



Preliminary study on antioxidant properties, phenolic contents, and effects of *Aesculus hippocastanum* (horse chestnut) seed shell extract on *in vitro* cyclobutane pyrimidine dimer repair

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ABSTRACT

Aim: Human skin exposure to solar ultraviolet (UV) radiation induces cells to produce reactive oxygen species that directly cause DNA damage via the formation of cyclobutane pyrimidine dimers (CPDs), which are the primary UVB-induced DNA lesions. CPDs are responsible for cell death, mutation, and neoplastic transformation. *Aesculus hippocastanum* (horse chestnut) seed extract has traditionally been used for venotonic treatments and also as a raw material for cosmetics. This study aimed to characterize the antioxidant properties of the phenolic contents of extracts from *A. hippocastanum* seed shell and endosperm and to investigate their effects on CPD repair in UVB-exposed human dermal fibroblasts *in vitro*. **Materials and Methods:** Crude 60% aqueous ethanol extracts (v/v) were prepared, and their total polyphenol contents, antioxidant activities, and oxygen radical absorbance capacity (ORAC) values were measured by Folin–Ciocalteu, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging capacity, and ORAC assays, respectively. The levels of CPDs induced by UVB irradiation were measured by cell-based immunoassays after 6 h treatment with the extract of *A. hippocastanum* seed shell at a concentration of 100 $\mu\text{g/ml}$ and 9 h treatment with it at concentrations of 50, 100, and 200 $\mu\text{g/ml}$. **Results:** *A. hippocastanum* seed shell extract had 602 ± 1.6 mg gallic acid equivalent per gram of extract and exhibited 4990 ± 70.9 and 7140 ± 835 μmol Trolox equivalents per gram of extract as DPPH radical-scavenging activity and ORAC value, respectively; these values were comparable to those of extracts from green tea and *Mallotus japonicus* leaves. However, these values of the seed endosperm extract were relatively low. A 9 h treatment of cells with seed shell extracts at concentrations of 50, 100, and 200 $\mu\text{g/ml}$ after UVB exposure significantly reduced CPD levels compared with those in UVB-exposed cells without treatment. **Conclusion:** This study indicated that *A. hippocastanum* seed shell extract possesses a potential to limit the harmful effects of human skin exposure to UVB and that this could be the first step toward identifying novel benefits of this extract.

KEY WORDS: Antioxidant activity, *Aesculus hippocastanum*, cyclobutane pyrimidine dimers, DNA repair, horse chestnut seed

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INTRODUCTION

Solar ultraviolet (UV) radiation is essential for Vitamin D production but is also detrimental to human skin. UV rays are divided into three types based on the wavelength range: UVC (100–290 nm), UVB (290–320 nm), and UVA (320–400 nm). UVC is predominantly filtered out by the ozone layer in the stratosphere, whereas UVB and UVA reach the earth's surface and thus interact with the skin. Although UVB is a minor

constituent of the total solar UV radiation, it is more active than UVA because it is 1,000 times more capable of causing photodamage [1]. UVB directly causes the formation of cyclobutane pyrimidine dimers (CPDs), which are a type of DNA damage resulting from the absorption of high-energy photons by cellular DNA. CPDs are bulky DNA lesions involving adjacent pyrimidine nucleotides of the same DNA strand. They are normally recognized and removed by photoreactivation and the nucleotide excision repair (NER) system [2]. Conversely,

a defect of the NER system causing the hereditary disorder xeroderma pigmentosum is characterized by an extremely high incidence of skin cancer in sun-exposed areas [3], indicating that CPDs are strongly correlated with cancer development. Human skin exposure to UV irradiation also leads to the generation of reactive oxygen species (ROS). ROS not only induce oxidative stress, including damage to the cell structure, DNA, proteins, and lipids but also activate intracellular signaling by stimulating mitogen-activated protein kinases. Such kinase activation initiates the expression of matrix metalloproteinases and reduces collagen synthesis, causing wrinkle formation on the skin surface, which is a clinical hallmark of photoaged human skin [4].

