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Antioxidant properties of fermented mango leaf extracts

ANNA PARK, TAEKYU KU, and ILSOU YOO, *Department of Advanced Organic Materials Engineering, Faculty of Engineering and Applied Science, Chonbuk National University, Jeonju, Chonbuk 561-756, Republic of Korea*.

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Synopsis

Antioxidant properties of mango (*Mangifera indica*) leaves were evaluated. Hydroalcoholic leaf extracts that were lyophilized were subsequently fermented with either *Lactobacillus casei* or effective microorganisms (EM) such as probiotic bacteria and/or other anaerobic organisms. Antioxidant properties were measured as a function of the mango leaf extract concentration in the fermentation broth.

Tests for radical scavenging using the 1,1-diphenyl-2-picrylhydrazyl radical showed higher antioxidant activity for Lactobacillus- and EM-fermented mango leaf extracts than for the synthetic antioxidant butylated hydroxytoluene. Antioxidant activity generally increased with increasing fermented extract concentration as did the fermented extracts' polyphenol and flavonoid contents.

Fermented extracts reduced reactive oxygen species generation by lipopolysaccharide in RAW 264.7 cells when measured via fluorescence of dichlorodihydrofluorescein acetate treated cells using flow cytometry. RAW 264.7 cells also showed a concentration-dependent cytotoxic effect of the fermented extracts using the 3-(4,5-dimethylthialol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Inhibition of mushroom tyrosinase activity as well as nitrite scavenging by the fermented extracts increased as fermented extract concentrations increased. Tyrosinase activity was assayed with 3,4-dihydroxyphenylalanine as substrate. Nitrite scavenging was assessed via measurement of inhibition of chromophore production from nitrite–naphthylamine–sulfanilic acid mixtures.

The antioxidant properties of fermented mango leaf extracts suggest the fermented extracts may be useful in developing health food and fermentation-based beauty products.

INTRODUCTION

Among the components of mango, mangiferin and catechol oxidase are recognized for their colon cleansing effects and capacity to enhance resistance against pathogens and diseases. Mango also contains high levels of tryptophan, a precursor of the neurotransmitter serotonin (1). Lupeol, a triterpene present in mango, is known as an effective inhibitor of cellular growth in both prostate and skin cancers (1).

Extensive research efforts have focused on the fermentation of natural bioactive substances using microorganisms. Microbial fermentation of natural substances is known to enhance

Address all correspondence to Anna Park at dranna@hanmail.net.

their bioavailability owing to the preservation of nutritional components during fermentation. In addition, microorganisms secrete various hydrolytic enzymes and release bioactive substances bonded to intracellular components (2).

Koreans traditionally consume high amounts of fermented food such as kimchi, miso, and soy sauce. With the recent unveiling of physiological activity of fermented foods, intensive research has been conducted to use such foods as health-promoting functional materials. Broadly speaking, fermentation can be regarded as the microorganism-mediated production of useful biomaterials. Microorganisms assimilate nutrients and contribute to the maintenance of life and growth, as well as generate energy through their enzymatic degradation of nutrients. Foolad *et al*. (3) reported that children with allergic skin diseases could benefit from the intake of food supplements containing probiotic components, which have preventive and inhibitory effects against diseases; *Lactobacillus rhamnosus* GG, a strain of *L. rhamnosus*, showed a substantial long-term preventative effect.

The *in vivo* formation of reactive oxygen species (ROS), oxygen-derived highly reactive metabolic substances such as hydrogen peroxide, superoxide anion, hydroxyl radical, and oxygen-free radicals, which inevitably occur during aerobic metabolism, generate toxicity that can disrupt normal physiology (3). ROS provoke damage to multiple cellular organelles and metabolic processes, thereby being involved in aging, cancer, cardiovascular diseases, and inflammation. Such free radicals are usually removed or suppressed *in vivo* by antioxidants. It has been reported that natural antioxidants such as vitamin C, carotenoids and phenolic compounds (i.e., flavonoids, tannins, cumminoids, etc.) are highly effective in inhibiting aging *in vivo* or preventing atherosclerosis, inflammation, degenerative diseases, and cancer (4–7).

