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Enhancement of Melanogenic Inhibitory Effects of the Leaf Skin Extracts of Aloe barbadensis Miller by the Fermentation Process

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Abstract: This work first showed that the skin-lightening effects of the leaf skin extracts of Aloe vera were significantly increased by the fermentation of Lactobacillus plantarum BN41. The fermented extract (BF) showed much higher antioxidant activities of DPPH scavenging effects and the reduction in intracellular ROS production than the water extract (BW), and even higher than Trolox as a positive control. High efficacy of the BF results was shown from the synergistic effects of higher elution of aloesin (2.96 ± 0.09 mg/g vs. 2.03 ± 0.02 mg/g in BF and BW, respectively) and bioactive substances from the fermentation processes. The inhibition of tyrosinase activities and melanin synthesis at 0.3% (w/v) optimal dosage of BF was much better than those of arbutin and aloesin, which are commercial skin-lightening ingredients. It was also first proved that BF effectively down-regulated all microphthalmia-associated transcription factors (MITF), tyrosinase-related protein-1 (TYRP-1) and TYRP-2, and tyrosinase (TYR) gene expression (p < 0.05), proposing melanogenesis inhibitory mechanism in the MITF/TYRP-1/TYRP-2/TYR pathway. However, aloesin and arbutin selectively suppressed the expression of TRY-1, TRY-2 or TYR. It was clearly demonstrated that the fermentation process reduces inherent cytotoxicity of aloe, showing much less cell cytotoxicity than BW. Conclusively, 0.3% (w/v) of the BF can be utilized as a competitive and sustainable natural skin-lightening ingredient.

Keywords: leaf skin of aloe; lactic acid fermentation; hyperpigmentation

1. Introduction
Continuous exposure to ultraviolet (UV) light has been considered to cause sunburns, inflammation, damage to skin matrix structures that cause many skin disorders, and eventually skin photo-aging associated with the darkness of skin colors [1,2]. Melanin, a nitrogenous polymer, helps to protect against the invasion of UV light into human skin by absorbing certain amounts of solar radiation. Melanin is synthesized in melanocytes located in the basal layer of the skin, and sunlight mainly stimulates its synthesis. However, overproduction of melanin due to long exposure to sunlight and skin aging causes hyperpigmentation, such as freckles, and results in various types of skin cancers [3–5]. Tyrosinase (TYR) is one of the key enzymes in synthesizing melanin polymers by converting L-L-tyrosine into L-dihydroxyphenylalanine (L-DOPA) and sequentially oxidizing to dopaquinone, and the microphthalmia-associated transcription factor (MITF) is also closely associated with synthesizing melanin by upregulating the genes related to producing melanin, such as tyrosinase-related protein-1 (TYRP-1) and TYRP-2 [6,7]. Therefore, it is absolutely necessary to control the activities of tyrosinase and the biosynthesis of proteins associated with producing melanin for properly managing hyperpigmentation of the skin.
The search for new tyrosinase inhibitors and/or the regulation of proteins related to melanin synthesis are of great interest in the cosmetic industry. To date, arbutin and kojic acid have most commonly been used as tyrosinase inhibitors, and L-ascorbic acid and glutathione are also considered to inhibit melanin synthesis. However, a well-known tyrosinase inhibitor, kojic acid, has been shown to be highly sensitizing to the skin, with cases of contact dermatitis reported, and some of the tyrosinase inhibitors are known to be unstable due to being easily broken down within cosmetic formulations [8,9]. Therefore, it is necessary to develop natural resources for skin whitening purposes, which should be more stable in the formulation and should not cause sensitivity or irritation in the skin.

Among many natural skin-whitening candidates, the extracts from aloe, a member of the Lily family, have been reported to inhibit 1-3,4-dihydroxyphenylalanine (l-DOPA) oxidation by mushroom tyrosinase [10]. An active component of the aloe extracts to show skin-whitening efficacy is considered to be aloesin, a natural hydroxymethyl chromone compound that is located mainly in the outer layers of skin leaves, not in the gels that have most often been used for cosmetic purposes for many centuries [11]. From several reports, aloesin has been shown to be a competitive tyrosinase inhibitor since aloesin has been proven to improve skin wound healing processes and showed synergistic effects on skin whitening with the cotreatment of arbutin [11,12]. However, obtaining a single component of aloesin from crude aloe extracts is quite expensive because the amounts of aloesin are not abundant enough to reduce the purification costs, and it is even more difficult to process very hard skin layers of aloe leaf, which is where aloesin exists in aloe [13,14].

Therefore, it is necessary to develop a cost-effective methodology for processing the relatively hard leaf skin of Aloe vera while maintaining its skin lightening effects. To accomplish this purpose, the fermentation associated with lactic acid bacteria could primarily be considered because the lactic acid fermentation can operate under mild conditions such as low extraction temperature and shear stress, etc., and which would not destroy the biologically active components existed in the leaf skin of aloe [15,16]. Moreover, the fermentation can increase the extraction yields of bioactive substances from hard cell walls of the plants, which enhance its efficacy by synergistic effects with various bioactive molecules existing in the fermentation extracts [17,18]. For this purpose, Lactobacillus family members have most been utilized since the plant extracts from the fermentation with Lactobacillus species show many promising results that treat various skin problems, possibly due to the metabolites obtained during fermentation processes [19–21]. Therefore, the leaf skin of A. vera fermented by L. plantarum BN41 newly isolated from Kimchi, the fermented vegetable was used to develop a natural cosmetic ingredient that effectively inhibits skin melanogenesis, and also to expand the use of sustainable resources.

