Changes in the antioxidant capacity and phenolic compounds of *maesil* extract during one-year fermentation

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Abstract This study aimed to identify the effects of soaking time and fermentation temperature and period on the total phenolics, total flavonoids, and antioxidant activity of *maesil* extract. Green *maesil* fruits were fermented with brown sugar at 15 or 25°C for one year. After soaking *maesil* for 90 days, the fruits were removed and the remaining liquid was further ripened for 275 days, whereas other samples continued to ferment with *maesil* until 365 days. Total phenolics, total flavonoids, and antioxidant activities of the extracts fermented with *maesil* for one year were higher compared with those wherein the fruits were removed at day 90. The total phenolic and total flavonoid contents were relatively higher at 15°C, whereas the ABTS and ferric-reducing antioxidant power (FRAP) activities were significantly higher at 25°C. The total phenolic and total flavonoid contents highly correlated with the antioxidant activities. These results show that fermentation temperature plays a critical role in enhancing the phenolic compounds and antioxidant activity of *maesil* extract.

Keywords: *maesil* extract, fermentation, antioxidant activity, total phenolics, total flavonoids

Introduction

Maesil is a fruit of *Prunus mume* Sieb. et Zucc., i.e., a member of the Rosaceae family. It is also known as Japanese apricot *Ume* and Chinese *Mei*. *Maesil* has attracted considerable attention both as a food and a medicine due to its antioxidant activity (1–3).

Oxidative stress from the reactive oxygen species is a central component of chronic and degenerative diseases including cancer, aging, and neurodegenerative diseases (4–6). Phenolic compounds are natural antioxidants that contribute to the prevention of the degenerative diseases. Previous investigators have reported the antioxidant activity of *maesil* extracts using methanol, ethanol, or ethyl acetate (2,3,7,8). However, *maesil* is mostly fermented under aqueous conditions to enhance the health-promoting effect. Therefore, *maesil* products are mostly composed of water and hydrophilic compounds extracted from the *maesil* during fermentation. Accordingly, the antioxidant activity of *maesil* extracts extracted with organic solvents could be different from that of the other *maesil* products consumed by people. Phenolic compounds, such as chlorogenic acid, caffeic acid, *p*-coumaric acid, and ferulic acid, have been found in Japanese *Ume* and Chinese *Mei* (2,9). The phenolic compound contents can be influenced by the manufacturing practices and fermentation periods. *Maesil* extracts are fermented up to a year at a household level using different practices. Nevertheless, to the best of our knowledge, the changes in antioxidant activity and phenolic

compounds of *maesil* extract during fermentation have not been reported.

This study aimed to monitor the total phenolics, total flavonoids, and antioxidant activity in *maesil* extract during the fermentation of *maesil* and brown sugar at 15 or 25°C for one year in order to elucidate the effects of soaking time, fermentation temperature, and fermentation period.

Materials and Methods

Chemicals DPPH, ABTS, TPTZ, Folin–Ciocalteu phenol reagent, ethanol, potassium persulfate ($K_2S_2O_8$), monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), aluminum chloride ($AlCl_3$), sodium bicarbonate ($NaHCO_3$), hydrogen chloride (HCl), ferric chloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), sodium nitrate ($NaNO_3$), sodium hydroxide (NaOH), acetic acid, ethyl acetate, ascorbic acid, and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation Green *maesil* fruits were purchased from a local farm (Hadong, Korea) in May 2014. The fruits were washed with distilled water, wiped with a cotton cloth, and stored at room temperature to remove the remaining surface water. Furthermore, approximately 400 g was mixed with brown sugar at a ratio of 1:1

(w/w) and placed into a 1 L transparent plastic bottle. Forty replicate bottles were placed into a thermostatic incubator (VS-1203PFC; Vision Scientific Co., Ltd., Daejeon, Korea) at 15 or 25°C. The total phenolics, total flavonoids, and antioxidant activities of the *maesil* extracts were determined every 15 days for the first 90 days. At day 90, the fruits were removed and the remaining liquid was ripened for 275 days in the same bottle. Other samples continued to ferment with *maesil* until 365 days in the same bottle. From day 90 onwards, the total phenolics and antioxidant activities were determined every 30 days.

