



Rhus semialata M. extract ameliorate *para*-phenylenediamine-induced toxicity in keratinocytes

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ABSTRACT

para-Phenylenediamine (PPD), a major component of hair dyeing ingredients, can induce allergenic sensitization and exert mutagenic, tumorigenic and cytotoxic effect. In this study, we determined the cytotoxic effect of PPD on human keratinocytes and evaluated the protective effect of *Rhus semialata* M. extracts (RSE) on PPD induced cytotoxicity for the first time. We observed that RSE is a strong inhibitory agent against PPD-induced toxicity in human keratinocytes. The results indicated that RSE pretreatment significantly could suppress PPD induced cytotoxic effects, including decrease of cell viability, accumulation in subG1 phase of cells, and relocation of phosphatidylserine on keratinocytes. Also, we found that PPD caused cytotoxicity was associated with mitochondrial membrane potential loss and subsequent activation of caspase and PARP degradation. However, pretreatment of RSE showed preventive activities against PPD induced mitochondrial membrane potential loss and ROS production in keratinocytes. In conclusion, the results of present study suggest that RSE was able to protect the skin from several cytotoxic effects of PPD and could be a meaningful material in many industries using PPD.

1. Introduction

para-Phenylenediamine (PPD), aromatic amine compound, is widely used in the industrial manufactures. For example, as an ingredient in hair dye, dark henna dye, and leather dye, or as a precursor to polymers including aramid plastics and Kevlar for textile, or as an antiozonants for production of rubber products [1]. Many people dye their hair to enhance attractiveness and to look younger. In previous research, 50.9 % of individuals had experienced hair coloring at least once during their entire lifetime, and among them 81.4 % were female in Europe [2]. Previous surveys conducted in Korea have suggested that more than 50 % of the population who have gray hair are in their early 40 s and are in the process of aging. Most of them continue to dye their hair periodically, despite side effects [3]. Permanent hair dye containing PPD comprise more than 70 % of the hair dye market share in Europe, United States, and East Asia [4,5].

Dye produced during the hair dyeing procedure, is formed through chemical reaction in hair shafts. Under oxidation condition, a precursor agent such as PPD reacts with a coupler agent such as resorcinol, to produce a colored polymerized compound with high molecular weight [6]. Conversely, in the process of PPD oxidation Bandrowski's base (BB) well known as mutagenic compounds inducing allergic reaction, also could be formed by autooxidation in the presence of air, or by promoting the oxidation reaction with oxidants including hydrogen peroxide [7,8]. Although, skin functions as the first line of defense system against toxic chemicals and foreign substances, PPD exposed through skin epidermal layer can be absorbed by percutaneous absorption during hair dyeing or a tattooing process, resulting in allergic contact dermatitis [9–12]. Additionally, many previous studies reported that PPD possessed mutagenic, cytotoxic and carcinogenic effects on human organs including liver, kidney and bladder [13–18]. Despite the cytotoxic effect of PPD, hair dye products are sold directly to untrained customers for

Abbreviations: PPD, *para*-Phenylenediamine; RSE, *Rhus semialata* M extracts; ROS, Reactive oxygen species; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; PI, Propidium iodide; MTT, 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; DMSO, Dimethyl sulfoxide; DiOC₆, 3,3'-dihexyloxycarbocyanine iodide; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate.

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self-application over the counter. Some case studies reported that PPD was used as a means of ideal suicidal poison because of easy availability in households, and low cost in India and Sudan [19,20]. *Rhus semialata* M., synonym of *Rhus chinensis* Mill, is widely distributed in China, Korea and India. Galls produced on the plant are known treatment for cough, diarrhea, dysentery and uterine bleeding. However, other parts of the plant including stems, leaves, fruits, and roots are also used as traditional medicines as well as a functional ingredient in foods [21]. Many previous studies reported that the extract of *Rhus semialata* M. possesses various pharmacological effects such as anti-bacterial, anti-viral, anti-oxidant and anti-inflammation activities [22–25]. In the present study, we evaluated the protective effect of *Rhus semialata* M. on PPD induced cytotoxicity and investigated the mechanism of action of *Rhus semialata* M. in human keratinocytes, major target cells exposed to PPD in the process of hair dyeing or tattooing.

2. Materials and methods

2.1. Materials and reagents

PPD was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in PBS pH7.4 to make stock solution (94 mM). In the experiment, PPD stock solution was diluted to desired concentration with growth media.

Dulbecco's modified Eagle's medium (DMEM) was purchased from Welgene (Daegu, Korea) and streptomycin and penicillin, fetal bovine serum (FBS), propidium iodide (PI) were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Proteins were detected using PicoEPD Western reagent (ELPIS-Biotech, Daejeon, Korea). Annexin V-FITC apoptosis kit was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA).

2.2. Preparation of extracts from *Rhus semialata* M

The leaves and branches of *Rhus semialata* M. were obtained from the Juksangol nongwon (Jeonnam, South Korea) and identified by comparison with voucher specimen (NIBRVP0000373346), which is already deposited at the National Institute of Biological Resources. The dried and pulverized raw material (100 g) was extracted overnight with 70 % (v/v) ethanol at room temperature, and the supernatant was collected by filtration. Ethanol was removed by rotary vacuum evaporation (Heidolph, Schwabach, Germany) from collected supernatant. The extract was lyophilized to yield *Rhus semialata* M. extract (2.1 g, yield 2.1 %). Lyophilized powder dissolved in DMSO at 1000-fold higher concentration of final concentration in medium.

2.3. High performance liquid chromatography

A Waters (Milford, MA, USA) HPLC system with a 600 Controller, 996 Photodiode Array Detector, 616 Quaternary pump, and a 717 Autosampler was used for analysis of gallic acid. Data acquisition was achieved using the Waters Empower software. All chromatographic separations were conducted on an Molecules 2015, 20 3558 ACE® 5 µM C18 column (250 mm × 4.6 mm, 5 µm, ACE, UK) at ambient temperature with detection at 290 nm. The mobile phase consisted of 0.1 % trifluoroacetic acid in water and acetonitrile (70:30, v/v) for 20 min.