Against the backdrop of recent intensive research on improving fermentation-induced physiological functionality, this study aimed to evaluate the antioxidative properties of fermented hot-water extracts of mango leaves. Spectrophotometry was used to estimate the total polyphenol and flavonoid contents in mango leaf extracts fermented with either lactic acid bacteria or other effective microorganisms (EM). EM refers to the combination of probiotic and/or anaerobic microbes in commercial agricultural products, pharmaceuticals, and nutritional supplements based on the trademarked product, EM-1® Microbial Inoculant (8,9). The antioxidant levels were also studied using various tests including 1,1-diphenyl-2-picrylhydrazyl (DPPH)-mediated electron-donating activity, nitrite-scavenging activity, ROS production, and cytotoxicity via the MTT colorimetric assay. The MTT assay uses the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, along with a NAD(P)H-dependent cellular oxidoreductase enzyme to assess cell viability (10).

MATERIALS AND METHODS

SAMPLES AND EXTRACTION

Mango leaves were harvested in September from a mango farm in Jeju-do, Korea, dried at room temperature (25°C) for 2 weeks with room relative humidity of 40–50%, placed into a grinder, and subjected to extraction. The dried leaves were accurately weighed using an analytical balance. Subsequently, the 10-fold extract (1 gram dried mango leaf in

10 ml 50% Ethanol) was prepared by heating the solution on Hot plate stirrer (PC-4200, Corning Inc., Corning, NY) for 4 hours at 80°C. The extract vapor was condensed and returned to extract liquid using a Reflux condenser (Labpia, Seoul, Korea) to control the rate of heating and to prevent loss of extract through evaporation.

The extracted leaves were cleared of impurities using filter paper (Advantec No. 2, Tokyo, Japan) and a rotary evaporator (Rotary Evaporator N-1000SW, Eyela, Tokyo, Japan). Mango leaf extracts were kept in a deep freezer (−62°C) (DE8525, Ilshin Lab Co. Ltd., Dongduchon, Korea) for a day, lyophilized at 0 mTorr in a vacuum between −70° and −60°C (FD5508, Ilshin Lab Co. Ltd., Dongduchon, Korea), and air-sealed until analysis.

PREPARATION OF MANGO LEAF FERMENTATION EXTRACT

To prepare mango leaf lactobacillus fermentation extract (MLFE), we inoculated *Lactobacillus casei* (KCTC2180, Sahmyook University, Seoul, Korea) in a 55 mg/ml sterile MRS broth and incubated the culture at 37°C for 1 day to be used as the main culture. Different amounts of mango leaf extracts were each added to 10 ml of distilled water. Approximately 0.1 ml of the seed culture with *Lactobacillus casei* concentration of 6.2×10 colony forming units (cfu)/ml was added, mixed thoroughly, and maintained in an incubator (Wonil Incu1, Wonil Tech., Jeonju, Korea) set at 37°C for 2 days to obtain a fermentation broth.

To prepare mango leaf effective microorganism fermentation extract (MEFE), we obtained an EM Activity Solution (EverMiracle, Jeonju University, Jeonju, Korea). EM Activity Solutions (5%) and sugar (molasses, 5%) were combined in distilled water (90%) and fermented (or activated) at 37°C for 7 days in a sealed container. Upon ensuring the activated EM solution's pH is under 3.5, we added 0.1 ml of seed culture with lactobacillus concentration of 6.2×10 cfu/ml to 10 ml of distilled water containing different amounts of mango leaf extracts to obtain fermentation broth of varying concentrations for antioxidant activity tests.

CELL CULTURE

The cell line utilized in this study was RAW 264.7 (mouse leukemic monocyte macrophage cell line (KCLB 40071)) obtained from Korean Cell Line Bank (Seoul, Korea). The cells were cultured at 37°C in a 5% CO_2 incubator (Thermo Scientific, Waltham, MA) using Dulbecco's modified Eagle's medium (Gibco® DMEM, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco® FBS, Life Technologies) and 1% antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) (11).

ANTIOXIDANT ACTIVITY TESTING

Polyphenol measurement. The amount of polyphenol per gram of extract was assessed using the Folin–Denis method (12). Approximately 100 µl of the extract and 1 ml of 2% Na_2CO_3 were mixed and reacted for 2 minutes at room temperature. After the reaction, 50% Folin–Ciocalteu's phenol reagent (100 µl) was added, vortexed, and incubated for

30 min at room temperature. Subsequently, the absorbance of the mixture was measured using the UV/VIS spectrophotometer (OPTIZEN 2020UV plus, Mecasys Co., Daejeon, Korea) at a wavelength of 750 nm. Using Tannic acid (Sigma-Aldrich Korea Ltd, Youngjin-city, Korea) as a standard substance, the replicates were tested three times for the analysis.

