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Antioxidative Constituents from *Paeonia lactiflora*

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The ethanol extract of the peony root (*Paeonia Lactiflora* Pall, Paeoniaceae) as well as its major active components including gallic acid and methyl gallate were evaluated for their protective effects against free radical generation and lipid peroxidation. In addition, the protective effects against hydrogen peroxide-induced oxidative DNA damage in a mammalian cell line were examined. The ethanol extracts of the peony root (PREs) and its active constituents, gallic acid and methyl gallate, exhibited a significant free radical scavenging effect against 1,1-diphenyl-2-picryl hydrazine (DPPH) radical generation and had an inhibitory effect on lipid peroxidation, as measured by the level of malondialdehyde (MDA) formation. The PREs did not have any pro-oxidant effect. They strongly inhibited the hydrogen peroxide-induced DNA damage from NIH/3T3 fibroblasts, as assessed by single cell gel electrophoresis. Furthermore, the oral administration of 50% PRE (50% ethanol extract of peony root), gallic acid and methyl gallate potently inhibited the formation of micronucleated reticulocytes (MNRET) in the mouse peripheral blood induced by a $KBrO_3$ treatment *in vivo*. Therefore, PREs containing gallic acid and methyl gallate may be a useful antigenotoxic antioxidant by scavenging free radicals, inhibiting lipid peroxidation and protecting against oxidative DNA damage without exhibiting any pro-oxidant effect.

Key words: *Paeonia lactiflora*, Peony root, Gallic acid, Methyl gallate, Oxidative stress, Antioxidant, Free radical scavenging, Single cell gel electrophoresis, Micronucleus assay, Potassium bromate

INTRODUCTION

The radical scavenging activity and antioxidant capacity of natural plant products have received a great deal of attention owing to their effects in reducing the risk of cancer and cardiovascular disease in humans. Oxidative stress including reactive oxygen species (ROS)-generating chemicals, ultraviolet (UV) radiation and ionization radiation can damage various cellular components such as the DNA, protein, and lipid leading to many pathological conditions. Free radicals can initiate lipid peroxidation to abstract hydrogen atoms from the unsaturated lipids to yield lipid hydroperoxides (Pryor *et al.*, 1978). The chain reaction by the auto-oxidative process of lipid peroxidation can be inhibited either by scavenging the free radicals of the unsaturated fatty acids or by removing the reactive oxygen species (Lynch *et al.*, 1978). DNA damage mediated by oxidative stress also plays a key role in carcinogenesis and aging (Dizdaroglu *et al.*, 1992; Guyton *et al.*, 1993; Feig *et al.*, 1994; Beckman *et al.*, 1998; Kasai *e al.*, 1997; Lu *et al.*, 1999). Therefore, any antioxidant that scavenges free radicals directly or somehow interferes with the generation of free radical-mediated events is likely to inhibit carcinogenesis and the aging process.

The peony root (*Paeonia Lactiflora* Pall, Paeoniaceae) is commonly used in Korean traditional medicine for its ability to cool the blood and invigorate the blood circulation, etc (Huh, 1966). This plant contains oxypaeoniflorin, paeoniflorin, albiflorin, benzoyl albiflorin, gallic acid, pentagalloylglucose, paeonol and benzoic acid as its bioactive constituents (Wu *et al.*, 1996). Recent studies have reported that this material improved the memory (Ohta *et al.*, 1993; Honokaa *et al.*, 1996), and exhibited antioxidant activity (Zhang *et al.*, 1994; Okubo *et al.*, 2000), hepatoprotection (Qi *et al.*, 1991), anti-atherosclerotic effects with lipid peroxidation (Zhang *et al.*, 1991), antimutagenic properties (Sakai *et al.*, 1990), and platelet aggregation inhibition (Lin *et al.*, 1999). However, the antigenotoxic mechanisms underlying antioxidant effect of the peony root have not been reported. The aim of this study was to

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examine and evaluated the effect of the Peony root and its constituents on scavenging the DPPH radical as well as inhibiting lipid peroxidation, DNA damage and chromosomal damage, and to establish a proper plant extract as an antigenotoxic antioxidant. The ethanol extracts of the peony root and their constituents were prepared and evaluated for their free radical scavenging effect, as well as their ability to inhibit lipid peroxidation and oxidative DNA damage in NIH/3T3 fibroblast in order to suggest the mechanisms of its protection against oxidative stress. The *in vivo* effect on micronucleus formation against a strong oxidant, $KBrO_3$-induced oxidative chromosomal damage, was also examined using mice.

MATERIALS AND METHODS

Materials

The fetal bovine serum (FBS), Dulbecco's modified eagles medium (DMEM), phosphate buffered saline (PBS), trypsin-EDTA, and antibiotics were obtained from Gibco BRL (Grand Island, NY). Normal melting point agarose (NMPA), low melting point agarose (LMPA), methyl linoleate, thiobarbituric acid (TBA), Triton X-100, ascorbic acid (Vit-C), gallic acid, methyl gallate and ethidium bromide were purchased from the Sigma Chem. (St. Louis, MO).

