

The root extract of *Paeonia lactiflora* Pall inhibits the oxidative damage via its anti-oxidant activity

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ABSTRACT

Objectives : Reactive oxygen species (ROS) have been associated with pathogenic processes including carcinogenesis through direct effect on DNA directly and by acting as a tumor promoter. Therefore, it has been regarded that ROS may be a major target for cancer prevention. The root of *Paeonia lactiflora* pall (PL), a traditional Chinese herb, has been a component of effective prescriptions for treatment of liver disease. Also, there are some reports about the antioxidant activities of the extracts from PL. However, little has been known about the effects of PL against oxidative damage. This work aimed to elucidate the anti-oxidant effects of *Paeonia lactiflora* pall (PL) in the non-cellular system and cellular system.

Methods : Antioxidant activities of PL were evaluated by hydroxyl radical scavenging assay and Fe²⁺ chelating assay. Anti-oxidative effect of PL was evaluated by ϕ X-174 RF I plasmid DNA cleavage assay in non-cellular system. In addition, DNA migration assay, expression level of phospho-H2AX, MTT assay and lipid peroxidation assay were performed for evaluate the anti-oxidative effect of PL in cellular system.

Results : PL had a dose-dependent hydroxyl radical scavenging and Fe²⁺ chelating capacity. In addition, PL inhibited oxidative DNA and cell damage induced by hydroxyl radical in non-cellular system and cellular system.

Conclusion : Taken together, *P. lactiflora* pall may be possible for the application to a potential drug for treating the oxidative diseases such as cancer.

Key words : Anti-oxidant activity, Oxidative DNA damage, Oxidative cell damage, *Paeonia lactiflora* Pall

Introduction

Reactive oxygen species (ROS) have been associated with pathogenic processes including carcinogenesis through direct effect on DNA directly and by acting as a tumor promoter¹⁾. Radicals have been demonstrated to be initiators of the oxidative process²⁾ and to be involved in the development of disease such as cancer³⁾. Catalase, superoxide dismutase, glutathione and uric acid are examples of antioxidants produced by organisms under normal conditions as part of defense system against ROS-mediated cellular injury. However, if this defense system is challenged or

overwhelmed by excessive generation of ROS, redox imbalance or oxidative stress may occur. This can result in damage to the organism and disease initiation⁴⁾. ROS have also been shown to play an important role in carcinogenesis by damaging DNA and acting as tumor promoters¹⁾. The health promoting effect of antioxidants from plants is thought to arise from their potential effects on the reactive oxygen/nitrogen species. In addition, antioxidants have been widely used in food industry to prolong the shelf life. However, there is widespread agreement that some synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene need to be replaced with natural antioxidants due to

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their potential health risks and toxicity⁵).

Medicinal plants have been used to treat various human diseases in the East for centuries because of their good therapeutic performance and low toxicity⁵. The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs.

Paeonia lactiflora pall (PL) root, a traditional Chinese herb, has been a component of effective prescriptions for treatment of liver disease⁶. Also, there are some reports about the antioxidant activities of the extracts from PL⁵. However, little has been known about the functional role of PL against oxidative damage.

Therefore, it is important to understand the inhibitory mechanism of the extracts from PL against oxidative damage since these is a hallmark of many tumors and ROS-mediated signaling and genomic instability contributing to the initiation and progression of cancer

Materials and Methods

1. Sample preparation

Paeonia lactiflora pall (PL) root, a traditional Chinese herb, was kindly provided by the Bonghwa Alpine Medicinal Plant Experiment Station, Korea. One kilogram of the root of PL was extracted with 2L of 80% methanol with shaking for 24 hours. After 24 hours, Extract with 80% methanol was filtered, concentrated to approximately 400 ml volume using by a vacuum evaporator, and fractioned with ethyl acetate in a separating funnel. Aqueous fraction (32 g, yield rate: 3,2%) after fractionation with ethyl acetate was freeze-dried and kept at -70°C until use.