2.4. Measurement of total polyphenolic content

The content of total polyphenol compounds in the extract was determined using the Folin-Ciocalteu phenol reagent [26]. The content of total polyphenolic compounds was expressed as mg gallic acid equivalents/g dried extract.

2.5. Measurement of total flavonoid content

The content of total flavonoid compounds in the extract was determined using a colorimetric assay adapted from [27]. A total of 150 µL of 5% aqueous AlCl₃ was added. A total of 1 mL of 1 M NaOH was added 1 min after the addition of aluminum chloride. The absorbance of the solution was measured at 510 nm. The content of total flavonoid compounds was expressed as mg catechin equivalents/g dried extract.

2.6. DPPH scavenging activity assay

The antioxidant activity of RSE was first determined by measuring the DPPH scavenging ability [28]. The extract at various concentrations (1–100 µg/mL) was added to 200 µL of DPPH (100 µM) solution. DPPH reaction with an antioxidant capable of hydrogen ion donation reduces DPPH. The resulting decrease in absorbance at 540 nm was recorded using a Gen 5™ UV-vis spectrophotometer (BioTek, Winooski, VT, USA).

2.7. Cell and culture condition

The human keratinocyte (HaCaT) was purchased from CLS (Eppelheim, Germany) and maintained in DMEM supplemented with 10 % FBS, 100 µg/mL streptomycin and penicillin under standard condition (37 °C, 5% CO₂ atmosphere). When cells reached approximately 80 % confluence, the cells were subcultured every 3 days. To investigate the following experiments, HaCaT cells were seeded on plastic dishes and grown to 70–80 % confluence. The cells were treated with various concentrations of RSE 2.5–10 µg/mL 4 h before PPD treatment.

2.8. Measurement of cell viability

Cytotoxic effect of PPD on HaCaT cells was determined by using the 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Duchefa, Haarlem, Netherlands) assay. Briefly, HaCaT cells (6 × 10³) were plated into 96 well plates and incubated overnight and then cells were treated with serial dilution of PPD. In order to determine the protective effect of RSE on PPD IC₅₀ value, cells were pretreated with various concentrations of RSE for 4 h, and they were exposed to PPD (IC₅₀ value) for 48 h. After treatment, MTT reagent (1 mg/mL) was added to each well and incubated for an additional 3 h. After then the supernatant was removed and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystal produced from MTT. Absorbance was measured at a wavelength of 570 nm using a spectrophotometer (Epoch™ microplate spectrophotometer, BioTek, Winooski, Vermont, USA).

2.9. Flow cytometric analysis

Flow cytometric analysis of HaCaT cells with or without RSE after treatment of PPD was performed to analyze cell cycle distribution with staining of propidium iodide. Extent of apoptosis was detected with an Annexin V-FITC apoptosis kit (Thermo Scientific, Pittsburgh, PA, USA) as manufacturer's instructions. Cell cycle distribution profiles and apoptotic cell death were analyzed by using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Changes in mitochondrial membrane potential ($\Delta\psi_m$) following treatment with PPD and the protective effect of RSE on PPD induced apoptosis, were measured after staining with 3,3'-dihydoxycarbocyanine iodide (DiOC₆) obtained from Sigma-Aldrich (St. Louis, MO, USA). After treatment, the cells were harvested and incubated with PBS containing 50 nM DiOC₆ for 15 min at 37 °C prior to flow cytometric analysis.

2.10. Determination of intracellular ROS production

For measurement of intracellular ROS, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used, which readily diffuses into cells to be used as fluorescent probe. HaCaT cells were pretreated with various concentrations of RSE for 4 h and then stained with 5 μM of DCFH-DA in serum-free medium for 30 min at 37 °C, 5% CO₂ atmosphere. Then, 200 μM of PPD was treated for 24 h in the presence of DCFH-DA. After incubation, the cells were washed with PBS and fluorescence intensity was analyzed by Tecan Infinite F200 PRO (Tecan, AG, Mannedorf, Switzerland) with specific excitation/emission wavelength of 485 nm and 535 nm respectively.

2.11. Hoechst 33,342 staining

The protective effect of RSE on PPD induced DNA damage was evaluated by Hoechst33342 staining. HaCaT cells were seeded in a 6 well culture plate with 4 × 10⁵/wells. After overnight incubation, cells were pretreated with various concentrations of RSE (0, 2.5, 5, 10 μg/mL) for 4 h before PPD treatment at 37 °C. 2 days later, the cells were washed and stained with Hoechst staining solution (5 μg/mL) in PBS for 10 min at room temperature in the dark condition. Then cells were rinsed with PBS, and nuclear morphological changes were observed under an EVOS fluorescent microscope (Advanced Microscopy Group, Bothell, WA,

USA).

2.12. Preparation of cell lysates and western blot analysis

To prepare cell lysates, HaCaT cells treated with previous identical experiment condition were harvested and rinsed with ice cold phosphate buffer saline. And cells were resuspended in PRO-PREP™ protein extraction solution (iNtRON biotechnology, Seoul, Korea), and disrupted by sonication. Protein concentration of each sample was measured by Coomassie Plus™ Assay kit (Thermo scientific, Pittsburgh, PA, USA), and an equivalent amount of protein lysate (20 μg) was subjected to precast 4–12 % SDS gradient polyacrylamide gel with MOPS buffer, followed by electrotransfer to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). The specific antibodies for caspase-9, caspase-3, PARP and β-actin were used for immunostaining and detection was conducted with a PicoEPD Western reagent kit (ELPIS-Biotech, Daejeon, Korea) according to manufacturer's instruction.

