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Toxicity and Toxicokinetics of Amygdalin in Maesil (*Prunus mume*) Syrup: Protective Effect of Maesil against Amygdalin Toxicity

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Supporting Information

ABSTRACT: Maesil (*Prunus mume*, green plum)-based products have been widely used in Asian cooking, which may contain amygdalin enzymatically converted to hydrogen cyanide after oral ingestion. In this study, the toxicity of Maesil syrups matured with and without Maesils was evaluated by focusing on relationship between amygdalin toxicity and its metabolic change. The cytotoxicity of amygdalin was highly related to its metabolites converted by β-glucosidase, and the metabolic change was retarded in Maesil syrup. Toxicokinetics revealed extremely low oral absorption and short half-life of amygdalin standard and Maesil syrups, and delayed metabolic change of amygdalin in Maesil syrup was found. It seems that complex Maesil syrup components play roles against amygdalin degradation. Maesil syrup matured with Maesils had higher total polyphenols, lower amygdalin, and shorter half-life in bloodstream than Maesil syrup without Maesils, suggesting more safety benefit. No significant oral toxicity of Maesil syrups was found after 14-day repeated administration, implying their safety.

KEYWORDS: *amygdalin, metabolite, Maesil syrup, toxicity, toxicokinetics, oral toxicity*

INTRODUCTION

Amygdalin (D-mandelonitrile-β-gentiobioside) is a cyanogenic glycoside isolated from the seeds of bitter almonds (*Prunus dulcis*).[1−3] It is also found in many food plants, such as apricots, cherry, peaches, apple, plum, various beans, and other rosaceous plants, and is present in more than 2500 species.[3−5] Amygdalin is degraded into prunasin by digestive enzymes after oral ingestion, and then, decomposed into glucose and mandelonitrile by an endogenous enzyme, β-glucosidase, which is found in food plants and in animal's small intestine.[6−8] Finally, mandelonitrile is further hydrolyzed to benzaldehyde and hydrogen cyanide (HCN).[9] The toxic metabolite of amygdalin, HCN inhibits oxygen consumption by mitochondrial cytochrome oxidase and blocks oxidative metabolism, leading to cell death.[10,11] It is known that cyanogenic glycosides play roles as defense compounds in plants,[12,13] whereas a high intake of amygdalin can induce acute cyanide poisoning, which results in hypotension, paralysis, coma, and even death.[14−16] Long-term intake of cyanogenic plants can cause subacute cyanide poisoning, with symptoms such as anxiety, headache, dizziness, and confusion.[17,18]

Orally ingested amygdalin is estimated to be about 40-times more toxic than its intravenously injected form because amygdalin is enzymatically converted to its toxic metabolite, HCN in the gastrointestinal tract.[15,19,20] On the other hand, the conversion of amygdalin to HCN is known to be promoted by the intake of amygdalin with foods containing β-glucosidase such as almonds, apricot kernels, bean sprouts, peaches, celery, and carrots.[21] Moreover, concurrent intake of amygdalin with high doses of vitamin C, mineral acids, and heat treatment can enhance the metabolic conversion of amygdalin.[22] However, HCN is a volatile compound, which can be easily eliminated during food processing at more than 26 °C.[23,24]

Amygdalin is also found in economically important food plants, Korean green plums, so-called Maesils (*Prunus mume*, Chinese green plum or Japanese ume). Korean green plums have been widely applied for Maesil-ju (green plum wine), Maesil syrup (green plum syrup), Maesil-jangajji (green plum pickles), and Maesil-cha (green plum tea) in the last 10 years. Along with a wide range of applications of Maesil-based products in cooking and recipes, public concern about their potential toxicity is growing, although humans generally do not consume Maesil seeds. However, a systematic study on the determination of amygdalin and its metabolites in Maesil-based products has not been performed, and information about *in vitro* and *in vivo* toxicity of Maesil-based products is extremely limited. Moreover, toxicokinetics of amygdalin and its metabolites after oral ingestion of amygdalin-containing food has not been well determined.

Accordingly, the aim of this study was to investigate the relationship between toxicity and metabolic change of amygdalin in Maesil syrup. The effects of amygdalin metabolites on cytotoxicity were compared by treating amygdalin in the presence of β-glucosidase. Moreover, toxicokinetics of amygdalin and Maesil syrups matured with and without Maesils was evaluated after a single dose oral administration to rats to obtain information about oral absorption and half-life in the body. Finally, 14-day repeated oral toxicity of Maesil syrups with and without Maesils was performed in rats to determine the potential toxicity of amygdalin-containing food.

MATERIALS AND METHODS

Materials. Amygdalin, prunasin, mandelonitrile, benzaldehyde, β-glucosidase (G4511, 22.60 U/mg), Folin-Ciocalteu's phenol

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reagent, gallic acid, $NaNO_2$, quercetin, and crystal violet solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water (high performance liquid chromatography (HPLC) grade), acetonitrile, Na_2CO_3, and methanol were supplied from Samchun pure chemical Co., Ltd. (Pyeongtaek, Gyeonggi-do, Republic of Korea). NaOH and minimum essential medium (MEM) were purchased from Duksan pure chemicals Co., Ltd. (Ansan, Gyeonggi-do, Republic of Korea) and Welgene Inc. (Gyeongsan, Gyeongsangbuk-do, Republic of Korea), respectively. Water-soluble tetrazolium salt-1 (WST-1) and 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) were bought from Roche Molecular Biochemicals (Mannheim, Germany) and Molecular Probes Inc. (Eugene, OR, USA), respectively. Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were obtained from Invitrogen (Carlsbad, CA, USA). Maesil syrups matured with and without Maesils for 1 year were provided by SlowFood Farming Corp (Gyeongsangnam-do, Republic of Korea); Maesil syrup matured with Maesils for 3 months and then further matured without Maesils for 9 months was used as Maesil syrup without Maesils, and Maesil syrup continuously matured with Maesils for 1 year was used as Maesil syrup with Maesils (Figure 1).

