Food Research 6 (Suppl. 4) : 81 - 89 (2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Effect of *Aspergilus oryzae*-fermented broken rice, brewers' rice and rice bran on melanogenesis in highly pigmented human melanoma, MNT-1

[1,*]Jamaluddin, A., [2]Mohd Yusof, N.Y., [2]Mohd Abdul Rahman, S.M., [2]Mohd Pilus, N.S., [1]Abd. Razak, D.L., [1]Abdul Manan, M., [2]Amran, A.S., [1]Abd. Rashid, N.Y. and [1]Abd. Ghani, A.

[1]*Food Science and Technology Research Centre, Malaysian Agricultural Research and Development Institute (MARDI), Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia*
[2]*Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43650 Bangi, Selangor, Malaysia*

Article history:
Received: 5 April 2022
Received in revised form: 9 May 2022
Accepted:2 August 2022
Available Online: 25 March 2023

Keywords:
Brewers' rice,
Broken rice,
Rice bran,
Tyrosinase,
Melanogenesis

DOI:
https://doi.org/10.26656/fr.2017.6(S4).011

Abstract

The food and beauty sectors are developing strategies to establish a link between nutrient consumption and skin health, as nutraceuticals offer a promising treatment option for some skin disorders such as hyperpigmentation and premature ageing. As a result, the consumption of food ingredients and supplements claiming to lower the risk of developing such skin problems is increasing. In this study, anti-tyrosinase potential of *Aspergillus oryzae*-fermented rice by-product water extracts namely fermented broken rice (FBR), brewers' rice (FBrR), and rice bran (FRB) was assessed through *in vitro* study using highly pigmented human melanoma MNT-1 cells. The fermented extracts were evaluated for cytotoxicity, anti-tyrosinase activity using intracellular tyrosinase assay as well as melanin content analysis. Their effect on the gene expression of three melanogenic enzymes: tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) was also assessed by qPCR analysis. As a result, all extracts at tested concentrations of 50 and 100 µg/mL have a low cytotoxicity effect. Together with the positive control kojic acid, the FBR at both concentrations and the FRB at 50 µg/mL significantly decreased intracellular tyrosinase activity. However, their melanin content was not significantly reduced. The qPCR analysis indicated that FBR at 50 and 100 µg/mL significantly reduced TYR gene expression by up to 71% and 61%, respectively. On the other hand, FBR (100 µg/mL) increased TRP-1 gene expression, whilst FRB (100 µg/mL) elevated both TRP-1 and TRP-2 gene expression. The positive control kojic acid significantly reduced TYR, TRP-1 and TRP-2 gene expression. However, FBrR demonstrated no significant effect in any of the analyses. Based on these findings, FBR and FRB at 50 µg/mL possess anti-tyrosinase potential. However, further investigation is needed to dissect deeper mechanisms underlying their potential as functional bioingredients for nutraceutical or cosmeceutical applications.

1. Introduction

The skin is the largest organ in the body which participates in sensitivity and protects against microorganisms, chemicals, and ultraviolet (UV) radiation. As a result, the skin may undergo changes such as inflammation, immune dysfunction, and photoaging, which can have a significant impact on human health and appearance. The food and cosmetic industries are developing new strategies to establish a link between nutrient consumption and skin health. As a result, the use of food ingredients and supplements that claim to reduce the risk of skin disorders is increasing (Boelsma *et al*., 2001). Among the most commonly used ingredients are bioactive peptides and oligosaccharides, plant polyphenols, carotenoids, vitamins, and polyunsaturated fatty acids. The majority of bioactive food compounds responsible for health benefits are derived primarily from plants, with only a few derived from animal sources (Kussmann *et al*., 2007).

Nutraceuticals are the promising strategy for preventing, delaying, or minimising premature skin ageing, as well as treating certain skin disorders. The term "nutraceutical" was coined Stephen DeFelice, from the combination of "nutrition" and "pharmaceutical."

*Corresponding author.
Email: *anisj@mardi.gov.my*

eISSN: 2550-2166
Published by Rynnye Lyan Resources

According to DeFelice, nutraceutical is "a food or a component of it that provides medical or health benefits, including disease prevention and/or treatment" (Brower, 1998; Kalra, 2003). Nutraceuticals also can be used to enhance human health, slow the ageing process as well as support the structure or function of the human body. Nowadays, nutraceuticals have sparked a strong interest due to their nutritional potential, safety, and therapeutic effects. Recent market research proposed that the global nutraceutical market size was valued at USD 454.55 billion in 2021 and is expected to expand at a compound annual growth rate (CAGR) of 9.0% from 2021 to 2030 (Grand View Research, 2022). The rising demand for dietary supplements and functional foods is expected to be a major driving force in the market during the forecast period. In Malaysia, people are concerned about health and dietary habits, which is a positive factor for the growth of the nutraceuticals market in Malaysia. Malaysia's nutraceutical market is projected to reach USD 1382.3 million by 2027 from 794.4 million in 2020 (Research CMFE, 2021).

Nutraceuticals have been studied over the years to delay and combat these internal and external factors, which are mostly found in foods and by-products consumed naturally. A recent trend has been observed in the use of the solid-state fermentation (SSF) process for the bioconversion of agro-industrial by-products into value-added products. The reuse of these by-products is of particular interest due to their availability and low cost, besides being an environment-friendly alternative for their disposal. Furthermore, their appealing composition, combined with the growing interest in finding natural ingredients as an alternative to synthetic substances, has determined them an economically attractive source for the production of high-value products in a variety of industrial fields, including food, nutraceuticals, pharmaceutics and cosmetics.