Animals

Male ICR mice (5 weeks old) obtained from Daehan Biolink Co. (Seoul, South Korea) were used. The animals were given lab chow (Purina Korea, South Korea) and water *ad libitum*. They were acclimatized in a specific pathogen free facility under the conditions of 20-22°C, 40-60% relative humidity and 12/12 h (light/dark) cycle for at least 7 days prior to the experiments.

Apparatus

The melting points were determined using a Fisher-Johns melting point apparatus and were uncorrected. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian 200 MHz NMR using TMS as an internal standard. TLC was carried out using plates coated with silica gel 60 F254 (Merck). Silica gel column chromatography was performed on silica gel 60 (70-230 mesh, Merck).

Extraction and isolation of constituents

The dried root of *paeonia lactiflora* was obtained from a local market (Seoul, South Korea) and soaked in 30, 50, and 70% ethanol at room temperature for 7 days. The three ethanolic extracts were filtered to remove the solid material. The filtrates were evaporated *in vacuo* to dryness and these dried powders were used throughout the chemical and biological experiments. On the other hand, the oxypaeoniflorin, benzoyl paeoniflorin, paeonol, gallic acid and methyl gallate were isolated from the 80% methanolic extract of the dried root of *paeonia lactiflora* and were identified by IR, NMR, Mass, and HPLC etc. using the following procedures (Kitagawa *et al.*, 1979; Yoshikawa *et al.*, 1992; Kang *et al.*, 1993).

In order to isolate the pure compounds, the 80% methanolic extract (350 g) was mixed with distilled water and partitioned with *n*-hexane, ethyl acetate and *n*-butanol. After each fraction had been dried, the n-butanol fraction was dissolved in a small amount of methanol and poured onto a silica gel column. Subfractions were made using a stepwise gradient of chloroform : methanol (19:1-4:1) as the mobile phase. Compounds I, II, and III were isolated using chloroform : methanol (6:1) followed by 50% methanol or benzene : acetone : methanol (7:2:1). From the *n*-hexane fraction, compound IV was isolated using silica gel column chromatography and recrystallized in 50% ethanol. Gallic acid (Compound V) and methyl gallate (Compound VI) were separated by HPLC and identified by comparing them to two commercial compounds (Sigma Co.). Fig. 1 shows the chemical structures isolated from the root of *paeonia lactiflora.*

Paeoniflorin (I)

White powder; ^{1}H-NMR (Acetone-d_6) δ 1.56 (3H, s, CH_3), 4.76 (1H, d, J = 8.0 Hz, H-1'), 4.90 (2H, s, H-8), 5.49 (1H, s, H-9), 7.59-7.80 (3H, m, H-2", H-3", H-4"), 8.14 (2H, d, J=7.0 Hz, H-5", H-6"); ^{13}C-NMR (Acetone-d_6) δ 18.82 (C-10), 22.37 (C-7), 43.09 (C-5), 43.65 (C-3), 60.53 (C-8), 62.14 (C-6'), 70.78 (C-4'), 70.88 (C-6), 74.01 (C-2'), 76.63 (C-5'), 77.23 (C-3'), 85.26 (C-2), 88.17 (C-1), 99.06 (C-1'), 100.94 (C-9), 104.94 (C-4), 128.63 (C-3", C-5"), 129.54 (C-2", C-6"), 130.33 (C-1"), 133.20 (C-4"), 166.12 (C-7").

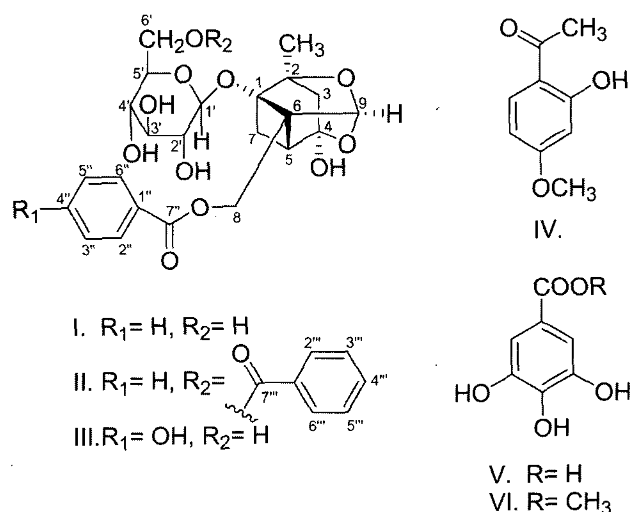

Fig. 1. Structures of the major constituents isolated from the root of *paeonia lactiflora*

Benzoyl paeoniflori (II)