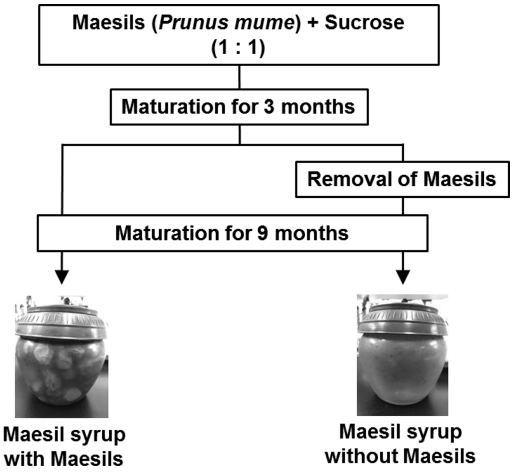

Figure 1. Flowchart for preparation of Maesil syrups matured with and without Maesils.

HPLC Analysis. Concentrations of amygdalin and its metabolites in Maesil syrups were quantified by HPLC using Agilent 1100 series (Agilent Technologies, Santa Clara, CA, USA), equipped with diode-array detector (DAD), on a Supelcosil LC-18 (250 mm × 4.6 mm i.d., 5 μm, Supelco Inc., Bellefonte, PA, USA). The wavelength of UV detector was set at 215 nm. The mobile phase was water/acetonitrile (80:20, v/v, Samchun pure chemical Co., Ltd.) and flow rate was set at 1 mL/min. Column temperature was maintained at a constant 25 °C and injection volume of sample was 20 μL. The samples were analyzed after filtering through a syringe filter (Advantec, Tochigi, Japan). The metabolic changes were monitored by incubating amygdalin (10 mg/mL) or Maesil syrup (matured without Maesils) in the presence of β-glucosidase (3.7 U/mL). After designated incubation times, two volumes of acetonitrile were added, and centrifuged at 10 000 × *g* for 3 min to precipitate proteins. The contents of amygdalin or metabolites in supernatants were analyzed by HPLC as described above.

Saccharide concentrations in Maesil syrups were quantified by HPLC using a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan), equipped with refractive index detector (RID), on a Hypersil APS-2 column (250 mm × 4.6 mm i.d., 5 μm, Thermo Fisher Scientific, MA, USA). The mobile phase was acetonitrile/water (80:20, v/v, Samchun pure chemical Co., Ltd.) and flow rate was set at 1 mL/min. Column temperature was maintained at 40 °C. Each experiment was repeated three times on separate days.

Total Phenolic Content. Total phenolic contents of Maesil syrups were measured with Folin-Ciocalteu's phenol reagent (Sigma-Aldrich). Briefly, 20 μL of 50% Folin-Ciocalteu's phenol reagent was added to 80 μL of Maesil syrups and allowed to stand for 5 min at room temperature in the dark. Then 100 μL of 2% Na_2CO_3 (Samchun pure chemical Co., Ltd.) was added. After incubation for 30 min at room temperature in the dark, absorbance was measured at 750 nm using a plate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA, USA). Gallic acid (Sigma-Aldrich) was used as a standard. The total phenolic contents of the Maesil syrups were expressed as gallic acid equivalent (GAE)/mL.

Total Flavonoid Content. Total flavonoid contents of Maesil syrups were determined using a spectrophotometric method. Briefly, 30 μL of 5% $NaNO_2$ (Sigma-Aldrich) was added to 50 μL of Maesil syrups. After incubation for 5 min, 60 μL of 2% $AlCl_3{\cdot}6H_2O$ (Sigma-Aldrich) was added and incubation was continued for 6 min at room temperature in the dark. Finally, 100 μL of 1 N NaOH (Duksan pure chemicals Co., Ltd.,) was added. After incubation for 11 min at room temperature in the dark, absorbance was measured at 510 nm using a plate reader (SpectraMax M3, Molecular Devices). Quercetin (Sigma-Aldrich) was used as a standard and results were expressed as quercetin equivalent (QE)/mL.

Cell Culture. Human intestinal epithelial INT-407 cells were provided by Dr. Tae-Sung Kim at Korea University (Seoul, Republic of Korea) and liver epithelial HepG2 cells were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). The cells were cultured in MEM (Welgene Inc.) under a humidified atmosphere (5% CO_2/95% air) at 37 °C. The medium was supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Welgene Inc.).

Cell Proliferation. The effect of materials on cell proliferation was measured with water-soluble tetrazolium salt-1 (WST-1; Roche, Molecular Biochemicals). Cells (1×10^4 cells/100 μL) were incubated with amygdalin (1, 2.5, 5, and 10 mg/mL) in the absence or presence of β-glucosidase (3.7 U/mL) for 24 h. Then 10 μL of WST-1 solution (Roche) was added to each well, and cells were further incubated for 4 h. Absorbance was measured using a plate reader at 440 nm (SpectraMax M3, Molecular Devices). Cells incubated without amygdalin were used as controls.

Clonogenic Assay. INT-407 cells (5×10^2 cells/2 mL) were seed in six-well plates and incubated overnight at 37 °C under a 5% CO_2 atmosphere. The medium in the plates was then replaced with fresh medium containing various concentrations of amygdalin with and without β-glucosidase, and incubation was continued for 7 days. For colony counting, cells were washed with phosphate buffered saline (PBS), fixed with 90% methanol, and then, stained with 0.5% crystal violet solution (Sigma-Aldrich) for 1 h. After cell washing with distilled and deionized water (DDW) and air-drying, colonies consisted of more than 50 cells were counted. Each experiment was done in triplicate and colony numbers without test materials were used as controls.

