Research article

Submerged fermentation with Lactobacillus brevis significantly improved the physiological activities of Citrus aurantium flower extract

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1. Introduction

Plant-based bioactives have nutraceutical potential and multifunctional properties and have attracted attention, particularly phenolic acids, flavonoids, and terpenoids. These natural compounds have low toxicity and few side effects; exert anticarcinogenic effects; and have health-promoting characteristics, including antiaging, antioxidative, and antimicrobial properties (Dosokey and Setzer, 2018; Degirmenci and Erkurt, 2020).

The skin and hair pigment melanin protects the skin from radiation. Melanogenesis occurs when melanin accumulates in the skin's epidermal layer and is related to skin aging. The production of melanin is facilitated by the enzyme tyrosinase (Mapunya et al., 2012). Overactivity of tyrosinase and its related proteins results in hyperpigmentation. Signs of aging, wrinkles in particular, are visible manifestations of proteolytic degradation of the extracellular matrix (ECM). Scholars have discovered significant associations of ECM degradation with wrinkly formation and dermal collagenase, elastase, interstitial collagenase [matrix metalloproteinase (MMP)-1], and 72-kDa gelatinase (MMP-2) activity (Maity et al., 2011).

The Rutaceae species Citrus aurantium L., or bitter orange is resistant to several viral diseases, is tolerant of cold, and can be used to improve the fruit quality of grafted plants (Sarrou et al., 2015). It also has numerous therapeutic properties. The entire immature fruit of this species is dried and used in traditional Chinese medicine (Stohs, 2017). Its peel, flowers, and leaves contain many bioactives—for example, essential oils, vitamins, flavonoids, and phenolics—and are thus used in the treatment of digestive and cardiovascular system disorders (Moraes et al., 2009; Haj Ammar et al., 2012). Phenolics and flavonoids are crucial because they are beneficial to health. Furthermore, the fruit, flowers,

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ABSTRACT

The physiological activity of the 50% ethanolic extract of Citrus aurantium flower before and after fermentation was investigated in this study. C. aurantium flowers grown in Taiwan were extracted using 100% methanol or 50% ethanol and then fermented by one of six microbes: four species of lactic acid bacteria (Bifidobacterium bifidum, Bifidobacterium lactis, Lactobacillus acidophilus, and Lactobacillus brevis) anaerobically cultivated in MRS broth and two species of mold (Aspergillus oryzae and Aspergillus niger) aerobically cultivated in potato dextrose broth. The 50% ethanolic extract of C. aurantium flowers exhibited higher tyrosinase inhibition (IC50: 200.8 ± 11.6 mg/L) and antioxidative activity than did a 100% methanolic extract (IC50: 274.1 ± 15.7 mg/L). The 50% ethanolic extract fermented by L. brevis (L. brevis–fermented extract) exhibited the highest yield (86.2% ± 1.2%) and physiological activity. The L. brevis–fermented extract exhibited over 5.2-, 13.5-, 12.5-, 3.17-, and 4.29-fold higher antityrosinase activity, antioxidative activity, antibacterial activity, total flavonoid content, and antiwrinkle activity than did the unfermented extract. The L. brevis–fermented extract can be considered safe because it exerted no toxic effect on CCD-966SK or HEMn cells at concentrations of 400 and 200 mg/L, respectively. The fermented extract (40 mg/L) inhibited melanin formation, reducing it to 50.8% ± 2.3%. Furthermore, the L. brevis–fermented extract exhibited excellent antiaging and antiwrinkle activity, as determined from MMP-1, MMP-2, elastase, and collagenase activity. The improvement in physiological characteristics, especially the considerable formation of neohesperidin, is mainly attributable to biosynthesis or biotransformation by L. brevis during fermentation. In conclusion, the 50% ethanolic extract of C. aurantium flowers fermented with L. brevis can be used in cosmetics applications aiming for skin-whitening or antiwrinkle properties.

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phytoconstituents, and essential oils of *C. aurantium* were discovered in a bioactivity study to exert antioxidative, anti-inflammatory, and antimicrobial effects (Suntar et al., 2018).

The physiological activity of biochemical products can be enhanced through their fermentation, which modifies their naturally occurring molecular constituents. Moreover, the cytotoxicity of herbal extracts can be reduced through fermentation by microorganisms (Wang et al., 2017). However, no study has investigated the physiological activity or characteristics of a fermented extract of *C. aurantium* flowers. We previously discovered that the bioactive and antioxidative activity of some herbal extracts were significantly improved when those extracts were fermented by probiotic bacteria, indicating that different bioactive compounds can be produced through various metabolic pathways during fermentation (Wang et al., 2016, 2017; Wu et al., 2018).

*C. aurantium* flowers were extracted with 100% methanol or 50% ethanol in this study. The extracts were fermented by various lactic acid bacteria or fungi. We evaluated whether the unfermented and fermented extracts had toxic effects on CCD-966SK cells and determined the extract's tyrosinase inhibitory, antioxidative, antimicrobial, and anti-wrinkle activity. The composition of unfermented and fermented extracts, including the levels of flavonoids and phenolics, was analyzed to elucidate the mechanisms underlying the extracts' high physiological activity. Furthermore, to examine the skin-whitening potential and safety of the fermented extract, we investigated its toxic and tyrosinase inhibitory effects on human epidermal melanoma (HEMn) cells as well as its effect on the melamin content of these cells.

2. Material and methods

2.1. Plant material

*C. aurantium* cultivated in the mountainous area (at 500-m altitude) of Miaoli County, Taiwan, was obtained. *C. aurantium* flowers were collected in May 2020. The petals were carefully removed. Subsequently, the flowers were washed with distilled water, left to dry naturally, and then ground into a powder (particle size: 800 μm; modified from the method used by Razeghi et al., 2020). The dried powder was stored at 4 °C.