Total phenolic content determination The total phenolics were evaluated using a colorimetric method by Singleton and Rossi (10). The extracts were diluted 10-fold with distilled water and 0.5 mL of each was mixed with 0.9 mL of 10-fold diluted Folin–Ciocalteu reagent and 3.6 mL of saturated NaHCO₃ solution. The mixtures were then incubated at room temperature for 1 h, and the absorbance was measured at 700 nm (Biochrom Libra S22; Biochrom, Cambridge, UK). The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per mL of the extract. All experiments were repeated three times.

Total flavonoid content determination The total flavonoid content

was determined using the AlCl₃ colorimetric method by Debnath *et al.* (3) and Marinova *et al.* (11). Standard solutions of catechin for calibration were prepared at concentration of 0, 20, 40, 60, 80, and 100 µg/L in distilled water. The extracts (100 µL) were mixed with 400 µL of distilled water and 60 µL of 5% NaNO₃ solution. After 5 min at room temperature, 120 µL of 10% AlCl₃ solution and 400 µL of 1 N NaOH solution were added into the mixture. Absorbance was measured at 510 nm. Total flavonoid content was expressed as mg catechin equivalent (CE)/L. All experiments were repeated three times.

DPPH radical scavenging activity determination The antioxidant activity of the *maesil* extract was determined using the method by Xia *et al.* (2) with some modifications. Two mL of the 10-fold diluted samples was mixed with 2 mL of 0.2 mM DPPH in ethanol. The mixture was vigorously shaken and allowed to stand at room temperature for 30 min in the dark, and the absorbance was measured at 517 nm (Biochrom Libra S22; Biochrom). The control was prepared using distilled water without the *maesil* extract. Reference positive controls were 0.1% ascorbic acid and 0.1% gallic acid. The percent scavenging activity of the DPPH radical was calculated using the following equation.

Fig. 1. Changes in the total phenolics of *maesil* extract during one year fermentation at 15 and 25°C. With *maesil* indicates that the *maesil* fruits were soaked in the liquid throughout the year. Without *maesil* indicates that the *maesil* fruits were taken out of the bottle at day 90 and then ripened until day 365. The total phenolics are expressed as a gallic acid equivalent (GAE).

$$\text{DPPH radical scavenging activity (\%)} = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

In this equation, A_{blank} and A_{sample} are the control and extract absorbance, respectively. All experiments were conducted in triplicate.

ABTS radical scavenging activity determination The ABTS^{•+} assay followed the procedure of Shi *et al.* (7) with slight modifications. The extracts were diluted 10-fold with distilled water and the ABTS radical cation (ABTS^{•+}) was dissolved in distilled water to a 7 mM ABTS^{•+} solution. ABTS^{•+} radicals were formed by the reaction of ABTS solution with 2.45 mM K₂S₂O₈. The mixture was incubated in the dark at room temperature for 24 h. The ABTS^{•+} solution was 5-fold diluted using 0.1 M phosphate buffer (pH 7.0) to reach 0.70±0.02 absorbance units at 734 nm. The ABTS solution (3.6 mL) and sample (0.4 mL) were mixed, and the absorbance was measured at 734 nm for 10 min. Positive reference controls were 0.1% ascorbic acid and 0.1% gallic acid. The ABTS radical scavenging percentage of *maesil* extracts was calculated as follows.

$$\text{ABTS radical scavenging activity (\%)} = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

In this equation, A_{blank} and A_{sample} are the absorbance of the control and extract, respectively. All experiments were conducted in triplicate.

Ferric-reducing antioxidant power (FRAP) assay This assay followed

Ardestani and Yazdanparast's method (12) with slight modifications. FRAP reagent was freshly prepared using 300 mM acetate buffer (pH 3.6), 20 mM FeCl₃, and 10 mM tripyridyltriazine (TPTZ) in 40 mM HCl. These three solutions were mixed together at a ratio of 10:1:1 (v/v/v). Standard FeSO₄ solution (1,000 μM) prepared using distilled water and used for calibration. Working solutions of 0, 100, 300, 500, 900, and 1,000 μM were added to 3 mL of FRAP reagent and incubated at 37°C for 30 min; furthermore, the increases in absorbance at 593 nm were measured. The extracts (0.1 mL) were mixed with 3 mL of FRAP reagent, and after 30 min incubation at 37°C, the absorbance was measured at 593 nm. The antioxidant capacity based on the ability to reduce ferric ions in the sample was calculated from a linear calibration curve and expressed as mM FeSO₄ equivalents per mL of sample. All experiments were repeated three times.