The same scenario happen in the rice processing industry as Malaysia is one of the world's rice-producing and consuming countries where the production volume of rice in 2020 was approximately 1.51 million metric tonnes (Hirschmann, 2022). In 2015, it was reported the milling of 400, 906 tonnes of paddy produced about 60.7% graded rice only with the remainder becoming by-products (Ministry of Agriculture, 2017). Ironically, the utilization of these by-products is very limited despite they are a good source of secondary metabolites. The presence of carbon sources, nutrients and moisture in these wastes opens up great possibilities for their reuse in bioprocessing for the production of value-added products. These by-products have been recognized as an excellent source of nutrients and bioactive compounds, such as gamma-oryzanol, ferulic acid, tocopherols and tocotrienols, which offer beneficial health properties and antioxidant activity.

Therefore, this study was carried out to characterize and valorize the rice by-products namely broken rice, brewers' rice and rice bran for their potential application in the nutraceutical industry. Our previous study, SSF of rice by-products such as broken rice, brewers' rice and rice bran with GRAS status *Aspergillus oryzae* resulted in the development of bioactive extracts of fermented rice-by-products: broken rice (FBR), brewers' rice (FBrR) and rice bran (FRB) with greater antioxidant and anti-tyrosinase activity than their unfermented counterparts. The SSF procedure successfully elevated the total phenolic content in these substrates. The anti-tyrosinase activity was evaluated using mushroom tyrosinase as a model and demonstrated higher tyrosinase inhibition showing their potential as functional bioingredients for nutraceutical and cosmeceutical purposes (Abd. Razak *et al*., 2019; Abd. Ghani *et al*., 2021). However, it has been reported that the regulation of mushroom tyrosinase differs significantly from that of mammalian tyrosinase in several aspects (Pomerantz, 1963; Hearing *et al*., 1980). Some compounds that inhibit mushroom tyrosinase do not have comparable effects on mammalian tyrosinase (Galindo *et al*., 1987; Jacobsohn and Jacobsohn, 1992; Funayama *et al*., 1995; Goenka and Simon, 2020). Hence, our study aimed to investigate the anti-melanogenic potential of FBR, FBrR and, FRB through *in vitro* study by evaluating their effect on intracellular tyrosinase activity, and melanin content as well as the possible underlying mechanism using highly pigmented human melanoma, MNT-1 cell line.

2. Materials and methods

2.1 Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM) was purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS), AIM-V medium, non-essential amino acids (NEAA), sodium pyruvate, trypLE^{TM}, and phosphate buffer saline (PBS) were all purchased from Thermo Fischer Scientific (Yokohama, Japan). MTT reagent was purchased from Nacalai Tesque (Kyoto, Japan). Kojic acid, sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), Triton X-100, Tris-HCl, sodium phosphate buffer, protease inhibitor cocktail**,** bicinchoninic acid (BCA), bovine serum albumin (BSA), and L-3,4-dihydroxyphenylalanine (L-DOPA) were all purchased from Sigma-Aldrich (MO, USA).

2.2 Preparation of fermented extracts

The fermented extracts were prepared by the Malaysian Agricultural Research and Development

https://doi.org/10.26656/fr.2017.6(S4).011

Institute (MARDI). Briefly, thirty grams of rice by-products (*Oryza sativa* L. *indica*) (broken rice, Brewers' rice, and rice bran) were added to Erlenmeyer flasks and autoclaved for 15 mins at 121°C. Approximately 35 mL of sterilized distilled water was mixed into each flask to adjust the substrate moisture content to 50%. Fungal spores of *Aspergillus oryzae* were added at an initial concentration of 5×10^6 spores/g of substrates and incubated at 32°C. Samples were harvested and oven-dried at 50°C for 24 hrs. All experiments were performed in triplicate. All samples were subjected to hot water extraction by mixing 1 g of sample with 5 mL distilled water and boiling for 15 mins in a water bath. The samples were then centrifuged for 15 mins at 10,000 rpm, and 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.

2.3 Cell culture procedure

Highly pigmented human melanoma, MNT-1 cells were a kind gift from Prof. Michael Marks, University of Pennsylvania through Dr Cheah Shiau Chuen (UCSI University, Malaysia). Cells were maintained and passaged in Dulbeccos's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) containing 20% fetal bovine serum, 10% AIM-V medium, 1% non-essential amino acids, and 1% sodium pyruvate (ThermoFisher Scientific, Massachusettes, USA). The cell line was grown and maintained at 37°C in 5% carbon dioxide and regularly passaged at a density of 80%.

2.4 Cell viability analysis via MTT assay

Cell viability was determined using tetrazolium dye colourimetric test (MTT). Briefly, the MNT-1 cells were cultured in a 96-well plate at a density of 1×10^5 cells/well for 24 hrs. The cells were then replenished with 200 mL of fresh media containing different concentrations of FBR, FBrR, and FRB extracts ranging from 1 mg to 500 mg and incubated for 48 hrs. A total of 20 µL MTT reagent was added to each well and the plate was incubated at 37°C for 4 hrs to allow the MTT to metabolize. After the incubation, the media was removed, and the formazan produced was dissolved in 100% dimethyl sulfoxide (DMSO). The absorbance was determined at 570 nm using a microplate reader (BioTek®, USA). The percentage of cell viability was calculated against non-treated controls based on the equation below which *A* represents the absorbance of the treated sample and *B* represents the non-treated control.