2.2. Microbial strains, cells, and tyrosinase

The *C. aurantium* flower extract was fermented by one of four lactic acid bacteria—namely, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, *Lactobacillus acidophilus*, or *Lactobacillus brevis* (American Type Culture Collection (ATCC) codes 29521, 25741, 4356, and 8287, respectively)—or two fungi, namely, *Aspergillus oryzae* (Bioresource Collection and Research Center (BCRC) 32288) or *A. niger* (ATCC 42418). The activity of the fermented and unfermented extracts against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus brasiliensis* (ATCC codes 8739, 6538, 9027, 10231, and 16404, respectively) was evaluated on the basis of the USP 51-antimicrobial effectiveness test. These microbial strains were purchased from the BCRC (Hsinchu, Taiwan). HEMn cells from neonatal foreskin were obtained from Cascade Biologics (Cascade cat. C-102-5C, Portland, OR, USA) and cultured in human melanocyte growth supplement (HMGS)-supplemented Medium 254 (Cascade Biologies). For a comparison group, we obtained cells from the normal human skin fibroblast line CCD-966SK (ATCC CRL-1881) from the BCRC. We sourced mushroom tyrosinase and all other chemicals employed from Sigma-Aldrich (St. Louis, MO, USA) at analytical grade (purity >99%).

2.3. Microbial cultivation and extraction, and fermentation of *C. aurantium* flowers

The four lactic acid bacteria were anaerobically cultivated for 48 h in MRS broth at 37 °C. The mold species (*A. oryzae*, *A. niger*, and *A. brasiliensis*) were aerobically cultivated for 7 days in potato dextrose broth (PDB) at 25 °C while shaking at 300 rpm. *C. albicans* was aerobically cultivated for 3 days in a yeast mold broth at 24 °C while shaking at 300 rpm. Aerobic cultivation of the other bacteria (*P. aeruginosa*, *S. aureus*, and *E. coli*) was performed overnight in tryptic soy broth (TSB) at 37 °C with shaking at 350 rpm.

In the extraction, 1 g of dried *C. aurantium* flower powder was added to 90 mL of solution containing 10 mL of M HCl with 80 mL of either 50% ethanol or 100% methanol, and the solution was then gently stirred for 30 min. The pH of the resulting mixtures ranged from 1.8 to 2.2 (modified from a method used by Karimi et al., 2012). Subsequently, the methanolic and ethanolic extracts were filtered, and a rotary vacuum evaporator operated for 2 h at 45 °C (methanolic extract) or 60 °C (ethanolic extract) was employed to concentrate them. Until used, the concentrated extracts were maintained at 4 °C.

In the fermentation, 200 mL of phosphate-buffered solution (PBS, pH 6) containing 2 g of *C. aurantium* flower extract was mixed with 2 × 10⁹ cfu/mL lactic acid bacteria. Anaerobic incubation of the mixture (for 1–5 days) was then performed at 37 °C. In addition, 2 g of *C. aurantium* flower extract, dissolved in 200 mL of PBS (pH 5), was mixed with one of two fungi (*A. oryzae* and *A. niger*) at 2.0 × 10⁸ spores/mL. Aerobic incubation of the mixtures (for 2–8 days) was then performed at 25 °C with shaking at 300 rpm (Wang et al., 2019). Curves of the lactic acid bacteria and fungi growth during fermentation are presented in Figures S1 and S2, respectively. Fermentation was conducted for a fixed period. After 10 min of centrifugation (8000 × g), the supernatant of the fermented solution was collected, filtered using a 0.45-μm filter, and concentrated with a rotary vacuum evaporator operated at 60 °C until a paste had formed. The fermented *C. aurantium* extracts were kept at 4 °C until further experiments were performed. We then compared the physiological characteristics of the unfermented extracts with those of the fermented extracts.

2.4. Antityrosinase activity and cellular melamin content

The method described by Zheng et al. (2012) was employed to analyze the antityrosinase activity of the fermented and unfermented *C. aurantium* flower extract. Dimethyl sulfoxide (DMSO, 1 g/L)-based solutions containing the extract at differing concentrations were prepared. Subsequently, 30 μL of the DMSO-based solutions was mixed with 970 μL of 0.05 mM PBS, after which the mixture was added to a solution containing 1 mL each of 350 unit/mL mushroom tyrosinase solution and 100 mg/L L-tyrosine. Under darkness, 3 mL of this reaction solution was mixed until homogenous. After the solution had been incubated for 20 min, the solution's absorbance at 490 nm was determined through ultraviolet–visible (UV–vis) spectrophotometry (UV-2600i, Shimadzu, Japan). The control comprised distilled water instead of an extract, and the positive controls employed were α-arbutin and kojic acid. The IC₅₀ value was defined as the concentration of the extract when half the original tyrosinase activity was inhibited. The following equation, expressing the percentage of tyrosinase inhibition, was used to calculate an extract's antityrosinase activity (Wu et al., 2018):

\[
\text{Tyrosinase inhibition}(\%) = \frac{[A - B] - [C - D]}{A - B} \times 100
\]

where A, B, C, and D are the absorbance at 490 nm of no extract (control), no extract and enzyme (blank), extract and enzyme (experimental group), and extract but no enzyme (blank of C), respectively.

The method described by Wang et al. (2019) was employed to analyze the antityrosinase activity of the fermented extracts in HEMn cells. First, HEMn cells (2 × 10⁶ cells/well) were incubated in Medium 254 supplemented with HMGS at 37 °C for 24 h and grown in 24-well plates. Various concentrations of the fermented extract were applied to the cells for 24 h, after which the cells were collected and washed in PBS before then being lysed with a solution containing 1% Triton X-100, 0.1 M PBS,
and protease inhibitors. Lysates were obtained by sonicating the aforementioned solution for 5 min at 4 °C and then centrifuging it for 15 min at 8000 × g. In 96-well plates, 2.5 mM L-DOPA in 0.1 M PBS was reacted with the lysates for 60 min. A microplate in an Epoch ELISA reader (BioTek Instruments, USA) was used to read the absorbance at 475 nm.

