Synergistic protective effects of Houttuynia cordata fermented with Aureobasidium pullulans against UVA and H2O2 induced oxidative stress in human skin keratinocytes

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Research Article

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Abstract

The biological activities of *Aureobasidium pullulans* fermented with a medicinal plant, *Houttuynia cordata*, was investigated for human skin keratinocytes induced chemical and photo oxidations. In this research, H_2O_2/UVA-induced HaCaT cell lines were treated with *H. cordata* water/ethanol extracts (HCW/HCE) and fermented with *A. pullulans* water/ethanol extracts (HCFW/HCFE). *A. pullulans* fermented with *H. cordata* (HCFW) increased in 5.4 folds of total polyphenol (HCFW 46.89 mg GAE/extract g), and 2.3 folds in flavonoids (HCFW 53.80 mg GAE/extract g) compare with water extracts of *H. cordata* (HCW). Further, no significant cytotoxicity for HaCaT cells showed all the extracts of *H. cordata* fermented with *A. pullulans*. HCFW extracts have significantly lowered inflammation factors such as COX-2 and Hsp70 proteins in oxidative stressed HaCaT cells induced by H_2O_2 and UVA treatments. All *H. cordata* extracts significantly down-regulated gene expression involved in oxidative stress and inflammation factors, including IL-1β, IL-6, COX-2, TNF-α, NF-κB, and MMP-1 in the H_2O_2/UVA treated HaCaT cells. However, keratin-1 gene expression in the UVA treaed HaCaT cells was increased in two forlds by HCFW extracts. Further, *A. pullulans* fermentated *H. cordata* extracts (HCFW/HCFE) reduced effectively the genes involved in oxidative stresses than those of *H. cordata* extracts only. Overall, the pholyphenol rich extracts of *H. cordata* fermented with *A. pullulans* showed synergistic protective effects for human epidermal keratinocytes to prevent photoagaing and intrisinc aging by anti-oxidation and anti-inflammatory functions.

Introduction

Ultraviolet (UV) exposure to natural sunlight is helpful for the synthesis of vitamin D in human skin. Still, over-exposure to UV radiation causes a significant risk to human skin and eyes. UVA (320–400 nm), UVB (280–320 nm), and UVC (100–280 nm) are the three type of ultraviolet radiation that causes sunburn, photo-ageing, and skin melanoma [1]. UV exposure is one of the prominent etiological factors for skin cancer, and the prevalence of human skin cancer accumulated considerably worldwide over the last few decades [2]. Furthermore, UVA-induced cytotoxicity and mutagenesis caused increased intracellular reactive oxygen species (ROS) levels production that may be linked to damage to extracellular membrane lipid, protein, and DNA polysaccharides [3]. In addition, UV light produces oxidative stress in the skin dermis, resulting in a high number of matrix metalloproteinases (MMPs) and the degradation of extracellular matrixes, resulting in a variety of photoaging symptoms. Therefore, UV radiation-induced photoaging is one of the extrinsic skin ageing by mediated MMPs and lipid peroxidation in skin cells [4].

Exposure to UVA radiation causes skin inflammation, contributing significantly to the extrinsic skin ageing process. UVA light emits from non-natural sources and treats inflammatory skin conditions such as atopic dermatitis, skin cancer, and connective tissue problems for cosmetic purposes [5]. The production of inflammatory cytokines such as interleukin-1 and 6 (IL-1 and 6) and tumour necrosis factor (TNF-α), which can aggregate and destroy collagen in cells, has been linked to the formation of reactive oxygen species (ROS). In addition, it contributes to the pathophysiological process in photo-carcinogenesis and photoaging [6]. The ROS are produced by the electron transport activities and are a

byproduct of various metabolic pathways in cellular compartments. However, high amounts of ROS in human cells can be induced by toxic environmental stress incluiding UV exposure and chemicals [7].

ROS's oxidative stress activates the inflammatory mediating gene nuclear factor-kappa B (NF-κB) to induce an inflammatory response in the human cells [8]. Briefly, NF-κB is a representative transcriptional factor that regulates the inflammatory response in cells and exists in the cytoplasm. NF-κB plays the induction of immune response to microbial infection [9]. Breifly, NF-κB migrates to the nucleus and attaches to the κB binding site to activate the down-regulating genes involved in inflammation such as cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β) and interleukin 6 (IL-6) [10]. COX-2 is an inducible protein, and its expression is increased in the different types of cells when exposed to inflammatory cytokines, tumour promoters, and mitogen activation factors [11]. Multiple lines of scientific evidence suggest that genetic depletion of COX-2 decreases skin inflammation and cancer in response to UV irradiation [12]. UV can also activate collagenases such as MMP-1 and MMP-13, which degrade collagen and cause collagen shortage and wrinkles in the dermis [13].

Numerous synthetic products have been commercialized and utilized as anti-agents that promote collagen synthesis, and skin inhibits the expression of MMPs [14, 15]. In the recent past, vitamin D, retinoids, corticosteroids, and other synthetic drugs have been utilized as anti-ageing agents. However, it causes severe side effects such as skin, liver toxicity, and pharmaco-resistance property [16]. Therefore, several natural and medicinal plants with fermented occurring phytochemicals are being considered for their ROS scavenging ability to prevent harmful side effects [17, 18]. Thus, natural sources can reduce the inflammatory response in skin disorders, safe anti-photoaging agents, and no side effects for novel ingredients for cosmeceuticals.

Houttuynia cordata Thunb. is a leafy vegetable used in cosmetics and folk medicines in Korea, Japan and China [19–21]. Several phytochemicals, including flavonoids and essential oils such as bornyl acetate, methyl nonyl ketone, β-myrcene, β-pinene, and limonene are present in *H. cordata* [22, 23]. *H. cordata* has antibacterial, antiviral [23], anticancer [24], anti-inflammatory [25]antibiofilm activities [26]. Also, it suppresses the expression of inflammatory mediators in HMC-1 cells [27, 28]. Besides, some reports demonstrated that *H. cordata* successfully inhibited the inflammation and regulation of mucosal immunity [29, 30].

