ORIGINAL ARTICLE

Antioxidative and Photoprotective Effects of *Pleurotus flabellatus* (Pink Oyster Mushroom) Polysaccharides Against UVA Radiation-Induced Cytotoxicity in Human Dermal Fibroblast (HS-27) Cell Line

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ABSTRACT

Introduction: Ultraviolet (UV) A is the longest wavelength of UV radiation, accounts for approximately 95% of the radiation reaching the earth's surface. It can penetrate deeply into the skin layer and able to induce photoaging and photocarcinogenesis through the activation of reactive oxygen species (ROS). Polysaccharides-containing *Pleurotus flabellatus* (known as a pink oyster mushroom) has antioxidative properties and may inhibit free radical activities generated from UV radiation. Hence, this present study was to evaluate the antioxidative and photoprotective properties of exopolysaccharides (ExPFE) and exopolysaccharides (EnPFE) of *Pleurotus flabellatus* extracts on UVA irradiated human dermal fibroblast (HS-27) cell line. **Methods:** The antioxidant level of ExPFE and EnPFE was determined using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, while both cytotoxicity and photoprotective effects of the extracts on the HS-27 cell line were determined using CellTiter-Blue® cell viability assay. The effects of ExPFE and EnPFE on the HS-27 cell migration was evaluated using the scratch assay. **Results:** Both ExPFE and EnPFE exhibited respectable antioxidant and scavenging activity in DPPH. The extracts also demonstrated a non-cytotoxicity, but photoprotective effects to the HS-27 cells by increasing the percentage of cell viability and enhancing cell migration activity upon UVA exposure. **Conclusion:** The ExPFE and EnPFE exhibit antioxidative and photoprotective effects on UVA irradiated HS-27 cell line. This study suggests that pink oyster polysaccharides could be a potential natural bioactive compound for skin protection against UVA radiation.

Keywords: Ultraviolet; *Pleurotus flabellatus*; antioxidant; photoprotective; polysaccharide

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INTRODUCTION

Ultraviolet (UV) radiation is contributed to the generation of free radicals, leading to acute effects of immediate pigment darkening and chronic effect of photoaging (1). Excessive exposure of human skin to UV radiation (UVR 280–400nm), particularly UVA (315–400 nm) and visible radiation (400–700 nm), promote the formation of reactive oxygen species (ROS) in the human biological systems (2). The cytotoxic and genotoxic effects of ROS on cells are mediated by oxidizing lipids and proteins, and through inducing base lesions, respectively. UVA could have more indirect effects on DNA compared to UVB via the generation of ROS which also leads to immunosuppression and photocarcinogenesis (3). Currently, the use of bioactive compounds from natural product derivatives for the treatment and prevention of UV-induced photoaging has become a trend in the cosmeceutical industry.

An antioxidant does not only hold the ability to scavenge the excessive and toxic ROS but also consists of potential photoprotective properties. *Pleurotus flabellatus* or locally known as Pink Oyster mushroom was found to contain a perceptible amount of antioxidant level (4). Moreover, a previous study by Mohamad et al. (5) specifies the anti-bacterial properties of *Pleurotus flabellatus* through the biological activities of its polysaccharides, one of the components in the mushroom cell wall which are indigestible biopolymers. This may be due to their ability in attaching to other surfaces like macrophages thus enhancing immunomodulatory effects (6). Besides, mushroom polysaccharides contain β-glucan which

was not only found to protect the host skin against UV radiation, but it also provides various complementary benefits including immunostimulator and anti-aging properties (7).

However, there is limited study on the photoprotective properties of mushroom, especially in *Pleurotus flabellatus*. Thus, this study aims to evaluate the antioxidative and photoprotective properties of the exopolysaccharides *Pleurotus flabellatus* extract (ExPFE) and endopolysaccharides of *Pleurotus flabellatus* (EnPFE) on human dermal fibroblast (HS-27) cell line using UVA radiation. This study hypothesized that ExPFE and EnPFE potentially have antioxidative and photoprotective effects against UVA radiation in the HS-27 cell line.