To examine the melanin content of the HEMn cells, we used a slight modification of the approach reported by Liao et al. (2012). First, the 2 × 10⁶ HEMn cells placed in each well of six-well culture plates and incubated for a period of 24 h. Subsequently, various concentrations of fermented extract were applied to the cells, which were subsequently incubated for another 72 h. Thereafter, trypsinization was performed to collect the cells, which were then centrifuged for 5 min at 2000 × g. After removal of the supernatant, we recovered the cell pellet and suspended it in 100 μL of 1 N NaOH containing 10% DMSO. After heating the suspension at 70 °C for 1.5 h, we used an ELISA plate reader to determine the suspension’s absorbance at 405 nm. Finally, through comparison with a synthetic melanin standard, we determined the cells’ melanin content.

2.5. Analysis of scavenging activity of 2,2-diphenyl-1-picrylhydrazyl and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

We employed a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay to determine the antioxidative capacity of the fermented and unfermented C. aurantium flower extracts. The method described by Wu et al. (2018) was used to estimate levels of DPPH. First, 100 μM DPPH solution in 97% ethanol was prepared. Various concentrations (0.4 mL) of C. aurantium flower extract were added to 0.8 mL of methanol and 1 mL of the DPPH solution. After 1 h of dark incubation, a UV–vis spectrophotometer was used to determine the absorbance at 517 nm of the extract solution and of a blank not containing the extract; the positive control was BHT. The scavenging activities of the DPPH radical of extracts were calculated as follows (Wu et al., 2018). The IC50 value of the DPPH scavenging activity of the extract was determined at 50% scavenging activity.

\[
\text{DPPH scavenging activity (\%) = } \left( \frac{A_0 - A}{A_0} \right) \times 100
\]

(2)

where \(A_0\) and \(A\) are the absorbance at 517 nm without and with the extract.

Scholars widely use the 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical to examine the antioxidative activity of natural compounds. ABTS can interact with both hydrophilic and lipophilic systems. We employed a slightly modified version of the method presented by Merchán Arenas et al. (2011) to determine the ABTS scavenging activity of the unfermented and fermented extracts. First, 2.45 mM potassium persulfate was reacted with 7 mM ABTS solution (1:1) for 18 h in the dark to produce the ABTS radical cation. Before the extracts’ antioxidative activity was measured, the prepared ABTS solution was diluted to an optical density of 0.7 at 734 nm. Various concentrations of the fermented and unfermented extracts of C. aurantium flowers (50 μL) were mixed with 950 μL of the diluted ABTS solution for 30 min. The positive control was again BHT. The ABTS scavenging activity of the extracts was calculated as follows (Merchán Arenas et al., 2011). The IC50 value of the ABTS scavenging activity by the extract was expressed at 50% scavenging activity.

\[
\text{ABTS scavenging activity(\%) = } \left( \frac{A_0 - A}{A_0} \right) \times 100
\]

(3)

where \(A_0\) and \(A\) are the absorbance at 734 nm without and with the extract.

2.6. Total reducing power

The method of Wang et al. (2016) was used to quantify the total reducing power. We mixed the fermented and unfermented extracts of C. aurantium flowers (1 mL) at various concentrations with 2.5 mL of 0.2 M PBS and 2.5 mL of 1% potassium ferricyanide, and the ensuing reaction proceeded for 20 min in a water bath at the temperature 50 °C. After the solution had been left to cool, 2.5 mL of 10% trichloroacetic acid was employed to terminate the reaction (in approximately 5 min). After 15 min of centrifugation of the reaction solution at 3500 × g, we collected the supernatant and mixed 2 mL with 0.4 mL of 0.1% ferric chloride and 2 mL of deionized water. The consequent reaction proceeded for 10 min, and subsequently, the UV–vis spectrophotometer was used to measure the solution’s absorbance at 700 nm. The positive controls were ascorbic acid and α-tocopherol. The IC50 value was defined as the concentration of the extracts at which the absorbance was 0.5.

2.7. Minimum inhibitory and fungicidal concentrations

The minimum inhibitory concentrations (MICs) of the fermented and unfermented extracts of C. aurantium flowers for P. aeruginosa, S. aureus, and E. coli were determined using a serial tube dilution method in accordance with Rahman et al. (2013). In brief, 2 mL of extract (the concentration of which was varied), 2 mL of TSB, and 1 mL of inoculum (5 × 10⁶ cfu/mL) were combined in a test tube and incubated for 18 h at 37 °C. The lowest extract concentration for which the growth of E. coli, S. aureus, or P. aeruginosa was visibly prevented was considered the MIC.

We used the conventional plate count method to evaluate the extracts’ antifungal activity. In brief, 1 mL of extract (the concentration of which was varied), 1 mL of inoculum (2 × 10⁷ cfu/mL), and 100 mL of specific broth (YM for C. albicans and PDB for A. brasiliensis) were combined in a conical flask and incubated for 5 days at 25 °C. The lowest concentration killing 99.9% of the inoculum (C. albicans or A. brasiliensis) was considered the minimum fungicidal concentration (MFC).

2.8. Total phenolic and flavonoid content

The approach of Kujala et al. (2000) was employed with minor modifications to quantify the total phenolic content (TPC) in terms of gallic acid equivalents (GAE) of the unfermented and fermented C. aurantium flower extracts. First, 0.5 mL of an extract was oxidized for 10 min with 1 mL of Folin–Ciocalteu phenol reagent (diluted 1:10); the mixture was then neutralized using 1 mL of 7.5% Na2CO3 solution for 3 h. This was followed by 15 min of centrifugation at 3000 × g. The UV–vis spectrophotometer was employed to measure the supernatant’s absorbance at 760 nm. The TPC of each extract (mg-GAE/g-dried extract) was determined by consulting a calibration curve of gallic acid standard solutions. For absorbance \(y\) and gallic acid concentration \(x\), the calibration curve could be expressed as \(y = 0.0525x + 0.0712\) (\(R^2 = 0.9912\)).

