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Aspergillus oryzae-Fermented Broken Rice and Rice Bran Exhibited Anti-photoaging Effect on UVB-irradiated Human Fibroblasts

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The current study evaluated the efficacy of *Aspergillus oryzae*-fermented broken rice (FBR) and rice bran (FRB) extracts in inhibiting photoaging in UVB-irradiated fibroblasts. The FBR and FRB extracts at 50 and 100 µg/mL markedly suppressed intracellular elastase activity and the FRB extract at 100 µg/mL significantly lowered the MMP-1 secretion in UVB-irradiated fibroblasts. Both FRB and FRB extracts also significantly downregulated the expression of MMP-1 and SFE genes in the UVB-irradiated fibroblasts. Furthermore, the substantial elevation of COL1A1 gene expression by FBR and FRB extracts at 50 µg/mL also indicated the potential of these extracts to enhance collagen type 1 synthesis. The present findings indicate that FBR and FRB extracts may potentially serve as functional components to mitigate skin damage caused by UVB radiation. However, further investigation is necessary to ascertain the precise anti-photoaging mechanism.

Keywords: broken rice; rice bran; anti-photoaging; solid-state fermentation; fibroblast

I. INTRODUCTION

Photoaging, or premature aging of the skin, is a condition in which the aspect of the skin is altered due to excessive ultraviolet (UV) exposure. Increasing consumer concerns regarding the safety of existing synthetic chemicals have resulted in a high demand for skin care products containing natural and safe therapeutic components or active ingredients that could alleviate this skin issue. Agricultural by-products, which produce a substantial amount of waste each year, have recently attracted scientific attention as raw materials for the production of functional bioingredients. Valorisation of these by-products could potentially mitigate their potential environmental impact (Sorrenti *et al.,* 2023).

Broken rice and rice bran are highly valuable sources of nutrients and bioactive substances, such as oryzanol, ferulic acid, tocopherols, and tocotrienols which derived from rice processing industry (*Esa et al.,* 2013). Utilisation of solid-state fermentation with *Aspergillus oryzae* has successfully improved the antioxidant and anti-elastase activity compared to their unfermented counterparts (Abd. Razak *et al.,* 2018; 2019). As both these functionalities are associated with preventing photoaging, this has prompted to the study of fermented broken rice (FBR) and rice bran (FRB) extracts for their anti-photoaging ability in UVB-irradiated human fibroblasts.

It is long known that excessive UV exposure would lead to skin photoaging which primarily manifests as wrinkles and sagging of the facial skin (Tanveer *et al.,* 2023). This photodamage has been attributed to the upregulation of matrix metalloproteinase-1 (MMP-1) and skin fibroblast elastase (SFE) in dermal fibroblasts, which leads to the degradation of major dermal components of collagen and elastin, resulting in sagging and wrinkles. Hence, numerous studies examining anti-photoaging mechanisms in human skin have focused on collagenase and elastase inhibitory activities. In this study, the FBR and FRB extracts were evaluated for their anti-photoaging ability by assessing their inhibitory potential on matrix metalloproteinase-1 (MMP-1) production and intracellular elastase activity, both of which

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play crucial roles in skin wrinkling and sagging. The total collagen content in human fibroblasts as affected by FBR and FRB treatments was also evaluated. The anti-photoaging mechanism of the studied extracts was also investigated using quantitative polymerase chain reaction (qPCR) analysis by measuring the gene expression level of MMP-1, skin fibroblast elastase (SFE) as well as type 1 collagen (COL1A1) and elastin (ELN).

II. MATERIALS AND METHOD

A. Preparation of Fermented Extracts

Thirty grams of broken rice and rice bran weighed in separate Erlenmeyer flasks were autoclaved at 121 °C for 15 mins. Then, 35 mL of sterile distilled water was added to achieve a substrate moisture level of 50%. Approximately 5×10^6 of *Aspergillus oryzae* spores/gram of substrate were added and subjected to incubation at 32 °C for 12 days. The substrates were then recovered and underwent oven-drying at 50 °C for 24 hours. Hot water extraction was performed by boiling 1 g of dried sample in 5 mL of distilled water for 15 mins and the mixture was centrifuged at 10,000 rpm. The supernatants were recovered and filtered using a filter paper (Whatman No. 1). The filtrates were freeze-dried and stored at -20 °C for further analysis.

