

Suppressive Effect of Polyphenols from Immature Pear Fruits on Blood Glucose Levels

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Abstract The agricultural practice of pear thinning involves the removal of immature pear fruits, which are usually treated as waste but can contain high levels of beneficial polyphenols. To assess whether these fruits provide commercial utility, we aimed to examine the inhibitory effects of polyphenols from the immature pear fruits of the Hosui and Kosui cultivars on α -glucosidase *in vitro* and blood glucose levels in mice after the oral administration of starch. Polyphenols were extracted from the immature pear fruits by 80% ethanol and 70% acetone, and the mixed extraction was purified by HP-20 column. The polyphenol contents of the Hosui and Kosui immature pear fruits were 28.5 and 20.9 mg/g D.W., respectively, and both were characterized by high percentages of oligomeric and polymeric polyphenols. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of the polyphenols from Hosui and Kosui fruits were 115.8 and 76.3 $\mu\text{mol/g}$ D.W., respectively, indicating a significant difference between the two cultivars. Moreover, compared with the polyphenols from immature Kosui fruits, which had an IC_{50} value of 66.4 $\mu\text{g/mL}$, those from Hosui fruits, with an IC_{50} value of 21.3 $\mu\text{g/mL}$, resulted in more potent inhibition of α -glucosidase activity. Polyphenols were fractionated with a Sephadex LH-20 column into fraction I (Fra.I; monomeric), fraction II (Fra.II; oligomeric), and fraction III (Fra.III; polymeric). Compared with Fra.I, Fra.II and Fra.III from immature Hosui and Kosui fruits showed higher inhibition of α -glucosidase activity, with the highest inhibitory activity in Fra.III. Thus, α -glucosidase inhibitory activity appears to be related to the degree of polyphenol polymerization. Moreover, polyphenols from immature Hosui fruits effectively suppressed increases in blood glucose levels after the oral administration of starch in mice. These results suggest that the immature pear fruits of the Hosui and Kosui cultivars have useful properties and, in particular, potential applications for the treatment of diabetes.

Keywords: immature pear, polyphenol, DPPH radical scavenging activity, α -glucosidase, blood glucose

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1. Introduction

Pear thinning is a common agricultural practice worldwide that involves the removal of immature pear fruits from trees to ensure better harvest quality. These immature pears are usually treated as agricultural waste. However, immature pear fruits can contain high levels of beneficial polyphenols [1] and may have commercial utility.

Diabetes has received considerable attention owing to the increasing number of people diagnosed with this chronic disease [2]. Postprandial hyperglycemia is an important risk factor for the development of type II diabetes [3]. Controlling blood glucose levels is the most effective method for preventing diabetes deterioration and hyperglycemia [4]. All dietary carbohydrates are hydrolyzed by enzymes to yield simple sugars, which can improve blood glucose levels [5]. α -Glucosidase is an

intestinal cell membrane enzyme that can hydrolyze polysaccharides; hence, inhibiting the activity of α -glucosidase may be an effective way to treat pre-diabetes and slow the progression of diabetes [6].

Several synthetic α -glucosidase inhibitors, such as acarbose and voglibose, have been developed, but these exhibit various side effects, such as digestion disorders, flatulence, and liver function disorders [7]. Recently, many studies have focused on natural α -glucosidase inhibitors from plants. Although it has been reported that polyphenols from fruits [8,9] may inhibit α -glucosidase activity, few studies have examined the inhibitory activity of the polyphenols from immature pear fruits against both α -glucosidase and blood glucose levels.

In the present study, we utilized pear fruits from the Hosui and Kosui cultivars (*Pyrus pyrifolia*), which are the major varieties in Japan. We examined the inhibitory effects of polyphenols from the immature pear fruits of these cultivars on α -glucosidase activity *in vitro* and blood

glucose levels in mice after the oral administration of starch.