2.13. Statistical analysis

All values were expressed as mean ± SD. All experiments were conducted in triplicate, and all data were evaluated on statistical significance by Student's *t*-test using Statview software (Abacus Concepts,

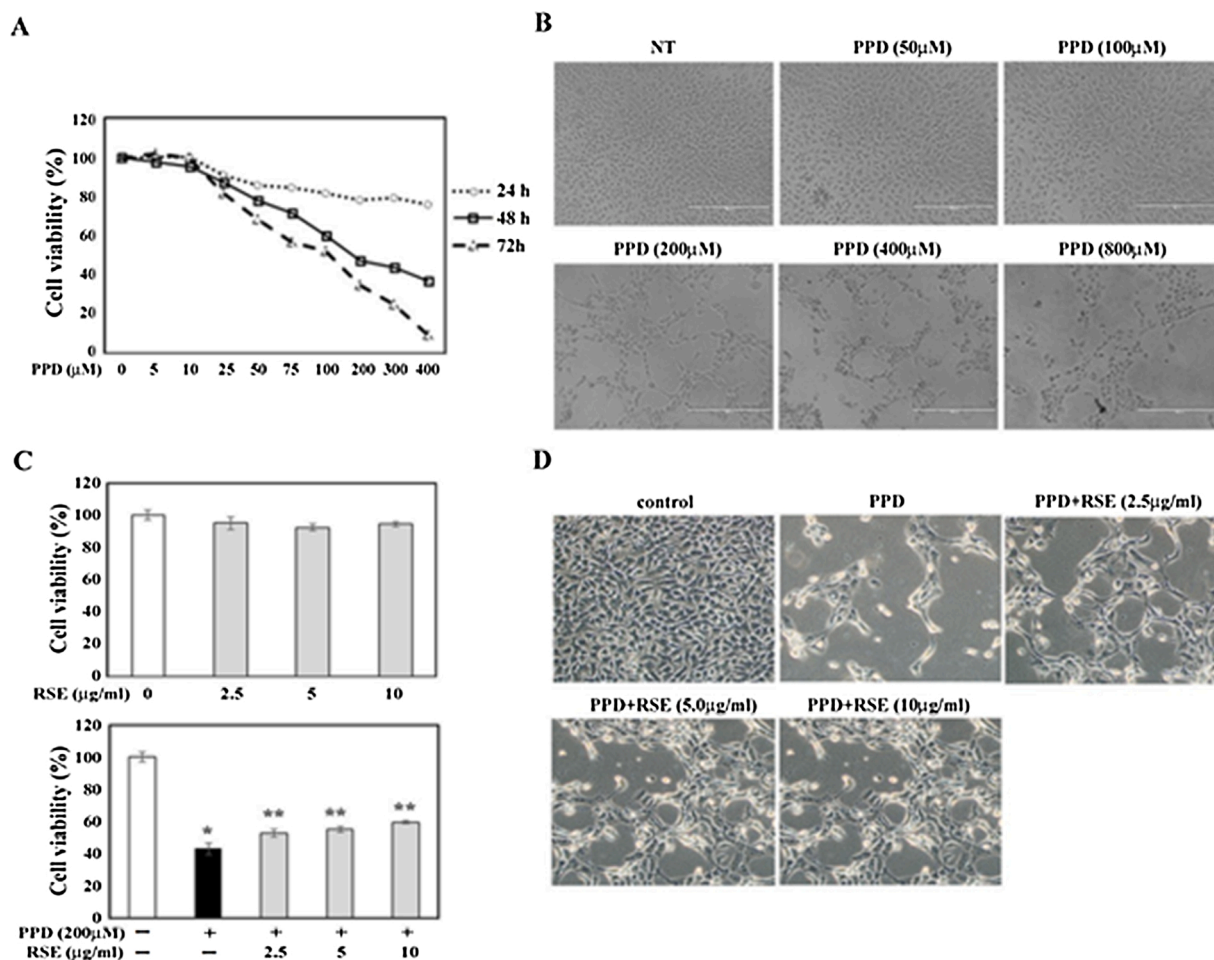


Fig. 1. Cytotoxic effect of PPD in HaCaT cells and protective effect of RSE on PPD- induce HaCaT cells. (A) PPD decreased cell viability of HaCaT cells in a dose and time dependent manner. HaCaT cells were treated with various concentration of PPD (0–400 μM) as indicated for 24, 48, and 72 h, respectively. (B) Morphological changes in HaCaT cells were observed using an inverted microscope after treatment of PPD for 48 h. (C) Cell viability was measured by MTT assay. For cell viability analysis, HaCaT cells were seeded on a culture plate, and treated with various concentrations of RSE prior to PPD induction. (D) Cell images of after exposure to PPD with or without RSE treatment for 48 h. The analysis was mean of triplicated measurements ± SD of three separate experiments. **p* < 0.05 vs PPD-untreated control, ***p* < 0.05 vs PPD-treated control.

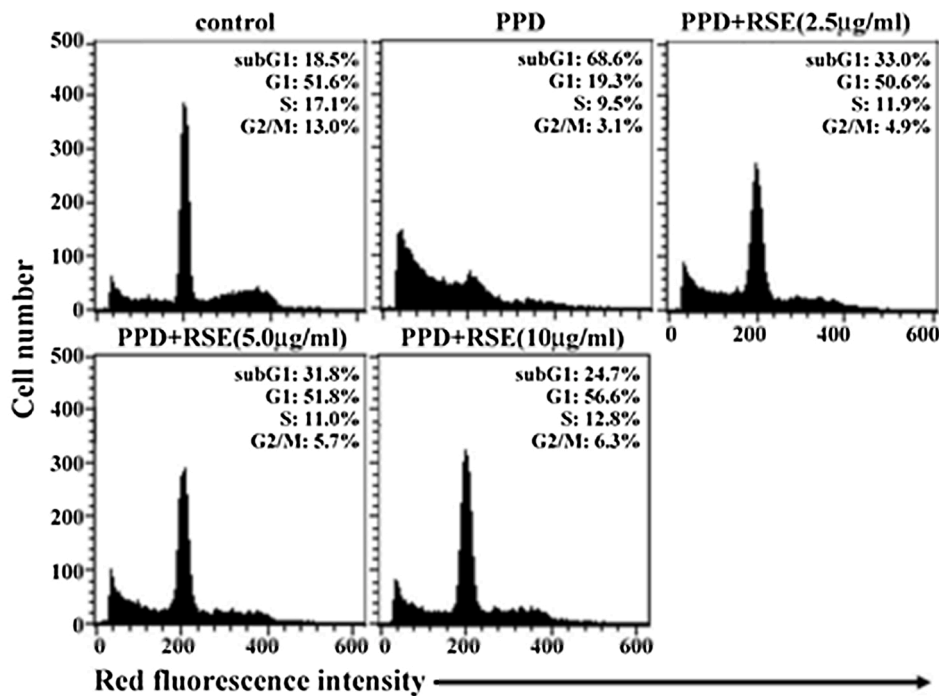
Piscataway, NJ, USA). $p < 0.05$ was statistically significant between data.