ROS Generation. Reactive oxygen species (ROS) levels were monitored using a peroxide-sensitive fluorescent probe, 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) (Molecular Probes Inc.), according to the manufacturer's guidelines. Cells (1×10^4 cells/100 μL) were incubated with amygdalin in the presence or absence of β-glucosidase for 24 h under standard condition as described above, washed with PBS, and incubated with 40 μM H_2DCFDA for 60 min at 37 °C. After washing with PBS, DCF fluorescence was immediately measured using a fluorescence microplate reader (SpectraMax M3, Molecular Devices). Excitation and emission wavelengths were 495 and 518 nm, respectively. Cells not treated without amygdalin were used as controls.

Apoptosis. Apoptotic cells were identified with annexin V-FITC and a dead cell marker, propidium iodide (PI) according to the manufacturer's protocol (Invitrogen). INT-407 cells (1×10^6 cells/2 mL) were exposed to amygdalin (2.5 mg/mL) in the presence or absence of β-glucosidase for 24 h, detached by scraping, and washed with cold PBS. Cells were resuspended at a density of 1×10^6 cells/mL in 100 μL of binding buffer, and 100 μg/mL of working solution containing 5 μL of annexin V-FITC and 1 μL of PI were added. After 15 min at room temperature in the dark, 400 μL of binding buffer was added to each sample. Apoptosis was analyzed by flow cytometry (Beckman Coulter,

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Brea, CA, USA) for at least 10 000 events. Cells incubated without amygdalin were used as controls.

Animals. Six-week-old female Sprague−Dawley (SD) rats weighing 130−150 g were purchased from KOATECH (Pyeongtaek, Gyeonggi-do, Republic of Korea). Animals were housed in plastic laboratory animal cages in a ventilated room maintained at 20 ± 2 °C and 60 ± 10% relative humidity under a 12 h light/dark cycle. Water and commercial laboratory complete food were provided *ad libitum*. Animals were environmentally acclimated 7 days before treatment. All animal experiments were performed in compliance with the guideline issued by the Animal and Ethics Review Committee of Seoul Women's University (SWU IACUC−2017A-1).

Toxicokinetic Study. Six female rats per group (~200 g) were administered a single dose (250 mg/kg) of amygdalin in distilled water (DW) or Maesil syrups with and without Maesils (10 mL/kg) by oral gavage. Blood samples were collected from tail veins at 0, 0.08, 0.25, 0.5, 1, 2, 4, 6, and 10 h after administration, and centrifuged at 3000 × *g* for 15 min at 4 °C to obtain plasma. Two volumes of acetonitrile were added to the plasma samples and mixed vigorously to precipitate proteins. After centrifugation at 10 000 × *g* for 3 min, the supernatants were than collected and analyzed by HPLC. The following toxicokinetic parameters were estimated using Kinetica software (version 4.4, Thermo Fisher Scientific, Waltham, MA, USA); maximum concentration (C_{max}), time to maximum concentration (T_{max}), area under the plasma concentration−time curve (AUC), half-life ($T_{1/2}$), and mean residence time (MRT).

Fourteen-Day Repeated Oral Toxicity. Five female rats per groups were daily administered Maesil syrups with and without Maesils (10 mL/kg) or equivalent volume of DW as a control by oral gavage for 14 consecutive days. Changes in body weight, behaviors, specific symptoms, and food or water consumption were daily recorded after administration. At the end of experiment, animals were sacrificed by CO_2 euthanasia and organs were collected. Organo-somatic indices were calculated by the following formula: [weight of the organ (g)/total body weight (g)] × 100.

Blood samples were collected from the posterior vena cava for hematological and serum biochemical analysis, as described previously.[25] Autohematoanalyzer (ADVIA 2120i, Siemens, Tarrytown, NY, USA), coagulometer (ALC 7000, Werfen Medical, IL, USA), and biochemical analyzer (TBA-120FR, Toshiba, Otawara, Japan) were used for hematological, aggregation time, and biochemical analysis, respectively. Histopathological examination was performed on kidneys, liver, lungs, and spleen fixed with 10% neutral buffered formalin and stained with hematoxylin and eosin.

Statistical Analysis. Results were expressed as means ± standard deviations. One-way analysis of variance (ANOVA) with Tukey's test in SAS Ver. 9.4 (SAS Institute Inc., Cary, NC, USA) was used to determine the significances of intergroup differences. Statistical significance was accepted for P values of <0.05.

RESULTS

Characterization. The contents of amygdalin and its metabolites in Maesil syrups matured with and without Maesils are presented in Table 1. The results show that Maesil syrup without Maesils had higher amount of amygdalin than Maesil syrup with Maesils. An amygdalin metabolite, prunasin, was also detected in Maesil syrups, but other metabolites including mandelonitrile, benzaldehyde, and HCN were not found. Limits of detection (LOD) for amygdalin and prunasin were 0.33 and 0.39 μg/mL, and limits of quantification (LOQ) for the former and the latter were 1.00 and 1.17 μg/mL, respectively. Recovery ranged from 99.39 to 102.32% for amygdalin and from 96.30 to 104.37% for prunasin. High levels (~20%) of saccharides were found in both Maesil syrups, and major two saccharides were determined to be fructose and glucose. On the other hand, the contents of total polyphenols and total flavonoids were significantly higher in Maesil syrup with Maesils than in Maesil syrup without Maesils.