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

2.5 Intracellular tyrosinase activity

The MNT-1 cells were plated in 6-well plates at a density of 10^5 cells/mL and left to attach overnight. The next day, the culture medium was replaced with fresh media containing 50 and 100 µg/mL of fermented extracts and kojic acid (100 µg/mL) as a positive control. After 48 hrs, the cells were washed twice with cold phosphate buffer saline (PBS) and lysed with 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and protease inhibitor cocktail. The lysate was centrifuged at 12,000 rpm for 20 mins at 4°C. The supernatant was recovered, and the amount of protein was determined using bicinchoninic acid (BCA) assay with BSA as a standard (McFeeters, 1980). An amount of 50 µL of lysate with the same amount of protein was placed in a 96-well plate, followed by 125 µL of 0.1 M sodium phosphate buffer (pH 6.8) and 25 µL of freshly prepared 10 mM L-DOPA in 0.1 M sodium phosphate buffer (pH 6.8). The plate was incubated at 37°C for 30 mins and the tyrosinase activity was determined at 475 nm using a microplate plate reader (BioTek®, USA). The tyrosinase activity (%) was calculated using the following equation where *A* represents the absorbance of the treated sample and *B* represents the non-treated control.

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

2.6 Analysis of melanin content

The MNT-1 cells were seeded into a 12-well plate and left to attach overnight. The culture medium was then replaced with new media containing 50 and 100 µg/mL of fermented extracts and cultured for 48 hrs. The cells were harvested, and the pellets obtained were dissolved in 400 µL of 1 M sodium hydroxide at 80°C for 1 hr and centrifuged at 10,000 rpm for 10 mins. The supernatants (200 µL) were transferred to a 96-well plate and the measurement of melanin level was determined at 405 nm. Kojic acid was used as the positive control. The melanin content (%) was calculated using the following equation which *A* represents the absorbance of the treated sample and *B* represents the non-treated control.

$$\text{Melanin content (\%)} = \left[\frac{A}{B}\right] \times 100$$

2.7 Gene expression analysis using qPCR

The qPCR analysis was carried out in 96-well plates with Bio-Rad CFX96 Real-Time PCR System and Bio-Rad CFX96 Manager Software (Bio-Rad, USA) using SYBR Green-based PCR assay. The preparation of the reaction mix was according to THUNDERBIRD SYBR qPCR Mix kit manufacturer (TOYOBO, Japan) and was subjected to the following conditions: 95°C for 2 mins, 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 analyzed at 65–5°C

https://doi.org/10.26656/fr.2017.6(S4).011

after 40 cycles. Each qPCR analysis was performed in triplicate and the mean was used for qPCR analysis. The gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Primer sets used for qPCR are listed in Table 1.

2.8 Statistical analysis

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

3. Results

3.1 Effect of FBR, FBrR and FRB extracts on cell viability of MNT-1 cells

After 48 hours of exposure to various doses ranging from 1 to 500 µg/mL, the effect of FBR, FBrR, FRB extracts, and kojic acid on MNT-1 cell viability was determined using the MTT colourimetric assay. The well -known tyrosinase inhibitor, kojic acid was used as a positive control. As illustrated in Figure 1, the FBR, FBrR, and FRB had a low cytotoxicity effect on MNT-1 cells. Kojic acid had no cytotoxic effect on human melanoma cells. Based on this outcome, concentrations of 50 and 100 µg/mL were chosen to be employed as treatments in the melanogenesis study. For comparison, kojic acid at 100 µg/mL was used as the positive control.

3.2 Tyrosinase inhibitory effect by FBR, FBrR and FRB extracts in human melanoma, MNT-1

Tyrosinase is the rate-limiting enzyme in the formation of melanin. As a result, the effect of FBR, FBrR, and FRB on the intracellular tyrosinase activity of the human melanoma cell line MNT-1 was evaluated. As illustrated in Figure 2, FBR at both concentrations as well as FRB at 50 µg/mL inhibited tyrosinase activity significantly. Additionally, FBrR and FRB at 100 µg/mL had a minimal effect on tyrosinase activity. The positive control kojic acid exhibited the most inhibitory action, with a 27% inhibition.

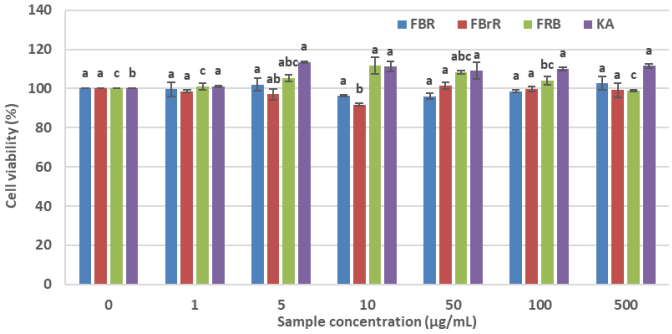

Figure 1. The effect of FBR, FBrR, FRB extracts, and kojic acid (as a positive control) on the viability of highly pigmented human melanoma cells, MNT-1. Cells were treated with different concentrations of the studied extracts (1-500 µg/mL). The values were expressed as mean of percentage ± SEM (n = 3). The values of each extract followed by the same letter are not significantly different from each other (p>0.05).