2. Materials and Methods

2.1. Chemicals and Regents

Murine melanoma (B16F10) was obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Thermo Fisher Sci., Waltham, MA, USA). RNeasy mini kit was purchased from Qiagen (Hilden, Germany) and cDNA synthesis kit was purchased from Fermentas (Burlington, ON, Canada). ReddyMixTM PCR master mix solution was purchased from Abgene (Surrey, UK). Oligonucleotide primers for TYR, TYRP-1, TYRP-2, MITF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were purchased from Biogen (Seoul, Korea). All other solvents and reagents applied in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of the Samples

For the control of the extracts, the outer layers of Aloe vera leaf were obtained by peeling off the leaf of the whole areal parts to remove the gels at the center of the Aloe barbadensis Miller (Aloe vera as common name) (Kimjeonmoon Aloe Farm Co., Jeju, Korea, 2021). Then, 100 g of freeze-dried outer layers of aloe leaf were extracted by a most conventional water
extraction process with distilled water (1:10, w/v) at 100 °C for 24 h using a vertical reflux condenser (TL-6, Misung Scientific, Yangjoo, Korea). Then, the extracts were centrifuged (Combi 514R; Hanil Science Medical, Daejeon, Korea) at 12,000 x g for 30 min at room temperature, and the supernatants were concentrated in a rotary vacuum evaporator (EV-101, Eyela, Tokyo, Japan). Then, the concentrates were freeze-dried with a lyophilizer (PVTFA, 10AT, IlShin, Suwon, Korea) to obtain the powder (BW).

For lactic acid fermentation, 5% (v/v) of 1 × 10^6 CFU/mL of L. plantarum BN41 (deposited as 11919P in Korean Federation of Culture Collections, Seoul, Korea) isolated from the fermented vegetable, Kimchi, was inoculated into 1 L of distilled water containing 10 (%, w/v) dried powder of Aloe vera leaf skins and MRS culture medium (Sigma, St. Louis, MO, USA) and grown for three days at 37 °C in a shaking incubator (KB-105, Korea Biotech., Seoul, Korea) at a shaking speed of 100 rpm. After cultivation, the culture was further extracted by an ultrasound generator equipped with a circulating water bath (AUG-R3-900, Asia Ultrasonic, Bucheon, Gyeonggi-Do, Korea) to maintain a constant temperature of 20 °C at a fixed frequency of 20 kHz with 1500 W of input power for 4 h, and the culture broth was centrifuged at 12,000 x g for 30 min at room temperature. Then, the supernatant was concentrated with a rotary vacuum evaporator and freeze-dried to prepare a powder designated the Lactobacillus/Aloe vera leaf ferment extract filtrate (BF). All extracts were stored at −20 °C before use.

2.3. Determination of Aloesin Contents in the Extracts

The concentrations of aloesin in the two samples, BW and BF, were measured using high-performance liquid chromatography (HPLC, E2695, Waters, Milford, MA, USA) as follows [14]: 10 µL of each sample was injected into an HPLC equipped with a diode-array detection (DAD) detector (2998, Waters, Milford, MA, USA) and a C18 column (4.6 × 150 mm, 5 µm, Luna, Phenomenex, Torance, CA, USA). The mobile phase was composed of water (A): methanol (B), and elution was operated under a 0–70% (B) linear gradient for 50 min at a 0.8 mL/min flow rate. The wavelength of the detector was 293 nm, and ambient column temperature was used. The concentrations of aloesin in the samples were calculated using a linear regression curve from the peak areas of 10 to 100 ppm of standard aloesin (Sigma, St. Louis, MO, USA) and expressed as mg aloesin/g of sample.

2.4. Measurement of the Cell Cytotoxicity of the Extracts

The cytotoxicity of two samples, BW and BF, was determined by using the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as follows [22]: First, 1.0 × 10^4 murine melanoma (B16F10, American Type Culture Collection (ATCC), Manassas, VA, USA) were inoculated into a 96-well plate and grown with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Bend, OR, USA), 1% gentamycin sulfate (Sigma, St. Louis, MO, USA), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Sigma, St. Louis, MO, USA) in a 5% CO2 incubator at 37 °C for 24 h. Then, 200 µL of various concentrations of BW and BF were added to the cells and cultured for 24 h. Then, a 5 µg/mL MTT solution was added to each well, and the supernatant was removed 4 h later. Therefore, 10 µL of acid-isopropanol (0.04 N HCl in isopropanol) was added to the wells, and the absorbance of the solution was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at a wavelength of 565 nm. Cytotoxicity was estimated as the percentage of the ratio of cell growth of the treated samples to the cell growth of untreated cells as a control using the following Equation (1):

\[
\text{Cytotoxicity (\%)} = \left(1 - \frac{\text{Sample group}}{\text{Untreated group}}\right) \times 100
\]
2.5. Measurement of the Antioxidant Activity of the Extracts

For the analysis of the antioxidant activities of the two samples, 2,2 diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activities were measured as follows [23]: First, 80 µL of various concentrations of the two samples, BW and BF, 5 µg/mL of aloesin or 10 µg/mL of Trolox as a positive control were added to 200 µL of 0.1 mM DPPH dissolved in ethanol in a 96-well plate and incubated at 25 °C for 20 min in the dark. Then, the absorbance of the mixture was measured at 525 nm using a microplate reader. The DPPH free radical scavenging activity (%) of the samples was estimated using Equation (2):

\[
\text{DPPH radical scavenging activity (\%)} = \left( \frac{\text{Control O.D.} - \text{Sample O.D.}}{\text{Control O.D.}} \right) \times 100 \quad (2)
\]