2. Hydroxyl radical scavenging activity

Hydroxyl radical scavenger ability was measured according to a literature procedure⁷ with a few modifications. Hydroxyl radical was generated from Fenton reaction between 1,5 mM FeSO₄ and 6 mM H₂O₂ (1,4:1, v/v) at 37 °C for 30 min before the assay and detected by their ability to hydroxylate salicylate. The reaction mixture (1 ml) contained 760 μl of hydroxyl radical, 40 μl of varying concentrations of the extracts (0,125, 0,25, 0,5, 1 and 2 mg/ml) and 300 μl of sodium salicylate (20 mM). After a reaction for 30 min at 37 °C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. Hydroxyl radical scavenger ability was calculated from the log-dose inhibition curve. All determination was carried out in triplicate. Ascorbic acid was used as a positive control.

3. Fe²⁺ chelating activity

This assay was measured according to a literature procedure⁸ with a few modifications. The reaction mixture (800 μl) contained 120 μl of 2 mM FeCl₂, 40 μl of varying concentrations of the extracts (0,125, 0,25, 0,5, 1 and 2 mg/ml) and 640 μl of distilled water. The mixture was shaken vigorously and left at room temperature for 5 min. After 5 min, 200 μl of 5 mM ferrozine was added and mixed. The absorbance of the Fe²⁺-ferrozine complex was measured at 562 nm. Fe²⁺-chelating activity assay was calculated from the log-dose inhibition curve. All determination was carried out in triplicate. Deferoxamine was used as a positive control.

4. ϕ X-174 RF I plasmid DNA cleavage assay

Conversion of the supercoiled form of plasmid DNA to the open-circular and further linear forms has been used as an index of DNA damage⁹. For DNA cleavage assay by hydroxyl radical and ferrous iron, reaction mixtures (90 μl) contained 10 μl of ϕ X-174RF I plasmid DNA, 4 μl of varying concentrations of the extracts (0,25, 0,5, 1 and 2 mg/ml), 76 μl of hydroxyl radical generated from Fenton reaction between 250 μl of 1,5 mM FeSO₄ and 175 μl of 6 mM H₂O₂. The mixtures were incubated at 37 °C for 30 min. For DNA cleavage assay by ferrous iron, The mixtures contained 4 μl of varying concentrations of the extracts, 12 μl of 2 mM FeCl₂, 64 μl of distilled water and 10 μl of ϕ X-174 RF I plasmid DNA. The mixtures were incubated at 37 °C for 30 min. After the reaction for 30 min, 10 μl of a solution containing 50% glycerol (v/v), 40 mM EDTA and 0,05% bromophenol blue was added to stop the reaction and the reaction mixtures was electrophoresed on 1% agarose gel. The DNA in the gel was visualized and photographed under ultraviolet light after ethidium bromide staining.

5. Cell culture

NIH 3T3 cell, mouse fibroblast cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Cell was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml of penicillin, 100 lg/ml of streptomycin and 10% fetal bovine serum. The cells were incubated in an atmosphere of 5% CO₂ at 37 °C. In all experiments, cells were grown to 80-90% confluence and subjected to no more than 20 cell passages.

6. Intracellular DNA migration assay

NIH3T3 cells (2×10^6) were cultured in 6-well

plates for 24 hours at 37 °C in an incubator with a humidified atmosphere of 5 % CO₂. The cells were treated with the varying concentration of the extracts (0.5, 1 and 2 mg/ml) for 30 min and then added with the total concentration of 1 mM FeSO₄ and 1 mM H₂O₂ for 24 hours. Each cell was harvested and after the centrifugation at 1250 rpm, the supernatant was discarded. Each cell was resuspended with 20 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS and 0.5 mg/ml proteinase K) by pipetting cells to ensure complete lysis and then incubated at 55 °C for 60 min. After the centrifugation, 5 µl of RNase A was added to the supernatant and each cell was incubated at 55 °C for another 60 min. After 60 min, each cell was spun briefly to remove any further cell debris and collect the supernatant. Each lysate was heated at 70 °C for a few minutes and mixed with 10 µl of loading buffer (50% glycerol (v/v), 40 mM EDTA and 0.05% bromophenol blue). The reaction mixtures were electrophoresed on 2% agarose gel, and the DNA in the gel was visualized and photographed under ultraviolet light after ethidium bromide staining.