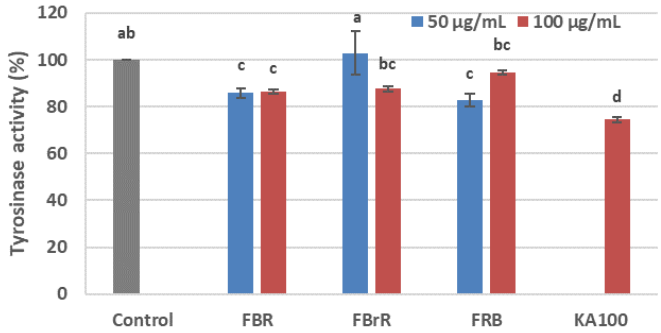

Figure 2. Intracellular tyrosinase activity in human melanoma cells, MNT-1 upon treatment with 50 and 100 µg/ml FBR, FBrR and FRB extracts. Kojic acid (100 µg/mL) was used as a positive control. The values were expressed as mean of percentage ± SEM (n = 3). The values followed by the same letter are not significantly different from each other (p>0.05).

3.3 Effect of FBR, FBrR and FRB extracts on melanin formation in MNT-1 cells

The modification of melanin content in MNT-1 cells treated with the fermented extracts was determined. As illustrated in Figure 3, cells treated at a concentration of 50 µg/mL had no effect on the melanin content of MNT-1 cells. On the other hand, all 100 µg/mL-treated cells displayed an insignificant decrease in melanin concentration. Their effects are parallel with the positive control, kojic acid.

Table 1. Primer sets used for qPCR analysis.

Primer	Sequence
Tyrosinase (TYR)	forward: 5'-AGATTCAGACGCAGACTCTTTTCA-3' reverse: 5'-GACACAGCAAGCTCACAAGC-3'
Tyrosinase-related protein 1 (TRP-1)	forward: 5'- TCT CTG GGC TGT ATC TTC TTC C-3' reverse: 5'- GTC TGG GCA ACA CAT ACC ACT-3'
Tyrosinase-related protein 2 (TRP-2)	forward: 5'-CTT GGG CTG CAA AAT CCT GC-3' reverse: 5' – CAG CAC TCC TTG TTC ACT AGG -3'
GAPDH	forward: 5'- GCA AAT TCC ATG GCA CCG T-3' reverse: 5'- TCG CCC CAC TTG ATT TTG G-3'

https://doi.org/10.26656/fr.2017.6(S4).011

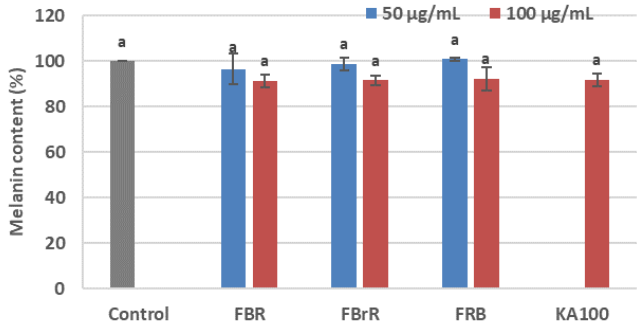

Figure 3. Melanin content of highly pigmented human melanoma cells, MNT-1 upon treatment with 50 and 100 µg/mL FBR, FBrR, and FRB extracts. Kojic acid (100 µg/mL) was used as a positive control. The values were expressed as mean of percentage ± SEM (n = 3). The values followed by the same letter are not significantly different from each other (p>0.05).

3.4 Effect of FBR, FBrR and FRB extracts on gene expression of melanogenic enzymes TYR, TRP-1 and TRP-2 in human melanoma, MNT-1

Tyrosinase (TYR), tyrosinase-related protein-1 (TRP -1) and dopachrome tautomerase (TRP-2) are key factors in melanin synthesis (Kobayashi *et al*., 1994). The qPCR analysis was used to determine whether FBR, FBrR, and FRB extracts have an effect on the gene expression of these melanogenic enzymes. As illustrated in Figure 4, FBR significantly lowered TYR gene expression at both concentrations tested. Additionally, the FBrR and FRB extract reduced TYR gene expression in a non-significant manner. All treatments lowered TRP-1 and TRP-2 insignificantly, except for FBR and FRB at 100 µg/mL, which raised TRP-1 and TRP-2. Meanwhile, the positive control kojic acid significantly decreased the expression of TYR, TRP-1 and TRP-2.

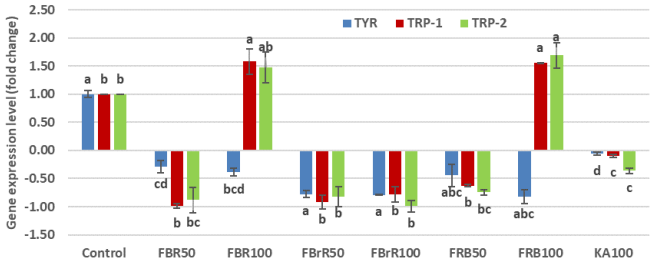

Figure 4. Effect of FBR, FBrR and FRB extracts on gene expression of TYR, TRP-1 and TRP-2 in human melanoma cells, MNT-1 upon treatment with 50 and 100 µg/mL of FBR, FBrR and FRB extracts. Kojic acid (100 µg/mL) was used as a positive control. The gene expression level was evaluated using qPCR analysis and GAPDH was used as an internal control. The values were expressed as means ± SEM (n = 3). The values of each gene followed by the same letter are not significantly different from each other (p>0.05).

4. Discussion

Melanin, a pigmented biopolymer generated in the melanosome of melanocytes, plays a critical role in both UV light protection and phenotypic appearance. However, an abnormal build-up of melanin pigments would lead to aesthetic problems as well as serious diseases related to hyperpigmentation. Mammalian melanin was formed in two chemically different types: black to brown eumelanin and yellow to reddish-brown pheomelanin. Tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2) are melanogenic enzymes that regulate melanin formation. Tyrosinase, a copper-containing enzyme, catalyses the initial rate-limiting processes in melanin synthesis: tyrosine hydroxylation and 3,5-dihydroxyphenylalanine (L-DOPA) oxidation to o-dopaquinone (Masum *et al*., 2019). Although three enzymes (TYR, TRP-1, and TRP-2) are involved in the melanogenesis process, only TYR is exclusively necessary as rate limiting catalyst for melanogenesis. As a result, tyrosinase inhibitors have long been sought as a technique for treating pigmentation disorders.