To measure intracellular Reactive Oxygen Species (ROS) production in H₂O₂-treated murine melanoma cells (B16F10), a method using 2',7'-dichlorofluorescein diacetate (DCF-DA) was employed as follows [24]: First, 1.0 × 10⁵ cells/well B16F10 cells were inoculated into a 96 well plate and cultured for 24 h with DMEM supplemented with 10% FBS at 37 °C. Thereafter, various concentrations of the two samples, BW and BF, 5 µg/mL of aloesin or 10 µg/mL of Trolox as a positive control were added and further cultivated for 1 h. Then, 1 mM H₂O₂ was added to each well and incubated for an additional 30 min at 37 °C. After finishing the cultivation, the well was washed with phosphate-buffered saline (PBS) (Gibco, Paisley, Scotland, UK) three times, and 10 µM DCF-DA in Hank’s balanced salt solution was added and reacted in a dark room for 30 min. After that, the cells were washed with PBS, and the absorbance was measured by a fluorescence microplate reader (Biotek FLx800, Agilent Tech., Santa Clara, CA, USA) at an excitation wavelength of 490 nm and an emission wavelength of 525 nm.

2.6. Assessment of the Inhibition of Tyrosinase Activities

To evaluate tyrosinase inhibition activity of two samples, 0.3 mg/mL of L-tyrosine solution dissolved in 950 µL of 0.1 M potassium phosphate buffer and 50 µL of the various concentrations of the samples, and 5 µg/mL of aloesin and 2% (w/v) of arbutin (Sigma, St. Louis, MO, USA) as a positive control were mixed together. Then, 200 units/mL mushroom tyrosinase (Sigma, St. Louis, MO, USA) was added to the mixture, and the mixture was allowed to stand for 20 min in a 37 °C water bath. Then, the reactions were terminated by subjecting the mixture to 0 °C for 5 min, and then the absorbance of the solution was measured at 475 nm by a microplate reader. The inhibition ratio was calculated by the following Equation (3) [25].

\[
\text{Inhibition ratio (\%)} = (1-\frac{B}{A}) \times 100 \quad (3)
\]

where A is the absorbance of the control with the enzyme and B is the absorbance of the samples with the enzyme.
2.7. Assessment of the Inhibition of Cellular Tyrosinase Activities and Melanin Synthesis

The inhibition of cellular tyrosinase activities in murine melanoma cells (B16F10) was measured as follows [26]: First, $1.0 \times 10^5$ cell/mL B16F10 cells were inoculated into a 96-well plate and cultured with DMEM supplemented with 10% FBS and 1% gentamycin-sulfate for 24 h in an incubator at 37 °C with 5% CO₂. After that, various concentrations of the samples, BW and BF, and 5 μg/mL of alosin and 2% (w/v) of arbutin were added and further cultivated for 2 h. Then, 200 nM α-melanocyte-stimulating hormone (α-MSH, Sigma, St. Louis, MO, USA) was added and further cultivated for 48 h. Cells were washed with PBS two times and lysed with 100 μL of lysis buffer containing 90 μL of PBS, 5 μL of PMSF and 5 μL of 1% Triton X-100. Then, the cell lysates were centrifuged at 10,000× g for 30 min. The supernatant was mixed with 2 mg/mL of L-DOPA and incubated at 37 °C for 20 min. Optical density of each sample was estimated at 475 nm by a microplate reader (Tecan, Männedorf, Switzerland), comparing with the controls with and without α-MSH.

The inhibition of melanin synthesis by the samples was estimated as follows [27]: $1.0 \times 10^5$ cell/mL B16F10 cells were inoculated into a 96-well plate and cultured with DMEM supplemented with 10% FBS for 24 h at 37 °C. Then, the culture medium was removed, and the cells were treated with various concentrations of the samples and 200 nM α-MSH, and 5 μg/mL of alosin and 2% (w/v) of arbutin and further cultured for 2 days in an incubator under the same conditions mentioned above. Then, removing the culture medium and washing with PBS two times, the cell numbers of the collected cells by trypsinization were measured by a hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany). Then, the cells were lysed with 200 μL of 1 N NaOH containing 10% dimethyl sulfoxide (DMSO) at 60 °C for 10 min, and the supernatant was collected by centrifugation at 1200× g for 15 min. Then, the absorbance of the solution was measured at 490 nm by a microplate reader, and the amounts of melanin were calculated by comparison with a linear regression line using standard melanin (Sigma, St. Louis, MO, USA). The amounts of melanin produced were measured as the weight of melanin per cell.

2.8. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The expression levels of four primary genes related to melanin synthesis from murine melanoma cells, tyrosinase (TYR), tyrosinase-related protein-1 (TYRP-1) and tyrosinase-related protein-1 (TYRP-2), and microphthalmia-associated transcription factor (MITF) were analyzed on ethidium bromide (EB, Sigma)-stained 2% agarose gels (BioRad, Berkeley, CA, USA) through electrophoresis with 1–5 V/cm applied voltage. The amount of mRNA corresponding to each gene was quantified by Image Processing Analysis in Java (ImageJ).
1.46r, National Institute of Mental Health, Bethesda, MD, USA) by normalizing to the housekeeping gene GAPDH; the values were expressed as the relative ratio of the number of genes expressed by the treatment to that of the control (not treated).

2.9. Statistical Analysis

All experiments were repeated three times, and the results are presented as the means ± SD (standard deviations). Statistical significance was analyzed using one-way analysis of variance (ANOVA) (SAS 9.1, SAS, Cary, NC, USA). Differences at * \( p < 0.05 \) were considered significant (* \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \)).