Table 1. Contents of Amygdalin, Its Metabolites, Saccharides, Total Polyphenols, and Total Flavonoids of Maesil Syrups with and without Maesils

constituents	Maesil syrups[c]	
	with Maesils	without Maesils
amygdalin (μg/mL)	134.98 ± 5.96a	166.82 ± 4.16b
prunasin (μg/mL)	26.05 ± 0.15a	31.57 ± 0.16b
fructose (mg/mL)	238.72 ± 0.72a	200.36 ± 8.10b
glucose (mg/mL)	210.80 ± 1.82a	205.28 ± 3.18b
total polyphenol (μg GAE/mL)[a]	461.30 ± 8.12a	376.41 ± 12.18b
total flavonoid (μg QE/mL)[b]	252.58 ± 17.21a	201.06 ± 2.62b

[a]Total polyphenol content was expressed as gallic acid equivalent (μg GAE/mL). [b]Total flavonoid content was expressed as quercetin equivalent (μg QE/mL). [c]Significant differences between Maesil syrups with and without Maesils indicated by different letters in the same row.

Metabolite Analysis. The metabolic changes of amygdalin standard or amygdalin in Maesil syrup in the presence of β-glucosidase are shown in Figure 2 and Supplementary Figure 1.

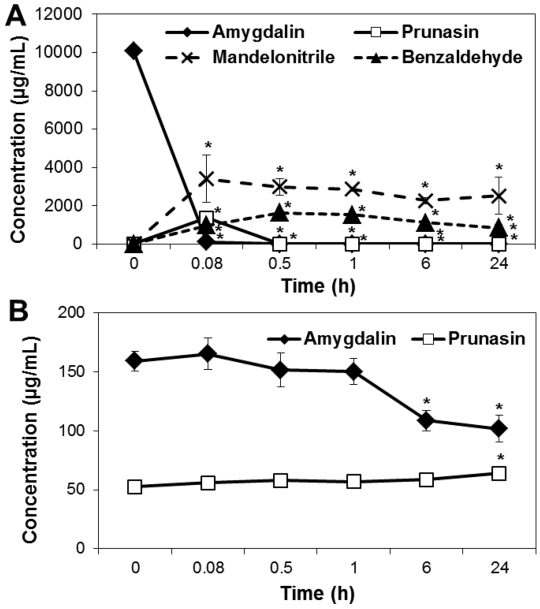

Figure 2. Metabolic changes of (A) amygdalin standard and (B) amygdalin in Maesil syrup in the presence of β-glucosidase. *, Significant differences from nontreated sample (0 h) at $P < 0.05$.

Amygdalin standard was rapidly converted to prunasin, mandelonitrile, and benzaldehyde within 5 min, and then only mandelonitrile and benzaldehyde were detected after 30 min (Figure 2A). On the other hand, the levels of amygdalin and prunasin in Maesil syrup remained constant during 1 h, and then amygdalin content slightly decreased after enzyme treatment for 6−24 h. Consequently, significant increase in prunasin level was found after 24 h in Maesil syrup (Figure 2B). However, no retarded degradation of amygdalin was found when it was incubated with only glucose and fructose mixture, polyphenol (as gallic acid), organic acids (citric acid, oxalic acid, fumaric acid, and malic acid), or all of above mixture in the presence of the enzyme (Supplementary Figure 2).

Inhibition of Cell Proliferation. The effect of amygdalin on short-term cell proliferation was evaluated with WST-1 assay in two different cell lines, human intestinal INT-407 and liver HepG2 cells. Moreover, the effect of amygdalin metab-

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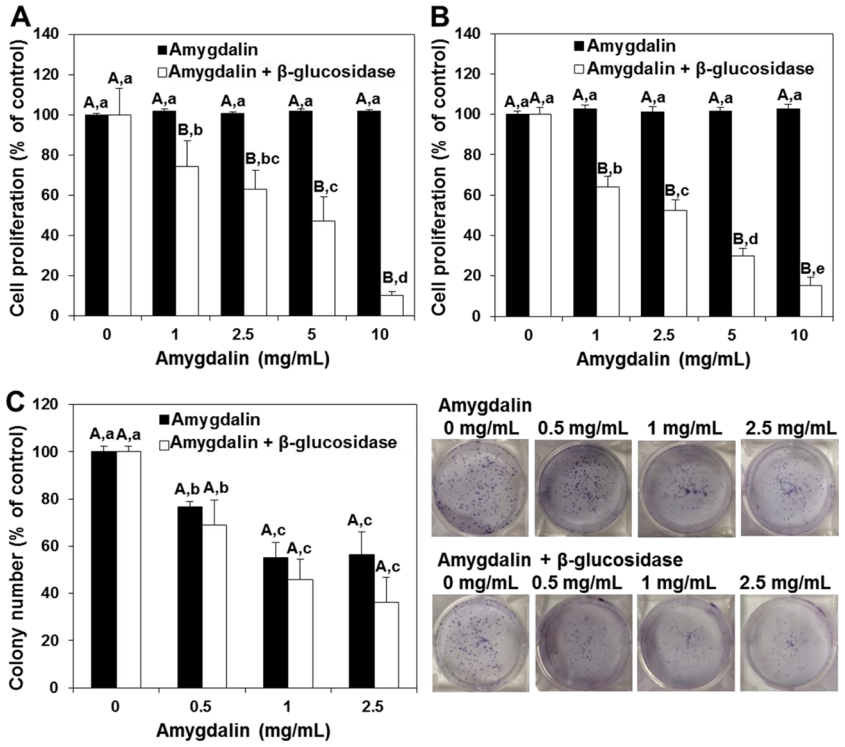

Figure 3. Short-term effect of amygdalin on cell proliferation of (A) INT-407 cells and (B) HepG2 cells in the absence or presence of β-glucosidase after 24 h. (C) Long-term effect of amygdalin on colony-forming ability of INT-407 cells in the absence or presence of β-glucosidase after 7 days. A, B indicate significant differences between absence and presence of β-glucosidase at $P < 0.05$. a, b, c, d, e indicate significant differences among different concentrations at $P < 0.05$.

olites on cytotoxicity was also compared by incubating amygdalin in the presence of β-glucosidase. The results show that amygdalin did not inhibit cell proliferation up to the highest concentration tested, 10 μg/mL, after incubation for 24 h, while amygdalin metabolites induced by β-glucosidase treatment remarkably inhibited cell proliferation in a concentration-dependent manner (Figure 3A,B). The same tendency was obtained in two different cell lines.