White powder; ^{1}H-NMR (Acetone-d_6) δ 1.35 (3H, s, CH_3), 4.59 (1H, d, J=7.8 Hz, H-1'), 4.82 (2H, s, H-8), 5.49 (1H, s, H-9), 7.56-7.73 (6H, m), 8.11-8.18 (4H, m); ^{13}C-NMR (DMSO-d_6) δ 18.85 (C-10), 22.01 (C-7), 42.82 (C-5), 43.55 (C-3), 60.47 (C-8), 64.15 (C-6'), 70.59 (C-4'), 70.75 (C-6), 73.89 (C-2', C-5'), 76.96 (C-3'), 85.22 (C-2), 88.24 (C-1), 98.89 (C-1'), 100.82 (C-9), 104.87 (C-4), 128.65 (C-3", C-3"', C-5", C-5"'), 129.44 (C-2", C-6"), 129.56 (C-2"', C-6"'), 130.23 (C-1"), 130.35 (C-1"'), 133.28 (C-4", C-4"'), 165.83 (C-7"'), 166.29 (C-7").

Oxypaeoniflorin (III)

White powder; ^{1}H-NMR (DMSO-d_6) δ 1.42 (3H, s, CH_3), 4.82 (1H, d, J=7.8 Hz, H-1'), 5.10 (2H, s, H-8), 5.67 (1H, s, H-9), 7.35, 8.16 (2H each, both d, J = 8.4 Hz, *p*-hydroxybenzoyl moiety); ^{13}C-NMR (DMSO-d_6) δ 19.11 (C-10), 22.10 (C-7), 42.13 (C-5), 43.65 (C-3), 61.26 (C-8), 62.21 (C-6'), 70.11 (C-4'), 70.27 (C-6), 73.48 (C-2'), 76.96 (C-3', C-5'), 85.01 (C-2), 87.50 (C-1), 98.68 (C-1'), 100.29 (C-9), 104.80 (C-4), 115.38 (C-3", C-5"), 120.29 (C-1"), 131.60 (C-2", C-6"), 162.17 (C-4"), 165.67 (C-7").

Paeonol (IV)

Recrystallized from 50% ethanol, white needles, m.p. = 49-50°C, ^{1}H-NMR ($CDCl_3$) δ 2.52 (3H, s, $COCH_3$), 3.82 (3H, s, OCH_3), 6.38 (1H, d, J= 2.4Hz, H-3), 6.42 (1H, dd, J = 2.4, 8.8 Hz, H-5), 7.61 (1H, d, J = 8.8 Hz, H-6); ^{13}C-NMR ($CDCl_3$) δ 25.52 ($CO\underline{C}H_3$), 54.92 (OCH_3), 100.23 (C-3), 107.02 (C-5), 117.43 (C-1), 131.75 (C-6), 164.70 (C-2), 165.56 (C-4), 202.03 ($\underline{C}OCH_3$).

Gallic acid (V)

Authentic compound from Sigma Co., yellow powder; ^{1}H-NMR (CD_3OD) δ 6.75 (2H, s, H-2, H-6); ^{13}C-NMR (CD_3OD) δ 110.81 (C-2, C-6), 122.95 (C-1), 139.90 (C-4), 146.80 (C-3, C-5), 171.33 (C=O).

Methyl gallate (VI)

Authentic compound from Sigma Co., recrystallized from acetone, white needles, ^{1}H-NMR (CD_3OD) δ 3.81 (3H, s, OCH_3), 7.05 (2H, s, H-2, H-6); ^{13}C-NMR (CD_3OD) δ 52.66 (OCH_3), 110.51 (C-2, C-4), 121.93 (C-1), 140.20 (C-4), 146.94 (C-3, C-5), 169.49 (C=O).

Quantifications of constituents in the extract

The total polyphenols and tannin content in the extract was determined using the Folin-Ciocalteu (Zheng and Wang, 2001) and Folin-Dennis method (Naczk M. *et al.*, 1992), respectively. The results are expressed as the gallic acid equivalent for the total polyphenols and the tannic acid equivalent for tannin. The following HPLC procedures were used to determine the paeonol, paeoniflorin, gallic acid and methyl gallate content. The HPLC system (Shimadzu, Japan) comprised of a solvent delivery pump equipped with a UV detector and a stainless steel ODS column (250 ×4.6 mm i.d., 5 μm spherical particle, Shimadzu, Japan).

The mobile phases were H_2O: CH_3CN (9:1) at 1.0 mL/min (UV 231 nm) for paeoniflorin, and H_2O : MeOH (98:2) at 1.0 mL/min (UV 372 nm) for gallic acid. Gradient programs were used to identify either methyl gallate or paeonol under the following conditions. For methyl gallate, solvent A: H_2O:HAc (96:4), solvent B : MeOH; flow rate 1 mL/min; gradient conditions, A = 100% and B = 0% at 0 min, A = 70% and B = 30% at 20 min, A = 70% and B = 30% at 40 min; A= 40% and B = 60% at 60 min. For paeonol, solvent A : H_2O, solvent B : acetonitrile; flow rate 1.0 mL/min; gradient conditions, A = 100% and B = 0% at 0 min, A = 20% and B = 80% at 40 min.