In recent years, both *in vivo* and *in vitro* studies have revealed that several phytochemicals exhibit chemopreventive potential against UVB radiation-induced adverse effects, including DNA damage, inflammation, oxidative stress, and photocarcinogenesis [5]. At present, natural products exhibiting a wide range of biological activities, such as antioxidant, anti-inflammatory, and antitumor effects, are promising candidates for treating UV-damaged skin. Previously, we had conducted a systematic screening focusing on antioxidant activities and had found that among 52 edible plant extracts, *Mallotus japonicus* leaf extract exhibits the strongest activity [6,7]. Our screening had also identified that the antioxidant activities of 60% aqueous ethanol extract of *Aesculus hippocastanum* (horse chestnut) seed shell extract are comparable to those of *M. japonicus* leaf extract. *A. hippocastanum*, commonly known as horse chestnut, is a native species of urban and rural landscapes and is widely distributed in temperate climates. It has been utilized in Europe as drugs and folk medicines, and its seed extract has traditionally been used for treating chronic venous insufficiency, phlebitis (inflammation of the veins), diarrhea, fever, enlargement of the prostate gland, rheumatism, neuralgia, and rectal complaints [8,9]. In addition, *A. hippocastanum* seed extract has been shown to exhibit anti-inflammatory [10], and antitumor [11] activities and the recent study indicated its beneficial effects on the functional properties of the kidney and microscopic improvements in diabetic nephropathy [12]. However, it has not yet been studied with regard to its beneficial effects in human skin against harmful UV damage. One study demonstrated that the incorporation of *A. hippocastanum* extract into 2% solution of a synthetic sunscreen containing octyl methoxycinnamate increases the sun protection factor value, although it does not display significant UV-absorbing properties when used alone [13].

In this study, we obtained the total phenolic contents, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activities, and oxygen radical absorbance capacity (ORAC) values of *A. hippocastanum* seed shell and endosperm extracts and compared them to those of *M. japonicus* and green tea leaf extracts. To assess whether they could be potential candidates for preventing UVB damage in human skin, their effects on CPD repair in cultured human dermal fibroblasts with or without extract treatments were also investigated.

MATERIALS AND METHODS

Chemicals

Hoechst 33342, propidium iodide, and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Dojindo Molecular Technologies, Inc., Kumamoto, Japan. Penicillin-streptomycin solution, *o*-phenylenediamine, Triton X-100, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Japan, Tokyo, Japan. Streptavidin-horseradish peroxidase (HRP), SuperBlock Blocking buffer in PBS and Alexa Fluor 488 Phalloidin were obtained from Thermo Fisher Scientific K. K., Yokohama, Japan. All other reagents and chemicals were of analytical grade and obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan, unless otherwise stated.

Sample Preparation

A. hippocastanum seeds from healthy trees growing at Dahlemer Weg in the district of Berlin-Zehlendorf, Germany, were collected in September 2014. Seeds were opened, and their shell and endosperm parts were separated. After freeze-drying, the samples were soaked in 60% ethanol water (v/v) at room temperature for 16 h in a flask with continuous mechanical stirring. The extraction ratio between the dried material and 60% ethanol water was 1:10 by mass. After centrifugation, each supernatant was collected and concentrated using a rotary evaporator at 40°C and finally lyophilized. For comparison, green tea (*Camellia sinensis*) and *M. japonicus* leaves were collected in September 2014 from Shimane Prefecture, Japan, and their extracts were prepared using the same procedure as that used for preparing *A. hippocastanum* extracts. The extraction yields of *A. hippocastanum* seed shells, endosperm, green tea leaves, and *M. japonicus* leaves were 8.35% (w/w), 20.7% (w/w), 10.0% (w/w), and 18.6% (w/w), respectively. All extracts were dissolved in 60% ethanol for performing the assays described below.

Antioxidant Activities

The levels of total soluble phenolics and DPPH radical-scavenging capacity of extracts were measured, as previously described [7]. ORAC assay was performed in accordance with the method described by Watanabe *et al.* [14]. All measurements were obtained using a plate reader (EnSpire; PerkinElmer, Inc., Wellesley, MA, USA).