When long-term effect of amygdalin and its metabolites on colony forming ability was evaluated in INT-407 cells (Figure 3C), both amygdalin and its metabolites remarkably inhibited colony formation, although the concentrations of amygdalin were relatively low compared to those used for short-term cell proliferation test. No statistical differences between amygdalin-treated cells with and without β-glucosidase were found ($P > 0.05$). The cytotoxicity of Maesil syrup was not tested due to its high saccharide content.

Oxidative Stress and Apoptosis Induction. The effects of amygdalin and its metabolites on ROS generation and apoptosis induction were evaluated to elucidate toxic mechanism. Figure 4A shows that amygdalin did not generate ROS after 24 h, whereas its enzymatically converted metabolites remarkably induced ROS in a concentration-dependent manner. The same tendency was obtained by flow cytometry analysis; amygdalin caused early and late apoptosis only when it was treated with β-glucosidase (Figure 4B).

Toxicokinetics. Plasma concentration−time profile of amygdalin standard is shown in Figure 5A. The dose was chosen to be much lower than LD_{50} values (522 mg/kg) but high enough to detect all metabolites.[26,27] Figure 5A shows that amygdalin was not detected in all plasma samples and only its first-step metabolite, prunasin was detected during 6 h postadministration. The peak concentration reached at 0.92 h postadministration and amygdalin remained in the circulation system during about 4.86 h, as evidenced by $T_{1/2}$ and MRT values (Table 2). On the basis of AUC values, total oral absorption was calculated to be extremely low (1.53 ± 0.50%).

When toxicokinetics of Maesil syrups with and without Maesils was evaluated, only amygdalin, but not its metabolites, was detected in the bloodstream (Figure 5B). Similar peak concentration and AUC values were obtained for both Maesil syrups with and without Maesils (Table 2). However, Maesil syrup without Maesils was found to be retained longer in the bloodstream than Maesil syrup with Maesils, showing significantly high $T_{1/2}$ and MRT values (Table 2). Oral absorption was estimated to be ~20% for both Maesil syrups without statistical significance ($P > 0.05$).

Repeated Oral Toxicity. *In vivo* 14-day repeated oral toxicity results of Maesil syrups with and without Maesils are presented in Supplementary Figure 3, Figure 6, Table 3, Table 4, Table 5, and Supplementary Table 1. No abnormal changes in body weight gain, food intake, and water consumption were observed in all treated groups (Supplementary Figure 3). Moreover, all relative organ weights of rats administered Maesil syrups, represented by organo-somatic indices, did not significantly change compared to those in untreated control group (Table 3) ($P > 0.05$). No hematological (Table 4), serum biochemical (Table 5), and histopathological abnormalities (Supplementary Table 1 and Figure 6) were found in rats administered Maesil syrups with and without Maesils.

DISCUSSION

The present study investigated the cytotoxicity, *in vivo* oral toxicity, and toxicokinetics of amygdalin standard and

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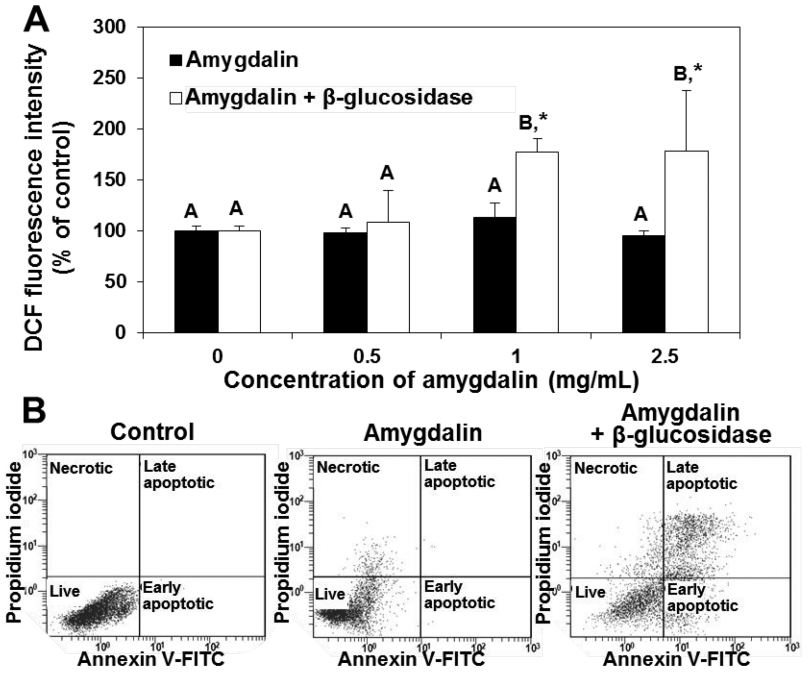

Sample	Live cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Necrotic cells (%)
Control	99.4	0.6	0.0	0.0
Amygdalin	94.2	0.3	0.0	5.4
Amygdalin + β-glucosidase	38.8	23.6	30.7	6.9

Figure 4. Effect of amygdalin on (A) intracellular ROS generation and (B) apoptosis in the absence or presence of β-glucosidase in INT-407 cells after 6 h. A, B indicate significant differences between absence and presence of β-glucosidase at $P < 0.05$. ∗, significant differences from nontreated sample at $P < 0.05$.