Free radical scavenging activity

The free radical scavenging activity was measured using 60 μM DPPH. The absorbance was measured at 520 nm after incubating the test sample at 37°C for 30 min according to the method reported by Fugita *et al.* (1988).

Inhibition of lipid peroxidation

The level of lipid peroxidation inhibition was measured according to the method reported by Ohkawa *et al.*(1979). Each test sample (0.1 mL) and ethyl linoleate (10 μM, Sigma Co.) were added to an incubation medium (4.89 mL) containing 2% sodium dodecyl sulfate, 1 μM ferrous chloride and 0.5 mM hydrogen peroxide. The incubation medium was kept at 55°C for 16 h. Each reaction mixture (0.3 mL) was transferred into a test tube, which was followed by the addition of 4% BHT (50 μL) in order to prevent further oxidation. 1 mL of 0.67% TBA was added to the reaction mixture. The samples were vortexed and incubated at 95°C for 30 min. After cooling, 4 mL of a 15% methanolic butanol solution was added and stirred. The reaction mixture was centrifuged (2,500 rpm) for 10 min and the absorbance of the supernatant was measured at 532 nm. The extent of lipid peroxidation was determined by measuring the quantity of the thiobarbituric acid reactive substance (TBARS). 1,1,3,3-Tetraethoxypropane was used as the standard, and the lipid peroxide concentration is expressed as the amount (μmol) of malondialdehyde (MDA), and is shown as the percentage inhibition of MDA formation.

Pro-oxidant effect

1 mg/mL calf thymus DNA (100 μL) was mixed with 500 μg/mL bleomycin 100 μL, 0.25 mM $FeCl_3$ 100 μL, 50 mM $MgCl_2$ 100 μL, 60 mM KH_2PO_4-KOH buffer (pH 7.0) (500 μL). The reaction was started by adding 100 μL of the sample solution. The tubes were incubated at 37°C for 1 h

followed by the addition of 1 mL of 25% HCl and 1 mL TBA reagent (1 w/v % TBA in 0.05 M NaOH). The tubes were then heated at 100°C for 10 min in order to develop the MDA-TBA chromogen, which was read at 532 nm after cooling (Laughton *et al.*, 1989).

NIH/3T3 fibroblast culture

NIH/3T3 fibroblasts obtained from the American Type Culture Collection were grown as monolayers in DMEM with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin at 37°C under 5% CO_2. The cells were plated at 5×10^4 cells/well in 24-well plates, and then incubated for 30 min with H_2O_2 (10^{-3} M) or H_2O_2 (10^{-3} M) plus the test compounds one day after the initial seeding. The medium was replaced by fresh complete medium. Two hours later, the cells were harvested and subjected to single cell gel electrophoresis.

Single cell gel electrophoresis (comet assay)

The cells were embedded in agarose on frosted microscopic slides according to the procedure reported by Sing *et al.* (1988). 0.65% NMPA in PBS (100 mL) at 65°C was dropped onto the slides, which were then covered with a glass coverslip (18×18 mm, No.1). The cover slip was removed after leaving the slides on ice for 10 min. The cells were mixed with 200 μL of 0.5% LMPA and 50 μL of a cell suspension was immediately pippeted onto the agarose layer on the same slide. After covering with a coverslip, the slide was left on ice for 10 min. A final layer of agarose (100 μL of 0.5% LMPA) was applied in the same manner. The slide without the coverslip was immersed in an ice-cold lysis solution (10 mM Tris, pH 10.0, containing 2.5 M NaCl, 100 mM EDTA, 10% DMSO, and 1% Triton X-100) at 4°C for 1 h. Electrophoresis was carried out in a tank containing 300 mM NaOH, 1 mM EDTA, pH 13.0 for 15 min under 25 V and 300 mA. The slides were then transferred to a 0.4 M Tris buffer (pH 7.5), washed three times and gently dried. Ethidium bromide (2 μg/mL) was dropped onto the gel to stain the DNA. The slides were examined at a X400 magnification using a BH2 fluorescence microscope (Olympus, Japan) equipped with a 20BG-W2 dichromatic mirror (excitation filter: 515 nm, barrier filter: 590 nm). Image analysis was performed using the Komet software (version 3.1, Kinetic Imaging, Liverpool, U.K.) on 50 randomly selected cells. The level of DNA damage was quantified by the increase of the tail moment, which was defined as a product of the comet length and the amount of DNA in the tail according to the original procedure reported by Olive *et al.* (1990).

Micronucleus assay

Working solutions of PRE and $KBrO_3$ were prepared in distilled water. The test material was administered orally once each day for 4 days followed by the $KBrO_3$ treatment intraperitoneally. Blood samples were obtained by piercing the tail blood vessel 24 h after the final $KBrO_3$ treatment. Preparations of the AO-coated glass slides and peripheral blood cells were performed according to the method described elsewhere (Hayashi *et al.*, 1990). The collected blood was placed in the center of an AO-coated glass slide and covered with a 24 mm×40 mm coverslip. The AO supravitally stained reticulocytes (RETs) were examined using fluorescence microscopy with a blue excitation and a yellow barrier filter. The frequencies of the micronucleated reticulcytes (MNRETs) were recorded based on 1,000 RETs per mouse.

