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Biochemical and gene expression studies reveal the potential of *Aspergillus oryzae*-fermented broken rice and brewers' rice water extracts as anti-photoageing agents

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ABSTRACT

In this study, the anti-photoageing efficacy of two water extracts from *Aspergillus oryzae*-fermented broken rice (FBR) and brewers' rice (FBrR), were investigated using UVA- and UVB-irradiated human fibroblasts . As UVA and UVB can damage the dermal and epidermal layers, two UV radiation approaches were utilised: i) direct UVA irradiation on fibroblasts, and ii) UVB-irradiated keratinocytes indirectly co-cultured with fibroblasts to observe their epithelial-mesenchymal interaction during UVB-induced photoageing. The anti-photoageing properties were tested using biochemical tests and quantitative polymerase chain reaction (qPCR). UV-irradiated human fibroblasts treated with FBR and FBrR dramatically downregulates MMP-1 and SFE gene expression. MMP-1 secretion was inhibited by FBR and FBrR with more substantial decreases in UVB-treated co-cultures, ranging from 0.76- to 1.89-fold compared to the untreated control. However, in UVA-treated fibroblasts the elastase-inhibiting activity of FBR and FBrR is up to 1.63-fold and 2.13-fold more than the control. In addition, post-UV irradiation treatments with FBR and FBrR were able to repair and enhance collagen formation in UVA-irradiated fibroblasts. Both FBR and FBrR upregulated elastin gene expression in fibroblasts under both culture conditions, especially at 50 µg/mL. The pro-inflammatory cytokines TNF-α, IL-1ß, and IL-6 were also lowered by FBR and FBrR, which may have contributed to the anti-photoageing effect in the UVB-treated co-culture. These results indicate that FBR and FBrR inhibit photoageing in human fibroblasts under both UV induction conditions. In conclusion, FBR and FBrR could be attractive bio-ingredients for cosmeceutical use in the cosmetic industry.

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Introduction

Skin ageing is a complex biological process that affects both the function and appearance of the skin. Skin ageing can be classified into two types, chronological ageing and photoageing, which are influenced by internal and external factors respectively. Chronological ageing or intrinsic ageing is defined by a decline in biological function, a decrease in stress adaptation and structural damage caused by reactive oxygen species (ROS) produced during cellular metabolism. Photoageing, also called extrinsic skin ageing, is caused mostly by ultraviolet (UV) radiation and partly by other factors such as smoking, infrared light, and air pollution (1).

Solar UV radiation is comprised of UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm). Whilst UVC is mostly absorbed by the stratospheric ozone, UVA and UVB could reach the surface of the earth and subsequently affect human skin. UVB which has a greater intensity than UVA, is absorbed primarily in the epidermis and believed to be the causative factor of numerous UV-related skin problems such as photoageing. UVA on the other hand penetrates deeper into the dermis than UVB and affects the connective tissues (2). In general, both UVA and UVB irradiation can cause skin damage by inducing oxidative stress in the skin which leads to increased activator protein (AP)-1 and MMPs expression, resulting in skin photoageing.

Repetitive UV exposure results in skin photoageing that primarily manifests as wrinkles and sagging of the facial skin (3). This photodamage has been attributed to the upregulation of matrix metalloproteinase (MMP)-1 and skin fibroblast elastase (SFE) in dermal fibroblasts. This leads to the degradation of major dermal components of collagen and elastin which results in sagging and wrinkles. MMP-1, a major collagenolytic enzyme, is part of a family of zinc-containing proteinases that are responsible for degrading extracellular matrix (ECM) proteins which form the dermal connective tissue. MMP-1 plays an important role in the photoageing process as it initiates the hydrolyzation of dermal collagen fibrils in ECM (4). Skin fibroblast elastase (SFE), a 94 kDa membrane-bound metalloprotease, has also been reported to be capable of destroying the three-dimensional architecture of elastic fibres, thus impairing skin elasticity and resulting in wrinkle formation (5,6). Due to this, numerous studies examining anti-photoageing mechanisms in human skin have focused on collagenase and elastase inhibitory activities.

Many studies on UV skin damage and anti-photoageing have been reported. The demand for cosmetics free of

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harmful synthetic substances has raised interest in using natural substances as active ingredients in cosmeceutical manufacture (7,8). To meet this demand, fermented broken rice (FBR) and fermented brewers' rice (FBrR) with improved antioxidant and anti-elastase functionality were produced via solid state fermentation (SSF) with generally recognised as safe (GRAS) *Aspergillus oryzae*. In our previous study, these fermented extracts were shown to have higher antioxidant and anti-elastase activity compared to their unfermented counterparts (9,10,11). As both these functionalities are associated with combating photoageing, this study sought to assess the potential of FBR and FBrR water extracts as anti-photoageing agents through an *in vitro* approach.