Flavonoid measurement. The flavonoids content per gram of extract was measured using the colorimetric method in the presence of diethylene glycol (13). Approximately 100 µl of the extract, 100 µl of 1 N NaOH, and 1 ml of diethylene glycol were combined using a vortex mixer and incubated for 1 h in a 30°C water bath. Following the reaction, the absorbance of the mixture was measured using a UV/VIS spectrophotometer at a wavelength of 420 nm. Catechin (Sigma-Aldrich Korea Ltd) was used as a standard substance for three repeat examinations.

DPPH radical scavenging activity measurement. The DPPH radical scavenging activity was measured using a modified version of the method described by Blois (14). First, we produced 500 m*M* of DPPH using 0.1 *M* Tris base-HCl buffer (Tris buffer) at pH 7.4 and methanol. Then, a sample aliquot of 100 µl, 400 µl of 0.1 *M* Tris buffer and 500 µl of 500 m*M* DPPH were combined and vortexed. The mixture was reacted for 30 min in the dark at room temperature and the absorbance of the mixture was analyzed using a UV/VIS spectrometer at 517 nm in three replicate runs.

The artificial antioxidant butylated hydroxytoluene (BHT) was used as a control and the DPPH radical scavenging activity was expressed as electron donation ability (EDA) percentage, a percentage based on the differences in absorbance of DPPH solutions between absence (A_initial) and presence (A_final) of antioxidant samples (BHT, MLFE, or MEFE) (15):

$$\text{EDA}\ (\%) = (\text{A\_initial} - \text{A\_final/A\_initial}) \times 100\%.$$

Cell viability estimation using MTT assay. Cell viability was measured using the methodology of Mosmann (16). RAW 264.7 cells were seeded onto 96-well plates at a density of 3×10^5 cells/well and cultured at 37°C in a 5% CO_2 incubator. The cells were cultured for another 24 h after treatment using various concentrations of the MLFE and MEFE extracts. After culturing, 100 µl of MTT solution was placed into each well, cultured for 4 hours, and the formazan precipitate was then dissolved in 100 µl of dimethylsulfoxide after the removal of the supernatant. An ELISA microplate reader (E-max, Molecular Device, Sunnyvale, CA) was used to measure the absorbance at 570 nm in three replicate viability assays. Cell viability is expressed in percentage using the following formula (17):

$$\text{Cell viability}\ (\%) = (\text{A}-\text{B})/\text{A} \times 100\%;$$

A: Absorbance of untreated RAW 264.7 cells at 570 nm

B: Absorbance of extract sample at 570 nm.

ROS production measurement. RAW 264.7 cells (4×10^5 cells) were seeded in a glass bottom dish (MatTek Corp., Ashland, MA) and incubated for 24 h. Cells were treated with a various amounts of the MLFE or MEFE for 10 hours and incubated for 20 h with 1 ml of 1 µg/ml LPS (Sigma-Aldrich Korea Ltd: E. Coli 0111:B4, L3024). Then, cells were treated with 10 µ*M* of dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich) for 0.5 h and harvested with trypsin/EDTA (Gibco) for three separate flow cytometric analyses.

DCFH-DA is a non-fluorescent material that can permeate cell membranes. Within cells, it hydrolyzes to dichlorodihydrofluorescein (DCFH), which in the presence of ROS is oxidized to fluorescent dichlorofluorescein (DCF), a high-performance fluorescent substance. To quantify the fluorescent cells, flow cytometry was also performed using a Flow Cytometry Caliber (Becton Dickinson, Franklin Lakes, NJ) with CellQuest Pro software. The percentage of cells in positive events was calculated as the events within the gate divided by total number of events then subtracting percentage of the control sample (untreated cells) (18).

Tyrosinase inhibitory activity estimation. Tyrosinase inhibition activity was assessed using the modified protocol of Masamoto *et al.* (19). To determine *in vitro* mushroom tyrosinase inhibition activity, 0.3 ml of 2.5 m*M* 3,4 dihydroxyphenylalanine (L-DOPA), 0.05 ml of the mango extract, and 1.5 ml of 0.1 *M* phosphate buffer solution (pH 6.8) were combined using a vortex mixer and pre-incubated at 25°C.