2. Materials and Methods

2.1. Materials

Immature pear fruits of the Hosui and Kosui cultivars were obtained from Yamamoto Orchard in the town of Yoichi (Hokkaido, Japan). For each immature pear fruit, the diameter was approximately 2 cm, moisture content was 82%, and weight ranged from 42.1 g to 43.3 g. The immature pear fruits were harvested approximately 70 days after flowering. Diaion HP-20 columns and Sephadex LH-20 columns for chromatography were obtained from the Mitsubishi Chemical Corporation (Tokyo, Japan) and GE Healthcare Bio-Sciences AB (Uppsala, Sweden), respectively. All other reagents and chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless otherwise stated.

2.2. Extract Preparation and Fractionation

Immature pear fruits were freeze-dried for 3 days and then crushed by blender, followed by extraction with 20 mL of 80% ethanol. After ultrasound treatment for 30 min, the mixture was centrifuged at $1,006 \times g$ for 10 min to obtain the supernatant. The same extraction process was repeated twice more. The residues were subjected to another three rounds of extraction with 70% acetone–water, and the supernatant was obtained. Then, the supernatant was mixed, concentrated by rotary evaporation in a vacuum, and purified by chromatography through a Diaion HP-20 column. The columns were washed with distilled water and then eluted with methanol. The methanol solution was concentrated by rotary evaporation in a vacuum and dissolved in 2 mL of methanol for the experiment. Part of the concentrate was dissolved in ethanol and fractionated by Sephadex LH-20 column chromatography. The column was successively eluted with ethanol, methanol, and 60% acetone to collect fraction I (Fra.I), fraction II (Fra.II), and fraction III (Fra.III), respectively.

2.3. Quantification of Polyphenols

Polyphenols were quantified using the Folin–Ciocalteu method [10]. The methanol fraction (HP-20 column; 100 μ L) was treated with 300 μ L of distilled water, 400 μ L of Folin–Ciocalteu reagent, and 400 μ L of a 10% Na_2CO_3 solution. The mixture was prepared in triplicate, incubated at 30 °C for 30 min, and centrifuged at $1,006 \times g$ for 10 min. The absorbance of the mixed supernatant was measured at 760 nm. The polyphenol content is expressed in milligrams of catechin equivalents per gram of dry weight of immature pear fruits (mg/g D.W.).

2.4. Estimation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

DPPH radical scavenging activity was evaluated by the method described by Brand-Williams et al. [11] with some

modifications. A 50- μ L aliquot of the methanol fraction (HP-20 column) was mixed with 100 μ L of ethanol, and the mixture was supplemented with 150 μ L of 0.5 mM DPPH in ethanol. The absorbance of the mixture was measured using a microplate reader at 517 nm. The DPPH radical scavenging activity is expressed in micromoles of trolox equivalents per gram of dry weight of immature pear fruits ($\mu\text{mol/g D.W.}$).

2.5. α -Glucosidase Inhibitory Activity

α -Glucosidase inhibition was analyzed following the methods of Matsumoto et al. [12] with modifications. Sucrose was broken down by α -glucosidase, and the amount of reducing sugar was calculated based on the α -glucose content. In total, 0.8 mL of enzyme reaction solution [50 μ L of 0.4% sucrose, 625 μ L of 0.1 mol/L sodium phosphate buffer (pH 6.8), and 125 μ L of 1% NaCl] was pre-incubated at 37°C for 30 min. The methanol fraction (HP-20 column) was concentrated by rotary evaporation in a vacuum and dissolved in distilled water. The distilled water fraction (polyphenol concentration, 0–200 $\mu\text{g/mL}$) was added to 0.1 U/mL α -glucosidase solution (EC3.2.1.20; Oriental Yeast Co., Ltd., Tokyo, Japan) at 37°C for 10 min. After pre-incubation, 200 μ L of the mixture (polyphenol extract and α -glucosidase) was added to the enzyme reaction solution and incubated at 37°C for 30 min. The reaction was terminated by adding 125 mL of 2 M NaOH and 1% dinitrosalicylic acid in boiling water for 10 min. After incubation, the mixture was analyzed at 540 nm at 25°C. Enzyme inhibitory reactions for all polyphenol extract concentrations were replicated three times. The α -glucosidase inhibitory activity is expressed as the percent inhibition. The concentration of inhibitors required for the inhibition of 50% of the enzyme activity under the assay conditions was defined as the IC_{50} value.