In this study, the FBR and FBrR extracts were assessed for their anti-photoageing effects on human fibroblasts using two modes of UV induction: i) direct UVA-irradiated monolayer fibroblasts and ii) UVB-associated co-culture of keratinocytes and fibroblasts, in order to mimic realistic UVA/UVB penetration in human skin. Evaluation of direct UVA-irradiation was carried out on the basis of deep penetration of UVA radiation into the dermis where fibroblasts are located. Unlike UVA, UVB radiation is almost fully absorbed by the epidermal layer where the keratinocytes are located and penetrates poorly into the dermis. Therefore, we carried out the indirect co-culture experiment by co-culturing keratinocytes exposed to UVB with fibroblast in order to see the interaction between these cells as well as the photoageing effect in the fibroblast. The FBR and FBrR were evaluated in both UV induction approaches to determine their potential for protecting against photoageing caused by UVA and UVB.

Materials and Methods

Chemicals and reagents

Iscove's Modified Dulbeccos's Medium (IMDM), keratinocytes serum-free medium (KSFM), fetal bovine serum (FBS), antibiotic-antimycotic, epidermal growth factor (EGF), phosphate buffer saline (PBS), trypLE™, and TRIzol™ were all purchased from Thermo Fischer Scientific (Yokohama, Japan). The MTT reagent was purchased from Nacalai Tesque (Kyoto, Japan). The N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (STANA), dimethyl sulfoxide (DMSO), Tris-HCl, Triton X-100, and protease inhibitor cocktail were all purchased from Sigma-Aldrich (MO, USA).

Preparation of fermented extracts

The fermented broken rice (FBR) and Brewers' rice (FBrR) water extracts were prepared by the Malaysian Agricultural Research and Development Institute (MARDI). Thirty grams of broken rice and Brewers' rice were added to Erlenmeyer flasks and autoclaved for 15 min at 121°C. Thirty-five mL of sterilised distilled water was mixed into each flask to adjust the substrate moisture content to 50%. Fungal spores of A. oryzae were added at an initial concentration of 5 x 10^6 spores/g of substrates and incubated at 32°C. Samples were harvested and oven-dried at 50°C for 24 h. All samples were subjected to hot water extraction by mixing 1 g of sample with 5 mL of distilled water and boiled for 15 min in a water bath. The samples were then centrifuged for 15 min at 10,000 rpm. The supernatants were collected and filtered using Whatman no. 1 filter paper. The filtrates were then freeze-dried and kept at -20°C for further analysis. Experiments were performed in triplicates.

Cell culture

Human fibroblasts, CCD1135sk (ATCC-CRL-2691) and human keratinocytes, HEK-001 (ATCC-CRL 2404) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The fibroblasts were grown in IMDM with 10% FBS and 1% antibiotic-antimycotic. The keratinocytes were cultivated in KSFM with 5 ng/ml EGF. All cell lines were grown and maintained at 37°C in a 95% air, 5% CO_2 atmosphere and regularly passaged at a density of 80%.

Cell viability analysis using the MTT assay

For cell viability analysis, the evaluation was done using a tetrazolium dye colorimetric test (MTT) (12). Fibroblasts and keratinocytes were individually cultured in 96-well plates at a density of 1´x 10^5 cells /well for 24 h. Cells were then replaced with 200 µL of growth media containing different concentrations of FBR and FBrR extracts ranging from 5 to 500 µg/mL and incubated for 24 h in the CO_2 incubator. Then, 20 µL MTT reagent was added to each well and the plate was put on a shaker at 150 rpm for 5 min, followed by incubation at 37°C for 4 h to allow the MTT to metabolize. The media was then removed and the formazan produced was dissolved in 100 µL DMSO. The plate was then shaken at 150 rpm for 5 min to fully solubilize the formazan and the absorbance was determined at 570 nm using a microplate reader (BioTek®). The percentage of cell viabilities was calculated against non-treated controls as described in the equation below, where A represents the absorbance of treated samples and B is the absorbance of non-treated controls.

$$\text{Cell viability (\%)} = \left[\frac{A}{B}\right] \times 100\,\%$$

UV irradiation

The UVA (5 J/cm^2) and UVB (100 mJ/cm^2) irradiation experiments were conducted using a BLX crosslinker, the Vilber-Lourmat™ BIO-LINK (France), which is equipped with 3 available wavelengths (UVA (365 nm), UVB (312 nm), and UVC (254 nm). Prior to the irradiation procedure, the tubes were changed to the corresponding wavelengths and the equipment was set to the required UV exposure.

Monolayer culture of fibroblasts and UVA irradiation

The fibroblasts were seeded in the Multiwell™ 6-well plates at a density of 5 x 10^6 cells/well (Beckton Dickinson, NJ, USA) and incubated for 24 h. The growth media was then removed and replaced with serum-free growth media and serum-starved for 16 h. Next, the growth media was removed and replaced with PBS followed by UVA (5 J/cm^2) exposure for 15 min. After UVA irradiation, the PBS was replaced with treatment media containing the 50 and 100 µg/mL FBR and FBrR extracts followed by incubation for 24 h. Both cells and supernatant were recovered for further analysis.