The FBR, FBrR, and FRB were produced via SSF with *A. oryzae* to address the demand for innovative and safe natural bioingredients for treating pigmentation disorders. Thus, their influence on intracellular tyrosinase activity, melanin content and expression of melanogenic-related genes TYR, TRP-1, and TRP-2 were investigated. The MTT assay was used to assess the effects of FBR, FBrR, and FRB on MNT-1 cell viability. The MTT assay detects the conversion of yellow 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase to a dark purple formazan product found only in metabolically active cells (van Meerloo *et al.,* 2011). The 50 and 100 µg/mL concentrations were chosen for further investigation as they showed no cytotoxicity effect on MNT-1 cells with kojic acid at 100 µg/mL as a positive control.

According to our findings, the tyrosinase activity was significantly inhibited by FBR at both concentrations, FRB at 50 µg/mL as well as the positive control, kojic acid (Figure 2). However, the melanin content was not significantly affected by these treatments. Similar findings have also been noted in the hydrolysable tannins which cellular tyrosinase activity was significantly inhibited without having any significant effect on melanin production level (Cho *et al.,* 2002). This finding also contradicts earlier studies on kojic acid, which has previously been associated with strong anti-melanogenic effects via tyrosinase inhibition (Lajis *et al.,* 2012; Saeedi *et al.,* 2019). As mentioned by Affenzeller *et al*. (2019), melanin which consists of eumelanin and pheomelanin is a complex polymer structure and high thermostability complicates their direct chemical identification. Since the studied extracts might have a different effect on one of the subtypes of

https://doi.org/10.26656/fr.2017.6(S4).011

melanins, another accurate measurement such as HPLC-UV-MS need to be considered (Affenzeller *et al.,* 2019). Therefore, further investigation into their actual effect on melanin content is warranted.

The effect of studied extracts on tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and dopachrome tautomerase (TRP-2) gene expression was also investigated using qPCR analysis, as these genes are crucial for mammalian melanin formation (Chang, 2009; Huang *et al.,* 2011). As seen in Figure 4, both FBR doses and the positive control kojic acid significantly reduced TYR gene expression. This result is consistent with the tyrosinase activity observed in this study. However, FBR had no influence on TRP-1 and TRP-2 expression, with the exception of FBR at 100 µg/mL, which significantly elevated TRP-1 expression. On the other hand, kojic acid significantly decreased the TRP-1 and TRP-2 expression. Based on this result, we could suggest that the tyrosinase inhibitory activity by FBR was through the downregulation of TYR gene expression. Although the FBR (100 µg/mL) increases TRP-1 expression while decreasing TYR expression, the TYR downregulation may be more essential than the TRP-1 upregulation, as tyrosinase is the rate-limiting enzyme in melanin production, as mentioned by Balcos *et al.* (2014). According to Masum *et al.* (2019), despite the fact that TYR, TRP-1, and TRP-2 are all involved in the melanogenesis pathway, TYR is crucial since it catalyses the initial step of melanogenesis, the oxidation of tyrosine to dopaquinone via the intermediate 3,4-dihydroxyphenylalanine (L-DOPA). This oxidation phase is the rate-limiting step in the synthesis of melanin, as the remainder of the reaction sequence can occur spontaneously at a physiological pH value (Halaban *et al.,* 2002).

The FRB at 50 µg/mL demonstrated significant tyrosinase inhibitory activity. This result is consistent with Chung *et al*. (2009) where co-fermentation of rice bran with *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae* decreased the intracellular tyrosinase activity in B16F1 melanoma cells. Although significantly inhibiting tyrosinase enzyme activity, FRB at 50 µg/mL displayed no significant changes in TYR gene expression. This result is in accordance with a study by Sugimoto *et al.* (2004) where α-arbutin (4-hydroxyphenyl a-glucopyranoside) demonstrates a strong inhibitory effect on human tyrosinase activity without affecting mRNA expression of the enzyme in cultured human melanoma cells and a three-dimensional human skin model. The inhibitory effect may be due to the direct inhibition of melanosomal tyrosinase activity, rather than the suppression of cell growth or tyrosinase gene expression (Sugimoto *et al.,* 2004). The expression of TRP-1 and TRP-2 was also not affected by the 50 µg/mL. However, at 100 µg/mL, FRB significantly increases TRP-1 and TRP-2 expression although no effect on melanin content or cellular tyrosinase activity was found. According to Ando *et al.* (2007), the TRP-1 and TRP-2, play a role in modifying the type of melanin synthesized whereby the second enzyme in the pathway, TRP-2 enables the conversion of dopaquinone to dopachrome, and then to 5,6-dihydroxyindole (DHI) or indole 5,6-quinone 2-carboxylic acid (DHICA). TRP-1 then catalyzes the oxidation of DHICA to produce eumelanin. Given that TRP-1 and TRP-2 are involved in eumelanogenesis or the formation of black melanin, we may postulate that FRB at a concentration of 100 µg/mL demonstrated contradictory results by stimulating eumelanogenesis through the upregulation of both TRP-1 and TRP-2 expression without affecting TYR expression.