Statistical analysis

All the *in vitro* experiments were performed in triplicate. The results are expressed as the arithmetic mean ± S.D. Six mice per group were used for the *in vivo* micronucleus assay, and the data is expressed as the arithmetic mean ± S.E. Statistical evaluation of the data was carried out using a Student's *t*-test.

RESULTS

Fig. 2 shows the contents of the constituents in PREs. In the 70% PRE, the contents were 7.60% tannin, 7.50% paeoniflorin, 7.10% total phenol, 0.75% methyl gallate, 0.36% gallic acid, and 0.06% paeonol. In the 50% PRE, the contents were 7.49% tannin, 6.02% paeoniflorin, 6.71% total phenol, 0.53% methyl gallate, 0.30% gallic acid, and 0.04% paeonol. In the 30% PRE, the contents were 6.75% tannin, 4.23% paeoniflorin, 6.25% total phenol, 0.56% methyl gallate, 0.49% gallic acid, and 0.01% paeonol. The order of abundance of the constituents was tannic acid, total phenols, paeoniflorin, methyl gallate, gallic acid, and paeonol. Their contents except for gallic acid increased

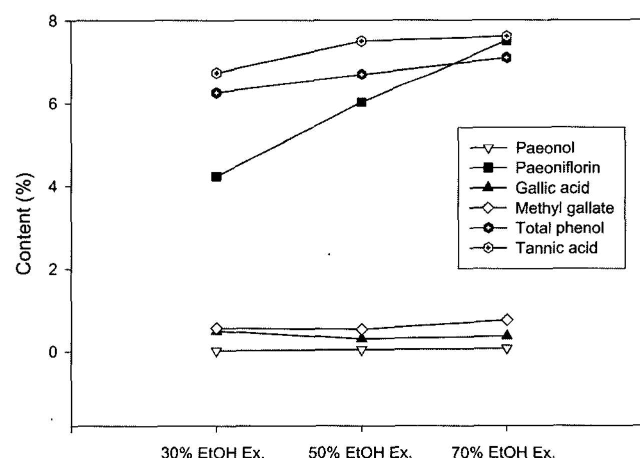

Fig. 2. Content of several constituents in the ethanol extracts of *Paeonia lactiflora*

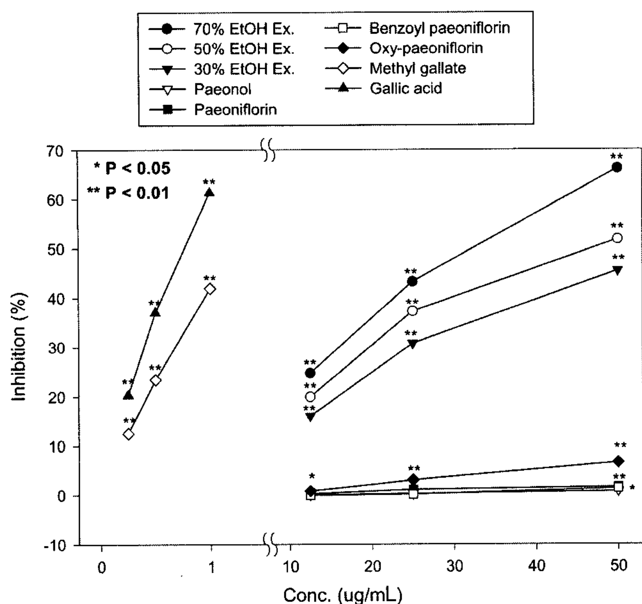

Fig. 3. DPPH free radical scavenging activity of the ethanol extracts of *Paeoina lactiflora* and its major constituents

when the concentration of ethyl alcohol, which was used as the extraction solvent, was increased.

Fig. 3 shows the free radical scavenging activity of PREs and its major components. Compared with the other test samples, both gallic acid and methyl gallate exhibited potent free radical scavenging activity in a concentration dependent manner. All the PREs showed free radical scavenging activity. The IC_{50} values were 0.78 μg/mL for gallic acid, 1.20 μg/mL for methyl gallate, and 34.12 μg/mL, 46.13 μg/mL, 54.70 μg/mL for 70%, 50%, 30% PRE, respectively. Among the three PREs, 70% PRE had the strongest activity. Gallic acid was most potent. The potency of the PREs under these experimental conditions was lower than that of methyl gallate. Oxypaeoniflorin, benzoylpaeoniflorin and paeonol exhibited almost no potency.