MATERIALS AND METHODS

Extraction of Exopolysaccharides (ExPFE) and endopolysaccharides (EnPFE)

Exopolysaccharides and endopolysaccharides of *Pleurotus flabellatus* strain Mynuk (PFSM) were obtained from and extracted at the Malaysian Nuclear Agency based on Vunduk et al. (8). The construction of a phylogenetic tree and biomolecular of PFSM was identified prior to the extraction procedure. PFSM sequence was found to be 99% similar to *Pleurotus flabellatus* strain CCRC 36222 (AY265827.1) and *Pleurotus flabellatus* strain ACCC51447 (EU424303.1) (8). The mushroom mycelia that previously cultured on potato dextrose agar (PDA) was inoculated in a mushroom complete media containing 20 g/L glucose, 2 g/L yeast extract, 2 g/L meat peptone, 1 g/L KH_2PO_4, and 0.5g/L $MgSO_4.7H_2O$. The culture was then incubated in a rotary incubator shaker for 2 weeks at 28 °C and 100 rpm. Next, the fermentation was submerged in a 5 L air-lift bioreactor for 4 days at 28 °C and 0.5-2.0 L/min flow rate. Biomass produced was separated into two parts and subsequently subjected to extraction for the production of EnPFE and ExPFE based on methods by Mohamad et al. (9) and Wan-Mohtar et al. (10).

For EnPFE, the dried mycelium was subjected to hot water extraction. About 100g of mycelium was heated for 2-4 hours at 100°C, filtered, and followed by centrifugation. Then, four volumes of 95% (v/v) ethanol were added and left overnight to get precipitation. The precipitate extract was concentrated and condensed using a rotary vacuum evaporator to remove the ethanol. The extract was then placed in a freezer approximately for 24 hours prior to freeze-drying using a freeze dryer at -60°C. For the ExPFE, the culture broth obtained from the fermentation process was filtered. The same volume of ethanol was added to the filtered broth followed by ethanol evaporation and freeze-drying process.

Diphenyl-2-picryl-hydroxyl (DPPH) assay

The efficiency of ExPFE and EnPFE on scavenging free radicals was evaluated using DPPH assay, as described by Rozila et al. (11), with slight modification. Briefly, the reaction mixture containing 4 mL of 0.06 mM DPPH solution, and 0.1 mL of the extracts or ascorbic acid ranging from 0.001 to 10 mg/mL was prepared in triplicate. The reaction mixture was then vigorously shaken and incubated in the dark for 30 minutes at room temperature. The absorbance was measured at 517 nm using a UV-VIS spectrophotometer. The percentage of free radical scavenging activity was calculated using the formula:

Free radical scavenging activity (%) =

$$\frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of 3mL DPPH solution without extract while Asample is the absorbance of sample with DPPH solution.

Cell Culture

The HS-27 cells (ATCC® CRL-1634™) were obtained from ATCC and maintained at 37°C and 5% CO_2 incubator in a complete Dulbecco's Modified Eagle's Medium F12 (DMEM-F12) containing 1% Penicillin/Streptomycin and 10% fetal bovine serum (FBS). The complete growth medium was replaced every two to three days. After reaching approximately 80% confluence, the HS-27 cells were detached using TrypLE™ Select (Trypsin-EDTA) and cells were seeded into the desired seeding density accordingly.

UVA radiation

The method used in this UVA dose determination method was adapted from Hseu et al. (12) with modification. Prior to the UVA irradiation procedure, it is crucial to determine the UV dose rate that can reduce the cell viability of HS-27 cells post-UVA-irradiation. The following is a formula by Zanatta et al. (13) to determine the UVA dose rate:

UV Dose (J/cm^2 or W·s/cm^2) = Intensity (W/cm^2) x Time (s)

The intensity of the UVA lamp can be calculated using the intensity formula (Intensity (W/cm^2) = Power (W) / (4ϖ x distance (cm)^2) where distance is the distance from the UVA source to the well plate surface. The cells were exposed to various UVA doses which were 15 J/cm^2; 20 J/cm^2; 23 J/cm^2; 25 J/cm^2; 37 J/cm^2; 50 J/cm^2; 53 J/cm^2, 76 J/cm^2, and 487 J/cm^2.

CellTiter-Blue® cell viability assay

The HS-27 cells were seeded in 96-well plates with a seeding density of 1.0 x 10^4 cells/well and the cells were allowed to attach overnight. Following treatment with ExPFE or EnPFE and/or UVA irradiation procedure, 20 µl of CellTiter-Blue® reagent was added to each well. The plates were wrapped with aluminum foil, shaken for 10 seconds, and then incubated for 5 hours. After incubation, the plates were shaken again for 10 seconds and the absorbance was taken using an ELISA microplate reader (Awareness Technology, Inc.) at 570 nm.