3. Results and Discussion

3.1. Amounts of Aloesin in the Extracts

Figure 1 compares the HPLC chromatograms of the aloesin standard and two extracts to estimate the contents of aloesin in the samples. The amounts of aloesin in the leaf skin of aloe conventional water extracts (BW) and its fermented extract (BF) were measured as 2.03 ± 0.02 mg/g and 2.96 ± 0.09 mg/g, respectively. The concentration of aloesin in the samples was similar to the concentration from other reports in the ranges of 0.59 to 3.84 mg/g in the leaf skin of *A. vera* [14,29]. Besides the increase in aloesin from the skin leaf, the fermentation process also increased the elution of other substances that was possibly attributed to its skin-lightening effects in comparing the intensities of various peaks in the latter parts of the chromatogram in Figure 1b,c from the water extract and the fermented extract, respectively.

![Figure 1](image_url)

**Figure 1.** HPLC chromatogram of two extracts and aloesin as a standard: (a) aloesin (20 ppm); (b) BW, the hot water extract of aloe skin leaf at 100 °C for 24 h; (c) BF, the fermented extract of aloe skin leaf fermented with *L. plantarum* BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h.
It is obvious that the fermentation can boost the elution of active substances from the leaf skin of *A. vera*, for this case, aloesin and other biologically active substances, where the results definitely demonstrate the advantages of the fermentation process. Moreover, this advantage will effectively work on processing the relatively hard leaf skin of aloe since it would not be easily extracted with conventional extraction processes. Other studies also support this hypothesis by significantly improving its effects as well as the concentrations of active substances through various fermentation processes [29–31]. Therefore, this result can expect that the BF shows higher skin-whitening efficacy than those from the conventional water extraction process (BW) because the BF contains higher amounts of aloesin, which is known to have a skin-lightening effect [32], as well as synergistic effects of aloesin and other bioactive components through the fermentation process.

3.2. Cell Cytotoxicity and Antioxidant Effects of the Fermented Extract

As shown in Figure 2, both BW and BF showed less or no cytotoxicity against murine melanoma at concentrations below 0.5% (*w/v*) of treatment concentration. However, at 1% of the highest addition of BW and BF, relatively high cytotoxicity was observed, especially up to 48% of cell death in adding 1% (*w/v*) of the BW and 15% in the same concentration of the BF, indicating that a high dosage of the extracts could affect the growth of skin fibroblasts since some components, such as various types of anthroquinones in the outer layers of *A. vera*, have been presumed to be toxic/harmful to skin, even though many of them work positively in the treatment of skin disorders [33,34]. However, the BF had lower cytotoxicity than the BW for all the ranges of the treatment, and the BF showed fairly high protection of cell growth at 1% of the highest dosage compared to the BW, such as 85% vs. 52% of cell growth for the BF and BW, respectively. These results clearly indicate that the fermentation of the leaf skin of aloe can reduce its toxicity, and similar results have also been found in other studies by showing less cell cytotoxicity and higher biological efficacy by various types of fermentation processes [35,36]. This result may reduce public concerns about the reputed cytotoxicity of the leaf skin of aloe and help to expand the use of a wide range of active constituents from many plants, which is a great advantage of the fermentation process. An upper limit of the dosages for treating both extracts might be found even though fermentation reduced its cytotoxicity well, implying that an optimal dosage of the BF would be considered to have its proper skin-lightening effects.

![Figure 2](image-url)

**Figure 2.** Cell cytotoxicity of the aloe skin leaf extracts from two different processes against murine melanoma cells. BW: Water extract of aloe skin leaf at 100 °C for 24 h. BF: Fermented extract of aloe skin leaf fermented with *L. plantarum* BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h. The mean values ± SD for separate triplicate experiments are shown, and error bars represent the SD. Statistically significant value was calculated by comparing with each group (*p* < 0.05, **p** < 0.01 and ***p*** < 0.001).
To compare the antioxidant efficacies of BW and BF, DPPH free radical scavenging effects were measured, as shown in Figure 3, since the antioxidant activities of many plant resources have been reported to be closely related to skin-lightening effects [37,38]. However, interestingly, aloesin itself showed much less of an antioxidant effect than two other extracts even though the treatment dosage (5 μg/mL of aloesin) was almost the same amount that existed in the BW and BF and is known to have skin-lightening effects, possibly indicating that the skin-lightening effect of aloesin itself would not be caused by its antioxidant effects but would be associated with other mechanisms. The conventional water extract of the leaf skin of A. vera (BW) noticeably did not show good antioxidant effects for all the ranges of the treatment concentrations, showing up to 80 ± 4.01% of 75.20 ± 2.92% DPPH free radical scavenging activity at 10 μg/mL Trolox of a usual dosage of many commercial cosmetics as a positive control in adding 0.5% (w/v) BW. In contrast, the BF showed significantly higher antioxidant activity than the BW for all doses in a concentration-dependent manner, and its activity was even higher than the activity of adding 10 μg/mL Trolox, such as 107 ± 8.25% and 119 ± 5.80% % free radical scavenging activity in treating 0.3% (w/v) and 0.5% (w/v), respectively. IC<sub>50</sub> of the BW and BF was also estimated as 0.39% (w/v) and 0.06% (w/v), respectively. However, at the highest dose of 1.0% of both extracts, the antioxidant activity of the BW and BF sharply decreased. The antioxidant activity of the BF was superior to that of the BW and even better than the antioxidant activity of Trolox, strongly indicating that the higher antioxidant effects of the BF were possibly due to the synergistic effect of various bioactive components such as polyphenols and flavonoids that were most commonly obtained from the lactic acid fermentation processes [16,20,21]. However, at above 0.5% addition of the BF, its antioxidant effects decreased to 85% of the control as well as the BW, and the patterns were similar to the cytotoxicity of the samples in Figure 2, also indicating that the highest dose of both samples should be considered a dose of 0.5% (w/v), and it is necessary to consider an optimal dose of the fermented extract to expect its high efficacy.