B. Cell Culture

The human fibroblasts, CCD1135sk (ATCC-CRL-2691) were cultured in IMDM medium supplemented with 10% FBS and 1% antibiotic-antimycotic. The cells were maintained at 37 °C in a 5% CO_2 incubator. The culture medium was replaced every 72 h, and the cells were routinely subcultured at a density of 80%.

C. Cell Viability Analysis

Cell viability analysis was done using the MTT colorimetric assay (van Merloo *et al.,* 2011). Fibroblasts were cultured at a density of 1 x 10^5 cells/well in a 96-well plate for 24 h in a 5% CO_2 incubator. Following incubation, the growth media were substituted with treatment media containing FBR and FRB extracts at varying concentrations (5, 10, 50, 100, and 500 µg/mL) and incubated for another 24 h. Post incubation, 20 µL of MTT reagent was added to each well and agitated at 150 rpm for 5 min followed by incubation at 37 °C for 4 h. After medium removal, 100 uL of DMSO was added to solubilise the formazan followed by shaken at 150 rpm for 5 min. The assay plate was analysed spectrophotometrically at 570 nm. The percentage of cell viability was determined against non-treated controls as in the equation below, where A denotes the absorbance of treated samples and B is the absorbance of non-treated controls.

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

D. UVB Irradiation and Extracts Treatment

The fibroblasts were grown in Multiwell™ 6-well plates at a density of 5 x 10^6 cells/well (Beckton Dickinson NJ, USA) and incubated for 24 h in a 5% CO_2 incubator. The growth media was then replaced with serum-free growth media and left serum-starved for 16 h. Post incubation, the growth media was substituted with PBS followed by UVB (100 mJ/cm^2) exposure using the Vilber-Lourmat™ BIO-LINK system. The PBS was then replaced with treatment media containing the 50 and 100 µg/mL of FBR and FRB extracts followed by incubation for 24 h. Both cells and supernatants were collected for further investigation.

E. MMP-1 Content Measurement via ELISA

The MMP-1 concentration in the cell culture supernatant was measured using the method described by Oh *et al.* (2020) using an enzyme-linked immunosorbent assay (ELISA) with the Human Total MMP-1 Duoset ELISA kit (R&D Systems) as per the manufacturer's instructions. An ELISA plate reader (BioTek®) was used to measure the absorbance at 450 nm. The results were determined by applying a four-parameter logistic (4-PL) curve-fit evaluation using recombinant human MMP-1 as a reference standard.

F. Analysis of Intracellular Elastase Activity

The untreated and treated fibroblasts were lysed in 500 µl of lysis buffer (0.1 M Tris-HCl (pH 7.6), 0.1% Triton X- 100 and protease inhibitor cocktail) for enzyme extraction (Suganuma *et al.,* 2010) and the protein concentration was quantified through BCA protein assay. For elastase analysis, 100 µl of enzyme solution was mixed with 2 µl of 62.5 mM STANA and incubated at 37 °C for 1 h. The release of p-nitroaniline was measured spectrophotometrically at 405 nm. The percentage of elastase activity in the treated sample relative to the untreated control was calculated using the equation provided

below, where A denotes the absorbance of the treated sample and B represents the untreated control.

$$\textit{Intracellular elastase activity (\%)} = [A_{sample}/B_{control}] \times 100\ \%$$

G. Determination of Soluble Collagen Content

The quantification of soluble collagen content in the supernatant was done using the Sircol™ Soluble Collagen Assay (Biocolor, Northern Ireland, UK). Initially, the collagen reference standard was prepared at various concentrations (0, 5, 10, and 15 µg/mL). To measure the total collagen content, 100 µL of collagen standard and test samples were individually mixed with 1 mL of Sircol dye reagent and agitated gently for 30 min followed by centrifugation at 12 000 rpm for 10 min. The collagen-dye pellet was rinsed with 750 µL of ice-cold acid-salt wash reagent and 250 µL of alkaline reagent was added followed by vortexing for 5 min to release the collagen bound dye into solution. Next, 200 µL of blank, standards and samples were transferred to a 96-well microplate, and the optical density measurement was conducted at 555 nm. The collagen concentration was determined from the standard curve generated.

H. Gene Expression Analysis via qPCR

The qPCR analysis was employed to examine the influence of FBR and FRB on the expression of photoaging-related genes (MMP-1, SFE, COL1A1, and ELN) in fibroblasts (Table 1). The TRIzol™ reagent was used to isolate the total RNA by following the manufacturer's protocol. The cDNA was then synthesised using the ReverTra Ace™ qPCR RT kit (Toyobo, Tokyo, Japan). The qPCR analysis was conducted using the Bio-Rad CFX96 Real-Time PCR System and Bio-Rad CFX96 Manager Software (Bio-Rad, USA) in 96-well plates using SYBR Green-based PCR assay. The reaction mixture was prepared according to THUNDERBIRD SYBR qPCR Mix kit manufacturer (TOYOBO, Japan). The gene expression levels were normalised using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, and the results were presented as a fold change.