Indirect co-culture of keratinocytes and fibroblasts and UVB irradiation

Indirect co-culture of keratinocytes and fibroblasts was

done with cell culture inserts (13). The keratinocytes were seeded in the permeable inserts (0.4 mm pore size PET track-etched membrane, Becton Dickinson, NJ, USA) at a density of 5 x 10^6 cells/insert on 6-well plates. The fibroblasts were seeded in the Multiwell™ 6-well plates at a density of 5 x 10^6 cells/well (Beckton Dickinson, NJ, USA). Prior to UVB exposure, both fibroblasts and keratinocytes were changed to serum-free and EGF-free growth media, respectively, and were serum-starved for 16 h. Upon initiation of the co-cultures, the keratinocyte growth media in the hanging inserts were replaced with PBS followed by UVB (100 mJ/cm^2) irradiation for 36s. After UVB irradiation, the PBS was replaced with treatment media containing the 50 and 100 µg/mL FBR and FBrR extracts. The inserts were then placed in the Multiwell™ where the fibroblasts had been seeded. The co-culture units were incubated at 37◦C in a 95% air and 5% CO_2 atmosphere for 24 h. Both cells and supernatant were recovered for further analysis.

MMP-1 content measurement via ELISA

The amount of MMP-1 in the cell culture supernatant was assessed by performing an enzyme-linked immunosorbent assay (ELISA) using the Human Total MMP-1 Duoset ELISA kit (R&D Systems, MN, USA) according to the manufacturer's protocol. The MMP-1 sandwich ELISA assays were performed by coating the 96-well microplate with the Human Total MMP-1 capture antibody followed by incubation overnight. Then, the biotinylated goat anti-human MMP-1 detection antibody was added followed by streptavidin-HRP B accordingly. Tetramethylbenzidine was used as the peroxidase substrate. The absorbance was read at 450 nm using an ELISA plate reader (BioTek®). The results were calculated by generating a four-parameter logistic (4-PL) curve-fit with the recombinant human MMP-1 as a standard.

Measurement of intracellular elastase activity

For elastase enzyme analysis, the untreated and treated cells were lysed in lysis buffer for enzyme extraction. The 6-well culture plate was placed on ice and the attached cells were washed with 1 mL of ice-cold PBS to remove the residual growth media. The PBS was aspirated and 500 µL of ice-cold lysis buffer (0.1 M Tris-HCl (pH 7.6), 0.1% Triton X-100, and protease inhibitor cocktail) was added into each well. The plate was shaken gently for 15 min on ice, and the cell suspensions were transferred to pre-cooled microcentrifuge tubes. The microcentrifuge tubes were then centrifuged at 12 000 rpm at 4°C for 20 min. The supernatants were recovered and used for enzyme analysis. Prior to enzyme analysis, the cell lysates protein concentration was analyzed using BCA protein assay.

To measure the intracellular elastase activity, 100 µl of enzyme solution was dispensed into a 96-well plate followed by pre-incubation at 37°C for 15 min. Then, 2 µl of 62.5mM STANA was added into each well and further incubation was done at 37°C for 1 h. The release of p-nitroaniline was measured at absorbance of 405 nm. The percentage of elastase activity was calculated against the non-treated control as described in the equation below, where A represents the absorbance of the treated sample and B represents the non-treated control.

$$\text{Intracellular elastase activity (\%)} = \left[\frac{A}{B}\right] \times 100\,\%$$

Determination of total collagen content

The total collagen content was determined using the Sircol™ Soluble Collagen Assay (Biocolor, Northern Ireland, UK). Collagen standards (0, 5, 10, 15 µg in 100 uL of cell culture medium) were prepared in microcentrifuge tubes using the collagen reference standard. Then, 100 µL of cell culture supernatants (samples) were added into another set of clean microcentrifuge tubes. One mL of Sircol dye reagent was added into each tube (standard and samples) and put on a gentle mechanical shaker for 30 min. The microtubes were then centrifuged at 12, 000 rpm for 10 min. The supernatants were then carefully discarded to avoid loss of any pellets during the draining of unbound dye.

For washing, 750 µL of ice-cold acid-salt wash reagent was added to the collagen-dye pellet in the microtubes to remove the unbound dye from the surface of the pellet and the inside surface of the microcentrifuge tube. The microcentrifuge tubes were then centrifuged at 12, 000 rpm for 10 min, and the supernatants were carefully discarded. The remaining fluid was carefully removed using cotton wool buds. Then, 250 µL of alkali reagent was added into tubes with the reagent blanks, standards, and samples followed by vortexing for 5 min to release the collagen bound dye into the solution. All reagent blanks, standards, and samples (200 µL) were transferred to a 96-well microplate and the optical density was read at 555 nm. The collagen concentration was obtained from the standard curve generated.