Cell Culture and UV Irradiation

Normal human dermal fibroblasts (NHDFs) were obtained from Lonza, Inc. (Walkersville, MD, USA) and incubated at 37°C in a humidified atmosphere of 5% CO₂ (v/v) with the Minimum Essential Medium Eagle (MEM; Sigma-Aldrich Japan, Tokyo, Japan) containing 10% (v/v) fetal bovine serum (FBS, Gibco, Invitrogen Japan, Tokyo, Japan) and 1% (v/v) penicillin-streptomycin solution. For experiments, cells from passages 5-7 were seeded at a density of 2.0×10^4 cells/well in 96-well plates and cultured in MEM for 2 days to obtain confluence. Just before UVB irradiation, the culture medium was replaced

by 100 μL phosphate-buffered saline (PBS) in every well to avoid the formation of medium derived toxic photoproducts induced by UV exposure. Subsequently, the UVB and sham treatments (UV light off) were performed. Cells were exposed to UVB irradiation from a lamp emitting a peak wavelength of approximately 306 nm (G15T8; Sankyo Denki, Japan) placed 25 cm above the cells. The irradiance of the lamp was measured using a Radiometer Sensor, UVX-31 (UVP Inc, Upland, CA, USA), and the total UVB dose to cells was found to be 6 mJ/cm² at 310 nm. After UVB or sham treatment, PBS was removed, and a part of the cells were immediately fixed in chilled methanol/acetone (1:1) for the 0 h treatment and the others were cultured in fresh culture medium with or without *A. hippocastanum* seed extracts at concentrations of 50, 100, and 200 $\mu\text{g}/\text{ml}$ (0.005%, 0.010%, and 0.020% [w/v]). The plate was further incubated in the 5% (v/v) CO₂ incubator. To measure CPD repair kinetics, cells were fixed 6 and 9 h after UVB exposure.

Cell-based Immunoassays for CPDs

CPDs caused by UVB irradiation in cells were measured using the microplate-formatted cell-based immunoassay [15] with slight modifications. Cells were washed 3 times with PBS containing 0.05% (v/v) Tween-20 (MP Biomedicals LLC, Solon, OH, USA) and were treated by 100 μL PBS containing 0.2 $\mu\text{g}/\text{ml}$ DAPI. After 20 min incubation at room temperature in the dark, cells were imaged at four fields in every well under $\times 4$ magnification using an imaging cytometer (Cytell Cell Imaging System, GE Healthcare UK Ltd., England). Imaged nuclei were enumerated as the cell number using an image analysis software (IN Cell Analyzer 1000 Workstation, GE Healthcare UK Ltd., England). After image acquisition, the levels of CPDs in NHDFs were determined by monoclonal antibodies of TDM-2 (Cosmo Bio, Tokyo, Japan) for capture in triplicate, as described previously [15,16]. Briefly, cells were treated with 2 M HCl to denature cellular DNA at room temperature for 30 min, and non-specific binding sites were blocked by SuperBlock Blocking buffer in PBS. 100 μL of a solution of the monoclonal antibody, diluted 1:1,000 in PBS, were added to well, and plates were incubated at 37°C for 30 min. Then, cells were treated with 100 μL of a solution of the biotin-labeled F(ab') fragment of goat anti-mouse IgG (H+L) (Abcam, Cambridge, UK), diluted 1:1,000 in PBS, and 100 μL of a solution of streptavidin-HRP, diluted 1:10,000 in PBS. Finally, after one wash with 100 μL of citrate-phosphate buffer pH5.0, 100 μL of substrate solution containing 0.04% (w/v) *o*-phenylenediamine and 0.007% (v/v) H₂O₂ in citrate-phosphate buffer were added. After 30 min incubation at 37°C, 50 μL of 2 M H₂SO₄ was added to stop the reaction and absorbances at 490 nm were measured using a plate reader (EnSpire; PerkinElmer, Inc., Wellesley, MA, USA). Each absorbance was subtracted from the average absorbance in each of three identically prepared wells with sham-irradiated cells and then normalized to the cell number.

Cell Viability

The cytotoxic effect of 6 mJ/cm² UVB exposure and *A. hippocastanum* seed shell extracts on NHDFs exposed to

6 mJ/cm² UVB for 6 and 9 h were evaluated by simultaneous double-staining in triplicate. After incubation period, 100 μL of MEM medium containing propidium iodide and Hoechst 33342 at concentrations of 1.0 and 2.0 $\mu\text{g}/\text{ml}$, respectively, were added to each well and stained at 37°C in a humidified atmosphere of 5% CO₂ (v/v) for 20 min. Cells were then imaged and analyzed as described above. Cell viability was calculated as the proportion of cells not stained with propidium iodide to all cells.