I. Statistical Analysis

The data is reported as the mean ± SEM (n = 3). The statistical significance was assessed using a one-way ANOVA followed by the Duncan multiple comparison test. Statistically significant results are defined as having p-values less than 0.05. The statistical studies were conducted using SPSS Version 26 (IBM Corporation, Chicago, USA).

Table 1. Primer sets used for qPCR analysis

Primer	Accession No.	Sequence
MMP-1	NM_001145938.2	forward : 5'AGAAAGAAGACAAAGGCAAGTTGA-3' reverse : 5'-TCAGTGAGGACAAACTGAGCC-3'
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'
COL1A1	NM_000088.4	forward : 5'- AGA GGT CGC CCT GGA GC-3' reverse : 5' – CAG GAA CAC CCT GTT CAC CA -3'
ELN	NM_001278939.2	forward : 5'- CTT TGG TGT CGG AGT CGG AG-3' reverse : 5'- TCC TGC AGC ACC GTA CTT G-3'
GAPDH	NM_002046.7	forward : 5'- GCA AAT TCC ATG GCA CCG T-3' reverse : 5'- TCG CCC CAC TTG ATT TTG G-3'

III. RESULT AND DISCUSSION

A. Effect of FBR and FRB Extracts on the Viability of Human Fibroblasts

The cytotoxicity effects of the FBR and FRB extracts on human fibroblasts were carried out using the MTT assay. The MTT colorimetric assay quantifies the amount of yellow 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) that is converted to a dark purple formazan product by mitochondrial succinate dehydrogenase in metabolically active cells (Ghasemi *et al.,* 2021). As exhibited in Figure 1, the FBR and FRB extracts displayed no cytotoxic effects towards fibroblasts at all concentration tested as the cell viability percentage ranging from 98.30% to 140.06% and 97.05 to 105.70%, respectively. The effect of the studied extracts on the UVB-irradiated fibroblasts was also investigated to ensure that the observed effect in the photoaging study is caused by the extracts and not by cell death. The 50 and 100 µg/mL of FBR and FRB extracts have been selected for further analysis and Figure 2 depicts their effect on the viability of UVB irradiated-human fibroblasts. The higher than 90% cell viability indicating that none of the treatments were cytotoxic.

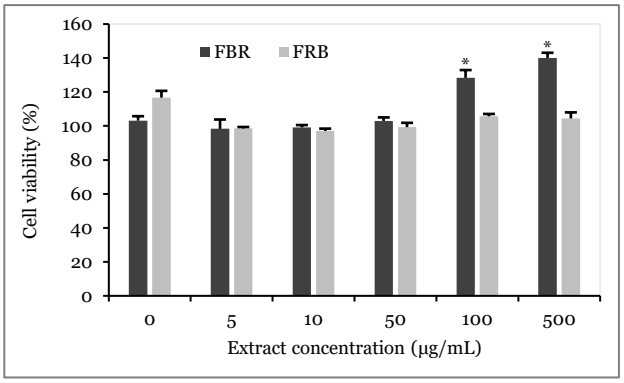


Figure 1. Effect of FBR and FRB extracts on the viability of human fibroblasts. Asterisks (*) indicate a significant difference of $p<0.05$ compared with untreated group, 0 µg/mL.

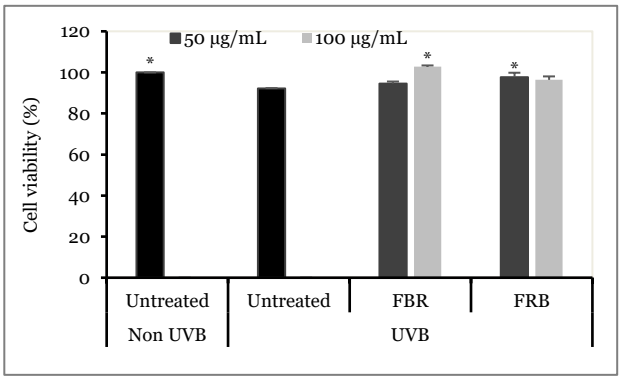


Figure 2. Effect of FBR and FRB extracts on the viability of UVB-irradiated human fibroblasts. Asterisks (*) indicate a significant difference of $p<0.05$ compared with untreated UVB-irradiated group.