Analysis of pro-inflammatory cytokines (TNF-α, IL-1ß, and IL-6) content via ELISA

The levels of TNF-α, IL-1ß, and IL-6 in the cell supernatant were determined using ELISA according to the manufacturer's protocol (Elabscience, USA). Briefly, 100 µL of sample and standard were added into 96-well plate and incubated at 37°C for 90 mins. The liquid was discarded and 100 µL of biotinylated detection antibody working solution was added into each well followed by incubation at 37°C for 60 mins. Each well was then washed three times with 1 x PBS followed by the addition of 100 µL of HRP conjugate working solution and incubated at 37°C for 30 mins. After three washes with 1 x PBS, 90 µL of substrate reagent was added to each well and the plate was incubated at 37°C for 15 minutes. Finally, 50 µL of stop solution was added into each well and the plate was read at 450 nm using an ELISA plate reader. Each protein's result was based on its standard plot fitted with a four-parameter logistic (4-PL) curve-fit.

Gene expression analysis using qPCR

Total RNA was isolated using the TRIzol™ reagent by following the manufacturer's protocol, and the cDNA was synthesised using the ReverTra Ace™ qPCR RT kit (Toyobo, Tokyo, Japan). The qPCR analysis was carried out in 96-well plates with the Bio-Rad CFX96 Real-Time PCR System and Bio-Rad CFX96 Manager Software (Bio-Rad, USA) using the SYBR Green-based PCR assay. The preparation of the reaction mix was according to the THUNDERBIRD SYBR qPCR Mix kit manufacturer (TOYOBO, Japan) and was subjected to the following conditions:

Table 1. Primer sets used for qPCR analysis.

Primer	Accession No.	Sequence
Matrix metalloproteinase-1 (MMP1)	NM_001145938.2	forward : 5'AGAAAGAAGACAAAGGCAAGTTGA-3' reverse : 5'-TCAGTGAGGACAAACTGAGCC-3'
Skin fibroblast-derived elastase (SFE)	NM_000902.5	forward : 5'-CTG CTG AGG GGT CAC GAT TTT A-3' reverse : 5'-ACA AGG ACC GAG AGG CTG AT-3'
Collagen-1 (COL1A1)	NM_000088.4	forward : 5'- AGA GGT CGC CCT GGA GC-3' reverse : 5' – CAG GAA CAC CCT GTT CAC CA -3'
Elastin (ELN)	NM_001278939.2	forward : 5'- CTT TGG TGT CGG AGT CGG AG-3' reverse : 5'- TCC TGC AGC ACC GTA CTT G-3'
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_002046.7	forward : 5'- GCA AAT TCC ATG GCA CCG T-3' reverse : 5'- TCG CCC CAC TTG ATT TTG G-3'

95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s in 96-well optical reaction plates (Bio-Rad, USA). The melting curves were analysed at 65–5°C after 40 cycles. Each qPCR analysis was performed in triplicates and the mean was used for the qPCR analysis. The gene expression levels were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, and the results were expressed as fold change. Primer sets used for qPCR are listed in Table 1.

Statistical analysis

All data is presented as means ± SEM (n = 3). The statistical significance of the data was determined by a one-way ANOVA and then by the Duncan multiple comparison test. P values < 0.05 are considered statistically significant. SPSS Version 26 was used to assess the statistical analyses (IBM Corporation, Chicago, USA).

Results

Non-cytotoxic effects of FBR and FBrR extracts on human fibroblasts and keratinocytes

The MTT assay was used to determine the cytotoxicity of the FBR and FBrR extracts on human fibroblasts and keratinocytes. The MTT colorimetric assay detects mitochondrial succinate dehydrogenase's reduction of yellow 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a dark purple formazan product which occurs only in metabolically active cells. As shown in Figure 1A, the percentage of viable cells varies between 95.00 and 140.06 %, indicating that FBR and FBrR extracts have no cytotoxic effect on fibroblasts at all the concentrations tested. In contrast, a distinct trend was found in keratinocytes, where the FBrR extract was cytotoxic at doses greater than 100 µg/mL (Figure 1B). The FBR extract on the other hand was not cytotoxic to human keratinocytes. Therefore, the concentrations of 50 and 100 µg/mL are selected for further investigation as no cytotoxic effects are exhibited on both fibroblasts and keratinocytes.

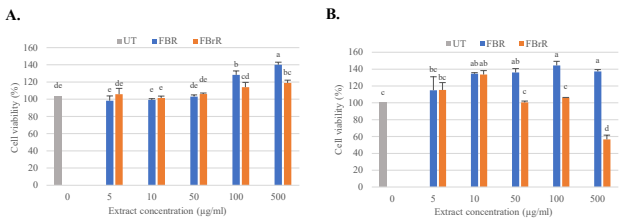

Figure 1. Effects of FBR and FBrR water extracts on the viability of (A) human fibroblasts and (B) human keratinocytes. Cell viability is presented as relative percentages compared to non-treated control (means ± SEM). Values followed by the same letter are not significantly different from each other (p>0.05).