We also discovered that FBrR had no significant influence on melanin content or tyrosinase activity in this investigation, despite the fact that it was previously reported to exhibit considerable tyrosinase inhibitory activity when examined using a mushroom tyrosinase model (Abd. Razak *et al.,* 2019). The TYR, TRP-1 and TRP-2 gene expressions were also not significantly affected by the FBrR treatment. It has been noted that inhibiting mushroom tyrosinase activity in a cell-free system does not always result in a decrease in cellular tyrosinase activity or melanin production, and vice versa (Song *et al.,* 2009). This discrepancy could be explained by the pharmacological features of the extracts, such as cell permeability and non-specific protein-ligand interactions that are cell type dependent. This disparity could also be explained by the structural differences between target proteins employed for enzyme and cellular activities. Moreover, the two proteins, mushroom tyrosinase and human tyrosinase share only approximately 30% sequence similarity (Choi and Jee, 2015).

The biological activities shown by any extract have frequently been ascribed to its compound content. According to Katina *et al*. (2007), the structural breakdown of cell walls induced by fermentation might lead to the liberation and/or synthesis of various bioactive compounds. According to Abd. Razak *et al.* (2018), ascorbic and gallic acids, as well as ferulic and p-coumaric acids, are among the bioactive components discovered in FBR and FRB extracts that have been reported to have anti-tyrosinase action (Shimada *et al.,* 2009; Lim *et al.,* 1999; Su *et al.,* 2013; Maruyama *et al.,* 2018). Besides, the kojic acid compound was also detected in all FBR and FRB as a result of the *A. oryzae*-fermentation which may contribute to the anti-tyrosinase

https://doi.org/10.26656/fr.2017.6(S4).011

activity found in this study (Abd. Razak *et al.,* 2018). Similar findings were reported in rice koji fermentation with a selected *A. oryzae* strain supplemented with *Astragalus radix* which demonstrated anti-tyrosinase activity with calycosin and kojic acid identified as major tyrosinase inhibitors (Kim *et al*., 2012). However, it is critical to note that the biological activities of these extracts cannot be directly linked to the type or quantity of a particular bioactive component contained in the extract (Ryan *et al.,* 2011). Different extracts contained a range of bioactive substances in varying concentrations. As a result, each extract exhibits unique biological properties, which are influenced by the intricate interactions – synergistic, additive, or antagonistic – between the bioactive components. This may explain the opposite effect seen in FRB at 100 µg/mL.

5. Conclusion

As a result of these observations, we found that FBR is the most promising extract for anti-tyrosinase activity, as it significantly inhibited tyrosinase activity and decreased TYR gene expression. FRB, on the other hand, decreased tyrosinase activity only at a concentration of 50 µg/mL by direct suppression of melanosomal tyrosinase activity but may promote eumelanogenesis at a concentration of 100 µg/mL via overexpression of TRP -1 and TRP-2 without affecting TYR gene expression. On the other hand, FBrR extracts lacked significant inhibitory activity against tyrosinase on both enzymatic and gene expression levels. Additionally, their effect on melanin content warrants further investigation using another high accuracy method. In conclusion, FBR and FRB at 50 µg/mL exhibit anti-tyrosinase activity, but further investigation is needed to elucidate the underlying processes of these examined extracts in order to establish their full potential as functional bioingredients for nutraceutical or cosmeceutical applications.

Acknowledgements

This study was funded by a RMKe-11 Developmental Project Research Grant (No. 21003004170001) from the Malaysian Agricultural Research and Development Institute (MARDI).

References

Abd. Ghani, A., Abd. Rashid, N.Y., Jamaluddin, A., Abd. Razak, D.L. and Abdul Manan, M. (2021). Improvement of cosmeceutical properties in rice by-products by solid state fermentation with *Aspergillus oryzae* and effects of different extracting conditions. *Food Research,* 5(1), 114 – 121. https://doi.org/10.26656/fr.2017.5(S1).025

Abd. Razak, D.L., Jamaluddin, A., Abd. Rashid, N.Y., Abd Ghani, A., Mansor, A. and Abdul Manan, M. (2018). Cosmeceutical potentials and bioactive components of fermented rice by-products. *International Journal of Pharmacy and Biological Sciences,* 9(Special Issue), 28-36.

Abd. Razak, D.L., Abd. Rashid, N.Y., Jamaluddin, A., Abd Ghani, A., Mansor, A. and Abdul Manan, M. (2019). Brewer's rice - a potential substrate for cosmeceutical bio-ingredient production by solid-state fermentation using *Aspergillus oryzae*. *Malaysian Journal of Microbiology*, 15(4), 160 – 166. https://doi.org/10.21161/mjm.191541

Affenzeller, S., Frauendorf, H., Licha, T., Jackson, D.J. and Wolkenstein, K. (2019). Quantitation of eumelanin and pheomelanin markers in diverse biological samples by HPLC-UV-MS following solid-phase extraction. *PLoS ONE*, 14(10), e0223552. https://doi.org/10.1371/journal.pone.0223552

Ando, H., Kondoh, H., Ichihashi, M. and Hearing, V.J. (2007). Approaches to identify inhibitors of melanin biosynthesis via the quality control of tyrosinase. *Journal of Investigative Dermatology*, 127(4), 751–761. https://doi.org/10.1038/sj.jid.5700683

Balcos, M., Kim, S., Jeong, Hs., Yun, H., Baek, K.J., Kwon, N.S., Park, K. and Kim, D. (2014). Docosahexaenoic acid inhibits melanin synthesis in murine melanoma cells *in vitro* through increasing tyrosinase degradation. *Acta Pharmacologica Sinica*, 35(4), 489–495. https://doi.org/10.1038/aps.2013.174