B. Effect of FBR and FRB Extracts on MMP-1 Level and Intracellular Elastase Activity

Excessive exposure of UV radiation increases the formation of reactive oxygen species (ROS) which promotes the production of collagen and elastin-degrading enzyme such as MMP-1 and skin fibroblast elastase which lead to imbalance and degradation of collagen and elastin synthesis, thereby resulting in skin collapse and wrinkles (Gromkowska-Kępka *et al.,* 2021). Thus, it is suggesting that inhibition of MMP-1 production and intracellular elastase activity could reduce UV-induced photoaging (Lee *et al.,* 2021). As tabulated in Figure 3, the 100 mJ/cm² of UVB irradiation had no significant influence on MMP-1 production in the untreated fibroblasts. The FBR extracts also displayed insignificant effect in reducing MMP-1 levels, while only FRB at 100 µg/mL significantly decreased ($p<0.05$) MMP-1 levels by 22.54% compared to the untreated UVB-exposed control. The reduction of MMP-1 level in fibroblasts by fermented rice bran has also previously been reported by Seo *et al.* (2010). On the other hand, the UVB radiation had different effect on the intracellular elastase in fibroblast as it significantly increased ($p<0.05$) the elastase activity in untreated UVB-exposed fibroblasts. Nevertheless, FBR and FRB extracts at significantly lowered elastase activity at all concentration tested as shown in Figure 4. FBR (100 µg/mL) demonstrated the most significant reduction ($p<0.05$) with 1.3-fold lower than the untreated UV-exposed control. According to these results, the inhibitory effect of FBR and FRB extracts on the induction of UVB is more pronounced for elastase activity than for the synthesis of MMP-1. This result is in concomitant with our previous findings where FBR and FRB extract demonstrated inhibitory effects in a larger degree for elastase activity than the MMP-1 production in UVA-irradiated fibroblasts (Jamaluddin *et al.,* 2023). In addition, FBR and FRB extracts also displayed elastase inhibitory activity evaluated in a cell-free analysis (Abd. Razak *et al.,* 2015; 2019). Thus, we may suggest that it is likely that the anti-photoaging effect of FBR and FRB at 50 ug/mL extracts in UVB-irradiated fibroblast was due to elastase inhibition rather than MMP-1 inhibition. The FRB at 100 µg/mL on the other hand exhibited anti-photoaging effect via significant inhibitory activity on both MMP-1 secretion and elastase.

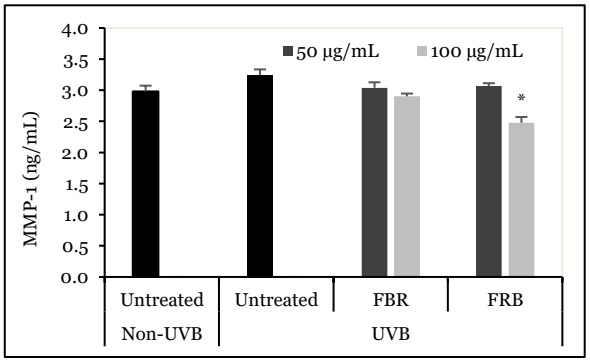


Figure 3. Effect of FBR and FRB extracts on MMP-1 content in UVB-irradiated fibroblasts. Asterisk (*) indicates a significant difference of $p<0.05$ compared with untreated UVB-irradiated group.

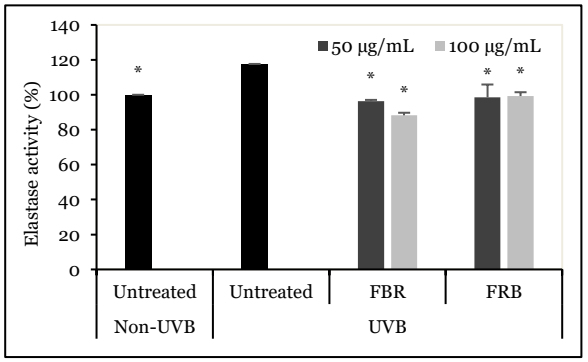


Figure 4. Effect of FBR and FRB extracts on elastase activity in UVB-irradiated fibroblasts. Asterisk (*) indicates a significant difference of $p<0.05$ compared with untreated UV-irradiated group.