Approximately 0.05 ml of mushroom tyrosinase (1.380 units/ml, Sigma-Aldrich) was placed into the vortex mixer and allowed to react for 2 min at 25°C. The absorbance was then measured using a UV/VIS spectrophotometer at a wavelength of 475 nm. The following equation was used in the calculations for the percentage of tyrosinase inhibition:

$$\text{Tyrosinase inhibition (\%)} = [(A-B)/A] \times 100\%;$$

A = Difference in absorbance between 0.5 and 1 minutes in the reactant without the sample;

B = Difference in absorbance between 0.5 and 1 minutes in the reactant with the sample using 0.05 ml of 1380 units/ml mushroom tyrosinase.

Nitrite-scavenging ability assessment. Nitrite scavenging was assessed using a modified version of Gray and Dugan's methodology (20). We combined 0.1 ml of 1 m*M* $NaNO_2$ and 0.3 ml of sample MLFE and MEFE, respectively. Then, 0.2 *M* citrate buffer-HCl (pH 2.5) was added to produce the final volume of 1 ml.

The components were combined using a vortex mixer and incubated in a water bath at 37°C for 1 h, after which Griess' reagents [30% acetic acid solution dissolved 1% sulfanilic acid and 1% naphthylamine = 1:1 (v/v)] and 3 ml of 2% acetic acid solution were added. After incubating for 15 min at room temperature, the absorbance of the mixture was measured using a UV/VIS spectrophotometer at a wavelength of 520 nm in three repeat examinations. The nitrite scavenging activity (%) was calculated using the following formula (21):

$$\text{Nitrite scavenging (\%)} = [1-(A-B)/C] \times 100\%;$$

A: Absorbance of test sample at 520 nm

B: Absorbance of sample blank with H_20 instead of $NaNO_2$ at 520 nm

C: Absorbance of control (H_20) without test sample at 520 nm.

STATISTICAL ANALYSIS

This study is conducted to test the null hypothesis of equality of antioxidant activities for the MLFE and MEFE. The antioxidant activities at each concentration were expressed as

mean ± S.D. The Student *t*-tests were carried out to test the difference in means with the significance level of $p < 0.05$ using SPSS 20.0 (IBM SPSS Statistics for Windows, Version 20.0, Armonk, NY).

RESULTS AND DISCUSSION

POLYPHENOL AND FLAVONOID CONTENTS

Polyphenols affect the skin in a variety of ways and neutralize harmful oxidative species, thus acting as strong antioxidants (22). Polyphenols do not stimulate the skin but suppress melanin production and, therefore, whiten the skin. This cosmetic effect may be due to a relationship between the amount of polyphenol used and the whitening effect (23).

As outlined in Table I, the polyphenol content increased as the MLFE concentration increased from 7.43 ± 0.09 mg/g at 2 mg/ml to 30.58 mg/g ± 0.05 at 50 mg/ml. In the MEFE, the polyphenol content increased from 8.51 ± 0.05 mg/g at 2 mg/ml to 35.21 ± 0.04 mg/g at 50 mg/ml. The statistically significant increases in polyphenol contents were detected for the MEFE in comparison to the MLFE for all concentration levels except at 10 mg/ml. However, a dose-dependent increase in polyphenol content was observed in both the MLFE and the MEFE.

Fermentation-dependent flavonoid contents are outlined in Table II. The flavonoid content in the MLFE increased from 1.35 ± 0.07 mg/g at 0.4 mg/ml to 8.89 ± 0.01 mg/g at 10 mg/ml. In addition, the flavonoid content in the MEFE increased from 1.91 ± 0.02 mg/g at 0.4 mg/ml to 9.80 ± 0.05 mg/g at 10 mg/ml. These results confirm that the flavonoid content increased with increasing the MLFE and MEFE concentrations. The MEFE again yielded statistically higher concentration of flavonoid at all concentration levels except at 50 mg/ml where the MLFE yielded a higher flavonoid content than the MEFE.

These results are consistent with the previous study (24). In our study, we estimated that the change in intensity of mango leaf fermentation concentration influenced the flavonoid content, thus confirming the antioxidative property of the mango leaf fermentation extracts (25).

