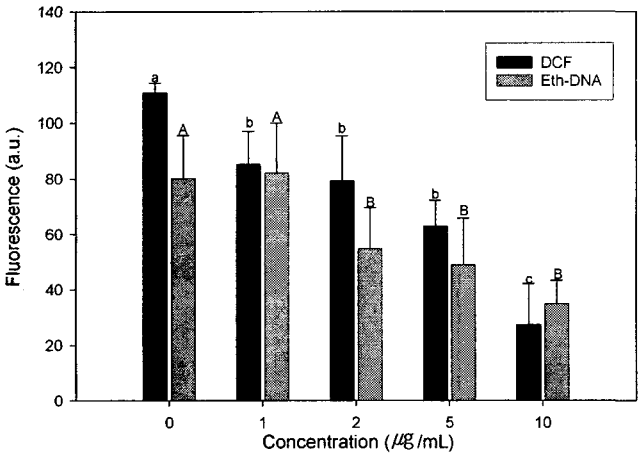

Fig. 5. Reductive effect of ethyl acetate extract of *R. javanica* on intracellular ROS level in H_2O_2-treated $HepG_2$ cells. Cells were incubated with EtOAc extract of *R. javanica* (1 ~ 10 μg/mL) for 1 hr, and then exposed to H_2O_2 (1 mM) for 1 hr. Data are presented as means ± SD (N=5). Different letters among various concentrations indicate significant differences from one another ($p < 0.05$).

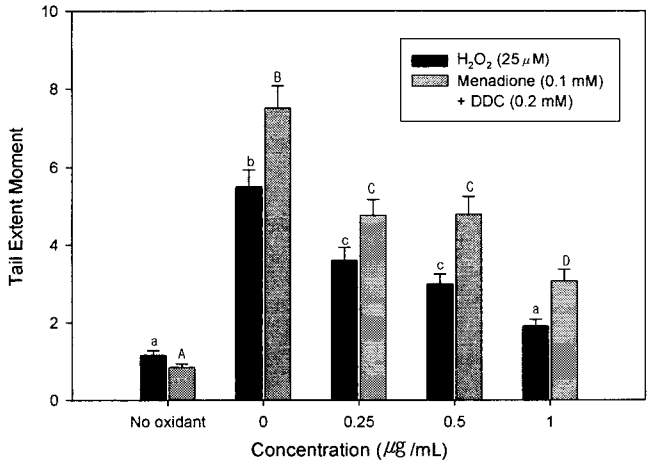

Fig. 6. Effect of ethyl acetate extract of *R. javanica* on DNA damage as measured by the comet assay. Cells were treated with various concentrations (0.25 ~ 1.0 μg/mL) of sample for 1 hr prior to exposure to the oxidant. Different letters among various concentrations indicate significant differenences from one another ($p < 0.05$).

nificantly decreased DNA damage of the cells exposed to H_2O_2 or menadione at all tested concentrations (0.25 ~ 1.0 μg/mL). Treatment with 1.0 μg/mL of the extract inhibited H_2O_2-induced DNA damage by 82%, and menadione-induced DNA damage by 67%, suggesting that DNA damage is prevented by the removal of a potential ROS, such as hydroxyl and superoxide radical. Treatment with the extract up to 1.0 μg/mL did not cause obvious DNA damage in $HepG_2$ cells (data not shown). It is well-known that ROS-induced oxidative DNA damage has been implicated in mutagenesis and carcinogenesis. These findings suggest that *R. javanica* has strong protective effect against ROS-induced DNA damage and is effective for antimutagenesis and anticarcinogenesis.

In conclusion, *Rhus javanica* might have biologically active components which have strong protective effects against ROS induced oxidative damages to the biomolecules, such as cell membranes and DNA, which might be the cause of various kinds of diseases and aging. The constituents of the stem bark of *R. javanica* were identified as gallic acid, methyl gallate, scopoletin, triterpenes and semialactic acid (20,21), while those of the leaves were not identified. Therefore, further studies for the identification of the active antioxidative constituents of *Rhus javanica* and their mechanisms of action in animal model system are needed.

ACKNOWLEDGEMENTS

This work was supported by a grant from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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(Received March 25, 2004; Accepted May 22, 2004)