Fig. 4 shows that gallic acid and methyl gallate also exhibited inhibitory activity against lipid peroxidation, but the PREs were lower than that of gallic acid and methyl gallate. The IC_{50} values were 68.52 μg/mL for gallic acid, 20.18 μg/mL for methyl gallate. However, the IC_{50} values of the PREs and the other compounds were much higher than that of either gallic acid or methyl gallate. The IC_{50} values of the PREs were 87.50 μg/mL, 160.02 μg/mL, and 317.50 μg/mL for 70%, 50%, and 30%, respectively. Among the three PREs, 70% PRE had the most potent activity. Oxypaeoniflorin, benzoylpaeoniflorin and paeonol did not exhibit any inhibitory activity of lipid peroxidation. These antioxidant profiles were similar to that of the above-mentioned free radical scavenging activities. Fig. 5 shows that the pro-oxidant effect of methyl gallate and the PREs were no higher than the well-known antioxidant, ascorbic acid, but gallic acid had a slightly higher pro-oxidant effect than the PREs.

NIH/3T3 cells were treated with H_2O_2 in order to determine the protective effect of the test materials against DNA oxidative damage. As shown in Fig. 6, the single cell gel electrophoresis assay showed that the gallic acid and methyl gallate treatment resulted in a significant reduction in the tail length in a concentration dependent manner. The IC_{50} values were 69.18 μg/mL for gallic acid and 39.42 μg/mL for methyl gallate. Fig. 6 shows that among the three PREs, 50% PRE gave the highest reduction at both 25 μg/mL and 100 μg/mL. Therefore, 50% PRE was selected to examine the *in vivo* micronucleus assay.

The micronucleus formation against $KBrO_3$-induced oxidative chromosomal damage was examined using mice. 50% PRE, gallic acid, and methyl gallate were administered orally for 4 days, which resulted in the potent

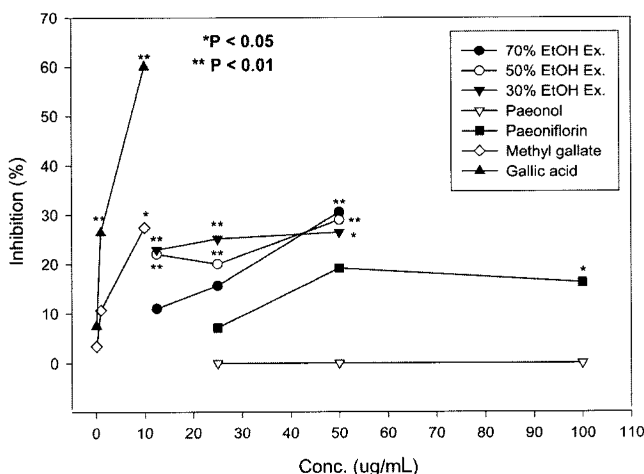

Fig. 4. Inhibition of lipid peroxidation by the ethanol extracts of *Paeonia lactiflora* and its major constituents

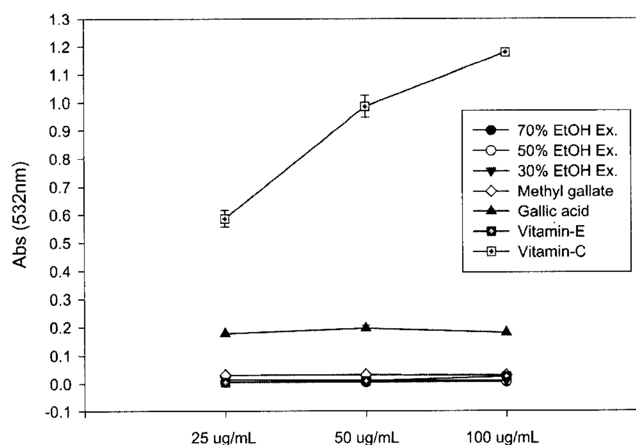

Fig. 5. Pro-oxidant effect of the ethanol extracts of *Paeonia lactiflora* and its major components and other well-known antioxidants