Fluorescence Microscopy

Cells treated with different concentration of *A. hippocastanum* seed shell extracts for 9 h after UVB (6 mJ/cm²) or sham irradiation as stated above were washed 2 times with PBS and fixed with 10% (v/v) formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 10 min. Then, cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min at room temperature. After blocking with 1% (w/v) BSA in PBS for 30 min, cells were stained with 5 units/ml Alexa Flour 488 Phalloidin in 1% (w/v) BSA, followed by nuclei staining with 0.4 $\mu\text{g}/\text{ml}$ DAPI. Each staining was performed for 30 min at 37°C in the dark. Cells were imaged under $\times 10$ magnifications using an imaging cytometer (Cytell Cell Imaging System, GE Healthcare UK Ltd., England).

Statistical Analysis

Statistical analyses were performed by one-way ANOVA with Tukey's *post hoc* test using R [17]. All $P < 0.05$ were considered statistically significant.

RESULTS

Antioxidant Properties and Phenolic Contents

The total phenolic contents, antioxidant activities, and ORAC values of *A. hippocastanum* seed extracts were measured by the Folin-Ciocalteu, DPPH radical-scavenging capacity, and ORAC assays method, respectively, as well as those of green tea and *M. japonicus* leaf extracts for comparison. The total phenolic contents of *A. hippocastanum* seed shell and endosperm extracts, expressed as mg gallic acid equivalent per gram of extract, were 602 \pm 1.6 and 10.66 \pm 0.2 mg/g, respectively, whereas the phenolic contents of green tea and *M. japonicus* leaf extracts were 331 \pm 7.7 and 288 \pm 5.2 mg/g, respectively. DPPH radical-scavenging activity of *A. hippocastanum* seed shell, endosperm, green tea, and *M. japonicus* leaf extracts, expressed as μmol Trolox equivalent per gram of extract, were 4990 \pm 70.9, 29.0 \pm 0.40, 3920 \pm 42.6, and 3010 \pm 70.6 $\mu\text{mol}/\text{g}$, respectively. The ORAC value of *A. hippocastanum* seed shell, endosperm, green tea, and *M. japonicus* leaf extract were 7140 \pm 835, 739 \pm 109, 5020 \pm 484, and 2280 \pm 103 $\mu\text{mol}/\text{g}$, respectively. The results of these analyses are summarized in Table 1.

CPD Repair *In Vitro*

NHDFs were irradiated with 6 mJ/cm² UVB and then treated with or without 100 $\mu\text{g}/\text{ml}$ of *A. hippocastanum* seed shell extract

Table 1: Antioxidant activity of extracts

Parts	<i>A. hippocastanum</i> seed shell	<i>A. hippocastanum</i> seed endosperm	<i>C. sinensis</i> leaves	<i>M. japonicus</i> leaves
Phenolic content (mg/g) ^{1,2}	602±1.6	10.66±0.2	331±7.7	288±5.2
DPPH radical-scavenging activity (μmol/g) ^{1,3}	4990±70.9	29.0±0.40	3920±42.6	3010±70.6
ORAC value (μmol/g) ^{1,3}	7140±835	739±109	5020±484	2280±103

¹Data are presented as mean±standard deviation of three separate measurements. ²mg gallic acid equivalent per gram of dry weight of extracts. ³μmol Trolox equivalent per gram of dry weight of extracts. *A. hippocastanum*: *Aesculus hippocastanum*, *C. sinensis*: *Camellia sinensis*, *M. japonicus*: *Mallotus japonicus*, DPPH: 1,1-diphenyl-2-picrylhydrazyl, ORAC: Oxygen radical absorbance capacity

in culture medium for 6 and 9 h, followed by quantification of CPD levels. The relative CPD levels in cells treated with *A. hippocastanum* seed shell extract were significantly decreased compared with those in cells that were not treated [Figure 1a]. Subsequently, the effects in cells treated with *A. hippocastanum* seed shell extract at concentrations of 50, 100, and 200 μg/ml were examined. The results showed that CPD levels reduced with the 9 h treatment in a dose-dependent manner [Figure 1b]. CPD levels in cells that underwent a 9 h treatment with extract at a concentration of 200 μg/ml, which was the highest concentration of *A. hippocastanum* seed shell extract used in this experiment, had decreased by approximately 40% compared with those at baseline. Treatments with UVB irradiation at 6 mJ/cm² and 50 μg/ml *A. hippocastanum* seed shell extract did not significantly inhibit cell viability at 6 and 9 h (without UVB: 99.81 ± 0.144% and 99.75 ± 0.20%; UVB: 99.75 ± 0.07% and 99.57 ± 0.16%; and 50 μg/ml treatment: 98.69 ± 0.86% and 98.67 ± 0.67% at 6 and 9 h, respectively; Figure 2) and had little effect on cell morphology [Figure 3]. Although treatments with at 100 and 200 μg/ml *A. hippocastanum* seed shell extracts significantly decreased cell viability and induced cell contraction slightly, most cells still remained viable (100 μg/ml treatment: 97.59 ± 1.19% and 97.64 ± 1.30% and 200 μg/ml treatment: 97.17 ± 1.56% and 97.25 ± 1.16% at 6 and 9 h, respectively; Figure 2) and attached to the well bottom [Figure 3]. The effect of *A. hippocastanum* seed endosperm extract on CPD repair was not able to evaluate under our experimental conditions because of cell detachment.