2.6. Oral Starch Tolerance Test in Mice

Male DDY mice (Japan SLC, Inc., Shizuoka, Japan) were housed in plastic cages at 23 °C under a 12 h/12 h light/dark cycle and a relative humidity of 60%. Mice had free access to feed (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water for 1 week before the experiment. The feed contained 344.9 kcal/100 g and was composed of (w/w %) 24.9% crude protein, 4.6% crude fat, 4.1% crude fiber, 6.6% crude ash, 51.0% NFE (nitrogen-free extract), and 8.9% moisture. After 1 week, the animals were randomly divided into experimental groups (8 mice per group). The mice were fasted for 16 h, and blood was drawn from the tail vein and subjected to assays to determine blood glucose levels. The polyphenols from the immature Hosui fruits were suspended in physiological solution and were administered in doses of 250 and 500 mg/kg. After the oral administration of the suspension of polyphenol compounds for 30 min, a single oral injection of 2 g/kg starch in physiological solution was administered. Blood was withdrawn from the tail vein at 0.5, 1, and 2 h, and blood glucose levels were analyzed using the OMRON Precision Exceed HEA-216 (Omron Healthcare Co., Ltd., Kyoto, Japan) according to the manufacturer's instructions. This study was approved by the regulatory authority of the National University Corporation Obihiro

University of Agriculture and Veterinary Medicine, and it adhered to the standard principles described in the Guide for the Care and Use of Laboratory Animals.

2.7. Statistical Analysis

Values are presented as the means \pm standard error of 5 out of 8 mice in each group. Statistical significance was evaluated by analysis of variance (ANOVA) and least significant difference (LSD) tests (SAS Enterprise Guide 5.1 system, Cary, NC, USA). Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Polyphenol Content, DPPH Radical Scavenging Activity, and Polyphenol Fractions

The polyphenol content of the immature Hosui pear fruits was higher than that of the immature Kosui pear fruits, and the same pattern was observed for the DPPH radical scavenging activities of the two types of fruits (Table 1). Thus, it appears that the polyphenols from both cultivars exhibited antioxidant activity. Similarly, it has been reported that the polyphenols from grape, apple, cacao, and persimmon exhibit antioxidant activity [13].

Table 1. Polyphenol content, DPPH radical scavenging activity, and α -glucosidase inhibitory activity in immature Hosui and Kosui pear fruits

Cultivar	Polyphenols (mg/g D.W.)	DPPH radical scavenging activity ($\mu\text{mol/g D.W.}$)	α -Glucosidase inhibitory activity IC ₅₀ ($\mu\text{g/mL}$)
Hosui	28.5 ^a \pm 0.6	115.8 ^a \pm 1.6	21.3
Kosui	20.9 ^b \pm 0.3	76.3 ^b \pm 5.8	66.4

D.W.: dry weight of immature pear fruits. Values represent mean \pm S.E.M. Different superscript letters indicate significant differences ($p < 0.05$).

We performed Sephadex LH-20 column chromatography to obtain three polyphenol fractions, i.e., Fra.I, Fra.II, and Fra.III, from the immature Hosui and Kosui pear fruits (Figure 1). The polyphenol contents in Fra.I, Fra.II, and Fra.III from Hosui immature pear fruits accounted for 6%, 63%, and 31% of the total weight, respectively,

while those from Kosui fruits accounted for 24%, 55%, and 21%, respectively. Thus, both Hosui and Kosui fruits had higher percentages of Fra.II than of the other fractions. According to Saito et al. [14], Fra.I contains monomeric polyphenols, Fra.II contains oligomeric polyphenols, and Fra.III contains polymeric polyphenols. Based on our results, the total amounts of non-monomeric (oligomeric and polymeric) polyphenols were 94% and 76% in immature Hosui and Kosui fruits, respectively. While the monomeric polyphenols arbutin and chlorogenic acid have been shown to be major polyphenols related to DPPH radical scavenging activity in Japanese pears [15], in our study, oligomeric and polymeric polyphenols were the major polyphenols in immature Hosui and Kosui pear fruits. Thus, we speculate that the DPPH radical scavenging activity of these polyphenols are mainly related to non-monomeric polyphenols. Notably, we previously reported that polymeric polyphenols may possess greater antioxidant activity [16].