DPPH RADICAL SCAVENGING ACTIVITY

DPPH radical scavenging activity allows the measurement of antioxidant activity based on EDA. Free radicals within the body react with proteins and other substances to

Table I
Polyphenols in the MLFE and the MEFE (n = 3)

	Polyphenol concentration (mg/g)			
MLFE concentration (mg/ml)	MLFE	MEFE	*t*	*p*-Value
0.4	3.38 ± 0.05	5.84 ± 0.02	−78.642	0.000
2	7.43 ± 0.09	8.51 ± 0.05	−18.262	0.000
10	22.52 ± 0.07	17.91 ± 0.06	86.552	0.000
50	30.58 ± 0.05	35.21 ± 0.04	−127.735	0.000

Table II
Flavonoids in the MLFE and the MEFE (n = 3)

	Flavonoid concentration (mg/g)			
MLFE concentration (mg/ml)	MLFE	MEFE	t	*p*-Value
0.4	1.35±0.07	1.91 ± 0.02	−13.382	0.000
2	3.62 ± 0.02	5.84 ± 0.01	−171.00	0.000
10	8.89 ± 0.01	9.80 ± 0.05	−31.691	0.000
50	20.91 ± 0.04	16.74 ± 0.03	144.72	0.000

accelerate aging, prompting researchers to explore natural substances that could neutralize free radicals (26).

The antioxidative effects of various concentrations of the MLFE and the MEFE were evaluated by estimating the level of DPPH radical scavenging activity, as manifested in its EDA percentage. Figure 1 illustrates the increase in DPPH radical scavenging activity as EDA percentage increase from 33% at 2 mg/ml to 69% at 50 mg/ml for the MLFE and from 61% at 2 mg/ml to 72% at 50 mg/ml for the MEFE, thus demonstrating that EM fermentation is more effective than lactobacillus fermentation at lower concentration levels but equivalent at higher levels (i.e., 50 mg/ml). The MLFE demonstrated significantly higher antioxidative properties compared to the synthetic antioxidant, BHT, at all concentrations except for10 mg/ml (EDA 45%). Compared to BHT, the MEFE showed statistically higher EDA level for all levels of mango leaf fermentation extract concentrations. The exact mechanism of antioxidant action by the mango leaf extract is unknown, although previous reports have suggested that the material itself could promote cell proliferation or impart a protective effect from external stimulants (27–29).

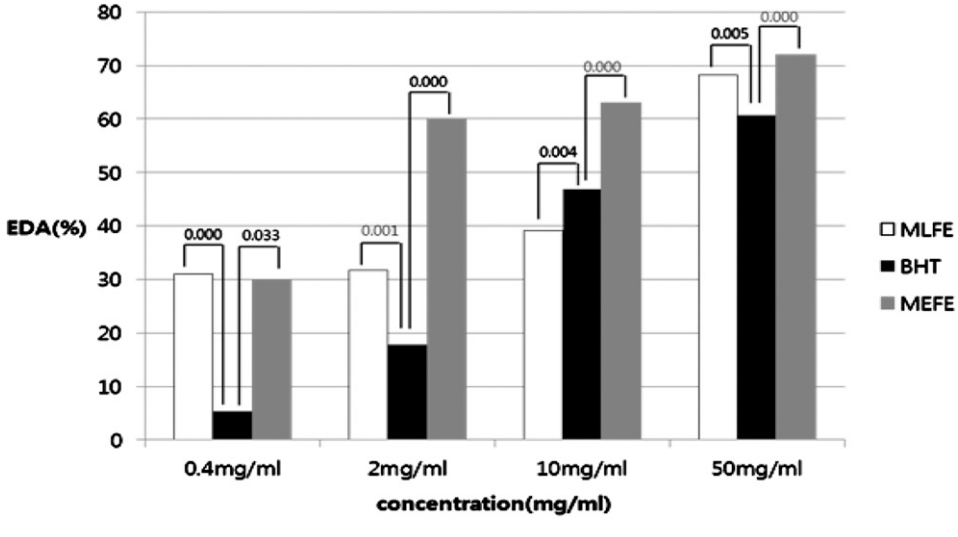

Figure 1. DPPH radical scavenging ability measured using percentage of EDA of the MLFE and the MEFE at different concentration levels (n = 3, mean ± S.D.).The synthetic antioxidant, BHT was used as control. P-values of *t*-tests comparing mean BHT and MLFE or MEFE are noted above.