Aureobasidium pullulans is a yeast-like saprophytic fungus as know black yeast. *A. pullulans* is a well-known biocontrol agent against pathogenic fungi and softens the tissues of plants and fruits. *A. pullulans* grow in various forms, and yeast itself is a source of fat or protein and is a significant food supplying pullulan, vitamin B and vitamin D [31, 32]. The β-glucan produced by *A. pullulans* is nearly as effective as the same type of glucans produced by various species. Therefore, the β-glucan contained in a cultured fluid of *A. pullulans* is authorized as a food additive and consumed as a health-promoting food. Beta-glucan is used to enhance the immune system and anti-allergy, anti-viral activity [33–36] and control inflammation induced by various pathogens [33]. Also, *A. pullulans* showed the anti-tumour activity by the beta-glucan, which stimulates the macrophages and causes tumour necrosis factor-related apoptosis-

inducing ligand (TRAIL) cancer cell death [34]. However, there is no report for changes in health functionality by conversion of substances of *H. cordata* fermented with *A. pullulans*. Therefore, we investigated the potential physiological effects of *H. cordata* extract fermented with *A. pullulans* against H_2O_2 or UVA-induced inflammatory reactions in keratinocyte (HaCaT) cells.

Materials And Methods

Cells and Chemicals

Human keratinocytes (HaCaT) cell lines were obtained from the American-style culture collection (ATCC, Manassas, USA). The Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), antibiotics, trypsin-EDTA and phosphate-buffered saline (PBS) were acquired from HyClone, Logan, USA. In addition, hydrogen peroxide (H_2O_2), Folin-Ciocalteu, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), methyl thiazolyl diphenyl-tetrazolium bromide (MTT) gallic acid, and quercetin were procured from Sigma Aldrich CO., USA.

Protein assay kit (Pierce, USA), polyvinylidene fluoride (PVDF) membrane (Neuroscience, Seoul, South Korea), Primary antibodies (MMP-1, Hsp70, COX-2, and β-actin), secondary antibodies, and Chemiluminescent substrate were procured from Thermo Scientific, (MA, USA). cDNA Synthesis and qPCR Master Mix were purchased from Promega (Madison, USA) and other fine chemicals and reagents utilized in the present experiment were analytical grades.

Non-fermentation and fermentation *H. cordata* water/ethanol extraction

H. cordata was provided by Stemforce Co., Inc. (Gyeongsan, South Korea). The dried plants were washed three times, then allowed to dry, weighed, and then crushed into the powder used in this study. *H. cordata* extracts of non-fermented or fermented with *A. pullulans* were prepared with hot-water extracts and 50% ethanol extract.

Briefly, dried *H. cordata* with about 200 g and water were combined in a ratio of 1:2 (w/v) and wetted. Next, add 0.6% peptone, and mix evenly with sterilizing for 15 min at 121 $^{\circ}$C. After cooling, dextrin was added in an amount corresponding to 1.5% of the sample and combined well. Next, $2.3x10^7$ CFU/mL of *A. pullulans* suspension was inoculated and fermented for 18 days at 30 $^{\circ}$C (Shin et al. 2019; Xia et al. 2017). Finally, the water extract, sterilized purified water equivalent to 5 times the weight of the fixed non-fermented and fermented sample, was added and bathed for 24 h at 60 $^{\circ}$C, followed by three extractions. First, the supernatant was filtered after centrifuging the sample at 8,000×g for 10 minutes. Then, again, the sample was centrifuged at 8000×*g* for 20 min. Next, the extracted sample was filtered via filter paper, and the dry extract sample was powder by a rotary evaporator. A dried extract sample was kept at 4°C for future use.

Determination of total phenolic and flavonoid content

The total phenol content of the *H. cordata* extract was analyzed by the Folin-Ciocalteu method [35]. The sample was 1 g and dissolved in 10 ml of the deionized distilled water (ddH_2O), followed by a centrifuge of the sample at 240×*g* for 5 min. The obtained supernatant was filtered using Whitman filter paper, and the filtrate was further centrifuged at 240×*g* for 10 min. Briefly, 200 µL of *H. cordata* extract mixed with 670 µL ddH_2O. Then. Folin-Ciocalteu's phenol reagent (30 µL) was added to the mixture and placed at room temperature for 5 minutes. Next, added sodium carbonate (600 µL) was then kept at room temperature for 30 minutes and absorbance was determined at 765 nm using a UV-Vis spectrophotometer (Optizen 2120UV, Republic of Korea). The total polyphenol content of the *H. cordata* extracts was represented as mg gallic acid equivalents per gram of extract sample (mg/g).

The total flavonoid content was estimated by a colourimetric assay [35]. *H. cordata* extracts of 200 µL were mixed with ddH_2O (790 µl), 10% aluminium chloride hexahydrate, 1M potassium acetate (30 µL), and 95% ethanol (450 µL) were mixed and kept to incubation for 40 minutes. After incubation, absorbance was measured by UV-Vis spectrophotometer at 510 nm. The total flavonoid content of the *H. cordata* extracts was represented as mg quercetin equivalents per gram of extract sample (mg/g).

DPPH and ABTS scavenging assay

The scavenging efficiency of *H. cordata* extracts was determined by Blois et al. [36]. The 200 µL of *H. cordata* extracts were diluted for each concentration with 100 µL of DPPH dissolved in ethanol solution mixed with 200 µL of *H. cordata* extracts sample. The reaction was allowed for 30 minutes and absorbance was determined at 517 nm using an ELISA reader (Infinite^{TM} F200, Männedorf, Switzerland).

The ABTS radical scavenging of *H. cordata* extracts was determined by Re et al. [37]. Briefly, 2.45 mM potassium persulfate and 7 mM ABTS were mixed at a 1:1 (v/v) ratio and kept darkroom for 24 h to generate ABTS radicals. The ABTS solution in which radicals were formed was diluted with ethanol, and the absorbance measured at 734 nm was adjusted to be 0.70 ± 0.02. *H. cordata* extracts sample, an equal volume of ABTS (150 µl) and the 0.2 % test sample was mixed well and allowed at room temperature for 6 minutes. Finally, the absorbance was determined at 734 nm using an ELISA reader. DPPH or $ABTS^+$ results were compared with ascorbic acid as a positive control. DPPH or $ABTS^+$ scavenging (%) was calculated using a formula depicted in our previous study [38].

Cell culture and cytotoxicity assay

The HaCaT cells were grown in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin) at 37 °C in a 5% CO_2 incubator. Next, the cell lines were subcultures at 2 ~ 3-day intervals.