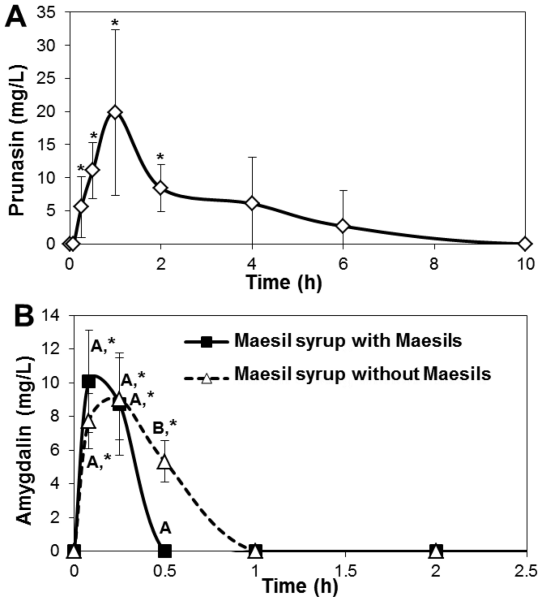

Figure 5. Plasma concentration−time profiles after a single dose oral administration of (A) amygdalin standard (250 mg/kg) and (B) Maesil syrups with and without Maesils to rats. A, B indicate significant differences between Measil syrups with and without Maesils at $P < 0.05$. ∗, significant differences from nontreated sample at $P < 0.05$.

Table 2. Toxicokinetic Parameters and Oral Absorption of Amygdalin Standard and Maesil Syrups with and without Maesils after Single Dose Oral Administration to Rats[a]

		Maesil syrups[b]	
biokinetic parameters	amygdalin standard	with Maesils	without Maesils
C_{max} (mg/L)	20.11 ± 11.04	11.71 ± 2.03a	9.57 ± 2.20a
T_{max} (h)	0.92 ± 0.20	0.14 ± 0.09a	0.19 ± 0.09a
AUC (h × mg/L)	73.24 ± 23.85	4.71 ± 1.78a	7.39 ± 2.71a
$T_{1/2}$ (h)	3.10 ± 1.89	0.25 ± 0.07a	0.47 ± 0.22b
MRT (h)	4.86 ± 2.36	0.40 ± 0.09a	0.75 ± 0.28b
absorption (%)	1.53 ± 0.50	18.23 ± 6.89a	24.89 ± 9.12a

[a]Abbreviation: C_{max}, maximum concentration; T_{max}, time to maximum concentration; AUC, area under the plasma concentration-time curve; $T_{1/2}$, half-life; MRT, mean residence time. [b]a and b indicate significant differences between Maesil syrups with and without Maesils.

amygdalin-containing Maesil syrups by focusing on determination of the effects of amygdalin metabolites on toxicity. Cytotoxicity of Maesil syrups could not be evaluated due to high concentration of saccharides. Maesil syrups matured with and without Maesils were tested to answer the question as to whether Maesil syrup matured with Maesils causes high toxicity. In general, traditional Maesil syrup is made with 1:1 ratio of sucrose and Maesils, and matured for 3 months. Then Maesils are removed from the syrup and further matured at least for 1 year (Maesil syrup without Maesils, Figure 1).

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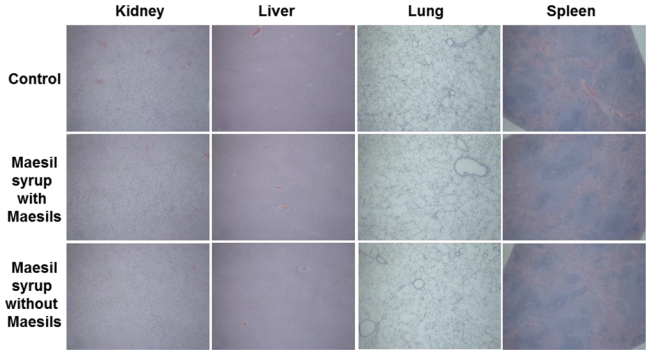

Figure 6. Normal histopathological sections of kidneys, liver, lungs and spleen of rats administered DW as a control or Maesil syrups with and without Maesils after 14-day repeated oral administration. Images magnification at 50×.

Table 3. Organosomatic Indices of Rats after 14-Day Repeated Oral Administration of Maesil Syrups with and without Maesils[a]

organ	control	Maesil syrup with Maesils	Maesil syrup without Maesils
brain	0.86 ± 0.04	0.90 ± 0.04	0.87 ± 0.03
heart	0.40 ± 0.01	0.40 ± 0.03	0.40 ± 0.03
kidney	0.87 ± 0.08	0.79 ± 0.02	0.83 ± 0.03
large intestine	1.39 ± 0.21	1.42 ± 0.07	1.37 ± 0.28
liver	3.68 ± 0.34	3.52 ± 0.25	3.73 ± 0.32
lung	0.58 ± 0.04	0.59 ± 0.17	0.53 ± 0.04
ovary	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
small intestine	3.45 ± 0.11	3.62 ± 0.11	3.48 ± 0.44
spleen	0.30 ± 0.03	0.31 ± 0.02	0.34 ± 0.05
stomach	0.89 ± 0.16	1.11 ± 0.22	1.23 ± 0.30

[a]No significant differences from the control at $P > 0.05$.