7. Lipid peroxidation assay

This assay was carried according to literature procedure¹⁰ with some modification. NIH3T3 cells were cultured in a 6-well plate at 2 × 10⁶ cells/well for 24 hours. Twenty four hours after plating, the cells treated with the varying concentrations of extract (0.5, 1 and 2 mg/ml) for 30 min. After 30 min, the total concentration of 1 mM H₂O₂ and 1 mM FeSO₄ was added to the plate and incubated for 24 hours. The cells were then washed with cold phosphate-buffered saline (PBS), harvested, and homogenized in an ice-cold 1.15% KCl. One hundred microliters of the cell lysates was mixed with 0.1 ml of 8.1% sodium dodecylsulfate, 0.75 ml of 20% acetic acid (adjusted to pH 3.5), and 0.75 ml of 0.8% thiobarbituric acid (TBA). The mixture was made up to a final volume of 4 ml with distilled water and heated to 95 °C for 2 hours. After cooling to room temperature, 2.5 ml of an n-butanol/pyridine mixture (15:1, v/v) was added and the mixture was shaken. After centrifugation at 1000 g for 10 min, the supernatant fraction was isolated and the absorbance was measured spectrophotometrically at 532 nm.

8. MTT assay

NIH3T3 cells (5 × 10³ cells/well) were cultured in 96-well plate at 37 °C for 24 hours. After 24 hours, the varying concentrations of the extracts (0.5, 1 and

2 mg/ml) were treated to each well, and then incubated at 37 °C for 30 min. After 30 min, the total concentration of 1 mM H₂O₂ and 1 mM FeSO₄ was applied to each well and then incubated at 37 °C for 24 hours. After 24 hours, 50 µl of MTT solution (1 mg/ml) was treated to each well for 4 hours, the supernatant was removed, and then 100 µl of DMSO was treated to each well. The absorbance was measured with a microplate reader at 570 nm.

9. Western blot of phospho-H2A.X

NIH3T3 cells (2 × 10⁶ cells/well) were cultured in 6-well plate at 37 °C for 24 hours. After 24 hours, the varying concentrations of the extracts (0.5, 1 and 2 mg/ml) were treated to each well, and then incubated at 37 °C for 30 min. After 30 min, the total concentration of 1 mM H₂O₂ and 1 mM FeSO₄ was applied to each well and then incubated at 37 °C for 24 hours. After 24 hours, each cell was harvested for the analysis. The cells were lysed with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM DTT) containing 1 % Triton X-100. Insoluble debris was removed by centrifugation at 15,000 g for 15 min three times. The protein extracted from the cells was separated on 15 % Tris-HCl ready gel (Bio-rad, Hercules, CA) following the manufacturer's instruction. Gels were transblotted on to PVDF membranes for western blot analysis. The membranes were blocked for non-specific binding for 90 min in block buffer (5 % non-fat milk and 0.1 % Tween 20 in 1 × Tris-buffered saline [TBS]) and then washed with 1 × TBS solution (0.1% Tween 20 in 1 × TBS). After washing with 1 × TBS solution, the membrane was incubated with phospho-H2A.X at 1:1000 dilutions in antibody dilution buffer (5% BSA, 1 × TBS, 0.1% Tween 20) with gentle shaking at 4 °C for 16 hours and then washed with 1 × TBS solution. After washing, the membranes were incubated with Phototope-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody as the secondary antibody at 1:1000 dilutions in antibody dilution buffer (5% non-fat milk and 0.1% Tween 20 in 1TBS) for 1 h and then washed again. After washing, the membranes were treated with the detection agent (Amersham Biosciences) and immediately developed in Polaroid film.