The HaCaT (5×10^3) were seeded into a 96-well plate at 37 °C in a 5% CO_2 incubator. For 24 hours. Next, different concentrations of HCE, HCW, HCFE, and HCFW (10, 100 µg/ml) were added and then incubated at 37 °C in a 5% CO_2 incubator for 24 hours. Then cells were induced with UVA (3 J/cm^2)/H_2O_2 (1 mM)

for 24 hours (table. 1). Herein, the old medium was removed, and add fresh DMEM (100 µL) and MTT (20 µL) solution and incubated for 3 hours in 5% CO_2 at 37 $^{\circ}$C. Afterwards, the sample optical density was measured at an ELISA reader using a 96-well plate (Infinite^{TM} F200, Switzerland).

Protein expression by Western blot

They were treated with *H. cordata* extract samples and then UVA or H_2O_2 exposed to HaCaT cells. After the incubation, the media was replaced by the PBS buffer. Protein extraction was performed by adding protein lysis buffer with an inhibitor cocktail. The sample was centrifuged at 12,000 rpm at 4 $^{\circ}$C for 15 minutes and the supernatant was collected. 30 µg of protein was electrophoresed on 7.5% SDS-PAGE and then attached to a PVDF membrane. After blocking the protein-attached PVDF membrane with NEO western enhanced buffer (Neuroscience, Seoul, Korea), the PVDF membrane was treated with primary antibodies COX-2, Hsp70, MMP-1 and β-actin shaken overnight at 4 °C, then washed 3 times for 5 minute each with TBST buffer. Next, the blot was incubated in secondary antibodies for one hour. Finally, the membranes were washed 3 times with TBST for 5 minutes, and protein bands were visualized by Chemiluminescent substrate (Thermo Scientific, MA, USA). After the secondary antibody reaction, the PVDF membrane was developed using the photosensitive film in the dark. The relative intensity of a specific protein band was quantified using Imager (SLB, Seoul, Korea) and lab works software (Upland, CA, USA).

RNA isolation and qRT-PCR analysis

According to the manufacturer's instructions, total RNA was isolated from all treated groups samples, using a Tri-RNA isolation kit (Favorgen Biotech, Ping-Tung, Taiwan). NanoDrop (Thermo Scientific, MA, USA) determined RNA content and purity. For cDNA Synthesis, a GoScript™ reverse transcription mix, oligo (dT) kit was used. Nuclease-free water, GoScript™ reaction buffer, oligo(dT), and GoScript™ enzyme mix were mixed with the reaction product so that the final volume was 10 µL. cDNA was synthesized as follows: 3 µg of RNA heated at 65 $^{\circ}$C for 5 minutes, and Nuclease-Free water was added to the reaction product to make the final volume of the development of 20 µL. Then the reaction sample was repeated 1 at 25 $^{\circ}$C for 5 minutes, 42 $^{\circ}$C for 60 minutes, and 70 $^{\circ}$C for 15 minutes, and then stored at -20 $^{\circ}$C.

For real-time quantitative PCR (qPCR), cDNA and primers were added to a qPCR master mix (Promega, Madison, USA) kit. All the primer sequences were designed from the GenBank database and the BLAST pick primers program (https://www.ncbi.nlm.nih. gov/tools/primer-blast/) was shown in Table 2. Average 50 pmoles of primers for each gene, template (300 ng/2 µL), and qPCR master mix were added to each PCR tube, and Nuclease-free water was added to make the final reaction product 20 µL. The qPCR conditions were pre-denaturation at 95 $^{\circ}$C for 1 minutes followed by 40 cycles of 10 s at 95 $^{\circ}$C (denaturation), 15 s at 60 $^{\circ}$C (annealing), and 20 s at 72 $^{\circ}$C (extension). It was repeated twice, and the melting curve was analyzed by scanning at 60~95 $^{\circ}$C for 1 $^{\circ}$C each for 1 s. The relative expression of the genes was calculated using the $2^{-\Delta\Delta Ct}$ methods. Then, ΔΔCt was calculated by the treatment group (Ct

target gene - Ct GAPDH gene) and control group (Ct target gene–Ct GAPDH gene) estimated. Finally, the optimal GAPDH gene was used as an internal control.

Statistical analysis

All data were analyzed through one-way analysis of variance (ANOVA), the software SPPSS (SPSS program, USA). The statistical significance was determined using a value of $p< 0.05$.

Results

Total phenolic and flavonoid profiles of H. cordata extracts

The extraction yields result was shown HCE (13.48%), HCW (15.89%), HCFE (18.07%) and HCFW (16.17%). When comparing the yield of (HCE and HCFE) and (HCW and HCFW), the fermented sample was increased to 4.59% and 0.28%, respectively. The total polyphenolic contents of the extract with fermented samples had shown the highest HCFW (46.89 ± 1.24) and HCFE (25.73 ± 0.71) when compared to HCW (8.64 ±0.72) and HCE (11.01 ± 0.63). Total phenolic compounds of HCFE were increased to 4.59% compared with non-fermented *H. cordata* water extract (HCW) (Table 3). The flavonoid amount which is contained in the extract the amount of quercetin was the highest in the HCFW (53.80±1.21), HCFE (47.26 ± 0.42), HCW (23.77 ± 1.33), and HCE (20.17 ± 0.27) in this consequence. As a result of comparison with fermentation and without fermentation, HCFE had increased by 2.34 times of HCE, and HCFW had increased by 2.26 times of HCW (Table 3).

Antioxidant activity of *H. cordata* extracts

In this study, DPPH and $ABTS^{\cdot+}$ radical scavenging activity was utilized to measure the antioxidant ability of the *H. cordata* extracts and fermented with *H. cordata*. It showed the concentration-dependent antioxidant activity. i.e., the percentage of DPPH scavenging activity showed the HCE (19.05 to 92.47%), HCW (7.08 to 86.61%), HCFE (15.02 to 89.48%), HCFW (11.01 to 93.51%) in 10-1000 µg/mL concentration-dependent manner. Comparing HCW with HCFW sample has higher antioxidant activity (Fig.1a). the scavenging effect of ABTS radical on various extracts was shown HCE (29.42% to 99.75%), HCW (30.01 to 99.70%), HCFE (7.50 to 100.31%) and HCFW (6.85 to 100.32%) indifference concentration (10-1000 µg/ml). Comparing HCW with HCFW, fermented *H. cordata* showed higher antioxidant activity (Fig. 1).

Cytotoxicity effect of *H.cordata* extracts in UVA/H_2O_2-induced HaCaT cell lines

The HaCaT cell lines were treated different concentration of HCE, HCW, HCFE and HCFW in free medium (50, 100, 1,000 µg/mL) for 24 h. However, the cells treated with HCFW showed increasing viability (79.2, 89.6, 86.7, 79.3, and 82.7%) compared to HCW, HCE, and HCFE. The viability was increased through the fermentation process (Fig. 2A).