CELL VIABILITY ESTIMATION USING MTT ASSAY

Using the MTT assay procedures performed in previous studies (30,31), this study evaluated cytotoxic effects of mango leaf fermented extracts. Figures 2 and 3 show the viabilities of RAW 264.7 cells for mango leaf fermentation extracts measured using the MTT assay. Compared to the control, the cell viability decreased from 97.70%, 93.26% to 85.82% as the MEFE concentrations increased from 0.5, 1.25 to 2.5 mg/ml (Figure 2). In the case of MLFE, cell viability measures were 102.78%, 94.88%, and 85.90% at concentrations of 0.5, 1.25, and 2.5 mg/ml, respectively, which clearly demonstrates a dose-dependent cytotoxicity effect (Figure 3). At 0.5 mg/ml, the MEFE and the MLFE both demonstrated similar safety profile to control. The higher concentrations of fermented extracts (1.25 and 2.5 mg/ml) showed significant increase in cytotoxicity expressed in decreasing EDA% in contrast to control.

REACTIVE OXYGEN SPECIES

Macrophages produce excessive amounts of ROS (32,33). Figures 4 and 5 show the antioxidant effects of the MLFE and the MEFE evaluated by estimating the degree of ROS generation in LPS-stimulated macrophages using flow cytometry. Untreated cells showed weak DCF fluorescence, indicating the minimal level of ROS generation. LPS treatment induced a large amount ROS, evidenced by the significant rightward shift of DCF fluorescence. Both MLFE and MEFE showed a concentration-dependent response. MLFE showed marginal inhibitory effect on ROS generation at a concentration of 0.125 mg/ml (Figure 4). However, the MEFE showed significant inhibitory effects of ROS generation, evidenced by the remarkable leftward shift of DCF fluorescence. At a concentration of 0.05 mg/ml, the MEFE showed the strongest antioxidative effects (Figure 5). The results show EM fermentation enhances the antioxidative effects of mango leaf extract.

ESTIMATION OF TYROSINASE INHIBITORY ACTIVITY

Tyrosinase inhibition may play an important role in the cosmetic industry and in the development of skin-whitening products (34). Tyrosinase is an enzyme involved in

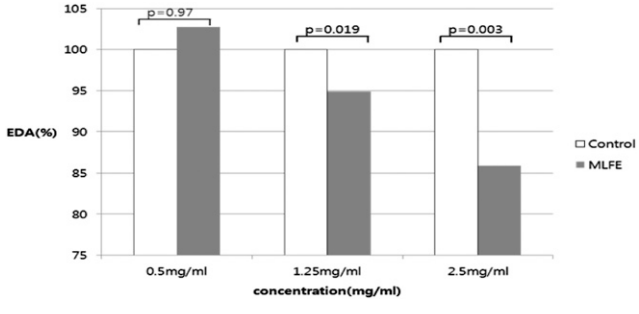

Figure 2. Effects of mango leaf extracts MLFE vs. Control on RAW 264.7 cell viability measured by MTT assay. Data expressed as mean ± S.D. (n = 3).

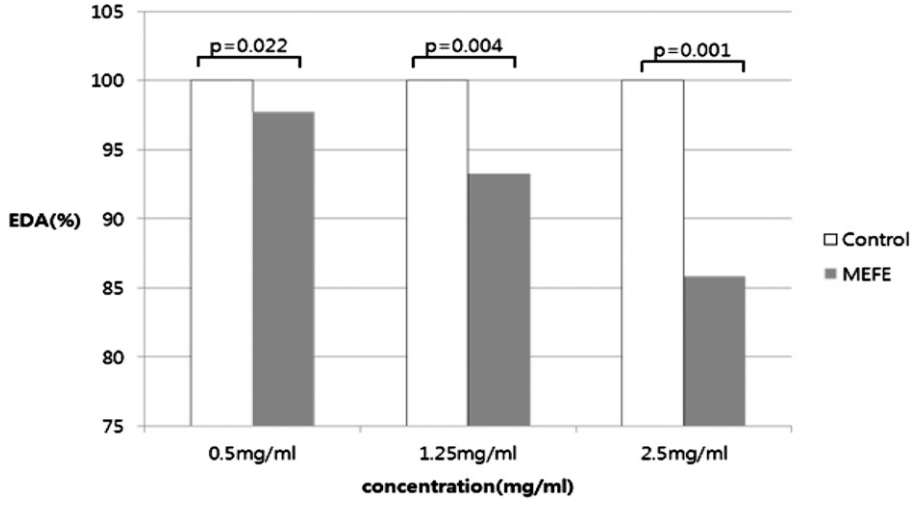

Figure 3. Effects of mango leaf extracts MEFE vs. Control on RAW 264.7 cell viability measured by MTT assay. Data expressed as mean ± S.D. (n = 3).

production of melanin, which increases the immunity of the skin but may also accelerate skin damage in excessive amounts (35).