DISCUSSION

The total phenolic content of *A. hippocastanum* seed shell extract was higher than those of green tea and *M. japonicus* leaf extracts. This is noteworthy since our previous systematic screening for antioxidant activity among 52 edible plant extracts had showed the *M. japonicus* leaf extract exhibits the highest antioxidant activity, followed by the green tea leaf extract. In addition, Dudonné *et al.* have also reported the total phenolic contents of extracts from plants used industrially for fragrance, cosmetic, and food-flavoring applications [18]. The phenolic content value of *A. hippocastanum* seed shell extract was found to be higher than their reported values. Further, a significant correlation between total polyphenol contents and antioxidant activities has been suggested [18-20]. As expected, *A. hippocastanum* seed shell extract, but not endosperm extract, exhibited higher DPPH radical-scavenging activity than green tea and *M. japonicus* leaf extracts. The ORAC value of *A. hippocastanum* seed shell extract was 7140 ± 835 μmol Trolox equivalents/g, which is

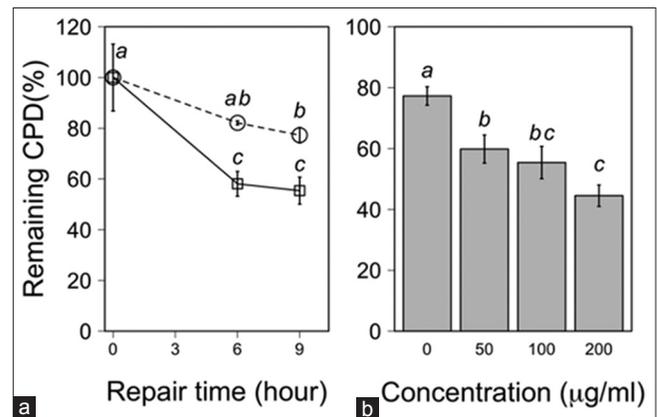


Figure 1: (a) Cyclobutane pyrimidine dimers (CPD) repair kinetics in normal human dermal fibroblasts. Cells were incubated for the indicated periods after ultraviolet B irradiation and processed for CPD detection. Open squares and open circles indicate relative CPD levels in cells treated with or without *Aesculus hippocastanum* seed shell extracts at a concentration of 100 μg/ml, respectively. (b) The relative CPD levels at 9 h after treatment with different concentrations of *A. hippocastanum* seed shell extracts. Results are expressed as mean ± standard deviation (% of the CPD level at 0 h) from three independent experiments and with different superscript letters are significantly different

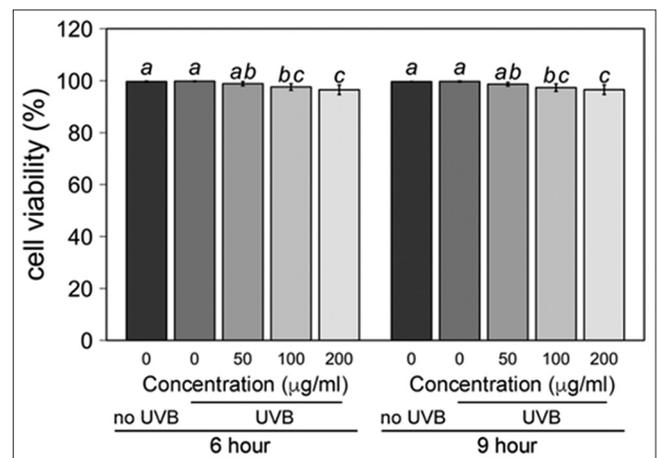


Figure 2: Cell viability of ultraviolet B (UVB)-irradiated NHDFs treated with *Aesculus hippocastanum* seed shell extracts. Before UVB irradiation, the culture medium was replaced with PBS, and cells were irradiated with 6 mJ/cm² UVB. PBS was then removed, and fresh medium containing different concentrations of *A. hippocastanum* seed shell extracts was added to the cells. At 6 and 9 h after treatment, percent cell viability was assessed by fluorescence imaging. Results are presented as a mean ± standard deviation from three independent experiments and with different superscript letters are significantly different