To investigate the effect of oxidative stress on human skin cells induced by H_2O_2, the extracts of HCW, HCE, HCFW, and HCFE (10 to 100μg/mL) were simultaneously treated on the HaCaT cells for 24 h. The cell viability rate of the HaCaT cells showed that the treatment of HCW (111.5 and 104.1%), HCE (105.4 and 94.4%), HCFW (105.0 and 90.0%), and HCFE (92.3 and 103.1%) reduced the oxidative stress of H_2O_2 (1 mM) compared to the H_2O_2 treatment group alone and increased the viable cells in a concentration-dependent manner (Fig. 2B). Resulting of this study, HCW, HCE, HCFW, and HCFE extracts in 100 μg/mL concentration reduced either UVA/H_2O_2-induced cell death significantly. Therefore, we choose the 100 μg/mL concentration for future protein and gene expression experiments.

We assessed the treatment of HCW, HCE, HCFW, and HCFE (10 to 100 μg/mL) against UVA (3 J/cm^2) mediated cell viability in HaCaT cells (Fig. 2C). We found that 100 μg/mL of HCW, HCE, HCFW, and HCFE treatment were significant (111.8, 79.1, 89.0, and 103.2%), respectively, protecting the UVA irradiated cells compared to both the control group and the UVA alone treated group.

Effect of *H.cordata* extracts against inflammation in UVA and H_2O_2-induced HaCaT cells

To determine whether the H. cordata extracts and the black yeast fermented *H. cordata* extracts can reduce skin inflammation in HaCaT cells were treated with UVA (3 J/cm^2) or H_2O_2 (1 mM). Both chemical and photo oxidant induced the protein levlels of inflammation factors such as COX-2, Hsp70 and MMP-1 proteins (Fig. 3).

The HaCaT cells cultuered with 1 mM H_2O_2 increased the COX-2 and Hsp70 protein levels (Fig. 3). The upregulation of COX-2 and Hsp70 proteins was reflected in the process of inflammation by the H_2O_2-induced oxidative stress. When inflammatory HaCaT cells were treated with 100 μg/mL extractions of HCW, HCE, HCFW and HCFE, the protein levels of COX-2 and Hsp70 proteins were significantly reduced (Fig. 3). The extracts of *H. cordata* fermented with *A. pullulans* (HCFE and HCFW) reduced COX-1 and Hsp70 proteins more than the extracts of *H. cordata* (HCF and HCW). Noticeably, the water extract of fermented *H. cordata* (HCFW) reduced COX-2 protein expression almost 5 fold more than the inflammatory HaCaT cells. Moreover, HCFW reduced Hsp70 protein expression nearly 10-fold that of the inflammatory HaCaT cells.

The UVA exposure HaCaT cells significantly over-expressed COX-2, Hsp70, and MMP-1 proteins compared to the control group (Fig. 4). The levels of COX-2, Hsp70, and MMP-1 proteins in HaCaT cells exposed to UVA were significantly reduced by treatment of HCW, HCE, HCFW, and HCFE extracts (Fig. 4). All *H. cordata* extracts showed the levels of COX-2 protein were reduced by more than 50% compared with that of UVA-treated cells. Noticeably, the water extraction of the fermented *H. cordata* (HCFW) decreased almost 70% of the COX-2 protein level.

Oxidative stress mediates UVA/H_2O_2-induced cellular inflammation by activating NF-κB and COX-2 pathways

We examined the treatment effect by the extracts of *H. cordata* (100 μg/mL) with inflammation-induced HaCaT cells by UVA in photo-aging mimics and H_2O_2 in stress-induced aging mimics. The oxidative stress was significantly recovered by *H. cordata* extracts (Fig. 2B and C). The treatment of H_2O_2 (1 mM) induced the expressions levels of inflammation-mediated genes such as NF-κB, IL-6, MMP-1, COX-2, TNF-α, IL-β, and MMP-1 in HaCaT cells (Fig. 5). The oxidative stress recovery in the figure 2 was indicated by significant reductions in mRNA expression levels of TNF-α, NF-κB, COX-2, IL-6, IL-β, and improved MMP-1 treated after HCW, HCE, HCFW, and HCFE (Fig. 5). The TNFα gene expression was reduced in H_2O_2 stressed cells treated with *H. cordata* extracts. Based on the qPCR results, the ethanol extracts of *H. cordata* fermented with with *A. pullulans* (HCFE) reduced NF-κB, TNFα, IL-6 and IL-1β gene expression significantly (Fig. 5).

UVA (3 J/cm^2) irradiated HaCaT cells enhanced the gene epression of COX-2, NF-κB, TNF-α, IL-6 and MMP-1 significantly. However, UVA urradiated HaCaT cells treated with HCW, HCE, HCFW and HCFE significantly reduced the mRNA transcript levles of COX-2, NF-κB, TNF-α, IL-6 and MMP-1 (Fig. 6). Remarkably, the COX-2 gene expression of UVA- irradiated HaCaT cells treated with the *H. cordata* extracts has reduced almost 4.5 folds than untreated extratcs.. Further, the MMP1 gene expression level was decreased from 4.6 to 7.8 folds after treaed with the extracts of *H. cordata*. Specifically, the HCFW treatment inhibited the gene expression of COX-2 and MMP-1 in the most effectively (Fig. 6).

Discussion

Human skin is vital for protecting the layer of the whole body from harmful materials, including toxic chemicals and UVA radiation, which leads to photoaging and skin cancer [39]. In recent years, natural sun-protective agents have been developed derived from medicinal plants [39, 40]. In the present study, we investigated *H. cordata* to be used as raw ingredients for cosmetic purposes. Furthermore, we found that extracts of *H. cordata* contained a high amount of total polyphenolics and flavonoids (Table 3). This might contribute to their *in vitro* antioxidant activity and demonstrate an additive effect against UVA/H_2O_2-related oxidative stress in human keratinocytes.

Based on the high amount of total polyphenolics and flavonoids in various extraction, we presumed that these phyto-phenolics serve as the main ingredients as the potent antioxidants. This study also demonstrated the effect of fermentation to extract from a sample. HCFE increased total polyphenolic content about 2.34 times that of HCE. Further, HCFW increased total polyphenolic content by about 5.43 times that of HCW. Also, HCFE increased flavonoid content by about 2.34 times that of HCE, and HCFW increased flavonoid content by about 2.26 times that of HCW. Many studies have shown that ethanol is a good solvent for phenolic extraction because it has a high extractive yield on a dry weight basis [41, 42]. Like in our study, a high amount of phenolic and flavonoid content was identified in the entire plant ethanol/waster extract of *H.cordata* fermentation yields [43, 44].