3. Results

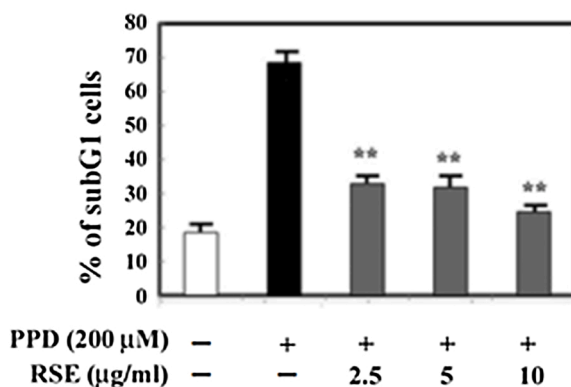
3.1. Cytotoxic effect of PPD on HaCaT cells

To investigate the cytotoxic effect of PPD on HaCaT cells, cells were treated with various concentrations of PPD from 0 to 400 μM for 48 h. When cells were treated with PPD 100 μM for 48 h or 72 h, the cell viability appeared to be reduced significantly in a dose dependent manner (Fig. 1A). The viability of HaCaT cells following the treatment of PPD 100, 200 and 400 μM declined to the level of 60.1 %, 47.4 %, and 37.2 % at 48 h and 52.4 %, 34.8 %, and 9.5 % at 72 h, respectively, as compared to the control. Based on these results, the IC_{50} value for PPD was about 200 μM at 48 h. Furthermore, the morphological change was also observed by increasing treatment of PPD (Fig. 1B). As shown in Fig. 1B, the number of cells significantly decreased, and morphological alteration including enlargement of the cells, forming a variety of shapes, cell shrinkage, blebbing of plasma membrane occurred.

A



B



3.2. RSE inhibits the toxicity of PPD on HaCaT cells

To examine whether RSE has protective effect on PPD induced cell growth inhibition on HaCaT cells, cells were pre-incubated for 4 h with RSE before PPD (IC_{50} value) treatment. After 48 h, cell viability was determined by MTT assay. In preliminary assay, we confirmed that RSE has no significant effect on cell viability at 10 $\mu\text{g}/\text{mL}$ but slightly decrease in viability at 20 $\mu\text{g}/\text{mL}$. As shown in Fig. 1C, the cell number of HaCaT cells treated with RSE from 2.5 to 10 $\mu\text{g}/\text{mL}$, significantly increased compared to PPD treatment alone. Under this concentration, cell viability was not influenced by the presence of RSE. Additionally, we also observed that PPD induced reduction of the number of cells and morphological alteration, were restored with RSE pretreatment under an inverted microscope (Fig. 1D).

3.3. RSE has protective effect in PPD induced cell death and cell cycle alternation on HaCaT cells

PPD was known to induce cell death. To investigate whether PPD induced cell viability reduction occurred because of cell cycle alteration,

Fig. 2. PPD induced cell death and protective effect of RSE in HaCaT cells.

(A) To determine cell death, HaCaT cells were treated, with or without various concentrations of RSE treatment before PPD induction. Cell cycle distribution was detected by using flow cytometry, after staining of DNA by propidium iodide. (B) Quantification of cell cycle distribution in RSE or PPD treated HaCaT cells. The analysis was mean of triplicated measurements \pm SD of three separate experiments. $**p < 0.05$ vs PPD-treated control.

the cells were treated with various concentrations of PPD for 48 h and then the DNA content of cells was stained with propidium iodide method. We observed that the percentage of cells in sub-G1 phase representing apoptotic cells increased in a dose dependent manner. On the other hand, pretreatment with RSE on HaCaT cells could reduce induction of apoptotic sub-G1 phase, following exposure to PPD (Fig. 2). These results indicated that RSE could restore cells from PPD induced cell death.

3.4. RSE inhibits the apoptotic effect of PPD on HaCaT cells

Morphological characteristics of apoptosis are shrinkage of cells, nuclear chromatin condensation, and finally karyorrhexis with DNA fragmentation and irregular chromatin distribution. To determine whether RSE could restore PPD induced apoptosis in HaCaT cells, we used Hoechst 33,342 dye to evaluate chromatin condensation, and Annexin V/PI dye to detect exposure of phosphatidylserine at the outer membrane leaflet as an indicator of apoptosis. Fig. 3A shows that treatment of HaCaT cells with 200 μ M of PPD, caused significant enhancement of early apoptosis (lower right) and late apoptosis (upper right). However, levels of early and late apoptotic cells, were decreased by RSE pretreatment. Furthermore, a higher number of apoptotic cells staining more brightly than normal cells, were observed by PPD treatment with Hoechst 33,342 staining. However, RSE pretreatment for 4 h could inhibit apoptotic morphological changes, following to PPD treatment (Fig. 3B). The results of western blot analysis induced by PPD treatment, showed that caspase-9 activity was increased slightly and caspase-3 activity was increased significantly (Fig. 3C). Activation of caspase-3 and caspase-9 induced by PPD was prevented by RSE pretreatment, especially in caspase-3 in a dose dependent manner.