Quantitative analysis shows that Maesil syrup without Maesils had significantly higher amygdalin content than Maesil syrup with Maesils (Table 1). Its first-step metabolite, prunasin, was only detected, indicating slight natural decomposition of amygdalin during maturation. Amygdalin degradation gradually occurs for several months to years, and it was reported that no amygdalin was detected in Maesil-based products after 3 months to 1 year.[28−31] It should be noted that Maesil syrups matured for 1 year were used in the present study, and commercially available Maesil syrups are on the market after maturation at least for several years.[32] On the other hand, it was demonstrated that Maesils and Maesil-based products have high contents of phenolic compounds, which contribute functional benefits.[33−35] In the present study, significantly higher amounts of total polyphenols and total flavonoids were found in Maesil syrup with Maesils than Maesil syrup without Maesils, implying that Maesil syrup continuously matured with Maesils had high levels of functional compounds, but low levels of amygdalin. It seems that maturation with Maesils can enhance natural amygdalin degradation in Maesil syrup because amygdalin content in Maesils was reported to decrease upon ripening and storage.[30,31] Meanwhile, high levels of glucose and fructose were detected in Maesil syrups, although sucrose was initially added to obtain Maesil syrups (Figure 1), indicating the hydrolysis of sucrose to glucose and fructose. It was also reported that Maesils extract contains about 12 μg/mL of organic acids (citric acid, malic acid, oxalic acid, and fumaric

Table 4. Hematological and Coagulation Time Values of Rats after 14-Day Repeated Oral Administration of Maesil Syrups with and without Maesils[a]

groups	WBC ($10^3/\mu L$)	WBC differential counting (%)					RBC ($10^6/\mu L$)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RETI (%)	PLT ($10^3/\mu L$)	PT (s)	APTT (s)
		NE	LY	MO	EO	BA										
control	6.50 ± 0.82	13.0 ± 3.2	81.3 ± 3.2	2.6 ± 1.1	2.0 ± 0.8	0.3 ± 0.1	6.90 ± 0.15	14.4 ± 0.1	46.3 ± 0.3	67.1 ± 1.7	20.9 ± 0.3	31.2 ± 0.5	3.09 ± 0.44	931 ± 139	16.4 ± 0.5	30.4 ± 4.7
Maesil syrupwith Maesils	7.44 ± 1.38	7.6 ± 1.3	88.2 ± 1.2	1.8 ± 0.7	1.0 ± 0.3	0.4 ± 0.1	6.85 ± 0.17	14.0 ± 0.2	45.3 ± 1.0	66.2 ± 1.1	20.4 ± 0.3	30.8 ± 0.5	3.58 ± 0.75	1202 ± 133	16.9 ± 0.5	25.2 ± 4.8
Maesil syrupwith-out Maesils	8.23 ± 1.69	9.0 ± 2.3	87.0 ± 2.6	1.7 ± 0.3	1.1 ± 0.3	0.3 ± 0.1	6.90 ± 0.20	13.9 ± 0.3	45.2 ± 1.2	65.5 ± 1.1	20.2 ± 0.4	30.9 ± 0.1	3.08 ± 0.17	1090 ± 83	16.7 ± 0.6	21.3 ± 1.7

[a]Abbreviation: WBC, white blood cell; NE, neutrophils; LY, lymphocytes; MO, monocytes; EO, eosinophils; BA, basophils; RBC, red blood cell; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RETI, reticulocyte; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time. No significant differences from the control at $P > 0.05$.

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Table 5. Serum Biochemical Values of Rats after 14-Day Repeated Oral Administration of Maesil Syrups with and without Maesils[a]

groups	TP (g/dL)	ALB (g/dL)	A/G	T-BIL (mg/dL)	ALP (U/L)	AST (U/L)	ALT (U/L)	CREA (mg/dL)	BUN (mg/dL)	CHOL (mg/dL)	TG (mg/dL)	GLU (mg/dL)	CA (mg/dL)	IP (mg/dL)	CK (IU/L)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)
control	6.2 ± 0.2	4.2 ± 0.1	2.1 ± 0.1	0.01 ± 0.01	591 ± 108	71 ± 4	40 ± 7	0.58 ± 0.03	18.5 ± 2.4	120 ± 8	64 ± 8	260 ± 42	12.0 ± 0.6	11.1 ± 1.0	163 ± 24	146.4 ± 1.2	5.29 ± 0.56	96.7 ± 1.7
Maesil syrup with Maesils	6.3 ± 0.3	4.2 ± 0.2	2.0 ± 0.1	0.01 ± 0.01	614 ± 102	73 ± 8	35 ± 5	0.55 ± 0.02	11.6 ± 1.0	89 ± 10	63 ± 17	296 ± 43	12.3 ± 0.3	10.8 ± 0.5	213 ± 57	147.3 ± 1.3	5.78 ± 0.17	97.4 ± 0.1
Maesil syrup without Maesils	6.3 ± 0.1	4.2 ± 0.0	2.0 ± 0.1	0.00 ± 0.00	602 ± 185	65 ± 5	31 ± 1	0.54 ± 0.08	15.3 ± 1.9	97 ± 17	73 ± 9	272 ± 131	12.4 ± 0.6	11.4 ± 1.1	161 ± 78	146.8 ± 1.0	6.48 ± 0.71	98.3 ± 1.1

[a]Abbreviation: TP, total protein; ALB, albumin; A/G, A/G ratio; T-BIL, total bilirubin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CREA, creatinine; BUN, blood urea nitrogen; CHOL, total cholesterol; TG, triglycerides; GLU, glucose; CA, calcium; IP, inorganic phosphorus; CK, creatine kinase; Na, sodium; K, potassium; Cl, chloride. No significant differences from the control at $P > 0.05$.

acid), 8 mg/mL of crude protein, 5 mg/mL of crude fat, 6 mg/mL of ash, 80 mg/mL of carbohydrate, 250 mg/100 g of minerals, 64 mg/mL of fatty acids, etc.[36,37]