Phytoconstituents have diverse biological activities, including free radical scavenging, anti-photoaging, and anti-inflammatory. Several in vitro assay models are available to associate the antioxidant activities of medicinal herbal extracts and their active metabolites. DPPH is a stable free radical that can take an electron or hydrogen from an anti-oxidant to form a stable product [45]. The natural byproduct compounds have neutralized free radicals by acting as rapid donators of a hydrogen atom to free radicals. The antioxidant activity of the DPPH was increased in the HCE (19.05 to 92.47%), HCW (7.08 to 86.61%), HCFE (15.02 to 89.48%), and HCFW (11.01 to 93.51%) in 10 – 1,000 µg/mL concentration-dependent manners (Fig. 1). The concentration-dependent ABTS radical activity was seen in various extracts of HCE (29.42–99.75%), HCW (30.01 to 99.70%), HCFE (7.50 to 100.31%) and HCFW (6.85 to 100.32%) in concentration-dependent manners. They noted that HCW and HCFW showed better antioxidants when compared with ascorbic acid. Overall, it could be suggested that the polyphenolic source from the *H. cordata* and with fermentation had strong antioxidant activity. In the current work, HaCaT cells, a human epidermal keratinocyte, were used as a model to estimate the protective property of *H. cordata* against H_2O_2/UVA-radiation that induces oxidative stress and cells damage in human skin. Here, we measured the detrimental effects of H_2O_2/UVA on HaCaT cell viability. We demonstrated that UVA-irradiation and H_2O_2 treatment decreased the cell viability by dose-dependent (Fig. 2). Therefore, the viability of HaCaT cells was reduced, and ROS generation increased after UVA-induced. However, *H. cordata* extarcts containg high amounts of the phenolics and flavonoids improve cell viability by scavenging ROS and protecting HaCaT cells from UVA-exposed cells (Fig. 2).These findings from previous studies also suggested that the growth inhibitory effects that occur through G1/S or G2/M arrest and anticancer activity of the *H. cordata* were attributed to the presence of phenolic compounds [46, 47].

UVA/H_2O_2 is commonly used to generate oxidative stress, which can induce excessive ROS production to skin constituents and may indirectly cause damage to cellular structural protein, leading to an imbalance of anti-oxidant/oxidative levels [52, 53]. In this study, we focused to changed genes responded in oxidative stress. UVA radiation induces the gene expression of MMPs in human skin cells. MMPs are zinc-dependent endogenous proteases associated with cell migration and proliferation and degrading extracellular matrix proteins such as fibronectin, collagen, and gelatin, causal to photoaging [48]. MMP-1 is a major protease that degrades collagen type I and III in the dermis, whereas MMP-2 degrades collagen type IV and gelatin [49]. Another stress induced protein, heat shock proteins (HSP-70) is responsible for the refolding or degradation of denatured proteins created by stress [50, 51]. Studies have shown that natural plants present polyphenol and flavonoids with diverse biological properties are a hopeful class of functional plants that inhibit extracellular matrix degradation and cellular alteration [54, 55]. Our present study also found that *H. cordata* fermentation extract with (HCFE/HCFW) were down-regulated proteins Hsp70, MMP-1 and COX-2, which indicates that *H. cordata* protects HaCaT cells from photodamage. Therefore, treatment with *H. cordata* extract rich in polyphenols results in protection of HaCaT cells from UVA-induced photoaging and inflammatory.

The activation of NF-κB, the nuclear factor, has an essential role in initiating inflammation response. The development of numerous inflammatory proteins such as IL-1β, IL-6, TNF-α, and COX-2 regulates cellular

senescence and photo-ageing [56]. The NF-κB activation to play a key function in the upregulation of numerous genes in inflammatory responses [57]. UVA irradiation is shown to have high activities of NF-κB and TNF-α (Fig. 6). Therefore, we predicted that the *H. cordata* extracts reduced the NF-κB mediated gene expression in inflammation, resulting in recovery of cell viability of the HaCaT induced photo-oxidative by UVA.

IL-6 can also reinforce the senescent growth arrest [58], including the MMPs that break down collagen in the presence of UV light [59, 60]. In addition, it has been reported that UVA induces dermal damage such as alteration of collagen and elastic and MMP-1 expression [61]. Our result indicated that UVA/H_2O_2 caused HaCaT cells were increased the mRNA expressions and the levels of COX-2, TNF-α, NF-κB, IL-6, IL-β, and MMP-1 production. It is be noted that the *H. cordata* extracts fermentied with *A. pullulans* (HCFW) down reualted the COX-2 and MMP-1 proteins in the photo- and chemical-oxidative stresses (Fig. 4). *H. cordata* extarcts inhibit significantly the production of inflammatory cytokines such as IL-6, IL-1β and TNF-α in the UVA/H_2O_2 treated HaCaT cells. Previous studies supported our results that *H. cordata* could suppress pro-inflammatory cytokine responses via inhibited the activation of NF-κB and dermal fibroblast production [28, 29]. Therefore, the decline of the NF-κB pathway by phenolics and flavonoids may involved in reducing the inflammeantion to attenuate photo and chmical oxidative response in HaCaT cells.

Further, the *H. cordata* extracts increased keratin (KRT-1) gene expression (Fig. 6). Among the extracts, water extract of *H. cordata* fermented with with *A. pullulans* (HCFW) enhanced highly the keratin gene expression of UVA- irradiated cells, indicating the *H. cordata* fermentation with *A. pullulans* showed synergistic protective effects of HaCaT cells for photo-ageing. Overall, the *H. cordata* fermented with *A pullulans* showed scavenging ablity againt to UVA radiation-induced ROS to prevent photoaging of skin via blocking inflammatory responses and collagen degradation.