3.5. RSE inhibits the PPD induced mitochondrial membrane potential collapse

To evaluate whether the protective effect of RSE on PPD was mediated by mitochondria dependent pathway, changes in mitochondrial membrane potential ($\Delta\psi_m$) was determined by flow cytometry used with DiOC₆ dye. DiOC₆ dye is a cell permeable lipophilic fluochrome dye, sensitive to mitochondria of live cells at low concentration. Significant loss of $\Delta\psi_m$ in PPD exposed cells were observed, as compared to continuously growing cells. However, mitochondrial membrane potential collapse was decreased with pretreatment of RSE for 4 h. Although, the growing HaCaT cells have only a small number of $\Delta\psi_m$ losing cells, 53.6 % of the cells lost their mitochondrial membrane potential in the presence of PPD. Whereas, mitochondrial membrane potential was recovered in RSE pretreated cells, after being induced with PPD (Fig. 4A). Additionally, 36.2%–29.3% of cells were observed with $\Delta\psi_m$ loss, in the presence of 2.5 μ g/mL to 10 μ g/mL of RSE co-treatment. These results demonstrated that RSE (2.5–10 μ g/mL) could protect disruption of $\Delta\psi_m$ induced by PPD.

3.6. RSE suppresses the production of intracellular ROS induced by PPD treatment in HaCaT cells

To determine whether RSE could protect them from oxidative stress induced by PPD, HaCaT cells were pretreated with RSE for 4 h before treatment with PPD. Intracellular level of ROS production was measured by using DCFH-DA as a fluorescent dye. As shown in Fig. 4B, changes in fluorescence intensity was observed with the fluorescence microscope. Tecan Infinite F200 PRO was used to analyze and quantify differences of fluorescence intensity. The PPD treated group had increased fluorescence intensity to 324.5 % compared to the control group. However, fluorescence intensity of RSE pretreated groups decreased by 40.9 % and 56.0 % at the concentration of 5 and 10 μ g/mL, respectively, compared to the PPD treated group (Fig. 4C).

3.7. The chemical components in the extract of *Rhus semialata* M

To our investigation revealed that the content of total phenolic compounds in RSE was 181.16 mg gallic acid equivalent/g dried extract. Meanwhile, the content of total flavonoids in RSE was 52.91 mg catechin equivalent/g dried extract. The chemical components in the extract of *Rhus Semialata* M. were analyzed by HPLC. As shown in Fig. 5, the major peak, tR of 7.95 min, was identified as gallic acid. Its content was calculated as over 2.81 % (w/w).

3.8. Gallic acid inhibits the toxicity of PPD on HaCaT cells

To clarify the roles of gallic acid, the pure compound was evaluated protective effect of RSE on PPD-induced HaCaT cells. In antioxidant assays, gallic acid showed potent free radical scavenging effect (Table 1), and such findings were consistent with previous reports [29]. In PPD-induced cell toxicity, gallic acid exhibited significant inhibitory abilities in a dose-dependent manner. (Fig. 6A, B). Hoechst 33,342 staining was performed to detect apoptotic cells. As shown in Fig. 6C, gallic acid pretreatment for 4 h could inhibit apoptotic morphological changes, following to PPD treatment. These results clearly proved that gallic acid is an active ingredient contributing to the antioxidant and cell protective activities of *Rhus semialata* M.

4. Discussion

Permanent hair dyes containing PPD were banned because of their multitude of health risks in the 1980's. Despite the possibility of inducing allergic contact dermatitis and cancer in many organs, PPD has been used as an ingredient of hair dyeing formulation up to the present for cosmetic reasons. In the United States, it is legal for PPD to be used up to 6% concentration in hair dyes [30]. Temporary exposure to PPD could cause symptoms of allergic reaction including eye irritation, itching, swelling, blistering, and renal failure within a few hours, or reaction may often be delayed by a few weeks after application [31]. Even if someone does not have a reaction to PPD initially, regular repeated exposure to PPD or other para-substituted substances, will make them sensitized to PPD [32,33]. The hair coloring and tattooing industries are continuously promoting individual expression, as well as a more youthful appearance with use of such products. Depending on demands, more people are seeking safer and natural ingredients for attenuating side effects, caused by PPD or PPD metabolites produced in the process of hair dyeing.

Most previous studies focus on the activation of immune reaction such as eosinophils, neutrophils, and dendritic cells involved in allergic contact dermatitis [34–36]. The major application site of PPD is skin epidermal layer, but cellular toxicity on keratinocytes in response to PPD has only recently been reported. Recent studies have demonstrated that up-regulation of genes involved in inflammatory response to PPD and cytotoxic effect with induction of DNA damage on keratinocytes against to PPD [37,38].

In HaCaT cells exposed to PPD (200 μ M) for 48 h, a significant reduction of cell viability with cell morphological change was induced with concentration dependent manner. In the process of hair dyeing, PPD triggers chemical reaction with the coupler reagent under the artificial condition accelerated by hydrogen peroxide. In this experiment, we observed that PPD alone treatment without the coupler reagent for 48 h could decline cell viability of HaCaT cells to 47.4 %, compared to non-treated control. In accordance with cell viability data, the sub G1 phase population meaning apoptotic cells increased after treatment of PPD. Also, mitochondrial membrane potential ($\Delta\psi_m$) loss was detected with cleavage of caspase-9, caspase-3 and PARP following treatment of PPD. This cytotoxic effect may be obtained from auto-oxidation of PPD.

In this study, we provided evidence that the ethanol extract of *Rhus semialata* M. (RSE), used as a traditional medicinal plant and in food