The FBR and FBrR extracts exhibit prominent MMP-1 secretion inhibition in UVB- irradiated co-cultures

The MMP-1 secretion, which is one of the major factors associated with skin ageing, was quantified using ELISA. As shown in Figure 2A, direct UVA radiation had no effect on the level of MMP-1 secretion in untreated fibroblasts. The MMP-1 content was also unaffected by the FBR and FBrR treatments, with the exception of the FBrR 50 µg/mL treatment, in which a considerable decrease was observed. Figure 2B demonstrates that UVB radiation on keratinocytes dramatically elevates MMP-1 secretion in the untreated group of the co-culture. However, treatment with FBR and FBrR significantly decreased MMP-1 secretion, with an equivalent effect at both concentrations. The lowest reduction was seen at 100 µg/mL FBR, which was 1.9-fold less than the untreated control. From this data, we can hypothesise that the anti-photoageing effect exhibited by FBR and FBrR in the UVB-irradiated co-culture is more strongly associated with the suppression of MMP-1 production.

Significant suppression of intracellular elastase activity by FBR and FBrR extracts in UVA-irradiated fibroblasts

The FBR and FBrR extracts were evaluated for their effect on elastase activity in fibroblasts, as elastase is pri-

marily responsible for the breakdown of elastin and subsequent loss of skin elasticity. As demonstrated in Figure 3A, UVA exposure resulted in a slight increase in elastase activity in the untreated control. On the other hand, FBR and FBrR treatments significantly decreased the activity of elastase in fibroblasts with comparable effects. In addition, UVB irradiation on the co-culture also had no influence on elastase enzyme activity in fibroblasts (Figure 3B). Treatment with FBR and FBrR slightly reduced elastase activity, with significant inhibition being only shown by FBR at 50 µg/mL. Thus, we may postulate that the FBR and FBrR reduce intracellular elastase activity to exert an anti-photoageing effect in UVA-irradiated fibroblasts.

Substantial collagen secretion in UVA-irradiated fibroblasts upon treatment with FBR and FBrR water extracts

UVA exposure reduced total collagen production by fibroblasts by 56.82% (Figure 4A). In contrast, treatment with FBR and FBrR significantly restored collagen content in UVA-irradiated fibroblasts. FBrR 100 µg/mL demonstrated the greatest collagen improvement, with a 2.8-fold increase over untreated cells. On the other hand, Fig. 4B indicates that UVB exposure to keratinocytes had no effect on collagen synthesis in co-cultures. There was no substantial increase or restoration of collagen in the FBR and FBrR-treated cells, with the exception of FBrR 50 µg/mL, which raised the collagen content by 2.1-fold in comparison to the untreated cells. According to these findings, the influence of collagen secretion by FBR and FBrR extracts is more pronounced in UVA-irradiated fibroblasts than in UVB-irradiated co-cultures.

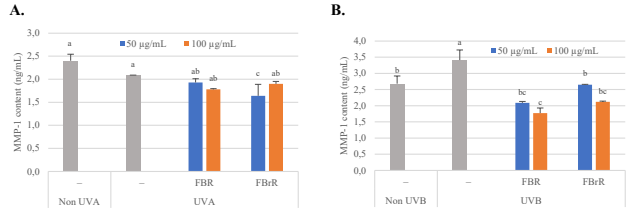

Figure 2. Effect of FBR and FBrR extracts on MMP-1 content in (A) UVA-irradiated fibroblasts and (B) UVB-irradiated co-culture of keratinocytes and fibroblasts. The unexposed and untreated cells were used as a comparison. Values were expressed as means ± SEM. Values followed by the same letter are not significantly different from each other (p>0.05).

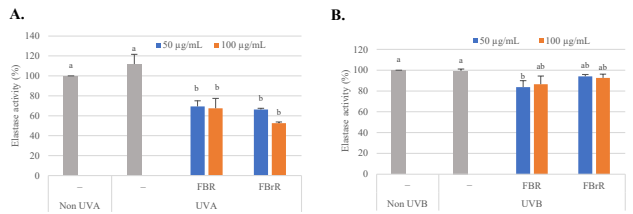

Figure 3. Effect of FBR and FBrR on intracellular elastase activity in human fibroblast upon (A) UVA-irradiated fibroblasts and (B) UVB-irradiated co-culture of keratinocytes and fibroblasts. Cells were treated with 50 and 100 µg/ml of the fermented extracts after UV irradiation. The unexposed and untreated cells were used as a comparison. Values were expressed as means ± SEM. Values followed by the same letter are not significantly different from each other (p>0.05).