Statistical analysis All statistical analyses were performed using the SPSS IBM version 21.0 (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA). Pearson's correlation analysis test was used to evaluate the significance of correlations. Differences between two temperature groups were analyzed for statistical significance using Student's *t*-test. Statistical significance is expressed as **p*<0.05, ***p*<0.01, and ****p*<0.001.

Fig. 2. Changes in the total flavonoids content of *maesil* extract during one-year fermentation at 15 and 25°C. With *maesil* indicates that the *maesil* fruits were soaked in the liquid throughout the year. Without *maesil* indicates that the *maesil* fruits were taken out of the bottle at day 90 and then ripened until day 365. Total flavonoid is expressed as a catechin equivalent (CE).

Results and Discussion

When *maesil* extract was manufactured at individual houses, the fruits are routinely fermented up to one year at ambient temperature (25°C) or a relatively cooler pantry (15°C). The fruits are mostly soaked in sugar for 90 days and then removed from the jar. However, some households continue the fermentation up to one year in order to improve the flavor and browning of the extract. Therefore, the *maesil* fruits were fermented at two different temperatures (15 and 25°C) up to one year in this study.

Total phenolic content The total phenolic contents in *maesil* extracts ranged from 1.3 to 139.2 µg GAE/mL (Fig. 1). It is lower than the result of Lee *et al.* (13), which demonstrated that the total phenolics in *Prunus mume* concentrate ranged from 806.6 to 1,768 µg of GAE/mL. Debnath *et al.* (3) found that the total phenolics in water extract of dried *Prunus mume* fruit powder were 67.8 µg GAE per g of dry weight fruit. Xia *et al.* (2) reported that polyphenols, such as chlorogenic acid (55.1 mg/g), neochlorogenic acid (20.3 mg/g), and cryptochlorogenic acid (26.7 mg/g), were found in dried powder of the ethyl acetate extract of *Prunus mume* fruits. Phenolic compounds including caffeic acid, *p*-coumaric acid, and ferulic acid were

identified in *Ume* (9). The total phenolic contents were higher in the extracts fermented with *maesil* throughout the fermentation period compared to those ripened after the removal of the fruit at day 90. This indicates that phenolic compounds continued to be released from *maesil* during fermentation. Total phenolic contents were higher at 25°C from the starting point to 45 days. After then, total phenolics were higher at 15°C than 25°C. Yu *et al.* (14) reported the changes in the total phenolics in *Prunus mume* fruits and fermentation broth at 30°C after the inoculation with *Lactobacillus fermentum* into blanched *Prunus mume* fruits. Water-soluble phenolics rapidly decreased in *Prunus mume* fruits for the initial 2 days, whereas it increased in the fermentation broth. This indicates that phenolics diffused into the broth from *Prunus mume* fruits. The total phenolics showed a slight fluctuation in the broth during the next 8 days. This trend is same as the trend observed in this study. Earlier investigations have found that fermentation of fruit juice with lactic acid bacteria degraded large polymeric phenolics and increased the content of small phenolics (15,16). These findings suggest that the fermentation temperature would affect the growth of lactic acid bacteria, which concomitantly resulted in the change of the total phenolic contents through the breakdown of polymeric phenolics in the *maesil* extract.

Fig. 3. Changes in the DPPH radical scavenging activity of *maesil* extract during one year fermentation at 15 and 25°C. With *maesil* indicates that the *maesil* fruits were soaked in the liquid throughout the year. Without *maesil* indicates that the *maesil* fruits were taken out of the bottle at day 90 and then ripened until day 365. (A) DPPH radical scavenging activity of the extract fermented with *maesil* is shown; (B) DPPH radical scavenging activity of the extract from which *maesil* was removed at day 90 is shown.