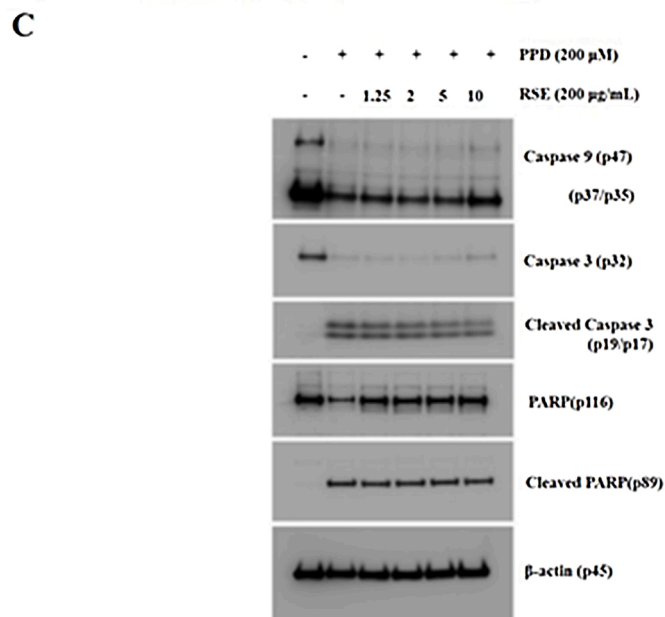
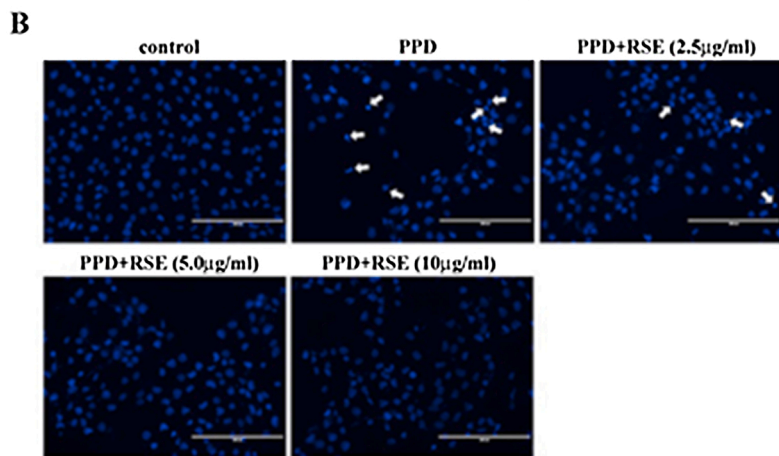
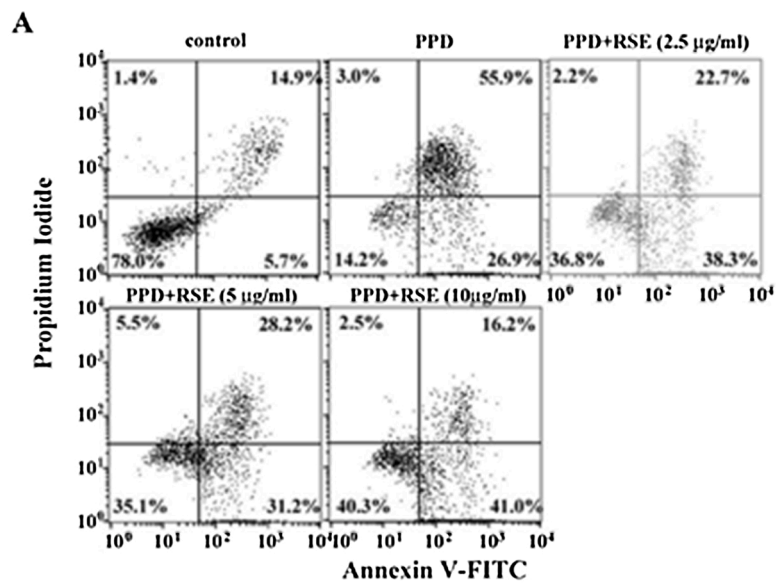


Fig. 3. Effect of RSE on PPD induced apoptosis in HaCaT cells. (A) Annexin V-FITC and PI staining of HaCaT cells following treatment with RSE prior to PPD induced apoptosis. Cells were incubated with varying concentrations of RSE for 4 h before PPD treatment. After 48 h, the cells were stained with Annexin V-FITC and /or propidium iodide and analyzed by using flow cytometry. (B) Equivalent cells were stained by hoechst 33,342 dye and visualized under the fluorescence microscope. (C) Western blot analysis of caspase-9, caspase-3, cleavage of PARP and β-actin.