FBR and FBrR extracts downregulate the MMP-1 and SFE genes, upregulate the ELN gene, but have no influence on the expression of COL1A1 in fibroblasts

The effect of FBR and FBrR on MMP-1 expression in UVA and UVB-irradiated fibroblasts is presented in Figure 5A. MMP-1 expression was significantly increased following both UV treatments. The elevated MMP-1 expression was significantly decreased by FBR and FBR extracts. The 100 µg/mL of FBR exhibited the greatest MMP-1 reduction in UVA-irradiated cells, being 61.54% less than the untreated control. In UVB-irradiated co-culture, the MMP-1 expression was reduced the most by FBR at 50 µg/mL, where it was 50.74% less than control. Contrarily, UVA and UVB radiation had no effect on the expression of the SFE gene in fibroblasts (Figure 5B). Nonetheless, the FBR and FBrR significantly lowered SFE expression in both UV-irradiated fibroblasts where their effects were equivalent. The expression of the COL1A1 gene in fibroblasts was found unaffected by UVA and UVB exposure as depicted in Figure 5C. The treatment of UV-irradiated cells with FBR and FBrR extracts also did not affect COL1A1 gene expression significantly. The UVA exposure also had no significant effect on the ELN gene expression (Figure 5D). Treatment with FBR and FBrR, however, significantly elevated the expression of ELN, with the exception of FBrR 100 µg/mL. In contrast to UVA, UVB exposure diminishes the expression of the ELN in the untreated cells. FBR and FBrR treatment resulted in

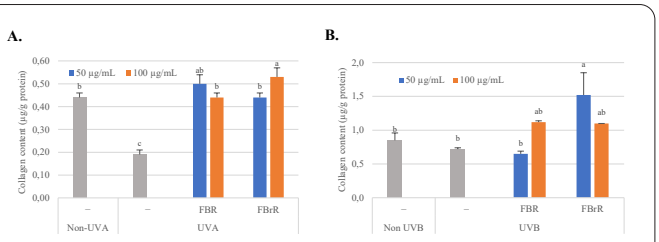

Figure 4. Effects of FBR and FBrR extracts on total collagen content in (A) UVA-irradiated fibroblasts and (B) UVB-irradiated co-culture of keratinocytes and fibroblasts. Cells were treated with 50 and 100 µg/mL of the fermented extracts after UVA/UVB irradiation. The unexposed and untreated cells were used as a comparison. Values were expressed as means ± SEM. Values followed by the same letter are not significantly different from each other (p>0.05).

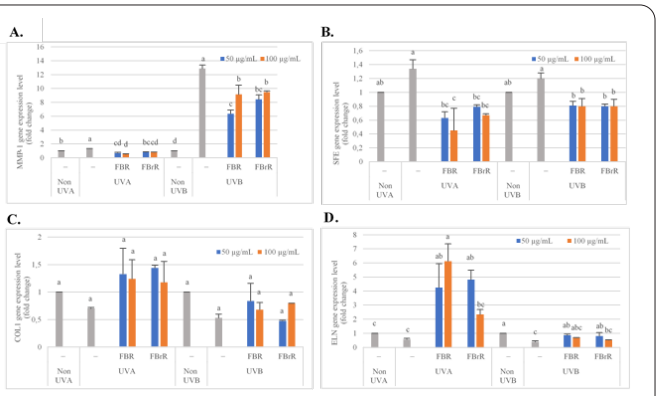

Figure 5. Effect of FBR and FBrR extracts on gene expression of (A) MMP-1, (B) SFE, (C) COL1, and (D) ELN in human fibroblasts upon treatment with UVA and in UVB-irradiated co-culture of keratinocytes and fibroblasts The unexposed and untreated cells were used as a comparison. Values were expressed as means ± SEM. Values followed by the same letter are not significantly different from each other (p>0.05).

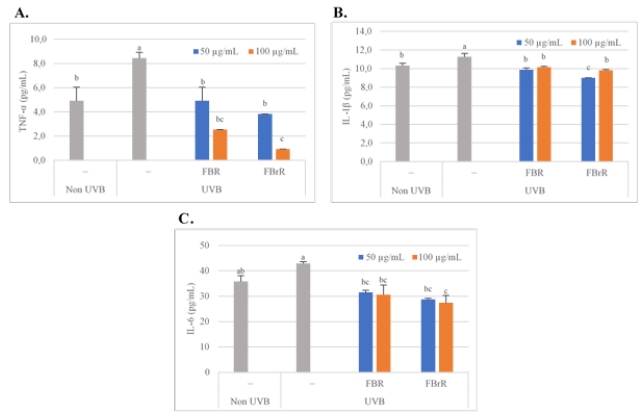

Figure 6. Effect of FBR and FBrR extracts on pro-inflammatory cytokines production (A) TNF-α, (B) IL-1ß and (C) IL-6 production in UVB-irradiated co-culture of keratinocytes and fibroblasts evaluated via ELISA. The unexposed and untreated cells were used as a comparison. Values were expressed as means ± SEM. Values followed by the same letter are not significantly different from each other (p>0.05).

significant increment in ELN expression particularly at a concentration of 50 µg/mL. Based on these results, we may infer that the anti-photoageing actions by FBR and FBrR extracts are by lowering the expression of the MMP-1 and SFE genes and increasing the expression of the ELN gene in the fibroblasts.