Conclusion

In *vitro* study demonstrated that HaCaT cells recovered H_2O_2 and UVA-induced oxidative stresses by treating *H. cordata* extracts fermented with *A. pullulans*. The total polyphenols and flavonoids of *H. cordata* were increased by *A. pullulans* fermentation. The polyphenols and flavonoids increased the antioxidant protective properties by scavenging UVA-induced ROS and anti-inflammation. It was noticed that the levels of inflammation mediated proteins including COX-2, Hsp70 and MMP-1 were significantly reduced in oxidative stressed HaCaT cells treated by *H. cordata* extracts fermented with *A. pullulans*. Photo and chemical stimulatory inflammation of the dermis proceed with the destruction of collagen proteins. Therefore, because the extracts of *H. cordata* inhibits the gene expression of COX-2 and MMP-1 effectively, it can be used as a material to prevent photoaging. In addition, *H. cordata* extracts reduced the expression levels of NF-κB mediated inflammatory cytokines IL-6, IL-β and TNF-α under photo-stresses induced by UVA. As demonstrated in this investigation, the fermented *H. cordata* with *A. pullulans* extract enhance synergistic protective effects for antioxidant and anti-inflammation to prevent photoaging for healthy skin.

Declarations

Acknowledgments

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Contribution of authors

Nakyoung Kwon: Perform experiment. **Ramachandran Vinayagam:** Writing-original draft and editing. **Geum Sook Do:** Data curation, writing and editing the manuscript. **Kyung Eun Lee:** Perform experiments, methodology development, data curation and financial support. **Sang Gu Kang:** Experiment design, wrting and editing the manuscript, project supervision.

Disclosure

The authors declare no conflict of interest. No ethical approval or informed consent was required for this study.

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Tables

Tables 1 to 3 are available in the Supplementary Files section

Figures

Figure 1

Free radical scavenging activity. (A) DPPH assay; (B) ABTS assay. A.A; L-ascorbic acid; HCE; *H. cordata* ethanol extract, HCW; *H. cordata* water extract, HCFE; *H. cordata* fermentation ethanol extract, HCFW; *H. cordata* fermentation water extract. Results are the means ± S. D of samples. *p< 0.05 indicates a significant difference from the control.

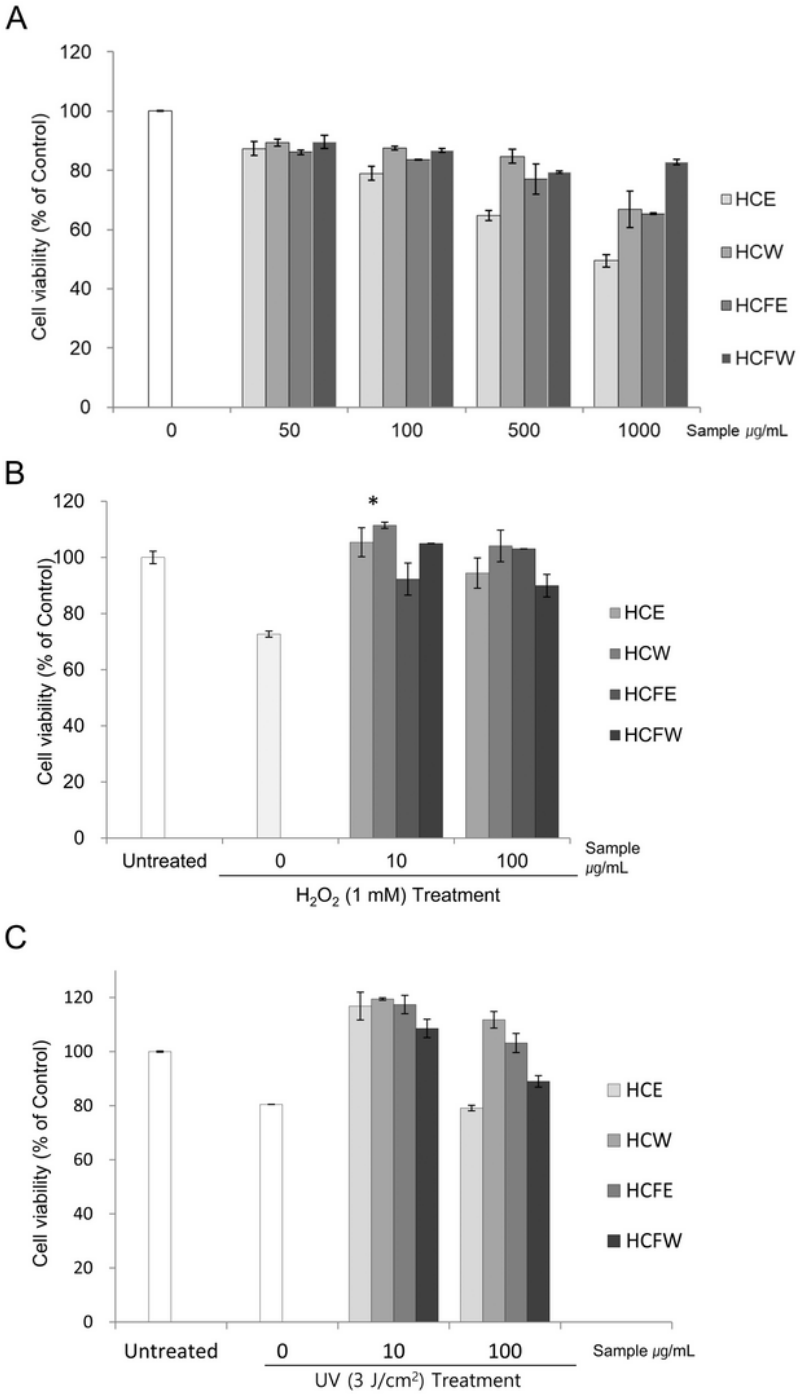

Figure 2

Cell viavilty assay of HaCaT keratinocyte cells. (A) Cell proliferation activities of extract of *H. cordata* and fermentation extract in HaCaT keratinocyte cells. (B) H_2O_2 treated HaCaT keratinocyte cells added *H. cordata* and fermentation extract. (C) UVA irradiated HaCaT keratinocyte cells added *H. cordata* and fermentation extract. HCE; *H. cordata* ethanol extract, HCW; *H. cordata* water extract, HCFE; *H. cordata* fermentation ethanol extract, HCFW; *H. cordata* fermentation water extract. Results are the means ± S. D

of samples. *p< 0.05 indicates a significant difference from the control. Results are the means ± S. D of samples. *p< 0.05 indicates a significant difference from the control.