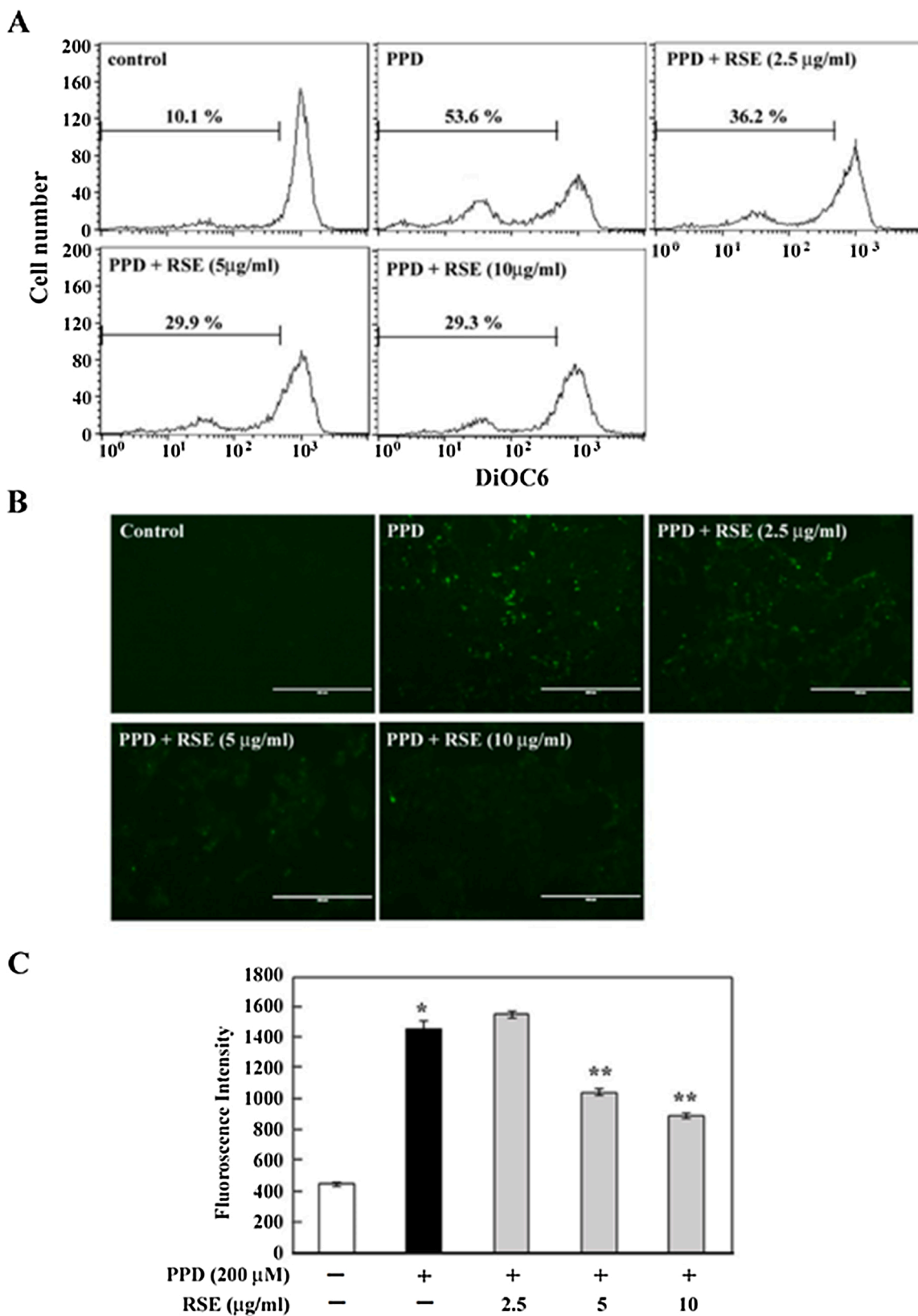


Fig. 4. RSE could attenuate the change of ROS and mitochondrial membrane potential ($\Delta\psi_m$) after treatment of PPD exposure in HaCaT cells. (A) HaCaT cells were treated with RSE for 4 h, and after that PPD was exposed for 48 h. Cells were stained with 0.1 μM of DiOC₆ for 15 min at 37 °C and loss of $\Delta\psi_m$ was analyzed by flow cytometry. (B) Changes in DCF fluorescence intensity of HaCaT cells induced by PPD in the presence or absence of RSE pretreatment was measured under a fluorescence microscope. (C) Intracellular ROS levels in HaCaT cells were measured by determination of fluorescence intensity at excitation wavelength of 485 nm and emission wavelength of 535 nm. The analysis was mean of triplicated measurements \pm SD of three separate experiments. The analysis was mean of triplicated measurements \pm SD of three separate experiments. * p < 0.05 vs PPD-untreated control, ** p < 0.05 vs PPD-treated control.

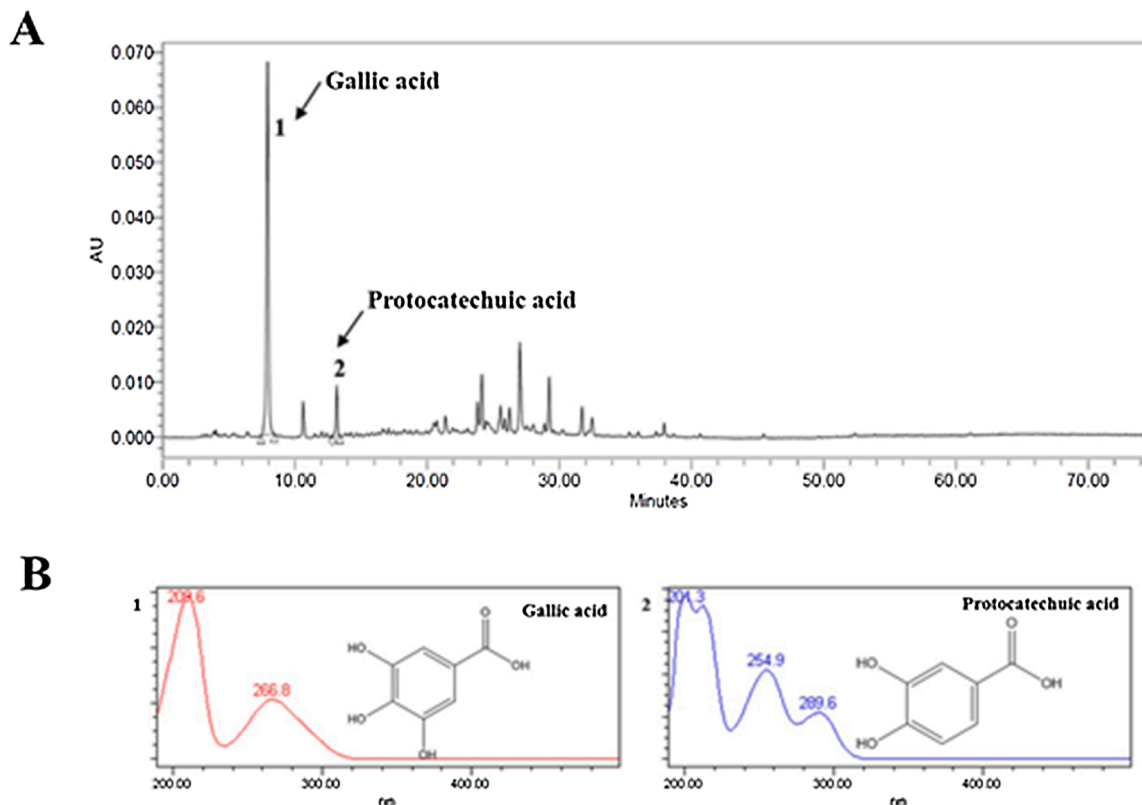


Fig. 5. The chemical components in the extract of *Rhus Semialata M.* (A) HPLC chromatogram of *Rhus Semialata M.* extract at 280 nm. (B) Structure of gallic acid and protocatechuic acid.

Table 1

EC₅₀ value of *Rhus Semialata M.* and gallic acid in the antioxidant assay compared to some pure chemicals commercially used.