![Figure 3. DPPH free radical scavenging activities of two aloe skin leaf extracts compared with 5 μg/mL aloesin and 10μg/mL Trolox. BW: Water extract of aloe skin leaf at 100 °C for 24 h. BF: Fermented extract of aloe skin leaf fermented with L. plantarum BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h. The mean values ± SD for separate triplicate experiments are shown, and error bars represent the SD. The significance of the difference was set to * p < 0.05, ** p < 0.01 and *** p < 0.001 compared with two samples, BW and BF.](image-url)

The effects of the samples on decreasing ROS production induced by H$_2$O$_2$ oxidative stress were also observed in Figure 4 since the accumulation of intracellular ROS within the cells causes many diseases and its production can be reduced by the antioxidants [39]. Similar to the DPPH scavenging effects shown in Figure 3, at 0.3% (w/v) dosage, the BF showed the highest reduction in ROS production, compared to the control treated with
H$_2$O$_2$. The decrease in ROS production by the BF was similar to that by 10 µg/mL Trolox (positive control), down to 109 ± 2.83% and 110 ± 8.02%, respectively, from 285 ± 7.24% of the control (+). However, no significant decrease in ROS production was observed at higher than 0.3% dosage, and whose results were also similar to those of the DPPH free radical scavenging effects shown in Figure 3. This strongly indicates that 0.3% BF would be an optimal dosage to maintain its highest efficacy. Compared to ROS scavenging effects of the BF, the BW did not show any apparent antioxidant activity, especially at below 0.1% (w/v) dosage where its activities were even less than aloesin. These results could postulate that anti-melanogenic effects of the BF were mostly caused by its high antioxidant effects.

![Figure 4](image-url)

**Figure 4.** Comparison of intracellular ROS production in H$_2$O$_2$-treated B16F10 cells in adding various concentrations of two different extracts of aloe skin leaf, and 5 µg/mL aloesin and 10µg/mL Trolox. BW: Water extract of aloe skin leaf at 100 °C for 24 h. BF: Fermented extract of aloe skin leaf fermented with L. plantarum BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h. The mean values ± SD for separate triplicate experiments are shown, and error bars indicate the SD. The significance of the difference was set to *p < 0.05, **p < 0.01 and ***p < 0.001 compared with the control (+).

### 3.3. Inhibition of Tyrosinase Activity and Melanin Synthesis by the Fermented Extract

First, for measuring the skin-lightening effects of both BW and BF, the inhibition of mushroom tyrosinase enzyme activities was observed, as shown in Figure 5, because tyrosinase plays an important role in synthesizing melanin in skin cells [6]. In general, both the BW and BF showed relatively good inhibition of tyrosinase activity, showing better than a 56% inhibitory effect of adding 2% arbutin, a well-known skin-lightening component from Vaccinium vitis-idaea var. minus LODD at doses higher than 0.3% (w/v) [40]. The mushroom tyrosinase inhibitory activity of the BF was also shown to be better than the inhibitory activity of the BW for all the ranges of treatment concentrations in a concentration-dependent manner. IC$_{50}$ of inhibiting tyrosinase activities of the BW and BF were also compared as 0.36% (w/v) vs. 0.10% (w/v) of the BW and BF, respectively. Contrarily, the efficacy of aloesin itself, an active substance in A. vera, in treating similar content existed in 1% (w/v) of the BF did not show a good effect on inhibiting tyrosinase activities, with an inhibitory effect of 49 ± 1.21% compared to 60 ± 6.81% and 76 ± 4.06% of the BW and BF, respectively, at 0.5% of treatment concentrations. The overall inhibitory effects of both the BW and BF also showed concentration dependency, indicating that the skin-lightening effects of the A. vera leaf skin would be caused not only by aloesin itself but also by a more complex mechanism of various substances within aloe. Even though the inhibitory effects of BW were better than the inhibitory effects of aloesin, the effects of BF were obviously significantly higher than the inhibitory effects of BW for all doses of the samples. Therefore, we can postulate that the higher skin-lightening effect of the BF would be caused by the synergistic effects of aloesin and other various components obtained from
fermentation. At 1% (w/v) of the highest treatment, the inhibition of mushroom tyrosinase activity of both BW and BF decreased to 54 ± 7.33% and 72 ± 9.18%, respectively, and the patterns were similar to the antioxidant effects of the samples, as shown in Figure 3. This result is also evidence that the skin-lightening activity of the extracts from A. vera are closely associated with its strong antioxidant activity. In Figure 6a, the inhibition of cellular tyrosinase activities in melanoma cells was also measured to confirm the skin-lighting effects of the BF. At above 0.3% (w/v) dosage, the BF showed the most significant reduction in tyrosinase activities, compared to the tyrosinase activities of the cells treated with α-MSH (Control (+)). The inhibitory effect of the BF was much higher than those of the BW, aloesin and even 2% arbutin. However, at above 0.3% dosage, the inhibitory effects of the BF did not increase even though its efficacy showed concentration dependency at lower dosages, whose results also imply that the optimal dosage of treating the BF would be in the range of 0.3% (w/v) treatment. It was also interesting that the inhibitory effect on cellular tyrosinase activities of the BF was higher than that of arbutin while the inhibition of mushroom tyrosinase activities of arbutin were similar to that of the BF at 0.3% dosage in Figure 5. This result indicates that the BF has an ability of inhibiting melanogenesis by effectively reducing tyrosinase activities. Contrarily, the inhibition of cellular tyrosinase activities by the BW was not better than that by aloesin, and whose pattern was also similar to the pattern of inhibiting mushroom tyrosinase activities. In this work, aloesin did not show impressive inhibitory effects on both mushroom and cellular tyrosinase activities even though aloesin is known to have skin-lightening efficacy [11]. The reason was that the amount of aloesin in the extracts (5 μg/mL) was not high enough to show its inhibitory effects, and these results also support the hypothesis that high efficacy of the BF was due to synergistic effects of aloesin itself and higher amounts of bioactive substances derived from the fermentation process as shown in Figure 1.