Table III shows the increase in tyrosinase inhibitory activity from 7.89% to 15.78% and from 8.64% to 29.82%, with increasing concentrations of the MLFE and the MEFE, respectively. Both fermentation extracts had increasingly stronger tyrosinase inhibitory activity with increasing concentrations. All concentrations of the MEFE demonstrated statistically significant increase in tyrosinase inhibitory activity compared to the MLFE (Figure 6).

NITRITE-SCAVENGING ABILITY

Nitrites can react with secondary amines (e.g., having a substituted hydrocarbon group R for two of the hydrogen atoms, and forming a R1–R2–NH compound), to produce a

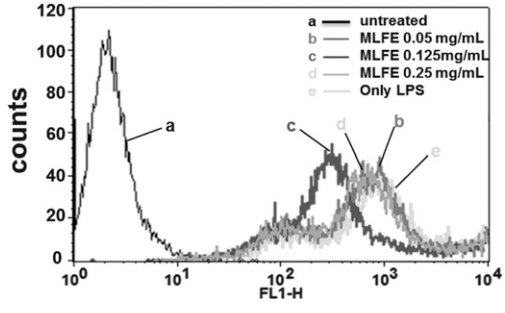

Figure 4. Effects of MLFE on the generation of ROS in LPS-stimulated macrophage cells. The data are representative of three independent experiments.

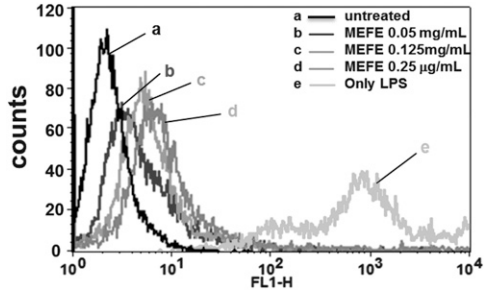

Figure 5. Effects of MEFE on the generation of ROS in LPS-stimulated macrophage cells. The data are representative of three independent experiments.

carcinogenic substance, nitrosamine (R1–N–R2–N=O). However, nitrite scavenging can effectively suppress the formation of these carcinogenic compounds (36). Higher concentrations of phenolic compounds at lower pH values increase nitrite scavenging, whereas this effect diminishes at high pH (37).

Table IV shows that the nitrite-scavenging activity increased from 0.08 ± 0.12% at 0.4 mg/ml to 94.97 ± 0.06% at 50 mg/ml in the MLFE, and from 0.19 ± 0.08% at 0.4 mg/ml to 95.89 ± 0.03% at 50 mg/ml in the MEFE, thereby demonstrated that higher concentrations of fermentation extracts resulted in enhanced scavenging ability. EM-fermentation imparted a superior effect versus lactobacillus fermentation at all concentrations. The statistically significant differences were observed between the two fermentation agents except at a low concentration level of 0.4 mg/ml, in supporting suppression of nitrosamine production by mango leaf extracts. Mango leaves, which are high in phenols, effectively break down nitrites at a low pH of 2.5.

CONCLUSIONS

We evaluated the concentration-dependent antioxidative properties of lactobacillus and EM fermentations of mango leaf extracts. We measured DPPH-radical inhibitory activity and ROS production to estimate the concentration-dependent activity of lactobacillus and EM fermentations. It was confirmed that the antioxidative activity significantly increased with increasing concentrations of the sample. Total phenolic and flavonoid contents also increased in a dose-dependent manner. EM fermentation exhibited higher antioxidative activity than lactobacillus fermentation, as well as the synthetic antioxidant, BHT.