EC ₅₀ value (μg/mL) (mean ± SD; n = 4)	
Product	DPPH ^a
<i>Rhus Semialata M.</i>	8.87 ± 0.18
Ascorbic acid	6.81 ± 0.13
Trolox	12.13 ± 0.21
Gallic acid	1.01 ± 0.04

^a EC₅₀ value : effective concentration at which 50 % of radicals are scavenged.

additives in Korea and China, could protect HaCaT cells from induction of apoptosis by PPD treatment. The most remarkable observation was that RSE pretreatment could prevent PPD induced cytotoxicity on HaCaT cells, with less morphological alteration. Consistent with the above results, subG1 phase cells representing apoptotic cells of PPD with co-treatment of RSE decreased to 24.7 % compared to 68.6 % of PPD alone treated cells. Additionally, results of Annexin-V/PI staining and Hoechst 33,342 staining show that RSE could suppress PPD induced apoptosis. Many previous studies have demonstrated that PPD induced ROS production could be attributable to activation of apoptosis via mitochondrial membrane potential collapse [39,40]. In our experiment, we determined change of mitochondrial membrane potential by PPD treatment, with or without RSE. As a result, RSE could inhibit the collapse of mitochondrial membrane potential in PPD treated HaCaT cells. Furthermore, through RSE pretreatment, we have demonstrated that PPD induced generation of ROS, which leads to oxidative stress in many organs, decreased by RSE treatment, and subsequently inhibited activation of caspase cascade.

Rhus semialata M. has been reported to contain gallic acid, methylgalate, protocatechuic acid, coumaric acid, β-sitosterol, morolic acid, (2S)-1-O-heptatriacontanoyl glycerol [41,42,29]. In the present study,

we found that gallic acid were mainly included in the ethanol extract of *Rhus semialata M.* (Fig. 5). This phenolic compound provided both antioxidant and protective effect against PPD cytotoxicity. Therefore, although it needs further study, there is the possibility that gallic acid are effective compounds of RSE for protecting PPD cytotoxicity.

5. Conclusion

In conclusion, results of current study demonstrate, that RSE attenuates the cytotoxic effect of PPD on human keratinocytes. We elucidated that the mechanism of RSE protecting cells from PPD was achieved by suppressing Δψ_m loss, activation of caspase-3, -9 and cleavage of PARP induced by PPD. These results suggest that RSE can be used as a valuable natural substance to protect human skin from toxicity of PPD in many industries such as hair coloring, tattooing and fur dyeing, which use PPD as a constituent.

Author contributions

H.W and E.J conceived and designed the experiments.
H.W and H.K performed the experiments.
H.W, S.S, E.J and D.P analyzed and interpreted the data.
H.W wrote the paper and E.S and S.S corrected the paper.
All authors read the manuscript and approved the final manuscript.

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CRediT authorship contribution statement

Hyunju Woo: Conceptualization, Methodology, Investigation.

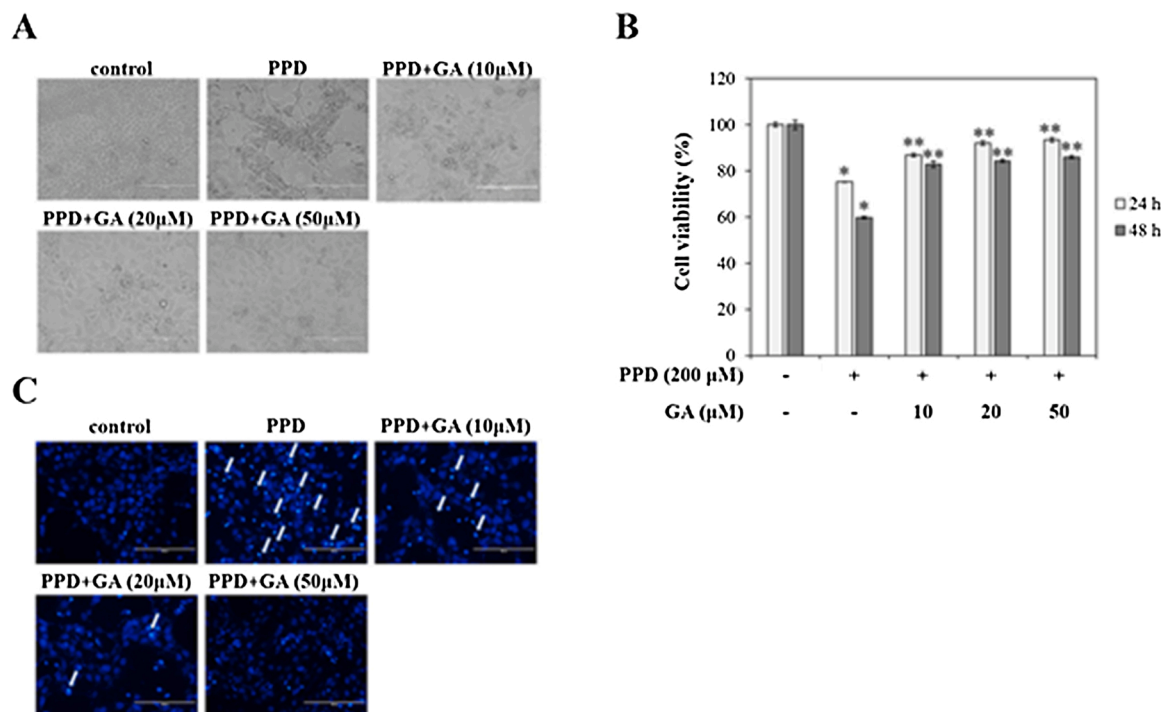


Fig. 6. Protective effect of gallic acid on PPD- induce HaCaT cells.

HaCaT cells were exposed to PPD (200 μM) with or without gallic acid for 48 h. (A) Morphological changes in HaCaT cells were observed using an inverted microscope after treatment of PPD for 48 h. (B) Cell viability was measured by MTT assay. For cell viability analysis, HaCaT cells were seeded on a culture plate, and treated with various concentrations of gallic acid prior to PPD induction. (C) Equivalent cells were stained by hoechst 33,342 dye and visualized under the fluorescence microscope. The analysis was mean of triplicated measurements ± SD of three separate experiments. * $p < 0.05$ vs PPD-untreated control, ** $p < 0.05$ vs PPD-treated control.

Hayeon Kim: Investigation. **Seungwoo Shin:** Investigation, Visualization, Writing - original draft. **Jong Heon Shin:** Formal analysis. **Dehun Ryu:** Formal analysis. **Deokhoon Park:** Supervision, Resources. **Eunsun Jung:** Data curation, Writing - original draft, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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