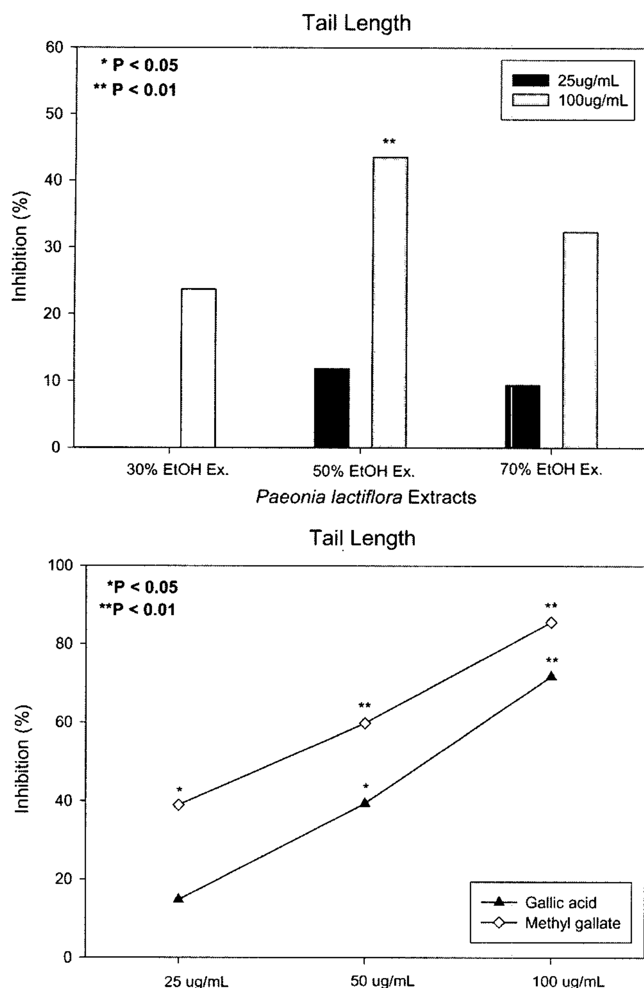

Fig. 6. Protective effect of the ethanol extracts of *Paeonia lactiflora*, gallic acid and methyl gallate on $H_2O_2(10^{-3}$ M)-induced DNA damage using the comet assay

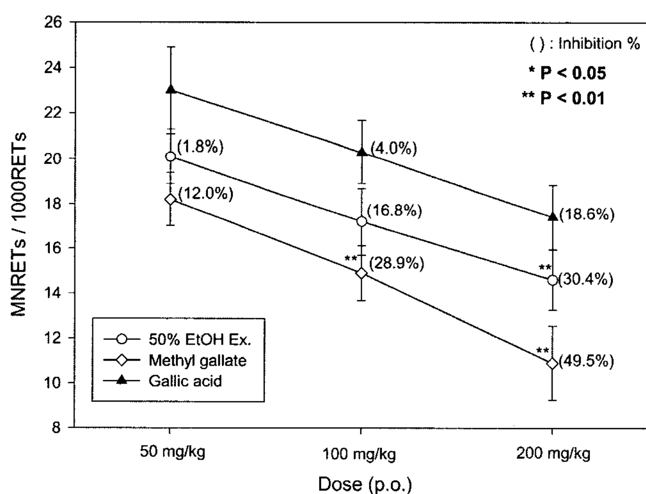

Fig. 7. Inhibition of 50% ethanol extract of *Paeonia lactiflora*, methyl gallate and gallic acid against the $KBrO_3$-induced micronuclei in the mouse peripheral reticulocytes. Bars mean ± S.E.

reduction of oxidative DNA damage against $KBrO_3$, as expressed by the frequency of MNRETs formation from the mouse peripheral blood (Fig. 7). Methyl gallate had a stronger activity than gallic acid. This tendency was similar to the inhibitory activity against DNA damage in the comet assay, but 50% PRE had a stronger effect than gallic acid.

DISCUSSION

The action mechanism involved in the protection of DNA and chromosome levels against oxidative stress by the constituents or ethanolic extracts of *Paeonia lactiflora* is not well described and it is not known whether there is any relationship with the ability to protect the oxidative stress and genotoxicity between the constituents and ethanolic extracts of *Paeonia lactiflora*. The aim of the present study was to compare the effects of ethanolic extracts of *Paeonia lactiflora*, gallic acid, methy gallate, and other constituents with respect to show the antigenotoxic antioxidant, assessed as DPPH free radical scavenging activity, lipid peroxidation inhibition and protection to H_2O_2 induced DNA damage in NIH3T3 cells and to $KBrO_3$ induced chromosomal damage in mice as well as to establish the proper extract of *Paeonia lactflora* as a natural antigenotoxic antioxidant.

A radical scavenging assay using DPPH was employed to evaluate the antioxidant activity *in vitro.* This is because radical trapping is one of the most important properties of the chain-breaking types of antioxidant (Niki, 1987). We found that the three PREs showed moderately potent free radical scavenging activity, and among them, the 70% ethanolic extract had the highest activity. However, its activity was less potent than either gallic acid or methyl gallate. The order of activity was gallic acid, methyl gallate, 70%, 50%, and 30% ethanolic extract. Oxypaeoniflorin exhibited slight activity, but paeonol, paeoniflorin, and benzoyl paeoniflorin exhibited the lowest activity. Therefore, the PREs might be anti-radical materials from natural plant products.

A possible target of radical oxidation not only in food but also in living cells is polyunsaturated fatty acids. The oxidation of polyunsaturated fatty acids can be used as a model to investigate the efficacy of polyphenols as chain breaking antioxidants (Rice-Evan *et al.*, 1996). The anti-oxidant activities of the PREs were examined using linoleic acid as the oxidation substrate. The PREs had a lower inhibitory effect on lipid peroxidation than gallic acid and methyl gallate. The order of activity obtained in this system was in good agreement with the data in the DPPH system. Gallic acid and methyl gallate also exhibited strong antioxidant activity. These results confirmed the reports by several investigators of the strong activity of

gallic acid on both DPPH radical scavenging and the inhibition of lipid peroxidation (Galato *et al.*, 2001; Schlesier *et al.*, 2002; Sroka and Cisowski, 2003). Therefore, PREs, which contain gallic acid and methyl gallate, could act as an inhibitor of lipid peroxidation with free radical scavenging activity.