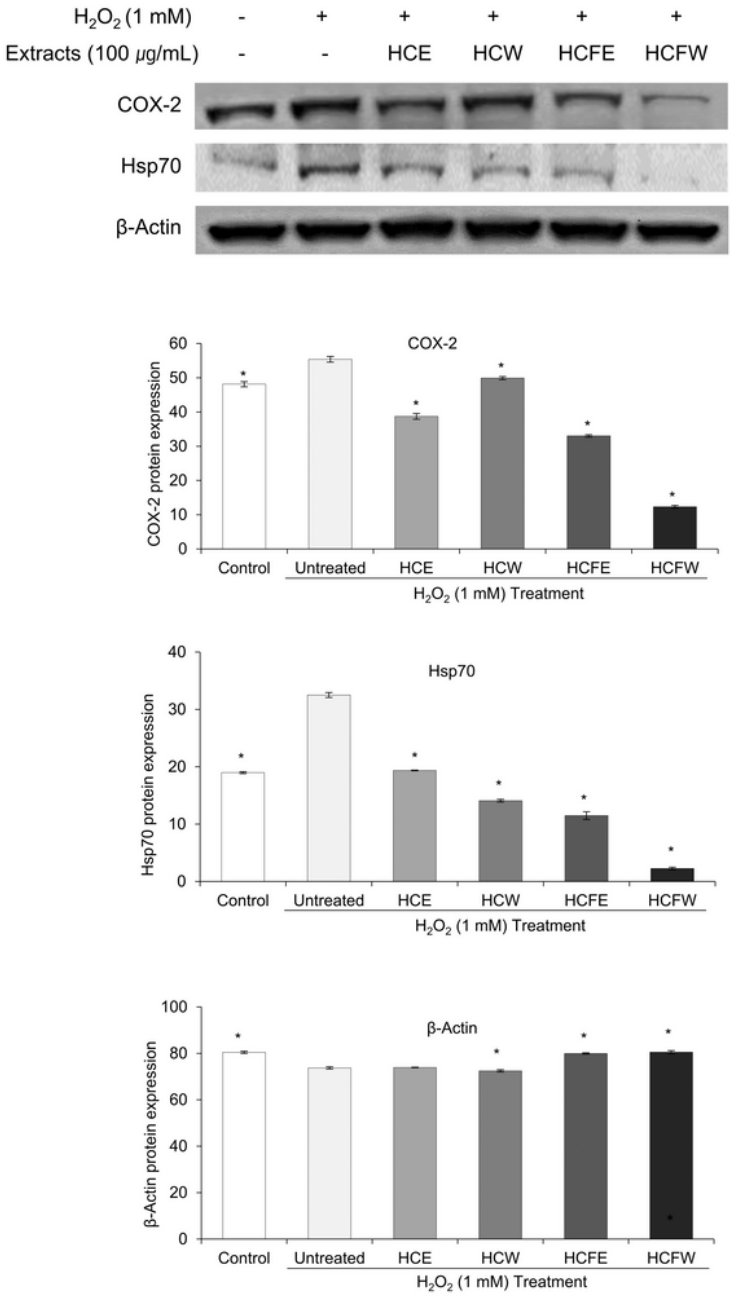

Figure 3

Chemical oxidative resistant effect of *H. cordata* extracts by western blot analysis. The protein levels of COX-2 and Hsp70 proteins were measured using their antibodies. HaCaT cells were treated on H_2O_2 (1

mM) and added with 100 ㎍/mL of each extract from *H. cordata*: Untreated; extract untreated cells, HCE; *H. cordata* ethanol extract, HCW; *H. cordata* water extract, HCFE; *H. cordata* fermentation ethanol extract, HCFW; *H. cordata* fermentation water extract. Control is no H_2O_2 treated HaCaT cells. The bar graphs show the quantitative relative level of proteins. Results are the means ± S. D of samples. *$p < 0.05$ compared with H_2O_2 treated group.