Photoprotective assay

The method used was adapted from Hseu et al. (12) with slight modification. The HS-27 cell line was seeded at 1.0 x 10^4 cells/well in a 96-well assay plate. The untreated cells are used as a negative control. Prior to UVA irradiation, the HS-27 cells were pre-treated with extracts or 0.05 mg/mL 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (positive control) for 24 hours. Following 50 min irradiation with a total of 487 J/cm^2 UVA dose, the cells were post-treated with the same concentration of extract or Trolox for another 24 hours. The viability of cells was analyzed using the Cell Titer-Blue® cell viability assay.

Cell migration assay

The method was adapted and modified from Silva et al. (14). The HS-27 cells were seeded in 24-well plates with a seeding density of 5.0 x 10^4 cells/well. The cells were treated with ExPFE or EnPFE in the presence or absence of UVA. Prior to UVA irradiation, the cells were pre-treated with various concentration of extracts or 0.05 mg/mL Trolox as positive control for 24 hours. A 100 W UV light was set up in the UV box with a distance of 7 cm from the plate. The cells were irradiated for 50 minutes with a total of 487 J/cm^2 UVA dose. The monolayer cells then were scratched gently with a 100 µL pipette tip across the center of each well. The migration of cells was observed under an inverted light microscope and the images were analyzed using ImageJ software version 1.50 (National Institutes of Health, USA). The migration rate was calculated as follows:

$$\text{Migration rate (\%)} = \frac{\text{Wound area at 0 hour} - \text{Wound area at 24 hour}}{\text{Wound area at 0 hour}} \times 100\%$$

Statistical analysis

The data was analyzed using One-Way analysis of variance (ANOVA) followed by Bonferroni post-hoc test via Statistical Package for the Social Sciences (SPSS) version 25. ImageJ software version 1.50 was also employed in image processing and analysis of scratch assay. A *p* value of less than 0.05 is considered significant.

RESULTS

Free radical scavenging activity of ExPFE and EnPFE

The antioxidant and scavenging activity of both ExPFE and EnPFE extracts were determined by the DPPH assay in comparison with ascorbic acid. Ascorbic acid shows the highest scavenging activity with the least IC50(0.021 mg/mL) compared to ExPFE (9.457 mg/mL) and EnPFE (1.090 mg/mL). Among the two extracts, the radical scavenging effect of EnPFE is higher (79.32%)compared to ExPFE (50.79%) at the highest concentration (10 mg/mL). This result shows that both ExPFE and EnPFE have the ability to scavenge the free radical to a certain extent in a concentration dependant manner (Figure 1).

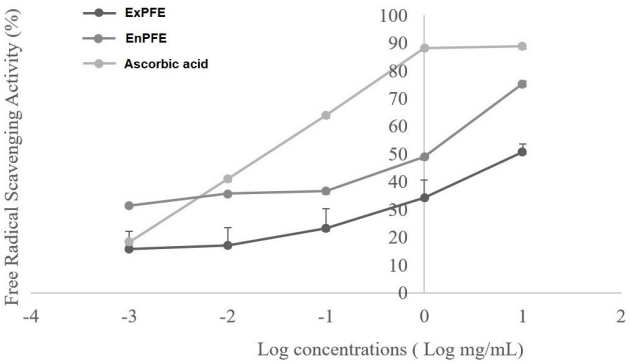

Figure 1: Free radical scavenging activity of ExPFE and EnPFE by DPPH assay. The graph shows the mean SD of three independent experiments. Log concentration of -3, -2, -1, 0, and 1 mg/mL represent 0.001, 0.01, 0.1, 1 and 10 mg/mL of the extract, respectively.

Cytotoxicity activity of the ExPFE and EnPFE

The mean percentage of HS-27 cell viability that was treated with various concentration of ExPFE and EnPFE for 24 hours were detected at above 90% (Figure 2). The result showed that the percentage of cell viability was not affected by the presence of a different concentration of ExPFE or EnPFE.

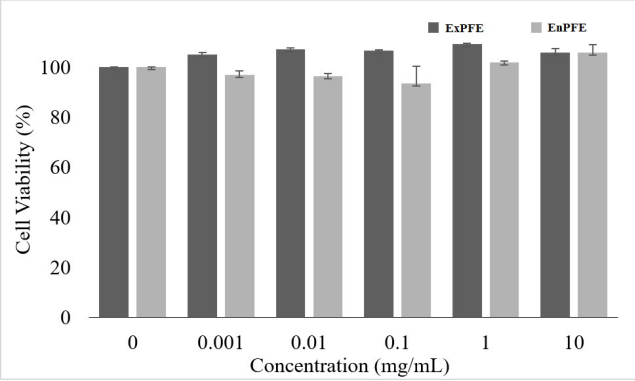

Figure 2: Cytotoxicity of ExPFE and MEPFE. The HS-27 cell line was treated with a serial concentration of ExPFE or EnPFE. The viability of cells was determined using Cell Titer-Blue® assay. Error bar represents ±SEM of three independent experiments.