10. Immuno-staining for phospho-H2A.X foci

NIH3T3 cells (5 × 10⁴) were cultured in 6-well plates with the cover glass for 24 hours at 37 °C. After 24 hours, the varying concentrations of water

extract (0.5 and 2 mg/ml) were treated to each well, and the cells were incubated at 37 °C for 30 min. After 30 min, the total concentrations of 1 mM H₂O₂ and 1 mM FeSO₄ were applied to each well and the cells were incubated at 37 °C for 24 hours. After 24 hours, the cells were fixed with 2% formaldehyde for 30 min and then washed with 1×PBS for 5 min three times. PBS with 10% fetal bovine serum (PBS/FBS) was then added to block non-specific binding for an hour. After washing, anti phospho-H2A,X polyclonal anti-body was diluted in 0.1% saponin/PBS/FBS solution at 1:500 dilutions for an hour, and then washed with 1×PBS for 5 min three times. Subsequent antibody, Alexa-Flour 488 goat anti-rabbit IgG, and DAPI were used against phospho-H2A,X antibody and nuclei, respectively and the cells were incubated in the dark for an hour. Then, the cells were washed with 1×PBS and mounted with antifade mounting medium. Mounted slides were viewed under a fluorescence microscope using a 60×oil immersion objective. The excitation wavelengths for DAPI and phospho-H2A,X were 359nm and 494nm, respectively.

11. Statistical analysis

All results were expressed as the mean ± the standard deviation of triplicate analysis. Statistical analysis was performed with the Student's unpaired t-test, with statistical significance set at *, P < 0.05.

Results

1. Antioxidant activities of PL extract

Antioxidant activities of ethyl acetate and aqueous fractions were evaluated by hydroxyl radical scavenging assay and Fe²⁺ chelating assay. In hydroxyl radical scavenging assay (Fig. 1A), aqueous fraction removed hydroxyl radical by 26% at 0.5 mg/ml, 84% at 1 mg/ml and 93% at 2 mg/ml. Ascorbic acid used as the positive control removed it by 57% at 0.125 mg/ml, 93% at 0.25 mg/ml, 97% at 0.5 mg/ml and 1 mg/ml, and 100% at 2 mg/ml. Although hydroxyl radical scavenging activity of the extract was lower than that of ascorbic acid, it showed a strong Fe²⁺ chelating activity.

In Fe²⁺ chelating assay (Fig. 1B), aqueous fraction chelated Fe²⁺ ion by 87% at 0.125 mg/ml, 95% at 0.25 mg/ml, 97% at 0.5 mg/ml and 1 mg/ml and 98% at 2 mg/ml. Deferoxamine used as the positive control chelated it by 37% at 0.125 mg/ml, 84% at 0.25 mg/ml and 99% at 0.5 mg/ml~2mg/ml.

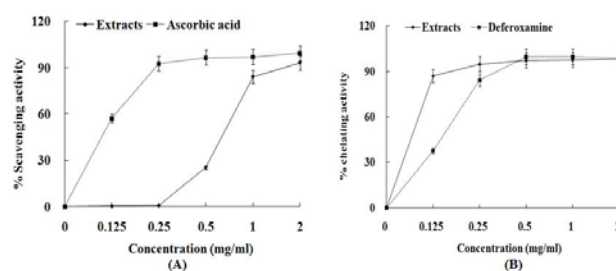


Fig. 1. Hydroxyl radical scavenging activity (A) and Fe²⁺ chelating activity (B) of PL extract. The absorbance values were converted to scavenging activity or chelating activity (%) and the data plotted as the mean of replicate scavenging effects or chelating effects (%) values ± 1 S.D. (n=3) against extract concentration in mg extract per ml reaction volume. Ascorbic acid and deferoxamine were used as the positive control.