FBR and FBrR extracts decrease TNF-α, IL-1ß and IL-6 secretion in UVB-irradiated co-cultures

It is well established that UVB does not penetrate the dermis layer and has a direct effect on fibroblasts. As a result, we investigated the effect of UVB as well as FBR and FBrR on the production of pro-inflammatory cytokines as it has been reported that UV exposure causes the release of pro-inflammatory mediators from a variety of skin cells, resulting in MMP activation (14). The TNF-α production was significantly increased upon UVB exposure (Figure 6A). Nevertheless, the TNF-α content was significantly decreased by FBR and FBrR at both concentration in a dose-dependent manner. UVB also significantly increased the IL-1ß content. But both FBR and FBrR significantly decreased IL-1ß content, particularly the FBrR 50 µg/mL, which was 1.2-fold less than the untreated control (Figure 6B). Although UVB exposure also results in a slight increase in IL-6, the effect is not statistically significant (Figure 6C). However, the FBR and FBrR treatments significantly decreased IL-6 production, and the effects of both extracts were comparable. These data indicate the decrease in TNF-α, IL-1ß, and IL-6 secretion by FBR and FBrR extracts may contribute in part to the anti-photoageing action in the UVB-irradiated co-culture.

Discussion

UV radiation induces epidermal keratinocytes and dermal fibroblasts to release MMP-1, a collagenase that destroys fibrillar collagen types I and II into specific fragments, which leads to photoageing (15). In our investigation, UVA at 5 J/cm^2 radiation had no effect on the MMP-1 release by fibroblasts as measured by ELISA. However, UVA radiation significantly increased MMP-1 gene expression in untreated fibroblasts. This is consistent with Suganuma et al. (2010) who observed a dosage of 5 J/cm^2 of UVA can boost the MMP-1 gene expression in human fibroblasts by 24 hours (16). Treatment with FBR and FBrR significantly downregulates the MMP-1 gene expression in fibroblasts. This finding is consistent with the rice koji fermented with two filamentous fungi, *Aspergillus oryzae* and *Aspergillus cristatus,* in which anti-ageing effect was exhibited through downregulation of MMP-1 in human fibroblasts (17). Nevertheless, no significant changes were observed in MMP-1 secretion except for FBrR at 50 µg/mL. The discrepancy between the level of mRNA and the level of protein may be explained by the complex and diverse post-transcriptional mechanisms involved in the conversion of mRNA to protein (18). In contrast, observation in the co-culture shows UVB exposure on keratinocytes significantly increases MMP-1 gene expression in human fibroblasts as well as MMP-1 secretion. The MMP-1 production was then significantly reduced by FBR and FBrR extracts, both transcriptionally and translationally.

Aside from MMP-1, elastase is another protease that is responsible primarily for the breakdown of elastin, an important protein found within the ECM. In our study, UVA radiation had no influence on elastase enzymatic activity as well as SFE gene expression in the fibroblasts. However, post-irradiation treatment with FBR and FBrR significantly decreased the elastase enzyme activity and downregulated the SFE gene expression in the UVA-radiated fibroblasts. The indirect UVB exposure also had no effect on SFE gene expression and elastase enzyme activity of human fibroblasts in the co-culture system. Nevertheless, the FBR and FBrR significantly downregulate the SFE gene expression in the UVB-exposed co-culture system. Yet significant inhibition of the intracellular elastase activity was only detected in FBR 50 µg/mL. The present results seem to be consistent with our previous finding that FBR water extract displayed elastase inhibitory activity evaluated using porcine elastase model (10). Another study by Abdul Razak et al. (2017) and Marto et al. (2018) also described the elastase inhibitory activity exhibited by *A.oryzae*-fermented rice bran and rice water respectively (9,19).

Although both FBR and FBrR significantly suppress MMP-1 and SFE gene expression in fibroblasts, their effects on MMP-1 secretion and elastase enzyme activity differed in both UV treatments. We discovered that the inhibitory effect of FBR and FBrR on UVA induction occurs to a larger degree for elastase than for MMP-1. In the UVB co-culture, however, there was a greater degree of MMP-1 attenuation by FBR and FBrR compared to elastase inhibitory activity. From this observation, we may suggest that it is likely that the anti-photoageing effect of FBR and FBrR in direct UVA exposure was due to elastase inhibition rather than MMP-1 inhibition and the opposite trend was observed in the UVB-irradiated cells.

The FBR and FBrR at both concentrations also displayed a significant increase in collagen synthesis in untreated UVA-radiated fibroblasts. In contrast, significant enhancement of collagen synthesis was only exhibited by FBrR 50 µg/mL in the UVB-expose co-culture. In comparison, analysis of COL1A1 gene expression demonstrated no significant changes by both UV exposures as well as treatments by FBR and FBrR. This discrepancy might be due to the ability of Sircol dye reagent to measure type I to type V collagen, not specifically to collagen type I.

The collagen measurement specifically for type I collagen needs to be done in order to observe the actual effect of FBR and FBrR on type I collagen protein production.