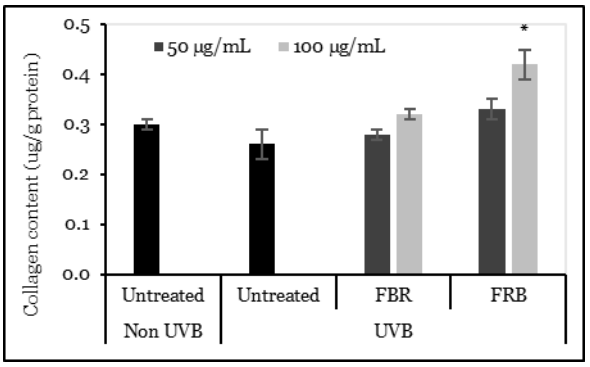


Figure 5. Effect of FBR and FRB extracts on total collagen content in UVB-irradiated fibroblasts. Asterisk (*) indicates a significant difference of $p<0.05$ compared with untreated UV-irradiated group.

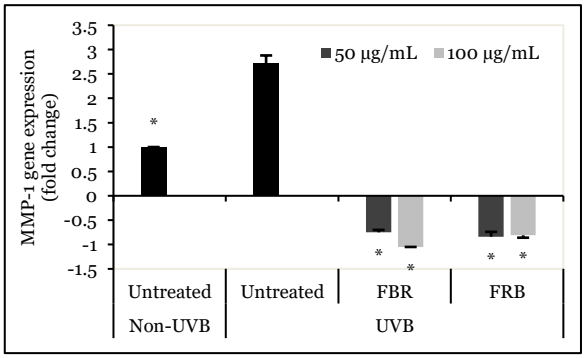
C. Soluble Collagen Content in FBR and FRB-treated UVB-irradiated Human Fibroblasts

The extracellular matrix of the skin dermis consists mainly of collagen, elastin, fibrin, and proteoglycans. Collagen is mainly composed of types I-IV (Seong *et al.,* 2017). In this study, the soluble collagen content in cell supernatant using the Sircol assay which assess the rate of newly synthesised collagen produced during cell growth. Nevertheless, this assay does not discriminate between collagen types as it measures mammalian collagen from type I to V. The effect of UVB irradiation on soluble collagen content in human fibroblasts is depicted in Figure 5. The analysis showed that direct UVB exposure on human fibroblasts had a mild effect on soluble collagen content Treatment with FBR and FRB extracts maintained the soluble collagen content in the fibroblast except for FRB where the soluble collagen content was significantly ($p<0.05$) augmented by the 100 µg/ml extract. Rice bran co-fermented with *Lactobacillus rhamnosus* and *Sacharomyces cerevisiae* also increased the collagen synthesis in UVB-irradiated human fibroblasts as reported by Seo *et al.* (2010), demonstrating the potential of fermented rice bran in enhancing collagen content.

D. Gene Expression Level of MMP-1, SFE, COL1A1 and ELN as Affected by FBR and FRB Treatments

UVB irradiation potentially enhance MAPK/AP-1 signalling pathway that promotes upregulation of MMPs expression, which subsequently results in breakdown of ECM components such as collagens, elastic fibres and glycosaminoglycans and lead to photoaging (Seo *et al.,* 2019). Hence, the effect of FBR and FRB extracts on MMP-1 and SFE gene expression were also investigated. As shown in Figure 6a, UVB irradiation on fibroblasts significantly enhanced ($p<0.05$) MMP-1 gene expression by 2.71-fold in comparison to the non-irradiated fibroblasts. The elevation of MMP-1 gene expression by the same dose (100 mJ/cm^2) of UVB was also reported in other human skin fibroblasts, Hs68 (Jung *et al.,* 2014). However, the MMP-1 gene expression was then significantly downregulated by all FBR and FRB treatments ($p<0.05$). SFE also plays a crucial role in the UVB-induced degeneration and tortuosity of elastic fibres (Tsukahara *et al.*, 2001). The UVB exposure also significantly increased ($p<0.05$) SFE gene expression in fibroblasts as tabulated in Figure 6b. Nevertheless, both FBR and FRB extracts significantly downregulate ($p<0.05$) the SFE gene expression at all concentration tested.