**Figure 5.** Comparison of inhibiting mushroom tyrosinase activities in adding various concentrations of two extracts of aloe skin leaf, and 5 μg/mL aloesin and 2% arbutin. BW: Water extract of aloe skin leaf at 100 °C for 24 h. BF: Fermented extract of aloe skin leaf fermented with L. plantarum BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h. The mean values ± SD for separate triplicate experiments are shown, and error bars indicate the SD. The significance of the difference was set to * p < 0.05, ** p < 0.01 and *** p < 0.001 compared with two samples, BW and BF.
Besides inhibitory effects on both mushroom and cellular tyrosinase activities, the inhibition of melanin synthesis in melanoma cells was also compared in Figure 6b. The addition of 2% arbutin as a positive control showed significance down to 48 ± 6.11% inhibition of melanin concentration compared to the amounts of the control treated with α-MSH (Control (+)). However, aloesin showed a relatively low efficacy of ca. 56 ± 3.37% inhibition of melanin synthesis. This inhibition was lower than the inhibition of arbutin and lower than the 49 ± 1.21% inhibitory effect on tyrosinase activity of aloesin, as shown in Figure 4, and this result seemed to be different from the pattern of inhibiting tyrosinase activity. We could presume that the skin-lightening effect of aloesin would be more attributed to the inhibition of tyrosinase activity than to melanin synthesis. Similar results were also reported in other studies, showing that aloesin competitively inhibits the conversion of tyrosine to DOPA and DOPA to dopachrome [41]. Compared to the efficacy of the BW and aloesin, the BF showed fairly high effects on inhibiting melanin synthesis for all doses in a concentration-dependent manner. IC50 of inhibiting melanin synthesis of the extracts were measured as 0.41% (w/v) and 0.07% (w/v) of the BW and BF, respectively. The effect of BW was much better than the effect of aloesin at the above 0.3% treatment, and even better than that of 2% arbutin. These results clearly proved that the skin-lightening effect of the BF was superior to the efficacy of arbutin, a well-known commercial skin-lightening ingredient. However, interestingly, at the highest dosage of 1.0%, the inhibition of melanin synthesis by the BF was not much increased, even increasing the melanin concentration up to 51 ± 3.08% from 40 ± 8.96% at 0.5% (w/v) addition. Similar results were consistently observed in Figures 2–4, indicating that an optimal dosage of the BF would be between 0.3 and 0.5% addition. This result would also indicate that the inhibitory effect of the BF seemed to be most likely associated with cell cytotoxicity as the sharp decrease in cell growth at 1.0% addition of the BF, as shown in Figure 2. In general, the skin-lightening effect of the fermented extracts of the leaf skin of A. vera must obviously be better than the skin-whitening effect of the BW and aloesin, and even arbutin, and these results also strongly support the hypothesis that the high efficacy of the BF was very closely correlated with the synergistic effects of various bioactive substances with high antioxidant activities through the lactic acid fermentation process.
3.4. Downregulation of TYR, TYPR-1, TYRP-2 and MITF Gene Expression by the Fermented Extract