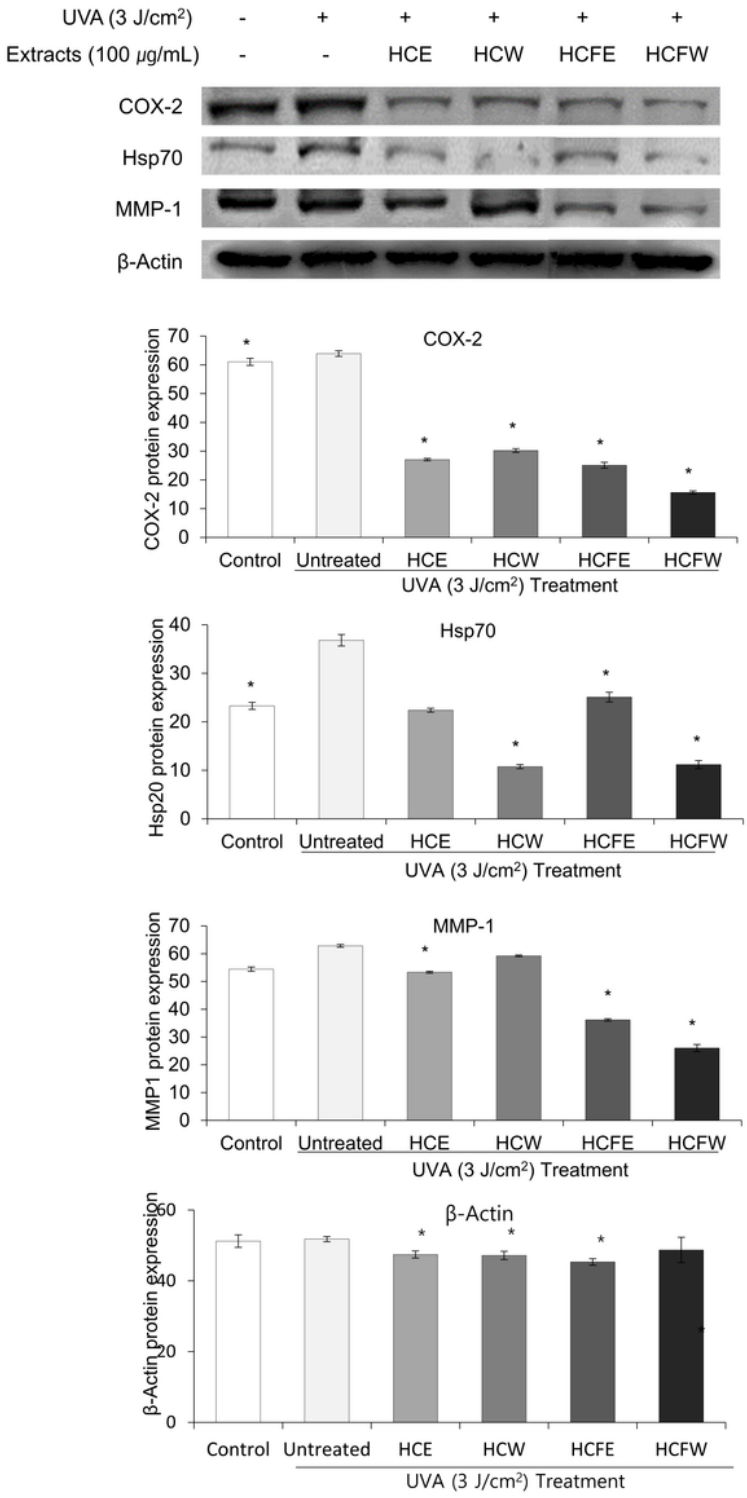

Figure 4

Photooxidative resistant effect of *H. cordata* extarcts by western analysis. The protein levels of oxidative stress related COX-2, Hsp70, and MMP-1 proreins were measured using their antibodies. HaCaT cells were irradiated on UVA (3 J/cm^2) and added with 100 ㎍/mL of each extract from *H. cordata*: Untreated; extract untreated cells, HCE; *H. cordata* ethanol extract, HCW; *H. cordata* water extract, HCFE; *H. cordata* fermentation ethanol extract, HCFW; *H. cordata* fermentation water extract. Control is no UVA expoused HaCaT cells. The bar graphs show the quantitative relative level of proteins. Results are the means ± S. D of samples. *$p < 0.05$ compared with H_2O_2/UVA treated group.

Figure 5

Quantitative gene expression ananlysis (qPCR) for the effect of *H. cordata* to H_2O_2-induced HaCaT cells. Transcribed mRNAs of COX-2, IL-6, TNF-α, NF-κB, MMP-1, KRT1, and IL-1β genes were subjected. Cells were added 100 ㎍/mL of each extract. HCE; *H. cordata* ethanol extract, HCW; *H. cordata* water extract, HCFE; *H. cordata* Fermentation ethanol extract, HCFW; *H. cordata* fermentation water extract. Control is no H_2O_2 treated cells. Results are the means ± S. D of samples. *$p < 0.05$ compared with H_2O_2/UVA-induced HaCaT cell lines.

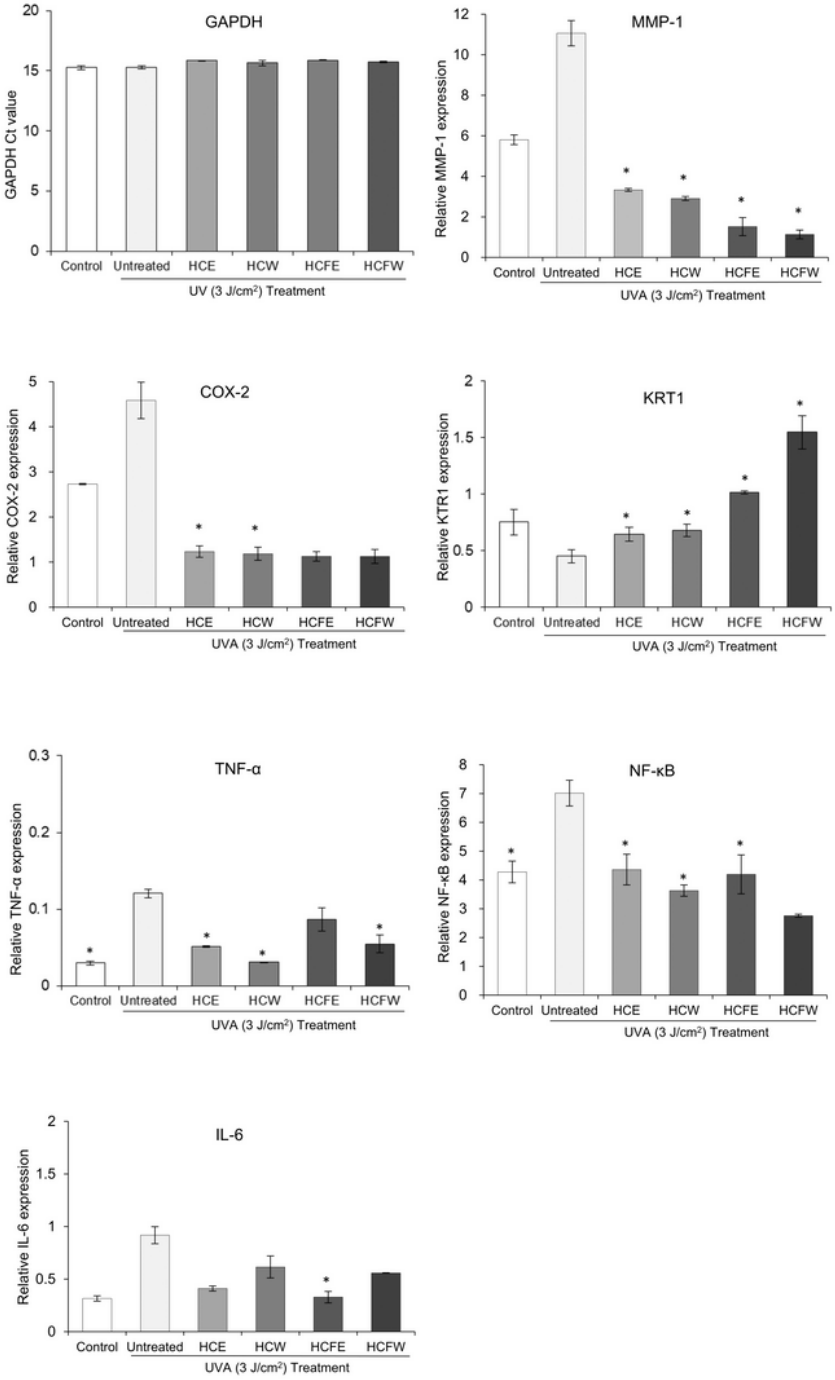

Figure 6

Quantitative gene expression ananlysis (qPCR) for the effect of *H. cordata* to UVA-irradiated HaCaT cells. Transcribed mRNA of COX-2, NF-κB, TNF-α, IL-6, MMP-1, and KRT1 genes were subjected. Cells were added 100 ㎍/mL of each extract. HCE; *H. cordata* ethanol extract, HCW; *H. cordata* water extract, HCFE; *H. cordata* fermentation ethanol extract, HCFW; *H. cordata* fermentation water extract. Results are the means ± S. D of samples. *p<0.05 compared with UVA treated group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- floatimage7.jpeg
- Table1.png
- Table2.png
- Table3.png