Table III
Tyrosinase Inhibitory Activity in Percentage for MLFE and the MEFE (n = 3)

	Tyrosinase inhibition(%)			
MLFE concentration (mg/ml)	MLFE	MEFE	*t*	*p*-Value
0.4	7.89 ± 0.90	8.64 ± 0.89	−2.387	0.037
2	10.52 ± 1.80	18.42 ± 2.1	−4.943	0.003
10	11.40 ± 2.30	23.68 ± 1.8	−7.282	0.001
50	15.78 ± 1.5	29.82 ± 0.7	−14.68	0.000

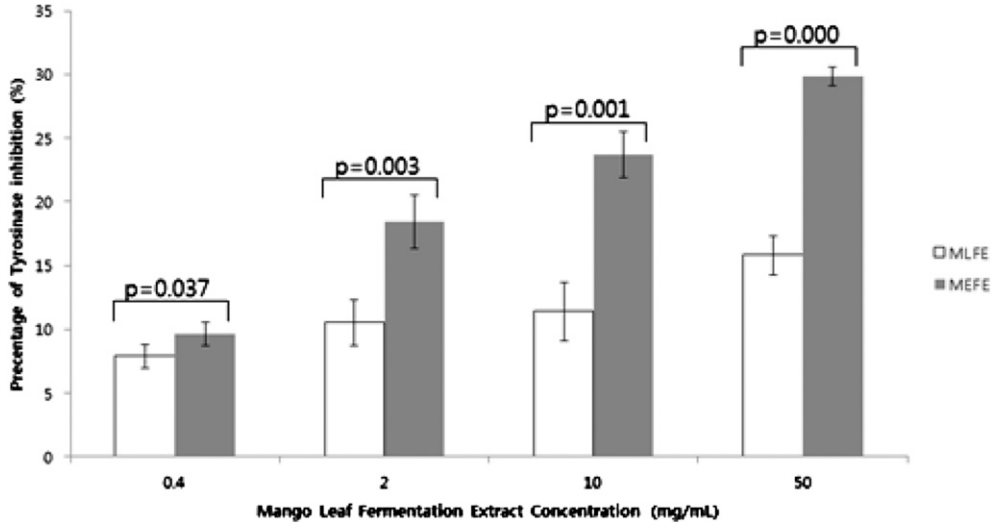

Figure 6. Inhibitory effects of the MLFE and MEFE on tyrosinase activity (%). Tyrosinase was used for the *in vitro* melanin synthesis. Data presented are mean ± S.D. (n = 3).

Both lactobacillus and EM fermentations showed increasing tyrosinase inhibitory activities with increasing concentrations of the extract.

Antioxidant activity increased with increasing concentrations of the extracts. EM fermentations showed a higher antioxidant effect over lactobacillus fermentations through increased inhibition of ROS production. Cytotoxic effect also augmented with increasing concentration of fermentation extracts as well. Both fermentations demonstrated stable cytotoxicity level demonstrated as cell viability percentage ≥85% at different concentrations tested (38). The results of this study show that mango leaf extracts fermented with lactobacillus and EM impart positive effects on RAW 264.7 cells by reducing ROS production as well as enhancing cytotoxicity-related stability and antioxidant activity.

One of the limitations of this study is differential time duration required to ferment each culture; the difference of 7 days for EM activation versus 2 days for Lactobacillus fermentation remains a significant challenge to the equivalent efficacy assumption despite equal seed culture concentration used to prepare the MLFE and the MEFE. Longer duration required for optimal fermentation should be balanced against additional antioxidant effect obtained from the use of EM. Another limitation is in the

Table IV
Nitrite Scavenging Abilities (%) of the MLFE and the MEFE at pH 2.5 (n = 3)

	Nitrite scavenging activity (%)			
MLFE concentration (mg/ml)	MLFE	MEFE	*t*	*p*-Value
0.4	0.08 ± 0.12	0.19 ± 0.08	−1.659	0.086
2	2.21 ± 0.07	26.03 ± 0.17	−182.5	0.000
10	46.51 ± 0.06	84.93 ± 0.01	−589.0	0.000
50	94.97 ± 0.06	95.89 ± 0.03	−12.54	0.000

range of cytotoxicity tests conducted. H.N. Kwon (38) reported the safety threshold of cell viability at 85% when comparing antioxidant effects of various mulberry root bark extracts. The mango leaf fermented extract concentrations for cell viability testing in this study ranged up to 2.5 mg/ml where the previous study's safety thresholds of 85% were demonstrated. Therefore, further cell viability tests beyond the ranges tested in this study would be necessary to establish the safety threshold for mango leaf fermented extracts.

In conclusion, the antioxidative effects of microbial fermentation products containing mango leaf extracts may serve as a basis for their use in developing a variety of health foods and fermentation-based beauty products.

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