Damage to DNA in the presence of a bleomycin-Fe complex has been used as a sensitive and specific method for examining potential pro-oxidant agents (Miranda *et al.*, 1989). As shown in Fig. 5, PREs, methyl gallate, and vitamin E, did not have any pro-oxidant effect at concentrations < 100 μg/mL, However, vitamin C had a pro-oxidant effect in a concentration-response manner. These results are similar to the report showing that vitamin C tends to act as a pro-oxidant (Ng *et al.*, 2000). Gallic acid had a slightly higher pro-oxidant effect than the PREs, but this effect was not higher than that observed for vitamin C. It was also reported that gallic acid has pro-oxidant action (Okezie *et al.*, 1993) and causes oxidative protein and DNA damage at concentrations > 1 μM (Labieniec *et al.*, 2005). Therefore, it appears that the PREs exhibited antioxidant activity without any pro-oxidant activity, whereas vitamin C and gallic acid have a pro-oxidant effect at the same concentration tested. The present data suggest that PREs did not show any pro-oxidant effect because these plant extracts contain lots of other antioxidative polyphenolic compounds beside gallic acid.

In addition, the PREs, gallic acid and methyl gallate also had a protective activity against oxidative DNA damage in the NIH/3T3 cells, as measured by the comet assay. The comet assay has been widely used in genetic toxicology research. The cellular mechanism to reduce the level of DNA damage is unclear. However, it is possible that PRE containing both gallic acid and methyl gallate may protect against DNA strand breakage induced by oxygen free radicals, because the comet assay detects DNA breaks in a single cell (Niki and Noguchi, 2000; Festa *et al.*, 2001). It was reported that gallic acid had an the inhibitory effect on the DNA strand breaks of human lymphocytes induced by hydrogen peroxide (Wu *et al.*, 2004), and also reduced the level of formamidopyrimidine-DNA glycosylase (FPG) and endonuclease III (Endo III) sensitive sites when the DNA repair enzyme, FPG and Endo III were used to examine the levels of oxidized pyrimidines and purines in the human lymphocytes induced by H_2O_2 (Wu *et al.*, 2004). The suppression of oxidative DNA damage by the peony root was attributed to the most active compound being galloyl-paeoniflorin. The paeoniflorin, oxypaeoniflorin, albiflorin, and caechin had little effect on the DNA cleavage using by *tert*-butyl hydroquinone (Okubo *et al.*, 1997). From these results and previous reports, PREs may be good source of antigenotoxic antioxidants and can protect against oxidative damage to the cellular DNA.

Several studies have shown the formation of micronuclei induced by $KBrO_3$ both *in vitro* (Robbino *et al.*, 1999; Sugisawa *et al.*, 2004) and *in vivo* (Sai *et al.*, 1992). The exposure of $KBrO_3$ *in vivo* provoked oxidative DNA and chromosomal damage partly through the formation of hydroxyl and NO radicals (Watanabe *et al.*, 2002). 50% PRE, gallic acid, and methyl gallate greatly reduced the *in vivo* formation of MNRETs induced by $KBrO_3$ when administered orally. The PREs, gallic acid, and methyl gallate also exhibited antigenotoxic activity against oxidative stress *in vivo*. Gallic acid prevented the H_2O_2-induced chromosomal damage in a dose-dependent manner with a significant effect detected at 1 μM using an *in vitro* cytokinesis-block micronucleus assay (Sugisawa *et al.*, 2004). Moreover, a co-treatment with well-known antioxidants, glutathione or cysteine, reduced the $KBrO_3$ induced micronuclei in rats (Sai *et al.*, 1992). One possible explanation for the inhibition of chromosomal damage 50% PRE is that this plant extract as well as its related polyphenolic compounds can reduce lipid peroxidation caused by scavenging free radical species (Kitagawa *et al.*, 1979; Yoshikawa *et al.*, 1992; Kang *et al.*, 1993).

This study showed in several *in vitro* and *in vivo* experiments that PREs and its major components, gallic acid and methyl gallate, possessed significant antioxidant activity. The mechanism of their antioxidant activity might involve the direct inhibition of the generation of reactive oxygen species, or the scavenging of free radicals. These results clearly show the antioxidant activity of PREs, which is due, at least in part, to their components, gallic acid, methyl gallate and other polyphenolic compounds. We found that the 50% PRE among PREs tested showed that the most antigenotoxic antioxidant activity without pro-oxidant effect. This study provides the scientific rationale of using the peony root as a natural antigenotoxic antioxidant. Further investigations will be needed to evaluate the value of PREs treating various conditions that involve oxidative stress as well as to characterize the active compounds in detail.

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