2. Protective effect of PL extract against oxidative damage in non-cellular system

The plasmid DNA cleavage assay using ϕ X-174 RF I plasmid DNA was used as an initial approach toward determining whether aqueous fraction with antioxidant activity could inhibit oxidative DNA damage induced by hydroxyl radical or Fe²⁺ ion. Conversion of the supercoiled form (SC) of plasmid DNA to the open-circular form (OC) and further linear form (LC) has been used as an index of DNA damage⁹.

It had no inhibitory effect on DNA damage induced by hydroxyl radical at the low concentrations (0.25 mg/ml and 0.5 mg/ml) while aqueous fraction (1 mg/ml and 2 mg/ml) inhibited DNA damage (Fig. 2A). But, aqueous fraction (0.25 mg/ml ~ 2 mg/ml) inhibited DNA damage induced by Fe²⁺ ion (Fig. 2B).

For the elucidation of the main mechanism for inhibitory effect on DNA damage at the low concentration (0.5 mg/ml) (Fig. 2C), the treatments were divided into three groups. In the first group, water extract was reacted with hydroxyl radical and DNA together for 30 min at 37 °C (lane c). In the second group, it was firstly reacted with Fe²⁺ ion for 10 min at the room temperature and then re-reacted with H₂O₂ and DNA together for 30 min at 37 °C (lane d). And in the third group, it was firstly reacted with H₂O₂ at 10 min at the room temperature and then re-reacted with Fe²⁺ ion and DNA together for 30 min at 37 °C (lane e). In this result, the simultaneous reaction of the extract with hydroxyl radical (lane c) and the pre-reaction with H₂O₂ (lane e) had no inhibitory effect on DNA damage, while the pre-reaction with Fe²⁺ ion inhibited the oxidative DNA damage by 90% (lane d). This result suggests that the extract may protect DNA from the oxidative damage by inhibiting the generation of hydroxyl radical through the blocking of Fenton reaction via Fe²⁺ ion chelation.

3. Protective effect of PL extract against oxidative damage in cellular system

The inhibitory effect of the extract against oxidative DNA damage was evaluated by intracellular DNA migration and the expression level of phospho-H2A,X in the cellular system. DNA migration assay is a sensitive biomarker of the DNA damage. The extract inhibited DNA migration induced by hydroxyl radical in a dose-dependent manner (Fig. 3A). In the expression level of phospho-H2A,X, a sensitive marker for breaks of double stranded DNA¹¹, the treatment of the extract in presence of H₂O₂ and FeSO₄ reduced the expression of phospho-H2A,X by 32% at 0.5 mg/ml, 53% at 1 mg/ml and 72% at 2 mg/ml, compared with the cells treated with H₂O₂ and FeSO₄ in absence of the extract (Fig. 3B). This result was confirmed by changes of phospho-H2A,X foci into the nuclear in immuno-staining (Fig. 3C). Therefore, the results of the present study indicate that PL can inhibit the oxidative DNA damage induced by excessive ROS. Protective effect of the extract against oxidative cell damage was evaluated by MTT assay and lipid peroxidation assay. In MTT assay (Fig. 3D), hydroxyl radical generated from Fenton reaction between H₂O₂ and FeSO₄ induced the oxidative cell death by 54%, compared with the untreated cells. However, the treatment of water extract in presence of H₂O₂ and FeSO₄ reduced the oxidative cell death, compared with the cell treated with H₂O₂ and FeSO₄ in absence of the extract. It is thought that this result correlates with the inhibitory effect of the extract against lipid peroxidation. In the lipid peroxidation assay (Fig. 3E), the extract inhibited the lipid peroxidation by 39% at 0.5 mg/ml, 69% at 1 mg/ml and 97% at 2 mg/ml compared with the cells treated with H₂O₂ and FeSO₄. From this result, it is thought that the extract from PL displays a significant protective capability against the oxidative cell damage.