Total flavonoid content (TFC) was quantified through aluminum chloride colorimetry and is expressed in rutin equivalents (RE; Pourmorad et al., 2006). In brief, 1 mL of each extract was combined with 0.3 mL of 5% NaNO2, 0.3 mL of 10% AlCl3 solution, and 4 mL of distilled water, after which the mixture stood for 10 min. Subsequently, 2 mL of 1 M NaOH followed by 2.4 mL of distilled water was added. The 10-mL solution was gently stirred and stood for 30 min. The UV–vis spectrophotometer was employed to measure the mixture’s absorbance at 510 nm. The TFC of the extract was calculated (mg-RE/g-dried extract) from a calibration curve of rutin standard solutions. For absorbance \(y\) versus rutin concentration \(x\), the calibration curve could be expressed as \(y = 0.0721x + 0.0362\) (\(R^2 = 0.9825\)).

2.9. Quantification of phenolics and flavonoids

A high-performance liquid chromatography (HPLC) system (Hitachi, Tokyo, Japan) was used to determine the main phenolics and flavonoids in the C. aurantium flower extracts and the concentrations of these compounds. For HPLC, a flow rate of 0.6 mL/min, injection volume of 25 μL, and 5-μm and 4.6 × 150-mm² Inertsil ODS-3 column (GL Science, Tokyo, Japan) were used. Separation was performed using gradient.
elution (solution A: deionized water; solution B: acetonitrile) with the following settings: 85% A followed by 40% A at 25 min, 15% A at 50 min, 40% A at 75 min, and 85% A at 100 min. Trifluoroacetic acid was employed to adjust the pH to 2.5. For detecting various compounds, the wavelength was varied from 230 to 400 nm. The wavelength used for the detection of flavonoids, alcohols, and phenolic compounds was 280 nm, whereas that used for the detection of flavonoids was 350 nm (Crozier et al., 1997). A compound’s retention time was compared with those of standard samples under the same conditions to determine the compound’s identity.

2.10. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldiazotetrazolium bromide assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Yang et al., 2017) was used to assess the extracts’ toxicity to CCD-966SK and HEMn cells. We cultivated the CCD-966SK and HEMn cells for 24 h in minimum essential medium containing 10% fetal bovine serum and HMGS-supplemented Medium 254, respectively, in a 96-well plate at 3 x 10⁵ cells/well; subsequently, either fresh medium (control) or extract (the concentration of which was varied) was applied for 24, 48, or 72 h. Next, 0.02% MTT was added, and the cells were incubated in a 5% CO₂ environment for 4 h at 37 °C. The solution was then removed, and 100 μL of DMSO was added. An Epoch ELISA reader was employed to measure the liquid’s absorbance at 570 nm. The following formula was used to calculate the viabilities of the cells (Yang et al., 2017):

\[
\text{Cell viability} = \left( \frac{A_{\text{abs},570\ \text{nm}}}{A_{\text{abs},570\ \text{nm}}} - 1 \right) \times 100
\]

(4)

2.11. Antwrinkle activity

Wrinkles are related to the inhibition of MMP-1, MMP-2, elastase, and collagenase activity (Azmi et al., 2014). Thus, we analyzed these four aging-related enzymes. The method proposed by Vandooren et al. (2011) was employed to measure collagenase activity by using a modified fluorogenic dye-quenched (DQ)-gelatin assay. First, extract (the concentration of which was varied) was added to a 96-well plate with 1 U/mL collagenase (100 μL/well) and 15 μg/mL DQ-gelatin added to each well. The mixture then stood for 20 min. The absorbance at 485 and 530 nm (excitation and emission, respectively) was measured to determine the gelatin proteolysis rate. A colorimetric assay with N-succinyl-Ala-Ala- Ala-p-nitroanilide (Suc-Ala) as the substrate was employed to assay elastase activity (Karim et al., 2014); specifically, 50 μL of extract (the concentration of which was varied) was reacted with 125 μL of 7 mM Suc-Ala (pH 8.0; prepared in 0.1 M Tris using gelatin zymography (Toth et al., 2012). First, the cells underwent a 2 h of incubation, the reacted mixture was spectrophotometrically analyzed at 420 nm. The MMP-2 activity in CCD-966SK cells was assayed via the orogenic dye-quenched (DQ)-gelatin assay. First, extract (the concentration of which was varied) was added to a 96-well plate, after which 25 μL of 0.3 U/mL neutrophil elastase was employed to determine the absorbance at 405 nm.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Yang et al., 2017) was used to assess the extracts’ toxicity to CCD-966SK and HEMn cells. We cultivated the CCD-966SK and HEMn cells for 24 h in minimum essential medium containing 10% fetal bovine serum and HMGS-supplemented Medium 254, respectively, in a 96-well plate at 3 x 10⁵ cells/well; subsequently, either fresh medium (control) or extract (the concentration of which was varied) was applied for 24, 48, or 72 h. Next, 0.02% MTT was added, and the cells were incubated in a 5% CO₂ environment for 4 h at 37 °C. The solution was then removed, and 100 μL of DMSO was added. An Epoch ELISA reader was employed to measure the liquid’s absorbance at 570 nm. The following formula was used to calculate the viabilities of the cells (Yang et al., 2017):

\[
\text{Cell viability} = \left( \frac{A_{\text{abs},570\ \text{nm}}}{A_{\text{abs},570\ \text{nm}}} - 1 \right) \times 100
\]

(4)

The antityrosinase activity of the extracts increased with their concentration (Figure 1). Such a dose-dependent increase was also discovered for the methanolic extract of pomegranate peel (Fawole et al., 2019). The highest and lowest yields recovered from fermentation were achieved by the 50% ethanolic extract of <i>C. aurantium</i> flowers fermented by <i>L. brevis</i> (86.2% ± 1.2%) and that fermented by <i>A. oryzae</i> (70.2% ± 5.3%), respectively. The yield of the <i>L. brevis</i>-fermented extract relative to that of dried <i>C. aurantium</i> flower powder was 7.4%. The optimal fermentation time for lactic acid bacteria and fungi was discovered to be 3 and 5 d, respectively.