Fig. 4. Changes in ABTS radical scavenging activity of *maesil* extract during one year fermentation at 15 and 25°C. With *maesil* indicates that the *maesil* fruits were soaked in the liquid throughout the year. Without *maesil* indicates that the *maesil* fruits were taken out of the bottle at day 90 and then ripened until day 365. (A) ABTS radical scavenging activity of the extract fermented with *maesil* is shown; (B) ABTS radical scavenging activity of extract where *maesil* was removed at day 90 is shown.

Total flavonoid content Total flavonoid contents of the extracts ranged from 4.81 to 266.99 mg CE/L (Fig. 2). These results were similar to the findings of Lee *et al.* (13) wherein the total flavonoids in *Ume* concentrate ranged from 132.2 to 222.5 mg CE/L. Debnath *et al.* (3) found that the total flavonoids in water extract of dried *Prunus mume* fruit powder were 442.0 mg equivalent/L. The matrix of the entire fruit could reduce the diffusion rate of flavonoids from the inner part of fruit. Total flavonoids contents increased at a relatively slower rate compared with the levels of the total phenolics. Total flavonoid contents in the extracts with *maesil* were higher at 15°C.

Radical scavenging activity DPPH and ABTS assays were used to evaluate the free radical scavenging activity of antioxidants in foods. As presented in Fig. 3A and 3B, DPPH radical scavenging activities showed a trend to increase with fermentation time, reaching a maximum value of 76.7%. The ABTS radical scavenging activities of *maesil* extract gradually increased for a year, which ranged from 25.7 to 80.5% (Fig. 4A and 4B). The ABTS radical scavenging activities of extracts fermented at 25°C were significantly higher than those at 15°C. Lee *et al.* (17) reported that the ascorbic acid content in *maesil* is 405.3 mg%. Ascorbic acid and phenol compounds were found to

have high free radical-scavenging activity (18).

Reducing ability The FRAP assay is used to detect the natural antioxidant activity wherein the compound donates electrons to reduce TPTZ-Fe (III) to TPTZ-Fe (II). As shown in Fig. 5, *maesil* extracts exhibited a good reducing power with higher values at 25°C compared with those at 15°C. The FRAP values of *maesil* extract dramatically increased at 15°C until day 30, and then remained below 600 mmol/mL. In comparison, the FRAP values of *maesil* extract at 25°C increased for 90 days, then reached 800 mmol/mL. The FRAP values of the extracts soaked with the fruits for one year gradually increased throughout fermentation.

Correlation between the total phenolic, total flavonoid and antioxidants The total phenolic and total flavonoid contents highly correlated with the antioxidant activities (Table 1). It seems that an enzymatic browning reaction occurred using phenolic compounds, subsequently resulting in the decrease of antioxidant activity. Correlation coefficients were higher in the extracts soaked with the fruit for one year. Yu *et al.* (14) reported that the total phenolics contents and antioxidant capacity (ORAC value) were correlated in

Fig. 5. Changes in FRAP (ferric reducing antioxidant power) of *maesil* extract during one year fermentation at 15 and 25°C. With *maesil* indicates that the *maesil* fruits were soaked in the liquid throughout the year. Without *maesil* indicates that the *maesil* fruits were taken out of the bottle at day 90 and then ripened until day 365. Error bars represent the standard deviations from the triplicate determinations.

Table 1. Pearson correlation coefficients between the total phenolics, total flavonoid, and antioxidant activity in *maesil* extract

		Total flavonoid	DPPH	ABTS	FRAP ¹⁾
With <i>maesil</i>	Total phenolics	0.822** ²⁾	0.796**	0.701**	0.569**
	Total flavonoid		0.819**	0.690**	0.622**
	DPPH			0.843**	0.799**
	ABTS				0.929**
Without <i>maesil</i>	Total phenolics	0.770**	0.661**	0.693**	0.534**
	Total flavonoid		0.681**	0.710**	0.641**
	DPPH			0.757**	0.710**
	ABTS				0.857**

¹⁾FRAP represents the ferric reducing antioxidant power.

²⁾** indicates a significant difference at $p < 0.01$.

the fermented broth of *Prunus mume* fruits. In addition, previous investigations have found a good correlation between antioxidant activity and total phenolics in various fruit fermented products (16,19,20). These results suggest that phenolic compounds in *maesil* extracts play an important role in antioxidant activity.

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Disclosure The authors declare no conflict of interest.

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