Boelsma, E., Hendriks, H.F. and Roza, L. (2001). Nutritional skin care: Health effects of micronutrients and fatty acids. *American Journal of Clinical Nutrition,* 73(5), 853–864. https://doi.org/10.1093/ajcn/73.5.853

Brower, V. (1998). Nutraceuticals: Poised for a healthy slice of the healthcare market? *Nature Biotechnology,* 16(8), 728–731. https://doi.org/10.1038/nbt0898-728

Chang, T.S. (2009). An updated review of tyrosinase inhibitors. *International Journal of Molecular Science,* 10(6), 2440–2475. https://doi.org/10.3390/ijms10062440

Cho, S.M., Kwon, Y.M., Lee, J.H. and Lee, M.W. (2002). Inhibitory effects of hydrolysable tannins on tyrosinase activities in B16 mouse melanoma cells. *Natural Product* Sciences, 8(4), 183–185.

Choi, J. and Jee, J.G. (2015). Repositioning of Thiourea-Containing Drugs as Tyrosinase Inhibitors. *International Journal of Molecular Sciences*, 16(12), 28534–28548. https://doi.org/10.3390/ijms161226114

Chung, S.Y., Seo, Y.K., Park, J.M., Seo, M.J., Park, J.K., Kim, J.W. and Park, C.S. (2009). Fermented rice bran downregulates MITF expression and leads to inhibition of alpha-MSH-induced melanogenesis in B16F1 melanoma. *Bioscience Biotechnology and Biochemistry,* 73(8), 1704-1710. https://doi.org/10.1271/bbb.80766

Funayama, M., Arakawa, H., Yamamoto, R., Nishino, T., Shin, T. and Murao, S. (1995). Effects of a- and b -arbutin on activity of tyrosinases from mushroom and mouse melanoma. *Bioscience Biotechnology and Biochemistry,* 59(1), 143–144. https://doi.org/10.1271/bbb.59.143

Galindo, J.D., Martínez-Liarte, J.H., Lopez-Ballester, J.A., Penafiel, R., Solano, F. and Lozano, J.A. (1987). The effect of polyamines on tyrosinase activity. *Biochemistry International,* 15(6), 1151–1158.

Goenka, S. and Simon, S.R. (2020). Organogold drug Auranofin exhibits anti-melanogenic activity in B16F10 and MNT-1 melanoma cells. *Archives of Dermatological Research,* 312(3), 213-221. https://doi.org/10.1007/s00403-019-01974-1

Grand View Research. (2022). Nutraceuticals Market Size, Share and Trends Analysis Report by Product (Dietary Supplements, Functional Food, Functional Beverages), By Region (North America, Europe, APAC, CSA, MEA), and Segment Forecasts, 2021 – 2030. Retrieved on January 27, 2022 from https://www.grandviewresearch.com/industry-analysis/nutraceuticals-market

Halaban, R., Patton, R.S., Cheng, E., Svedine, S., Trombetta, E.S., Wahl, M.L., Ariyan, S. and Hebert, D.N. (2002). Abnormal acidification of melanoma cells induces tyrosinase retention in the early secretory pathway. *Journal of Biological Chemistry,* 277(17), 14821-14828. https://doi.org/10.1074/jbc.M111497200

Hearing, V.J., Ekel, T.M., Montague, P.M. and Nicholson, J.M. (1980). Mammalian tyrosinase. Stoichiometry and measurement of reaction products. *Biochimica et Biophysica Acta (BBA)-Enzymology,* 611(2), 251–268. https://doi.org/10.1016/0005-2744(80)90061-3

Hirschmann, R. (2022). Production of rice in Malaysia from 2013 to 2020. Retrieved on January 27 2021 from https://www.statista.com/statistics/794700/rice-production-volume-malaysia/

Huang, H.C., Chiu, S.H. and Chang, T.M. (2011). Inhibitory effect of [6]-gingerol on melanogenesis in B16F10 melanoma cells and possible mechanism of action. *Bioscience Biotechnology and Biochemistry,* 75(6), 1067-1072. https://doi.org/10.1271/bbb.100851

Jacobsohn, G.M. and Jacobsohn, M.K. (1992). Incorporation and binding of estrogens into melanin: comparison of mushroom and mammalian tyrosinases. *Biochimica et Biophysica Acta,* 1116(2), 173–182. https://doi.org/10.1016/0304-4165(92)90114-A

Kalra, E.K. (2003). Nutraceutical–definition and introduction. *AAPS PharmSci,* 5, 25. https://doi.org/10.1208/ps050325

Katina, K., Liukkonen, K.H., Kaukovirta-Norja, A., Adlercreutz, H., Heinonen, S.M., Lampi, A.M., Pihlava, J.M. and Poutanen, K. (2007). Fermentation -induced changes in the nutritional value of native or germinated rye. *Journal of Cereal Science*, 46(3), 348–355. https://doi.org/10.1016/j.jcs.2007.07.006

Kim, A.J., Choi, J.N., Kim, J., Yeo, S.H., Choi, J.H. and Lee, C.H. (2012). Metabolomics-based optimal koji fermentation for tyrosinase inhibition supplemented with *Astragalus radix*. *Bioscience Biotechnology and Biochemistry,* 76(5), 863-869. https://doi.org/10.1271/bbb.110171

Kobayashi, T., Urabe, K., Winder, A.J., Jime´nez-Cervantes, C., Imokawa, G., Brewington, T., Solano, F., García-Borrón, J.C. and Hearing, V.J. (1994). Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. *The EMBO Journal,* 13(24), 5818–5825. https://doi.org/10.1002/j.1460-2075.1994.tb06925.x