3. Results and discussion

3.1. Solvent selection and yield

Fermentation by <i>Aspergillus</i> spp. and that by probiotic bacteria improve the physiological activity and reduce the cytotoxicity of herbal extracts (Yang et al., 2017, 2019). Thus, we investigated the physiological activity and cytotoxicity of the unfermented <i>C. aurantium</i> flower extract and extracts fermented by one of four lactic acid bacteria (<i>L. brevis</i>, <i>L. acidophilus</i>, <i>B. lactis</i>, and <i>B. bifidum</i>) or two fungi (<i>A. oryzae</i> and <i>A. niger</i>). Additionally, we determined the optimal fermentation time for various microbes to recovering the highest yield of fermented extract. During the extraction process, 50% ethanol (8.6% ± 0.4%) resulted in a higher extract yield than did 100% methanol (5.1% ± 0.7% (Table 1)). The highest and lowest yields recovered from fermentation were achieved by the 50% ethanolic extract of <i>C. aurantium</i> flowers fermented by <i>L. brevis</i> (86.2% ± 1.2%) and that fermented by <i>A. oryzae</i> (70.2% ± 5.3%), respectively. The yield of the <i>L. brevis</i>-fermented extract relative to that of dried <i>C. aurantium</i> flower powder was 7.4%. The optimal fermentation time for lactic acid bacteria and fungi was discovered to be 3 and 5 d, respectively.

3.2. Antityrosinase and antioxidative activity

The antityrosinase activity of the extracts increased with their concentration (Figure 1). Such a dose-dependent increase was also discovered for the methanolic extract of pomegranate peel (Fawole et al., 2012). Nonlinear regression analysis indicated that the antityrosinase activity of the 50% ethanolic extract of <i>C. aurantium</i> flowers (IC₅₀: 200.8 ± 11.6 mg/L) was higher than that of the 100% methanolic extract (IC₅₀: 274.1 ± 15.7 mg/L). One study discovered that the methanolic extract of Nepalese <i>C. aurantium</i> exhibited inhibited tyrosinase activity more effectively (IC₅₀: 48.2 mg/L) than did its Taiwanese counterpart (Adhikari et al., 2008). This finding indicates that the cultivation environment affects the physiological activity even when the same extraction technique is used. Because the antityrosinase activity of the 50% ethanolic extract was superior to that of the methanolic extract, we used the 50% ethanolic extract in the subsequent fermentation experiments. Table 2 lists the antityrosinase activity of the 50% ethanolic extracts of <i>C. aurantium</i> flowers fermented by various lactic acid bacteria or fungi. Among all the tested microbes, <i>L. brevis</i> resulted in the strongest tyrosinase inhibition, with the IC₅₀ being 38.2 ± 0.9 mg/L. The fermented extracts had much higher antityrosinase activity (5.2 times higher) than the unfermented extracts (IC₅₀: 200.8 ± 11.6 mg/L) had. However, after fermentation, physiological activity, increased nonsignificantly in some cases (e.g., after fermentation by <i>B. lactis</i> or even decreased (e.g., after fermentation by <i>A. oryzae</i> and <i>A. niger</i>). Thus, selecting an appropriate microphone is vital. For the positive controls kojic acid and α-arbutin, the tyrosinase IC₅₀ was 20.4 ± 1.2 and 276.1 ± 9.2 mg/L, respectively.

Figure 2 illustrates the DPPH scavenging activity of the methanolic and ethanolic extracts of <i>C. aurantium</i> flowers. The scavenging activity increased with the extract concentration. The DPPH scavenging activity of the methanolic extract (IC₅₀: 358.2 ± 16.1 mg/L)
Table 1. Yield of 100% methanolic and 50% ethanolic C. aurantium flower extracts and 50% ethanolic extracts fermented by one of four lactic acid bacteria or two fungi.

| Extraction/Recover yield (%) | 100% methanol | 50% ethanol | B. bifidum | B. lactis | L. acidophilus | L. brevis | A. oryzae | A. niger |
|-----------------------------|---------------|-------------|------------|-----------|---------------|-----------|-----------|---------|
| 5.1 ± 0.7%a | 8.6 ± 0.4%b | 78.2 ± 1.2%c | 76.5 ± 0.6%d | 83.1 ± 1.4%e | 86.2 ± 1.2%f | 70.2 ± 5.3%g | 73.5 ± 7.1%h |

Optimal fermentation time (d) – – 3 3 3 3 5 5

Yield1 = g of dried extract by solvent/g of dried C. aurantium flower powder.
Yield2 = g of dried fermented extract/g of dried 50% ethanolic extract.
In each row, letters (a–d) indicate P < 0.01.

Figure 1. Antityrosinase activity of extracts of C. aurantium flowers obtained using 100% methanol or 50% ethanol. Data are expressed as the mean ± standard deviation of three independent experiments.

Table 2. Tyrosinase inhibition and DPPH radical scavenging (expressed as IC50, mg/L) of the 50% ethanolic extract of C. aurantium flowers fermented by various lactic acid bacteria or fungi.

| B. bifidum | B. lactis | L. acidophilus | L. brevis | A. oryzae | A. niger |
|------------|-----------|---------------|-----------|-----------|---------|
| Tyrosinase inhibition | 120.8 ± 5.2a | 202.3 ± 8.8b | 48.2 ± 2.3c | 38.2 ± 0.9d | 536.3 ± 7.1e | 581.2 ± 4.6f |
| DPPH scavenging activity | 96.2 ± 3.8a | 275.8 ± 10.3b | 36.1 ± 1.2c | 18.6 ± 0.6d | 418.2 ± 6.5e | 462.3 ± 7.6f |

In each row, letters (a–f) indicate P < 0.01.

Figure 2. DPPH and ABTS scavenging activity of C. aurantium flower extract obtained using 100% methanol or 50% ethanol. Data are expressed as the mean ± standard deviation of three independent experiments.
was lower than that of the 50% ethanolic extract (IC\textsubscript{50}: 272.4 ± 8.6 mg/L). In addition, the 50% ethanolic extract had higher ABTS scavenging activity (IC\textsubscript{50}: 345.8 ± 16.3 mg/L) than did the methanolic extract (IC\textsubscript{50}: 446.3 ± 17.1 mg/L). These results indicated that 50% ethanol was the optimal extraction solvent for \textit{C. aurantium} flowers. Table 2 lists the DPPH scavenging activity of 50% ethanolic extracts fermented by various acid bacteria or fungi. The highest DPPH scavenging activity was achieved by the \textit{L. brevis}–fermented extract (IC\textsubscript{50}: 18.6 ± 0.6 mg/L). The fermented extracts had much higher DPPH scavenging activity (14.6 times higher) than the unfermented extracts (IC\textsubscript{50}: 272.4 ± 8.6 mg/L).