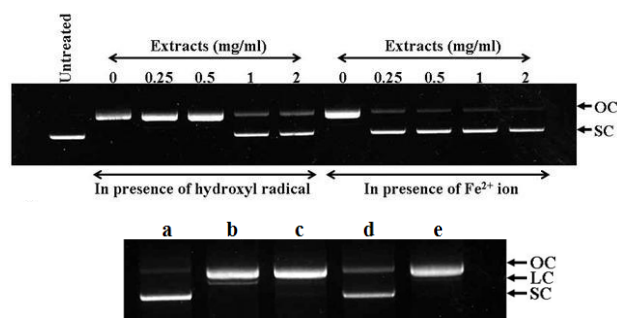


Fig. 2. Protective effect of PL extract against oxidative DNA damage in non-cellular system, (A) treatment of hydroxyl radical

in presence of aqueous fraction; (B) treatment of Fe²⁺ ion in presence of aqueous fraction; (C) Lane a was the normal DNA without hydroxyl radical and aqueous fraction. Lane b was treated with hydroxyl radical alone without the aqueous fraction. In the lane c, aqueous fraction was reacted with hydroxyl radical and DNA together for 30 min at 37 °C. In the lane d, aqueous fraction was firstly reacted with Fe²⁺ ion for 10 min at the room temperature, and then re-reacted with H₂O₂ and DNA together for 30 min at 37 °C. In the lane e, aqueous fraction was firstly reacted with H₂O₂ at 10 min at the room temperature, and then re-reacted with Fe²⁺ ion and DNA together for 30 min at 37 °C. In this assay, hydroxyl radical was generated from Fenton reaction between H₂O₂ and FeSO₄. Iron was generated from FeCl₂.