Kussmann, M., Affolter, M., Nagy, K., Holst, B. and Fay, L.B. (2007). Mass spectrometry in nutrition: Understanding dietary health effects at the molecular level. *Mass Spectrometry Reviews,* 26(6), 727–750. https://doi.org/10.1002/mas.20147

Lajis, A.F., Hamid, M. and Ariff, A.B. (2012). Depigmenting effect of kojic acid esters in hyperpigmented B16F1 melanoma cells. *Biomed Research International*, 2012, 952452. https://doi.org/10.1155/2012/952452

Lim, J.Y., Ishiguro, K. and Kubo, I. (1999). Tyrosinase inhibitory p-coumaric acid from ginseng leaves. *Phytotherapy Research,* 13(5), 371–375. https://doi.org/10.1002/(SICI)1099-1573(199908/09)13:5<371::AID-PTR453>3.0.CO;2-L

Maruyama, H., Kawakami, F., Lwin, T.T., Imai, M. and Shamsa, F. (2018). Biochemical characterization of ferulic acid and caffeic acid which effectively inhibit

melanin synthesis via different mechanisms in B16 melanoma cells. *Biological and Pharmaceutical Bulletin*, 41(5), 806–810. https://doi.org/10.1248/bpb.b17-00892

Masum, M.N., Yamauchi, K. and Mitsunaga, T. (2019). Tyrosinase inhibitors from natural and synthetic sources skin-lightening agents. *Reviews in Agricultural Science*, 7, 41-58. https://doi.org/10.7831/ras.7.41

McFeeters, R.F. (1980). A manual method for reducing sugar determinations with 2,2′-bicinchoninate reagent. *Analytical Biochemistry*, 103(2), 302–306. https://doi.org/10.1016/0003-2697(80)90614-4

Ministry of Agriculture (MOA). (2017). Agrifood statistics 2017. Retrieved on January 27, 2022 from MOA Website: https://www.moa.gov.my/documents/20182/29034/Perangkaan+Agromakanan+2017-ilovepdfcompressed.pdf/14f6dd00-f2a1-414b-98a3-251ef670d7fc

Pomerantz, S.H. (1963). Separation, purification and properties of two tyrosinases from hamster melanoma. *Journal of Biological Chemistry*, 238(7), 2351–2357. https://doi.org/10.1016/S0021-9258(19)67976-7

Research CMFE. (2021). Malaysia Nutraceuticals Market by Type of Nutraceutical (Functional Food, Functional Beverages, Dietary Supplements, Personal Care, Pharmaceuticals and Others), By Application (General Wellness, Beauty and Antiaging, Weight Management, Digestive Health, Sports and Energy, Others) Analysis and Forecasts to 2017-2027. Retrieved on January 27, 2022 from https://www.researchcmfe.com/report/21/Malaysia%20Nutraceuticals-market

Ryan, E.P., Heuberger, A.L., Weir, T.L., Barnett, B., Broeckling, C.D. and Prenni, J.E. (2011). Rice bran fermented with *Saccharomyces boulardii* generates novel metabolite profiles with bioactivity. *Journal of Agricultural and Food Chemistry*, 59(5), 1862–1870. https://doi.org/10.1021/jf1038103

Saeedi, M., Eslamifar, M. and Khezri, K. (2019). Kojic acid applications in cosmetic and pharmaceutical preparations. *Biomedicine and Pharmacotherapy,* 110, 582-593. https://doi.org/10.1016/j.biopha.2018.12.006

Shimada, Y., Tai, H., Tanaka, A., Ikezawa-Suzuki, I., Takagi, K., Yoshida, Y. and Yoshie, H. (2009). Effects of ascorbic acid on gingival melanin pigmentation *in vitro* and *in vivo*. *Journal of Periodontology*, 80(2), 317–323. https://doi.org/10.1902/jop.2009.080409

Song, T.-Y., Chen, C.-H., Yang, N.-C., Fu, C.-S., Chang, Y.-T. and Chen, C.-L. (2009). The correlation of *in vitro* mushroom tyrosinase activity with cellular tyrosinase activity and melanin formation in melanoma cells A2058. *Journal of Food and Drug Analysis*, 17(3), 4. https://doi.org/10.38212/2224-6614.2607

Su, T.R., Lin, J.J., Tsai, C.C., Huang, T.K., Yang, Z.Y., Wu, M.O., Zheng, Y.Q., Su, C.C. and Wu, Y.J. (2013). Inhibition of melanogenesis by gallic acid: possible involvement of the PI3K/Akt, MEK/ERK and Wnt/β-catenin signaling pathways in B16F10 cells. *International Journal of Molecular Sciences*, 14(10), 20443–20458. https://doi.org/10.3390/ijms141020443

Sugimoto, K., Nishimura, T., Nomura, K., Sugimoto, K. and Kuriki, T. (2004). Inhibitory effects of alpha-arbutin on melanin synthesis in cultured human melanoma cells and a three-dimensional human skin model. *Biological and Pharmaceutical Bulletin*, 27 (4), 510–514. https://doi.org/10.1248/bpb.27.510

van Meerloo, J., Kaspers, G.J. and Cloos, J. (2011). Cell sensitivity assays: the MTT assay. In Cree, I. (Ed.) Methods in Molecular Biology. Vol. 731, p. 237-245. USA: Humana Press. https://doi.org/10.1007/978-1-61779-080-5_20

https://doi.org/10.26656/fr.2017.6(S4).011