Hsouna et al. (2013) reported that the essential oil obtained from \textit{C. aurantium} flowers exhibited excellent DPPH scavenging ability (IC\textsubscript{50}: 15.3 mg/L), and this finding is similar to our finding for the \textit{L. brevis}–fermented extract. In the present study, the IC\textsubscript{50} for DPPH of the positive control BHT was 49.6 ± 2.8 mg/L. The present results demonstrate the promise of fermented \textit{C. aurantium} flower extract for use as an antioxidant. In conclusion the extract subjected to \textit{L. brevis} fermentation achieved the highest antityrosinase and DPPH scavenging activity, compared with those fermented by other microbes. Thus, \textit{L. brevis} was selected to ferment the 50% ethanolic extract in the subsequent experiments.

### 3.3. Antimicrobial activity

A previous study reported that antioxidative activity and antimicrobial activity are positively correlated (Wu et al., 2018). Because the \textit{L. brevis}–fermented extract in that study exhibited high antioxidative activity, we examined our extracts’ antimicrobial activity against five of the microbial species mentioned in the USP 51-antimicrobial effectiveness test. The MIC and MFC of the 50% ethanolic extract of \textit{C. aurantium} flowers were lower than those of the methanolic extract (Table 3); however, the antimicrobial activity of the 50% ethanolic extract was low (800–2500 mg/L). The antibacterial activity (MIC) of the \textit{L. brevis}–fermented extract against \textit{E. coli}, \textit{S. aureus}, and \textit{P. aeruginosa} significantly increased by 15, 12.5, and 16 times, respectively, after fermentation compared with before fermentation. Moreover, the antifungal activity (MFC) of the \textit{L. brevis}–fermented extract against \textit{C. albicans} and \textit{A. brasiliensis} increased by 6.7 and 13.9 times after fermentation compared with before fermentation (50% ethanolic extract). The fermented extracts were most effective against \textit{P. aeruginosa} (50 mg/L), followed by \textit{E. coli} (80 mg/L), \textit{S. aureus} (120 mg/L), \textit{C. albicans} (150 mg/L), and \textit{A. brasiliensis} (180 mg/L), in that order. Similar antimicrobial activity was reported for the essential oil of \textit{C. aurantium}; however, this oil was discovered to be ineffective against \textit{S. aureus} (Ammar et al., 2012).

Hsouna et al. (2013) reported the MICs of the essential oil of \textit{C. aurantium} against \textit{S. aureus} and \textit{P. aeruginosa} were 312 and 2500 mg/L, respectively. Teneva et al. (2019) reported that an essential oil obtained from \textit{C. aurantium} through steam distillation was relatively ineffective against Gram-negative bacteria (\textit{E. coli}, \textit{P. aeruginosa}, and \textit{Salmonella} sp.), with an MIC of >600 mg/L, but effective against Gram-positive bacteria (\textit{S. aureus} and \textit{B. subtilis}), with an MIC of 60 mg/L. Compared with the essential oils extracted in these previous studies, the \textit{L. brevis}–fermented extract obtained in our study was more effective, with an MIC of ≤120 mg/L, against the tested microbes.

### 3.4. Analysis of antioxidative characteristics

Wen et al. (2013) reported that fermented products have higher TPC and antioxidative and antibacterial activity than do unfermented extracts. We determined the possible sources of and mechanisms underlying the antityrosinase, antioxidative, and antimicrobial activity of our extracts. Table 4 lists the antioxidative activity of the 50% ethanolic extracts, unfermented or fermented by \textit{L. brevis}. According to our results, the ABTS scavenging activity, total reducing power, TPC, and TFC of the fermented 50% ethanolic extract were 13.5, 2.06, 1.79, and 3.17 times higher, respectively, than those of the unfermented extract. Thus, the \textit{L. brevis}–fermented extract appeared to have high antioxidative ability. The difference in TPC between the fermented versus unfermented extracts was greater than that in their TPC. Thus, the high physiological activity of the \textit{L. brevis}–fermented extract may be attributable to the high concentration of flavonoids. The IC\textsubscript{50} of the \textit{L. brevis}–fermented extract in the ABTS assay was 25.6 ± 1.4 mg/L; this value was superior to the IC\textsubscript{50} (672 mg/L) of a \textit{C. aurantium} extract obtained using boiled distilled water (Ammar et al., 2012) and that of the positive control, BHT (52.3 ± 3.7 mg/L). The TPC and TFC of the \textit{L. brevis}–fermented extract were 134.8 ± 20.6 mg-GAE/g-dried extract and 102.8 ± 10.8 mg-RE/g-dried extract, respectively; these values are higher than those of an 80% ethanolic extract of \textit{C. aurantium} flowers reported previously (4.55 mg-GAE/g-dried extract and 3.83 mg-RE/g-dried extract; Karimi et al., 2012). This finding indicates the superiority and therapeutic potential of the extraction–fermentation process developed in the present study.