The ELN gene expression in the untreated fibroblasts was also unaffected by the UVA radiation. However, post UVA-irradiation treatment with FBR and FBrR displayed a significant elevation of ELN gene expression, except for FBrR at 100 µg/mL. While in UVB-exposed co-culture, the ELN gene expression was significantly reduced but enhanced upon treatment with FBR and FBrR at 50 µg/mL. Based on this result, we may suggest that FBR and FBrR especially at 50 µg/mL have the ability to increase elastin production in fibroblasts. As mRNA levels should not be interpreted as the final output of gene expression (20), elastin protein analysis is necessary to determine their true effect on elastin formation. It has previously been reported that fermenting rice with *A. cristatus* and *A. oryzae* improves elastin gene expression (17).

The photoageing effect in UVB-irradiated co-culture might also be contributed by keratinocytes-secreted factors. Keratinocytes exposed to UVB radiation was reported to produce and secrete cytokines IL-1, IL-6, and TNF-α, which stimulate epidermal keratinocytes and dermal fibroblasts, in autocrine and paracrine manners respectively, and upregulate the levels of mRNA and MMPs 1, 2 (gelatinase A), 9 (gelatinase B) and 12 which degrade dermal collagen and elastic fibres (21, 22, 23). Therefore, we have also evaluated the pro-inflammatory cytokines of TNF-α, IL-1ß and IL-6 production in the UVB-irradiated co-culture. The TNF-α production was significantly increase upon UVB exposure in the untreated cells. The increase of TNF-α is an early response of keratinocytes to UVB and a crucial component of the inflammatory cascade in human skin (24). TNF-α expression is induced by UVB irradiation in both keratinocytes and dermal fibroblasts with TNF-α mRNA induction occurring as early as 1.5 hours after UVB exposure. Nevertheless, post-irradiation treatment with FBR and FBrR has significantly abrogated the production of TNF-α. UVB is also considered as one of the most potent inducers of cytokines such as IL-1, IL-6 (25) and IL-8 (26) release in epidermal cells. We found that the UVB exposure significantly increased the IL-1ß production but had no influence on the IL-6. However, both FBR and FBrR significantly attenuated the production of both IL-1ß and IL-6. The inhibition of TNF-, IL-1ß, and IL-6 by FBR and FBrR may have contributed in part to the anti-photoageing effect observed in the UVB-exposed co-culture.

The anti-photoageing effect of FBR and FBrR in both UV-induction modes could be attributed to bioactive compounds found in the studied extracts. The preparation of FBR and FBrR water extracts which involved the SSF fermentation process could induce the structural breakdown of cell walls and lead to liberation and/or synthesis of various bioactive compounds. Gallic acid and ascorbic acid are among bioactive compounds detected in the *A. oryzae*-fermented FBR (27). Gallic acid has been shown to have anti-photoageing activity by decreasing ROS activation, MMP-1 and IL-6 production, and increasing procollagen type I secretion in UVB-irradiated human fibroblast cells (28). Furthermore, ascorbic acid has free radical neutralising properties, interacting with superoxide, hydroxyl, and free oxygen ions, preventing inflammatory processes, carcinogens, and other processes that accelerate photoageing in the skin (29). While in the FBrR extract, ferulic acid, a potent antioxidant and anti-ageing agent (30) as well as caffeic acid were among phenolic acids detected (10). Both compounds are excellent anti-photoageing agents for the skin as UVA radiation accelerates elastase synthesis resulting in skin elasticity degradation and wrinkle formation (31,32). These compounds which detected in the studied extracts may partially contribute to the anti-photoageing effect evaluated in this investigation.

Conclusion

On the basis of the results obtained, these extracts exhibited anti-photoageing action via various pathways. The anti-photoageing efficacy of FBR and FBrR in UVA-irradiated fibroblasts was primarily due to elastase inhibition, whereas they exhibited greater MMP-1 inhibition in co-cultures exposed to UVB radiation despite the significant downregulation of SFE and MMP-1 gene expression observed in both UV irradiation modes. Although these extracts had little effect on the expression of the COL1A1 gene, they were able to increase the formation of soluble collagen, notably in UVA-irradiated fibroblasts. Moreover, both FBR and FBrR, particularly at a concentration of 50 µg/mL, upregulated the expression of the ELN gene in UV-irradiated fibroblasts. Attenuation of TNF-α, IL-1ß, and IL-6 cytokine production in co-cultures exposed to UVB further demonstrated the anti-photoageing potential of FBR and FBrR via inflammatory pathways. Taken together, both FBR and FBrR water extracts particularly at 50 µg/mL, have the potential to serve as anti-photoageing functional bioingredients. Nevertheless, further investigation is required to uncover the metabolic pathway and to dissect their deeper underlying mechanisms.

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Interest conflict

None.

Author contribution

AJ carried out all the experimental work and manuscript writing. NYMY assisted in experimental design, data interpretation, and manuscript revision. SMMAR assisted in experimental work and manuscript revision. MAM, DLAR, NYAR, and AAG prepared the fermented extracts and contributed to manuscript revision.

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