All the results clearly demonstrate that the BF reduced the hyperpigmentation process by more effectively inhibiting both tyrosinase activity and melanin synthesis, compared to the BW and aloesin. For a detailed understanding of the skin-lightening mechanism of the extracts, it is necessary to observe the regulation of the expression levels of mRNA related to the overall pathways of melanin synthesis, such as MITF, TYRP-1, TYRP-2 and TYR genes as shown in Figures 7–10. It has been proven that MITF is a key transcription regulator of genes for melanin synthesis and is also involved in regulating the differentiation, pigmentation, and proliferation of melanocytes [42,43]. The increase in MITF gene expression subsequently up-regulates the expression of genes for melanogenic enzymes such as TYRP-1 and TYRP-2, which results in increasing the production of tyrosinase [43]. This sequential process promotes melanin synthesis. First, the treatment concentrations for this experiment were used in the ranges of 0.1% to 0.5% (w/w) of the extracts because an optimal dosage could be considered between 0.3 and 0.5% treatment of both the BW and BF, as shown in the above results. PCR products that reflect mRNA expression of TYRP-1 (Figure 7a) and TYRP-2 (Figure 8a) in the melanoma cells treated with (control (+)) and without α-MSH (control (−)) are illustrated along with two positive controls, arbutin and aloesin, to compare with the cells treated with three different concentrations of BW and BF. To more easily compare the suppression levels of these genes over the suppression allowed by the pictures of electrophoresis bands in Figures 7a and 8a, the densitometric intensities of individual bands were normalized to the GAPDH band, which is a housekeeping gene, using a program that quantitatively compares the band sizes and is illustrated in Figures 7b and 8b. The 2% (w/v) addition of arbutin clearly down-regulated the expression of both TYRP-1 and TYRP-2 mRNA, as shown in Figures 7a and 8a, and the reduction was quantitatively observed down to 0.54 ± 0.03 and 0.49 ± 0.13 of relative intensity for TYRP-1 and TYRP-2 genes, respectively, compared to 1.0 of the densitometric intensity ratio of the negative control (control (−)) (Figures 7b and 8b). This result was supported by a clinical report that the topical treatment of 2.51% arbutin was found to be effective in improving melasma [44]. However, aloesin more effectively decreased the expression of TYRP-1 genes rather than of TYRP-2 down to 0.53 ± 0.01 and 0.68 ± 0.05, respectively, and these results were also supported by other studies showing that aloesin plays a more important role in inhibiting tyrosinase activity [41]. Overall, downregulation of both TYRP-1 and TYRP-2 gene expression in BW was not very effective compared to 2% arbutin and aloesin itself, even though the inhibitory effects of BW on tyrosinase activity and melanin synthesis were similar to the inhibitory effects of arbutin and even better than the inhibitory effects of aloesin at concentrations higher than 0.3%, implying that the skin-whitening mechanism of BW would be different from the skin-lightening mechanism of arbutin and aloesin. The aloesin in the BW would not play a main role in inhibiting melanogenesis as aloesin itself does since the BW does not contain enough to show its own efficacy. In contrast, the BF obviously showed a fairly high reduction in the expression levels of both TYRP-1 and TYRP-2 genes at all the ranges of treatments and was most effective in the 0.3% (w/w) treatment. Moreover, its efficacy was better than those of both arbutin and aloesin and was even higher than those of flavonoids and licorice from other plants [45]. However, at 0.5% of the highest dosage, little improvement in the downregulation of both genes was observed, which would also indicate that a proper dosage of BF should be considered as approximately 0.3% (w/w). These results seemed to be very much consistent with the results of antioxidant activities and inhibition of both tyrosinase and melanin synthesis, as shown in Figures 3–6.
Figure 7. Downregulation of mRNA expression of TYRP-1 from α-MSH treated B16F10 cells (a) and relative densitometric intensities corresponding to each PCR band normalized with a house keeping gene, GAPDH (b) in treating various concentrations of two extracts of aloe skin leaf, and 5 μg/mL aloesin and 2% arbutin as positive controls. BW: Water extract of aloe skin leaf at 100 °C for 24 h. BF: Fermented extract of aloe skin leaf fermented with L. plantarum BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h. The mean values ± SD for separate triplicate experiments are shown, and error bars indicate the SD. The significance of the difference was set to ** p < 0.01 and *** p < 0.001 compared with the control (+).

Figure 8. Downregulation of mRNA expression of TYRP-2 from α-MSH treated B16F10 cells (a) and relative densitometric intensities corresponding to each PCR band normalized with a house keeping gene, GAPDH (b) in treating various concentrations of two extracts of aloe skin leaf, and 5 μg/mL aloesin and 2% arbutin as positive controls. BW: Water extract of aloe skin leaf at 100 °C for 24 h. BF: Fermented extract of aloe skin leaf fermented with L. plantarum BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h. The mean values ± SD for separate triplicate experiments are shown, and error bars indicate the SD. The significance of the difference was set to * p < 0.05, ** p < 0.01 and *** p < 0.001 compared with the control (+).
Fermentation 2022, 8, x FOR PEER REVIEW...

Downregulation of mRNA expression of TYR from α-MSH treated B16F10 cells (a) and relative densitometric intensities corresponding to each PCR band normalized with a housekeeping gene, GAPDH (b) in treating various concentrations of two extracts of aloe skin leaf, and 5 μg/mL aloesin and 2% arbutin as positive controls. BW: Water extract of aloe skin leaf at 100 °C for 24 h. BF: Fermented extract of aloe skin leaf fermented with L. plantarum BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h. The mean values ± SD for separate triplicate experiments are shown, and error bars indicate the SD. The significance of the difference was set to * p < 0.05, ** p < 0.01 and *** p < 0.001 compared with the control (+).

Figure 10. Downregulation of mRNA expression of MITF from α-MSH-treated B16F10 cells (a) and relative densitometric intensities corresponding to each PCR band normalized with a housekeeping gene, GAPDH (b) in treating various concentrations of two extracts of aloe skin leaf, and 5 μg/mL aloesin and 2% arbutin as positive controls. BW: Water extract of aloe skin leaf at 100 °C for 24 h. BF: Fermented extract of aloe skin leaf fermented with L. plantarum BN41 at 37 °C for 3 days and further extracted by an ultrasound generator at 20 kHz for 4 h. The mean values ± SD for separate triplicate experiments are shown, and error bars indicate the SD. The significance of the difference was set to * p < 0.05, ** p < 0.01 and *** p < 0.001 compared with the control (+).