### 3.5. Identification of phenolic and flavonoid compounds

Wang et al. (2016) discovered high TPC in herb extracts after fermentation and indicated that the high TPC enhanced the extracts’ tyrosinase inhibition and DPPH scavenging activity. The high physiological activity of the \textit{L. brevis}–fermented extract may be attributable to the high flavonoid concentration after fermentation (results of section 3.4). The phenolics and flavonoids in the unfermented and fermented extracts were analyzed through HPLC to determine their phenolic and flavonoid compositions (Table 5). In the unfermented extract, the major phenolic compounds were ferulic acid, pyrogallol, syringic acid, and gallic acid and the major flavonoid compounds were rutin, naringin,

### Table 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 100% methanolic and 50% ethanolic \textit{C. aurantium} flower extracts and 50% ethanolic \textit{C. aurantium} flower extracts fermented by \textit{L. brevis} against various bacteria and fungi.

| MIC (mg/L) | S. aureus | E. coli | P. aeruginosa | MFC (mg/L) | C. albicans | A. brasiliensis |
|-----------|-----------|----------|---------------|------------|-------------|----------------|
| Methanolic extract | 2,000\textsuperscript{a} | 2,000\textsuperscript{a} | 1,500\textsuperscript{a} | 3,000\textsuperscript{a} | 5,000\textsuperscript{a} |
| 50% ethanolic extract | 1,500\textsuperscript{a} | 1,200\textsuperscript{a} | 800\textsuperscript{a} | 1,000\textsuperscript{a} | 2,500\textsuperscript{a} |
| \textit{L. brevis}–fermented extract | 120\textsuperscript{a} | 80\textsuperscript{a} | 50\textsuperscript{a} | 150\textsuperscript{a} | 180\textsuperscript{a} |

In each column, letters (a–c) indicate P < 0.01.
hesperidin, and neohesperidin. The major phenolic compounds in the *L. brevis*–fermented extract were pyrogallol, syringic acid, and gallic acid, and the principal flavonoid compounds were rutin, naringin, and neo-hesperidin. The extract's rosmarinic acid content, ferulic acid content, and hesperidin content were lower after fermentation. The hesperidin content significantly decreased to 14.6% after fermentation. The caffeic acid, rutin, apigenin, luteolin, and quercetin content differed only slightly after fermentation. Conversely the pyrogallol, syringic acid, gallic acid, naringin, rhoifolin, eriocitrin, and neohesperidin content increased with fermentation.

In particular, the content of neohesperidin significantly increased from 658 ± 7.81 to 1036 ± 12.3 µg/g-dried extract after fermentation. The results indicated that hesperidin biotransformed into neohesperidin during the fermentation process (Frydman et al., 2005). We also discovered that the major phenolics and flavonoids were more abundant in the fermented extract than the unfermented extract. Enzymes released from the cell wall of *C. aurantium* were inactive during extraction. Thus, the generation of phenolic and flavonoid compounds was attributed to fermentation and considered to result from modification of naturally occurring molecular constituents (Wang et al., 2016, 2017; Wu et al., 2018). Li (2012) reported that phenolic compounds namely pyrogallol, caffeic acid, and gallic acid, present in *C. aurantium* flowers extracts were favorable antioxidants. Flavonoid compounds—naringin, eriocitrin, and neohesperidin—exhibit high antioxidative and radical scavenging activity (Sicari et al., 2016). The high antioxidative activity of the *L. brevis*–fermented extract can thus be explained by the presence of these compounds. Similar flavonoid components—including rutin, naringenin, quercetin, rhoifolin, eriocitrin, hesperidin, and neohesperidin—were found in a Chinese *C. aurantium* extract (Shen et al., 2017), and common flavonoid compounds including apigenin, luteolin, naringenin, and hesperidin were discovered in a Korean *C. aurantium* extract (Santar et al., 2018). In a study of methanolic *C. aurantium* flower extracts, pyrogallol was found to be most abundant (541 µg/g-dry weight) phenolic compound, followed by syringic acid (269 µg/g-dry weight), caffeic acid (249 µg/g-dry weight), and gallic acid (212 µg/g-dry weight), whereas naringin was the most abundant flavonoid compound (688.1 µg/g-dry weight), followed by rutin (362 µg/g-dry weight) and quercetin (185 µg/g-dry weight; Karimi et al., 2012). The differences between the results of previous studies and ours are mainly attributable to the extraction solvents used.

### 3.6. Analysis of cell viability, antityrosinase activity, and melanin content

Safety is a more important consideration than pharmacological efficacy in the adoption of a new therapeutic substance. The toxicity to CCD-966SK cells of various concentrations of the unfermented and fermented extracts was examined using the MTT method (Figure 3). The cell viability of CCD-966SK cells was determined after 24, 48, and 72 h of treatment. When the treatment duration was 24 or 48 h, the *C. aurantium* flower extracts (both unfermented and fermented) were found to not have influenced cell viability. After 72 h of treatment, we discovered cell viability of >95.6% and found that the cytotoxicity of the 50% ethanolic extract did not significantly differ from the cytotoxicity of the control when the concentration was low (<300 mg/L). Significantly lower cell viability compared with the control (P < 0.01) was discovered when the concentration of the unfermented extract was ≥400 mg/L (viability of 92.3%-87.6%). However, the *L. brevis*–fermented extract nonsignificantly inhibited cell viability, even at 500 mg/L, suggesting that the *L. brevis*–fermented extract had low cytotoxicity; this finding is attributable to the biotransformation that occurred during fermentation (Wang et al., 2019).

The IC₅₀ was 38.2 mg/L for tyrosinase inhibition and 18.6 mg/L for DPPH scavenging activity (Table 2). The MIC value was 50–120 mg/L for bacterial inhibition, whereas the MFC value was 150–180 mg/L for fungal inhibition (Table 3) for the *L. brevis*–fermented extract. These concentrations had no negative effect on the viability of the investigated cells. According to the present findings, the *L. brevis*–fermented extract is relatively safe and can potentially be applied in the cosmetics industry.

We varied the concentration of the fermented extract and investigated its effect on the viability, antityrosinase activity, and melanin content of HEMn cells (Figure 4). When the concentration was 0–200 mg/L, the fermented extract nonsignificantly inhibited (P > 0.01) HEMn cell viability (>99.2%). Moreover, as the concentration was increased, the melanin content was discovered to gradually decrease, and the antityrosinase activity markedly increased. Melanin content and anti-tyrosinase activity exhibited a high degree of synchronization; thus, the *L. brevis*–fermented extract exerts a whitening effect, possibly through inhibition of tyrosinase activity (Harir et al., 2021). The fermented extract inhibited melanin formation, with the melanin content being 50.8% ± 2.3% at the extract concentration of 40 mg/L. When the concentration was higher than 160 mg/L, melanin was undetectable in HEMn cells. Tyrosinase activity was substantially inhibited, but the HEMn cells were not killed by the extract. In a previous study, 5-hydroxy-6,7,3',4'-tetramethoxyflavone and limonexic acid, which were isolated from *C. aurantium* var. *amara*, significantly inhibited B16 cells at 12.5 mg/L (Zha et al., 2012). The IC₅₀ value of antityrosinase activity in the in vivo HEMn cells in our study was 40.3 mg/L, which was slightly lower than the corresponding value for in vitro tyrosinase activity (IC₅₀: 38.2 mg/L). The slight difference between these two values indicates that the whitening mechanism may be related to the tyrosinase itself and unaffected by other enzymes and messenger pathways.