Cytotoxicity results reveal that amygdalin itself was not toxic after incubation for 24 h, but exhibited cytotoxicity in terms of inhibition of cell proliferation (Figure 3A,B), ROS generation (Figure 4A), and apoptosis induction (Figure 4B), when it was treated with β-glucosidase. These results clearly suggest that amygdalin metabolites produced by β-glucosidase play roles in cytotoxicity. Apoptosis induction by amygdalin was also reported,[38−40] which is in good agreement with our result. However, long-term exposure of amygdalin with and without β-glucosidase inhibited colony formation in a similar manner (Figure 3C), which may be associated with active cellular metabolic activity during 7 d. When the decomposition of amygdalin in the presence of β-glucosidase was quantitatively analyzed by HPLC (Figure 2 and Supplementary Figure 1), the levels of amygdalin standard rapidly decreased, but its metabolites, such as prunasin, mandelonitrile, and benzaldehyde, increased upon incubation for 24 h. The final metabolite, HCN was not detected, probably due to its volatility at more than 26 °C.[23,24] It was also reported that HCN was not detected in three different Maesil extracts,[41] which is comparable to our result. Hence, this result clearly confirms that the cytotoxicity of amygdalin is closely related to its metabolites. Meanwhile, the metabolic degradation profile of amygdalin in Maesil syrup slows retarded enzymatic conversion of amygdalin to prunasin only after long-term exposure, 6−24 h, which was not found when amygdalin was reacted with other main components, such as glucose and fructose mixture, polyphenol (as gallic acid), organic acids, or glucose, fructose, polyphenol, and organic acid mixture (Supplementary Figure 2). It is likely that complex functional Maesil syrup matrix play a role in defense against enzymatic degradation.[42,43] This tendency was also observed in toxicokinetic results.

Plasma concentration−time profiles demonstrate that the first-step metabolite, prunasin was only detected in the bloodstream following oral administration of amygdalin standard to rats, showing maximum concentration at 0.92 h postadministration and elimination from the circulation system within 5 h (Figure 5A and Table 2). The plasma concentration−time profile of amygdalin is highly consistent with the data obtained by Chen et al.[44] It is worth noting that oral absorption of amygdalin was extremely low (1.53%). On the other hand, toxicokinetics of orally administered Maesil syrups reveals that amygdalin was retained in the bloodstream without any degradation and could be rapidly eliminated from the body within 1 h (Figure 5B and Table 2), implying that Maesil syrups could reduce amygdalin toxicity. Although relatively high oral absorption was found for amygdalin in Maesil syrups (18.23 ± 3.89−24.89 ± 9.12%) compared to amygdalin standard (1.53 ± 0.50%), it should be noted that much lower AUC values was obtained for Maesil syrups (Table 2). This result suggests that absolutely low amount of amygdalin in Maesil syrups was absorbed into the body. Furthermore, amygdalin contents in Maesil syrups (134.98 ± 5.96−166.82 ± 4.16 μg/mL, which correspond to 1.40−1.61 mg/kg for animal experiments) were much lower than the dose used for toxicokinetic study of amygdalin standard (250 mg/kg), which may lead to relatively high absorption percentage and rapid absorption rate of the former. It is known that 1 g of amygdalin would potentially produce 59. One milligram of HCN and no observed adverse effect level (NOAEL) for HCN

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is 0.36 mg/kg.[45,46] When potentially produced amounts of HCN in the body were calculated based on AUC values of amygdalin, administered volume (10 mL/kg), animal weight (200 g), and theoretical HCN yield (5.9%) from amygdalin, 14−22 μg/kg of HCN could be produced, which were much lower than NOAEL. Moreover, estimated daily intake of Maesil syrup was reported to be extremely small, 20 mg/kg/day in South Korea, 2014,[47] supporting its safety. Meanwhile, amygdalin was significantly retained longer in rats administered Maesil syrup without Maesils (Figure 5B and Table 2). Considering high content of total polyphenols, low level of amygdalin, low oral absorption, and short residence time, it seems better that Maesil syrup is matured in the presence of Maesils. The discrepancy in toxicokinetics between amygdalin standard and amygdalin in Maesil syrups is likely to be related to high contents of other functional compounds in Maesil syrups, such as polyphenols and organic acids, as observed in retarded enzymatic degradation of amygdalin in Maesil syrup (Figure 2). Further study is required to elucidate the role of functional compounds in the safety aspects of amygdalin in Maesil-based products.

Fourteen-day repeated oral toxicity study demonstrates no significant toxicity of Maesil syrups in terms of changes in body weight, organo-somatic indices, food and water consumption, blood biochemical parameters, and histopathology, regardless of the presence of Maesils (Figure 6, Supplementary Figure 3, Table 3, Table 4, Table 5, and Supplementary Table 1). These results clearly show that Maesil syrups did not cause oral toxicity, which can be explained by low level, rapid clearance, low absorption, and little enzymatic conversion of amygdalin in Maesil syrups (Figure 5 and Table 2).

In conclusion, Maesil syrup matured with Maesils had high total polyphenol content, but low level of amygdalin. The toxicity of amygdalin was closely related to its enzymatically degraded metabolites, but the enzymatic degradation could be retarded when it was taken up in Maesil syrups. Toxicokinetic results reveal extremely low oral absorption and short half-life of amygdalin and Maesil syrups in the bloodstream. Potential protective effect of Maesil against amygdalin toxicity was also suggested. Moreover, no oral toxicity of Maesil syrups was observed following 14-day repeated administration to rats. Considering low concentration, low oral absorption, and low daily intake of amygdalin in Maesil syrups, it could be concluded that Maesil syrups do not cause oral toxicity at practical levels. These findings will be useful to predict and understand the potential toxicity of Maesil-based or amygdalin-containing food. Further study is required to determine the mechanism of action of Maesil syrup on defense against amygdalin degradation and toxicity as well as on metabolic change in the body.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b03686.

Summary of histopathological findings of rats after 14-day repeated oral administration of Maesil syrups with and without Maesils; HPLC chromatograms of amygdalin, prunasin, mandelonitrile, and benzaldehyde standards; HPLC chromatograms of metabolic changes of amygdalin standard incubated with fructose and glucose mixture, polyphenol, organic acids, or fructose, glucose, polyphenol, and organic acid mixture in the presence of β-glucosidase; changes in body weight gain, food intake, and water consumption in rats administered Maesil syrups with and without Measils or DW as a control (PDF)

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Notes

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