Evaluation of UVA dose rate reducing cell viability

The HS-27 cell line was exposed to various intensity levels of UVA. The mean percentage of HS-27 cell viability upon UVA radiation is shown in figure 3. The result indicates that there was a significant reduction ($p<0.05$) in cell viability of the UVA-irradiated HS-27 cell line at 487 J/cm^2 UV dose, thus it was used as an optimal dose for the subsequent UVA-irradiated procedure.

Photoprotective effects of ExPFE and EnPFE against UVA irradiation in HS-27 cells

Photoprotective analysis of ExPFE and EnPFE on UVA-irradiated HS-27 cells had shown a significant increase ($p<0.001$) in the mean percentage of cell viability compared to the untreated control (Figure 4). This data

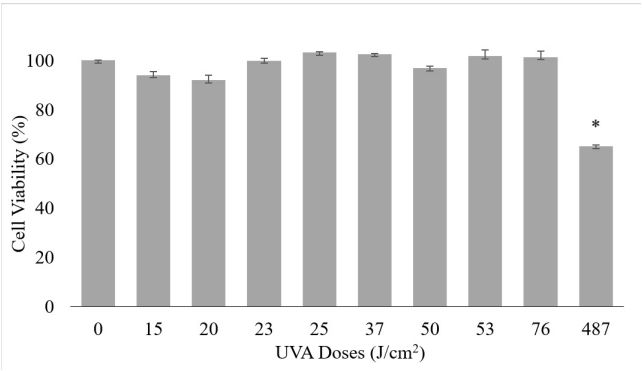

Figure 3: Effect of UVA on HS-27 cell viability. The effects of UVA exposure on viability of HS27 cells was investigated using Cell Titer-Blue® assay. The cells were irradiated with UVA ranging from 0 to 487 J/cm². Error bar represents ±SEM of three independent experiments. Statistical analysis using a one-way ANOVA with Bonferroni post-test was performed by comparison with the non-irradiated control (0 J/cm²), *p<0.05.

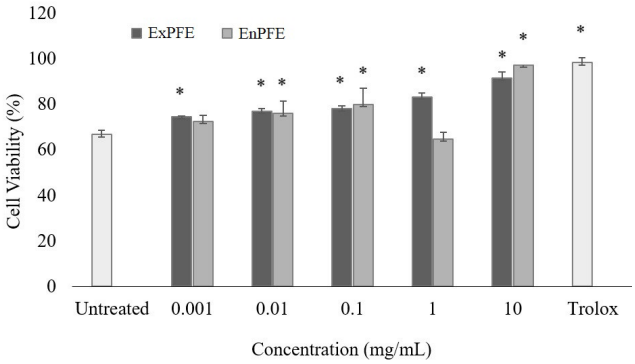

Figure 4: Photoprotective effect of ExPFE and EnPFE against UVA irradiation. The HS-27 cells were treated with ExPFE, EnPFE, or Trolox (positive control) prior to irradiation with 487 J/cm2 UVA. The cells were re-treated with the same extract for another 24 hours followed by cell viability measurement. Error bar represents ±SEM of three independent experiments. Statistical analysis using a one-way ANOVA with Bonferroni post-test was performed by comparison with the untreated control, *p<0.05.

suggests that both ExPFE and EnPFE have the ability to protect cells from the damaging effect of UVA.

Migration activity of UVA irradiated HS-27 cell line treated with ExPFE and EnPFE

Migration activity of UVA-irradiated HS-27 cells treated with ExPFE and EnPFE was observed after 24 hours using scratch assay (Fig. 5). There was a significant difference in the mean percentage of migration rate between untreated control with ExPFE and EnPFE treatment (Fig. 5a). The result also revealed that 1 mg/mL ExPFE and 0.1mg/mL EnPFE were the optimal concentration accelerates the migration of HS-27 cells. The bright-field images showed the migration of cells over 24 hours upon exposure to UVA radiation and treatment with the extracts (Fig. 5b).