### 3.7. Effect of *L. brevis*–fermented extract on antiwrinkle activity

Although studies have reported the high physiological activity and safety of *L. brevis*–fermented extracts, no study has focused on the antiwrinkle effects of *C. aurantium* flowers. The breakdown of collagen in the skin can be prevented by inhibiting collagenase activity. Furthermore, elastase activity can be inhibited to prevent skin aging (Karim et al., 2014). Collagen and gelatin are respectively digested by the MMP-1 and MMP-2 that skin fibroblasts secrete (Limtrakul et al., 2016); thus, inhibiting MMP-1 and MMP-2 may lead to antiwrinkle activity. Analysis of collagenase, elastase, MMP-1, and MMP-2 (antiwrinkle-related enzymes) inhibitory activity is crucial to evaluating antiaging and antiwrinkle activity. We discovered that treatment with the *L. brevis*–fermented extract resulted in considerably lower collagenase,

| Table 5. Compositions and contents of phenolics and flavonoids in 50% ethanolic *C. aurantium* flower extract, unfermented and fermented by *L. brevis*. |
|---|
| **Phenolics (µg/g-dried extract)** | 50% ethanolic extract | *L. brevis*–fermented extract |
| caffeic acid | 224 ± 2.31* | 206 ± 1.28* |
| rosmarinic acid | 75.4 ± 0.82* | 36.1 ± 1.12* |
| ferulic acid | 235 ± 1.89* | 52.3 ± 3.26* |
| pyrogallol | 526 ± 6.23* | 806 ± 8.61* |
| syringic acid | 278 ± 1.56* | 625 ± 9.33* |
| gallic acid | 206 ± 3.26* | 513 ± 7.21* |
| **Flavonoids (µg/g-dried extract)** | rutin | 405 ± 2.65* |
| | apigenin | 73.6 ± 0.63* |
| | luteolin | 86.3 ± 1.25* |
| | naringin | 361 ± 2.41* |
| | quercetin | 126 ± 0.95* |
| | rhoifolin | 21.6 ± 0.35* |
| | eriocitrin | 32.6 ± 1.45* |
| | hesperidin | 251 ± 3.48* |
| | neohesperidin | 658 ± 7.81* |

In each row, letters (a–b) indicate P < 0.01.
elastase, MMP-1, and MMP-2 activity than did treatment with the un-fermented extract (Table 6). The inhibitory activity of these four antiwrinkle-related enzymes was 4.29–5.90 times higher when the extract had been fermented. Maity et al. (2011) reported that syringic acid effectively inhibited elastase and MMP-1 activity. In addition, gallic acid is an effective inhibitor of collagenase, elastase, and MMP-2 activity (Wittenauer et al., 2015; Poomanee et al., 2021). Caffeic acid has been found to be an effective inhibitor of collagenase and MMP-1 activity (Shin et al., 2019; Saechan et al., 2021), and neohesperidin reduces MMP-1 activity (Karim et al., 2021). Naringin has been discovered to significantly inhibit the production of elastase or downregulate MMP-2 expression (Annapoorani et al., 2012; Aroui et al., 2016). Rutin was previously determined to be an effective inhibitor of collagenase activity or MMP-1 production (Hwang et al., 2014; Mota et al., 2020). The quantities of these phenolic and flavonoid compounds in our extracts were considerably higher or almost the same when fermentation was applied (Table 3), which explains the considerably higher antiwrinkle activity of the extract after fermentation. Wu et al. (2018) reported that antiwrinkle-related enzyme activity could be attenuated using a

| Activity (mg/L) | Collagenase | Elastase | MMP-1 | MMP-2 |
|----------------|-------------|----------|-------|-------|
| 50% ethanolic extract | 315.6 ± 27.4b | 502.8 ± 32b | 864.3 ± 36.8b | 912.0 ± 52.3b |
| L. brevis–fermented extract | 73.5 ± 11.3a | 85.2 ± 7.2a | 152.4 ± 14.8a | 175.2 ± 16.2a |

In each column, letters (a–b) indicate $P < 0.01$.
fermented extract. Thus, our results suggest that the *L. brevis*-fermented extract has potential for use in the cosmetics industry in formulations designed to slow the aging of the skin aging and prevent the development of wrinkles.

4. Conclusion

The phenolic and flavonoid compounds in *C. aurantium* flower extracts undergo biosynthesis or biotransformation during fermentation; this increases their TPC and TFC, in turn increasing the physiological activity of the product. Our fermented *C. aurantium* flower extract exerted a nontoxic effect on skin cells. Our study is the first to examine the antiaging and antioxidative activity of *C. aurantium* flowers. The results revealed that wrinkle-related enzymes are significantly inhibited by the *C. aurantium* flower extract after *L. brevis* fermentation. In addition, the extract exerts a nonsignificant effect on CCK-966SK cell viability, even at high concentrations. In this article, we report for the first time the suitability of *L. brevis*-fermented extract as a safe ingredient for inclusion in cosmetic formulations designed to have skin-whitening or antiwrinkle effects.

Declarations

**Author contribution statement**

Chih-Yu Chen: Conceived and designed the experiments; Wrote the paper.
Chi-Yao Hu: Performed the experiments; Analyzed and interpreted the data.
Ssu-Hsuan, Chen: Ya-Ting, Li: Performed the experiments.
Ying-Chien Chung: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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**Data availability statement**

Data included in article/supp. material/referenced in article.

**Declaration of interest’s statement**

The authors declare no conflict of interest.

**Additional information**

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