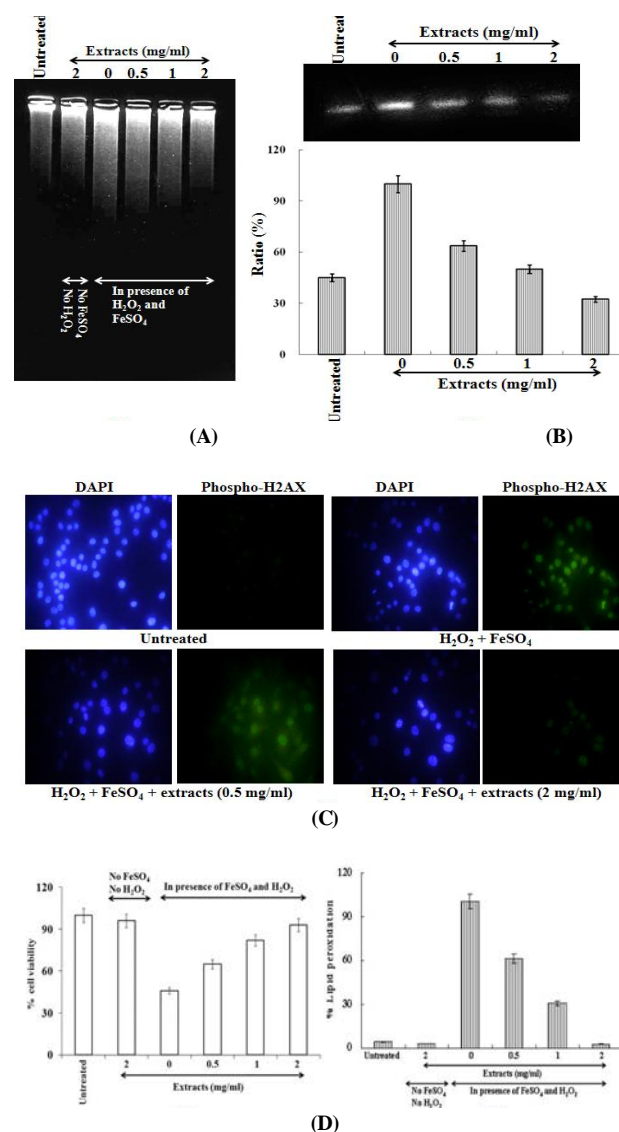


Fig. 3. Protective effect of PL extract against oxidative DNA and cell damage in the cellular system. The protective effect was evaluated by intracellular DNA migration assay (A), western blot for the level of phospho-H2AX expression (B), immuno-staining for the formation of phospho-H2AX foci (C), MTT assay (D) and lipid peroxidation assay (E). In western blot, each well was loaded with 25 µg protein. % ratio of the expression level of phospho-H2A,X was calculated by the density using the software Un-SCAN-IT gel Version 5.1 (Silk Scientific, Inc.). In MTT assay and lipid peroxidation assay, the absorbance values were converted to cell viability (%) and inhibitory effects (%), and the data plotted as the mean of replicate inhibitory effects (%) values ± 1 S.D. (n=3).

Discussion

Among ROS, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules¹²⁾. Hydroxyl radical scavenging is an important antioxidant activity because of very high reactivity of hydroxyl radical which enables it to react with a wide range of molecules found in living cells such as sugars, amino acids, lipids and nucleotides¹³⁾. Iron is essential transition metal elements in the human body required for the activity of a large range of enzymes and for some proteins involved in cellular respiration, O₂ transport and redox reactions. Unfortunately, they contain unpaired electrons that enable them to participate in one-electron transfer reactions. Hence, they are powerful catalysts of autoxidation reactions¹⁴⁾. Antioxidant factors can be divided into two groups such as scavenging of radicals formed during reaction and inhibiting the radical generation. From these results, its hydroxyl radical scavenging activity demonstrates the effectiveness against biologically generated radicals. Moreover, its Fe²⁺ chelating activity demonstrates the effectiveness for inhibiting the radical generation. Therefore, it suggests that PL extract can reduce ROS damage by scavenging the generated radical and inhibiting the radical generation.

The most important target for ROS in the carcinogenesis process is DNA¹⁵⁾. When present in a high enough concentration, these compounds can damage cellular proteins and DNA, or form DNA adducts that may promote carcinogenic activity¹⁶⁾. This DNA damage induced by hydroxyl radical has been shown to play a key role in the carcinogenesis¹⁷⁾. In addition, a possible mechanism of oxidative DNA damage induced by metals is thought to be the formation of hydroxyl radicals through Fenton-type reduction with hydrogen peroxide, which is naturally present in cells¹⁸⁾. Therefore, the inhibition of oxidative DNA damage is important to a chemoprevention of the carcinogenesis. In this study, we found that PL extract protected oxidative DNA damage in non-cellular and cellular system.

ROS damage involves injury to cellular membranes. Measurement of lipid peroxidation is used as an indicator or membrane damage in mammalian cells, resulting in the oxidative cell death. Lipid peroxidation can cause a cascade effect of lipid-derived radicals, thereby causing additional membrane damage. The products of lipid peroxidation, malondialdehyde and other group of aldehyde products may also cause DNA damage. It has also been proposed that free radicals derived from lipid peroxidation may be an important cause of cell membrane destruction and cell damage and function

as tumor initiator¹⁹⁾. In this study, we found that PL extract protected oxidative cell damage in cellular system.

Conclusion

In summary, the results of the present investigation indicate the below.

1. Aqueous fraction from PL scavenged hydroxyl radical and chelated Fe²⁺ ion
2. Oxidative DNA damage from hydroxyl radical produced by fenton reaction was inhibited by aqueous fraction from PL
3. Oxidative cell damage induced by exposure to hydroxyl radical was inhibited by aqueous fraction from PL.

In conclusion, these inhibitory effects on oxidative damage via its antioxidant activity may further explain that *Paeonia lactiflora* pall may be possible for the application to a potential drug for treating the oxidative diseases such as cancer.

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