DISCUSSION

Ultraviolet radiation is associated with various skin diseases including photoaging and skin cancer. The use of the natural compound as photoprotection has gained wide attention from the cosmeceuticals and pharmaceutical industries (15). UVA which profoundly penetrates the dermis layer usually associated with oxidative stress in human skin (16). Hence, the human dermal fibroblasts (HS-27) cell line was chosen in this study as a major target site of UVA radiation (17). As the role of antioxidant is to neutralize the free radicals (18), there are correlations between antioxidant activity and photoprotective effects (19).

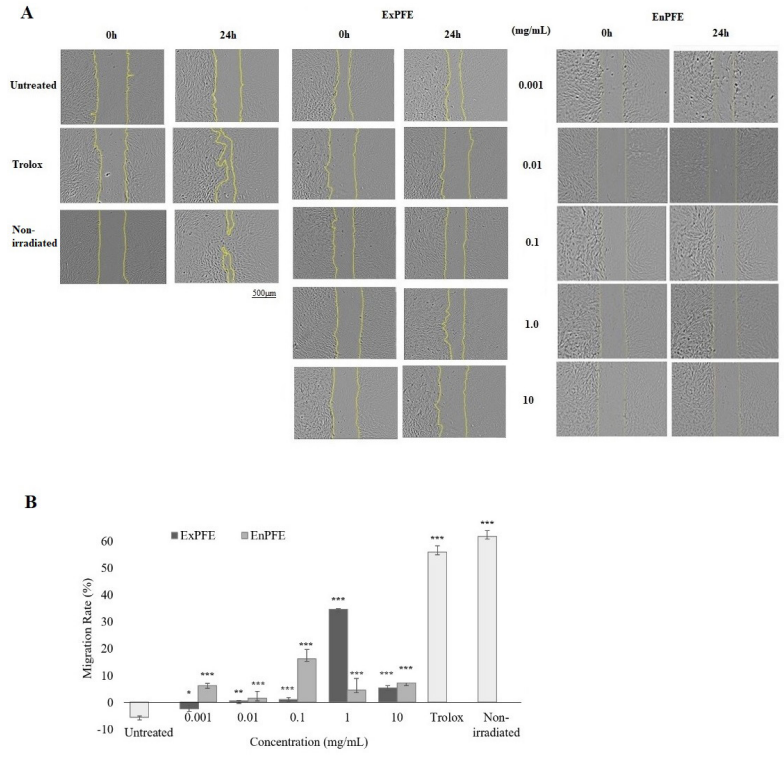

Figure 5: Effects of ExPFE and EnPFE treatment on HS-27-irradiated cell migration. A) The migration of HS27-irradiated UVA cells was assessed at 0- and 24-hours post-treatment with ExPFE, EnPFE or Trolox using scratch assay and documented by sequential digital photographs of the gap using bright-field microscopy. Representative of three images captured at Ч4 magnification on HS-27 treated with different concentration of ExPFE and EnPFE. B) The migration rate was evaluated as percentage of wound closure area using ImageJ software version 1.50 and analysed in comparison with the untreated control. Error bar represents ±SEM of three replicates, *p<0.05, **p<0.01 or ***p<0.001.

In this antioxidative and photoprotective study, the endo- and exopolysaccharides were extracted from edible *Pleurotus flabellatus*, which is a type of oyster mushroom. Pleurotus genus exhibits high nutrition and medicinal values through its variety of bioactive substances including polysaccharides, terpenoids, lectins, proteins, and peptides (20). Many studies have found the nutritional values and numerous biological activities of mushroom polysaccharides including the antioxidant activity (21–23). Most of the polysaccharides possessing biological properties are produced from mycelium (fungi's vegetative part) (24). The bioactive compounds of the mycelium and fruiting bodies of Pleurotus species display immunostimulatory, anti-inflammatory, anti-bacterial and anti-oxidative properties (20).

The antioxidant test that has been conducted in this study was DPPH scavenging activity, where the efficiency of capturing free radicals of ExPFE was evaluated using DPPH reagent. The result of this study has shown that the scavenging activity of EnPFE on free radicals was higher compared to ExPFE. Besides, a lower IC_{50} value represents a high antioxidant activity which comparable with ascorbic acid control. These results suggest that ExPFE and EnPFE are potentially good antioxidant and radical scavenger. Our investigation also in accordance with a previous study (25) which utilized polyphenols-containing extract that accountable to the total antioxidant activity of fruits (26) and medicinal plants (27).