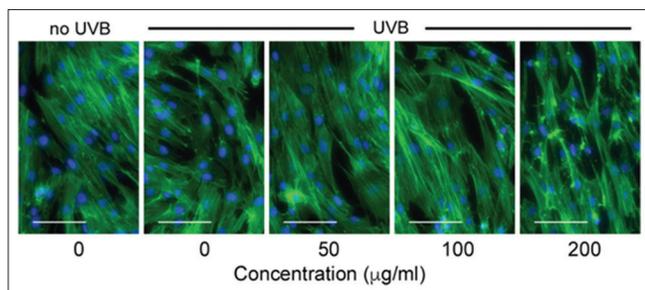


Figure 3: Effect of *Aesculus hippocastanum* seed shell extracts on ultraviolet B (UVB) irradiated NHDFs. Representative fluorescent images of cells treated with different concentration of *A. hippocastanum* seed shell extracts for 9 h after UVB (6 mJ/cm²) or sham irradiation were shown. Actin filaments were visualized as green through Alexa Fluor 488 Phalloidin, and cell nuclei stained by 4',6-diamidino-2-phenylindole were shown as blue. Scar bars=100 µm

not only greater than those of green tea and *M. japonicus* leaf extracts but also comparable with the second highest value of *Pinus maritima* commercial extract among those reported by Dudonné *et al.* [18]. Reportedly, *A. hippocastanum* seed shell is rich in polyphenolic components, including dimers, trimers, tetramers, oligomers, and monomeric flavonols such as catechins and epicatechin derivatives [21-23]. These compounds could contribute to the high values obtained here, and strong radical-scavenging activity might have made cells resistant to the cytotoxic effect of the increased ROS level induced by UVB irradiation.

One detrimental feature that occurs on the skin cell exposure to UVB irradiation is CPD formation. Therefore, we investigated whether the *A. hippocastanum* seed shell extract exerted a positive effect on the kinetics of CPD repair. As shown in Figure 1, seed shell extract reduced CPD levels compared to those without treatment. On the other hand, it remains unknown whether endosperm extract influences CPD repair. Naturally occurring compounds, specifically phenolics, are widely distributed in plants, including in their fruits, vegetables, seeds, nuts, flowers, and barks; they have the potential to exhibit substantial beneficial effects against skin aging and several diseases [24]. To date, (–)-epigallocatechin-3-gallate, one of the major polyphenols of green tea leaves, silymarin - a plant flavonoid from milk thistle (*Silybum marianum*) - and grape (*Vitis vinifera*) seed proanthocyanidins have been shown to exhibit the ability to aid DNA repair in UV-exposed skin in *in vitro* cell culture and *in vivo* animal models [25-27]. They promote UVB-induced CPD repair in XPA-proficient human fibroblast cells in a dose-dependent manner but not in XPA-deficient cells *in vitro*, suggesting the enhancement of DNA repair through a functional NER mechanism. In the present case of the application of *A. hippocastanum* seed shell extract, proanthocyanidins might have been responsible for the enhancement of UVB-damaged CPD repair *in vitro*. Although we have not yet elucidated the phenolic constituents of *A. hippocastanum* seed shell extract, proanthocyanidins are likely to be involved in the extract because 80% aqueous acetone extract of *A. hippocastanum* seed shells reportedly contains proanthocyanidins [23], and approximately 50% (v/v)

aqueous ethanol solvents are also commonly used to extract proanthocyanidins [28].

CONCLUSION

This preliminary study demonstrated that *A. hippocastanum* seed shell extracts had high total phenolic contents and exhibited relatively strong antioxidant properties, suggesting their potential utility against UVB-induced DNA damage via aiding CPD repair. Further, studies are necessary to elucidate whether the enhancement of CPD repair by *A. hippocastanum* seed shell extracts is mediated through the functional NER mechanism and also to identify the main active components. Such studies are now underway.

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