(a)



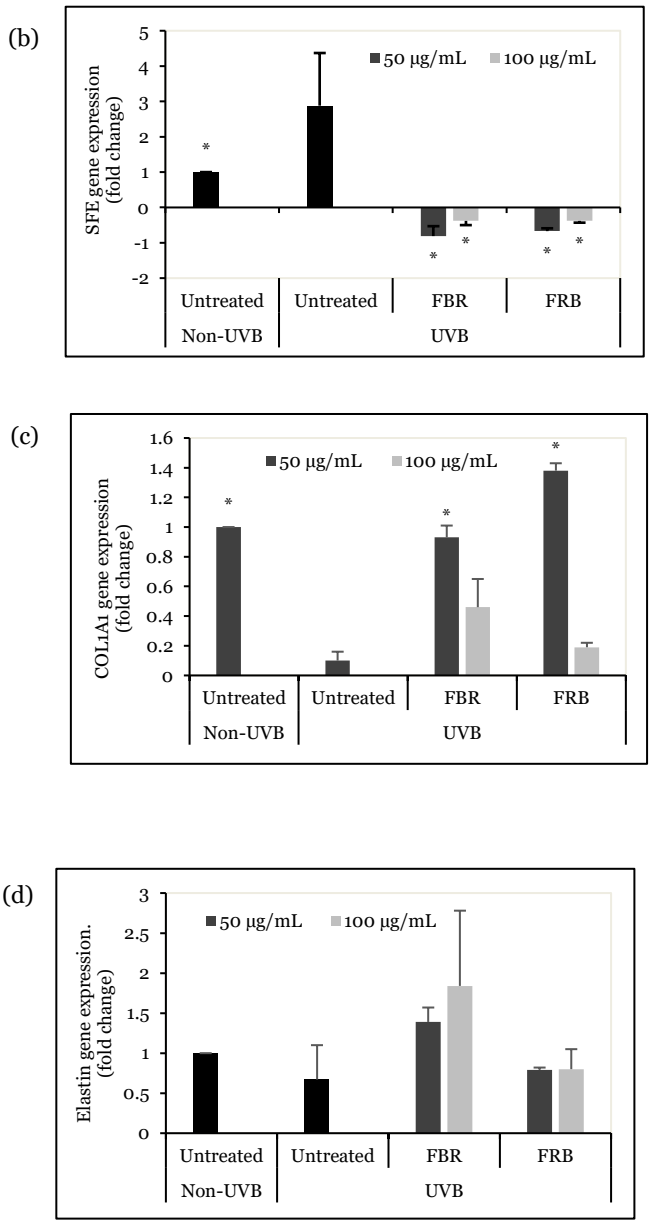


Figure 6. Gene expression of (a) MMP-1, (b) SFE, (c) COL1A1 and (d) ELN in UVB-irradiated fibroblasts upon treatment with 50 and 100 µg/ml of FBR and FRB extracts. Asterisks (*) indicate a significant difference of $p<0.05$ compared with untreated UV-irradiated group.

Excessive UVB irradiation also could lead to the breakdown of collagen, a major component of extracellular matrix (ECM), which also include type 1 collagen, elastin, proteoglycans, and fibronectin. Type I collagen is primarily synthesised by fibroblasts in the dermis, which is accountable for conferring strength and resiliency to the skin (Gelse *et al.,* 2003). It is an accordance with our finding which COL1A1 gene expression was significantly downregulated ($p<0.05$) upon direct UVB exposure on fibroblasts (Figure 6c). Nonetheless, the FBR and FRB extracts particularly at concentration of 50 µg/mL significantly upregulate ($p<0.05$) the COL1A1 gene expression. Analysis of ELN gene expression on the other hand, showed that the FBR and FRB extracts had no influence on the ELN gene expression of the UVB-irradiated fibroblasts (Figure 6d). The upregulation of COL1A1 gene expression demonstrated the potential of FBR and FRB extracts at 50 ug/mL in promoting collagen type 1 production. Nevertheless, the study of procollagen type 1 production is warranted since mRNA levels should not be taken as the final product of gene expression (Buccitelli & Selbach, 2020).

IV. CONCLUSION

The present study demonstrated the ability of FBR and FRB extracts in preventing photoaging in the UVB-irradiated fibroblasts. FBR and FRB extracts at both concentrations significantly downregulated the MMP-1 and SFE gene expression as well as inhibited the intracellular elastase activity. In addition, the FRB extracts at 100 ug/mL significantly lowered the MMP-1 level in the UVB-irradiated fibroblasts. The significant upregulation of COL1A1 gene expression by FBR and FRB extracts at 50 ug/mL demonstrated the potential of these extracts in promoting the collagen type 1 production. Although the exact anti-photoaging mechanism still needs to be elucidated, the current findings suggest that FBR and FRB extracts have the potential to be employed as functional ingredients to prevent skin damage induced by UVB radiation.

V. ACKNOWLEDGEMENT

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