Among a series of UVA dose that has been tested on HS-27 cells, our result demonstrated that UVA at 487 J/cm^2 produced a significant reduction in the HS-27 cell viability which indicates phototoxicity. The UVA dose ranged from 15 J/cm^2 to 76 J/cm^2 did not manage to induce sufficient phototoxicity to be used for the photoprotective study. Even though there are data from previous studies indicate that 20 J/cm^2 to 150 J/cm^2 of UVA irradiation can induce phototoxicity (12,28–30), meanwhile different methods and equipment might produce different results. This showed that the screening of the intensity or dose of UVA that can induce phototoxicity is important to obtain the optimal dose for the subsequent analysis in the photoprotective assay.

Cytotoxicity assay is an important method to determine the ability of cells to retain its survival upon treatment with a serial concentration of the bioactive compound (31). We utilized a Cell Titer-Blue reagent which contains the dye resazurin to measure the metabolic capacity of viable cells (11). The cytotoxicity analysis of ExPFE and EnPFE concentration ranging from 0.001 mg/mL to 10 mg/mL on HS-27 cells indicated that both extracts did not cause toxicity effects on the cells. Furthermore, ExPFE and EnPFE exhibited a significant proliferative activity of HS-27 cells in a dose-dependent manner which comparable with positive control. This finding is inline with Vunduk et al. (8), where the study has also shown that the *Pleurotus flabellatus* polysaccharide extracts ranging from 0.5 mg/ml to 2 mg/ml promote a significant proliferative activity without affecting the viability of the treated cells.

Trolox is a water-soluble derivative of vitamin E which contains antioxidants and an established ROS scavenger and it was used as a positive control in this study. It was proven in a previous study by Delinasios et al. (32) that both pre- and post-treatment with vitamin E offers significant skin cell protection against 20 J/cm^2 UVA dose. Moreover, the use of 0.05 mg/mL Trolox was sufficient to provide a photoprotective effect on UVA irradiated human dermal fibroblast cell line (33). The photoprotective assay was aimed to evaluate the effects of the photoprotective property of ExPFE and EnPFE against UVA irradiated HS-27 cell line. This study shows that the highest concentration of ExPFE and EnPFE extract may have photoprotective effects against UVA radiation since the mean percentage viability for both was above 90%. ExPFE and EnPFE had shown to contain photoprotective effects in a concentration-dependent manner on UVA irradiated HS-27. The photoprotective properties of ExPFE might be associated with the antioxidative activity of the extract which may have the ability to inhibit ROS through its scavenging activity (34). A study conducted by Lee et al. (35) demonstrated that the expression of matrix metalloproteinase (MMP)-1 induced by UV irradiation was inhibited by Fraxinus Chinensis extract. The antioxidant and inhibitory capacities were attributed to the coumarins contained in the Fraxinus Chinensis extracts. In addition, esculetin contained in the extracts exhibited strong scavenging activities against DPPH radicals and superoxide anions from xanthine, which contributed to a photoprotective effect. In addition, *Pleurotus flabellatus* extract has been revealed to have high flavonoid content (36,37). It has been well reviewed that flavonoids have significant photoprotective effects due to their UV absorbing capacity, direct and indirect antioxidant properties, and through the modulation of several signaling pathways (34,38–41).

UV radiation can be a cause of many skin disorders including photoaging, burn, and cancer. During the skin healing process, damaged skin needs to reconstruct through migration and proliferation of the endothelial cells. The scratch assay aims to evaluate wound healing rate after UVA irradiation and to investigate the influence of ExPFE and EnPFE on fibroblast migration (42). The observation and measurement in this study were taken for 24 hours to limit the study of migration cells and to minimize the contribution of cell proliferation to gap closure (43). The UVA-irradiated HS-27 cell line that was treated with 10 mg/mL of ExPFE and 0.1 mg/mL EnPFE showed a reduction in wound size as compared to the untreated control. This suggested that ExPFE and

EnPFE may have a photoprotective effect from UVA damage, through enhancement of cell proliferation and migration rate during the healing process (44) as compared to untreated control.

CONCLUSION

ExPFE and EnPFE showed antioxidative properties in DPPH radical scavenging activities when compared to standard ascorbic acid. Moreover, both ExPFE and EnPFE manifested a profound effect on UVA-irradiated HS-27 cells by exhibiting a photoprotective effect besides enhancing the migration of UVA-irradiated HS-27 cells. Thus, we suggest that the ExPFE and EnPFE exhibited antioxidant and photoprotective effects and it may be useful as an ingredient in cosmeceutical or pharmaceutical formulations for skin protection.

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