Besides the effects of the extracts on TYRP-1 and TYRP-2 gene expression, downregulation of the expression of MITF and TYR genes by the extracts, arbutin and aloesin, was compared in Figures 9 and 10 since MITF is known to be a key transcription factor in melanoma transcriptional regulation pathway and tyrosinase is a most affecting enzyme in melanin synthesis [42,46,47]. Similar to the results of Figures 7 and 8, the BF significantly downregulated the expression of TYR and MITF genes, compared to the BW for all the ranges of the treatment concentrations, and even higher than aloesin itself. However, specifically the reduction in TYR gene expression by arbutin was similar to that by 0.3% addition.
of the BF as 0.37 ± 0.02 vs. 0.36 ± 0.09 for the BF and arbutin, respectively, and even higher than the downregulation of TYR gene expression by 0.5% addition of the BF as 0.41 ± 0.03 vs. 0.36 ± 0.09 for the BF and arbutin, respectively as shown in Figure 9b. In contrast, it was very interesting that the downregulation of MITF by the BF was certainly higher than 0.43 ± 0.07 by arbutin, a well-known natural skin-lightening ingredient at all the dosages of the BF and much higher than the BW and aloesin in Figure 10b. It was also first found that arbutin suppress MITF gene expression less, compared to those of TYRP-1 and -2 and TRY genes, and which would imply that arbutin inhibited more effectively tyrosinase activities than transcriptional melanogenesis pathway. These results clearly indicate that the BF has a high ability for inhibiting the hyperpigmentation process by downregulating overall pathways of synthesizing melanin, especially effectively inhibiting MITF, the first step of melanin synthesis and sequentially regulating the rest of the pathways, compared to the arbutin. These results proposed melanogenesis inhibitory mechanism of the BF is involved in MITF/TYRP-1/TYRP-2/TYR while arbutin is TYRP-1/TYRP-2/TYR.

Compared to the results of the BW and arbutin, aloesin did not show significant downregulation of the expression of MITF genes in Figure 10 while relatively high reduction in the expression of TYR gene was observed. Again, these results strongly supports consistent results shown in the above that the BF more effectively inhibited hyperpigmentation process than the BW and aloesin because the BF significantly downregulated the expression of MITF gene that is known to be the most critical step in the melanogenesis process [47,48] while aloesin suppressed only TYRP-1 gene expression. It supports the results that aloesin competitively inhibit only tyrosinase activities by downregulating TYRP-1 gene expression as shown in Figures 3 and 7, and the other work also showed similar results [41]. This result hypothesized that melanogenesis inhibitory mechanism of aloesin is involved in TYRP-1/TYR pathway and arbutin is TYRP-1/TYRP-2/TYR pathway, which is quite different from those of the BF. That was why aloesin and arbutin did not show better performance of inhibiting skin-lightening effects than the BF in this work even though aloesin and arbutin have been used as skin-lightening ingredients so far. Generally speaking, downregulation of the expression of TYR and MITF genes by the BW was much lower than the BF and also arbutin, which implies that the skin-lightening efficacy of the leaf skin of aloe alone was not high enough to be applied to anti-melanogenic cosmetics.

These results strongly imply that BF would be a promising skin-lightening ingredient originating from plant resources since it showed a wide spectrum of preventing the hyperpigmentation pathway compared to the results of rucinol, a phenol derivative, that inhibits tyrosinase and TYRP-1, and cinnamic acid from ginseng inhibits tyrosinase. [41,46,49]. In addition, it was also encouraging that the fermentation process indisputably showed an enhancement of the skin-lightening efficacy of the leaf skin of A. vera byproducts of aloe processing, which can expand the application of plant resources in the cosmetic industry.

4. Conclusions

Traditionally, most of cosmetic applications of Aloe vera have used the gels inside, and the rest of them, almost half of whole leaves were discarded as byproducts due to the difficulties of processing them. In this study, the possibility of utilizing this valuable byproduct as a skin-lightening cosmetic was first introduced by fermenting with lactic acid bacteria, Lactobacillus plantarum BN41 isolated from fermented vegetable. It was very obvious that the overall skin-lightening efficacy of Aloe vera leaf skin is significantly increased through the fermentation process, compared to those of the conventional water extract of aloe leaf skin (BF) and even aloesin, a known skin-lightening substance that exists in A. vera. This increase is proven to be caused by the synergistic effects of higher elution of aloesin and other bioactive substances derived from fermentation processes. The synergistic effects of the fermented extract (BF) were also supported by the results showing that the skin-lightening efficacy of aloesin itself at the same dosage in the BF was lower than the skin efficacy in the BF. Moreover, aloesin showed less of an effect on inhibiting melanin synthesis even though the BF showed both high effects. Our results
strongly support the hypothesis that the skin-lightening effect of aloesin would be caused by selectively inhibiting tyrosinase activity within skin cells such as the inhibitory mechanism of the TYRP-1/TYR pathway, whereas the effects of BF were attributed mostly to its strong antioxidant activity. The results of this work also clearly showed that the BF was able to effectively downregulate all of MITF, TYRP-1, TYRP-2 and TYR genes in melanoma cells, compared to the expression levels of the BW, aloesin and arbutin, a well-known skin-lightening ingredient from Vaccinium vitis-idaea var. minus LODD. These results imply that the melanogenesis inhibitory mechanism of the BF was involved in the MITF/TYRP-1/TYRP-2/TYR pathway, and which was also first proven in this work. In contrast to the results of the BF, the overall skin-lightening effect of 2% arbutin was not much comparable to that of the BF either, suggesting the melanogenic inhibitory mechanism of TYRP-1/TYRP-2/TYR pathway. Our results certainly show that optimal treatment concentrations of the ferment extracts should be considered to expect high efficacy, for this case, within the range of 0.3–0.5% (w/v). The work also demonstrated the possibility that the fermentation process can reduce the inherent/potential cytotoxicity of the leaf skin of A. vera as well as improve skin-lightening effects, and which could appease concerns about the potential harmful effects of the leaf skin of aloe even though its negative effects have not yet been completely proven. In conclusion, the BF can preliminarily be considered a strong candidate as a cheap and effective natural ingredient with less side effects for replacement of many synthetic and chemical skin-lightening components by recycling the byproducts of aloe processing, and whose effects should be validated by clinical trials on human skin with topical ointments containing 0.3% of the BF.

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