3.2. α -Glucosidase Inhibitory Activity

We next analyzed the inhibitory effects of the polyphenols on α -glucosidase. Polyphenols from the Hosui and Kosui immature pear fruits inhibited the activity of α -glucosidase in a dose-dependent manner. Several studies have shown the α -glucosidase inhibitory activity of various fruits and vegetables [8, 9]. The IC₅₀ value of the polyphenols from immature Hosui fruits was 21.3 $\mu\text{g/mL}$, and that of the polyphenols from Kosui fruits was 66.4 $\mu\text{g/mL}$, indicating that the former exhibited greater α -glucosidase inhibitory activity (Table 1). Moreover, we analyzed and compared the inhibitory effects of 20 $\mu\text{g/mL}$ of polyphenols from Fra.I, Fra.II, and Fra.III on α -glucosidase (Figure 2). No inhibitory activity was detected in Fra.I. The rates of inhibition of α -glucosidase were 14.9% and 11.7% for Fra.II polyphenols derived from Hosui and Kosui fruits, respectively, and 18.5% and 15.7% for Fra.III polyphenols, respectively; thus, Fra.III showed more potent α -glucosidase inhibitory activity. We speculate that higher degrees of polymerization in polyphenols results in stronger inhibition of α -glucosidase. Consistent with this, we also found that oligomeric and polymeric polyphenols exhibited stronger α -glucosidase inhibitory activity than monomeric phenolic compounds in scarlet runner beans [17].

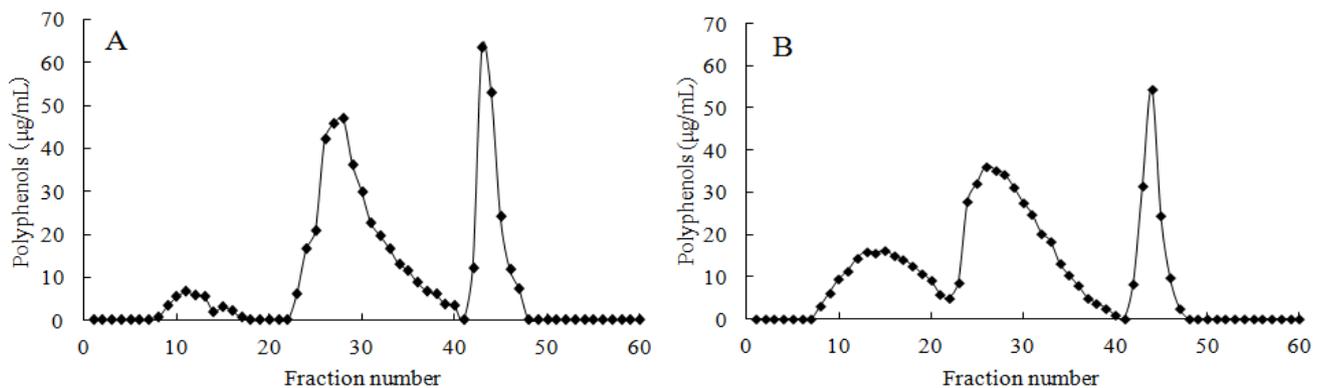


Figure 1. Sephadex LH-20 column chromatograms of polyphenols prepared from immature Hosui and Kosui pear fruits. The polyphenol fractions obtained by Diaion HP-20 column chromatography were applied to Sephadex LH-20 column chromatography and eluted with ethanol, fraction number 1-20 (Fra.I); methanol, fraction number 21-40, (Fra.II); and 60% acetone, fraction number 41-60, (Fra.III). (A) Immature Hosui pear fruits. (B) Immature Kosui pear fruits

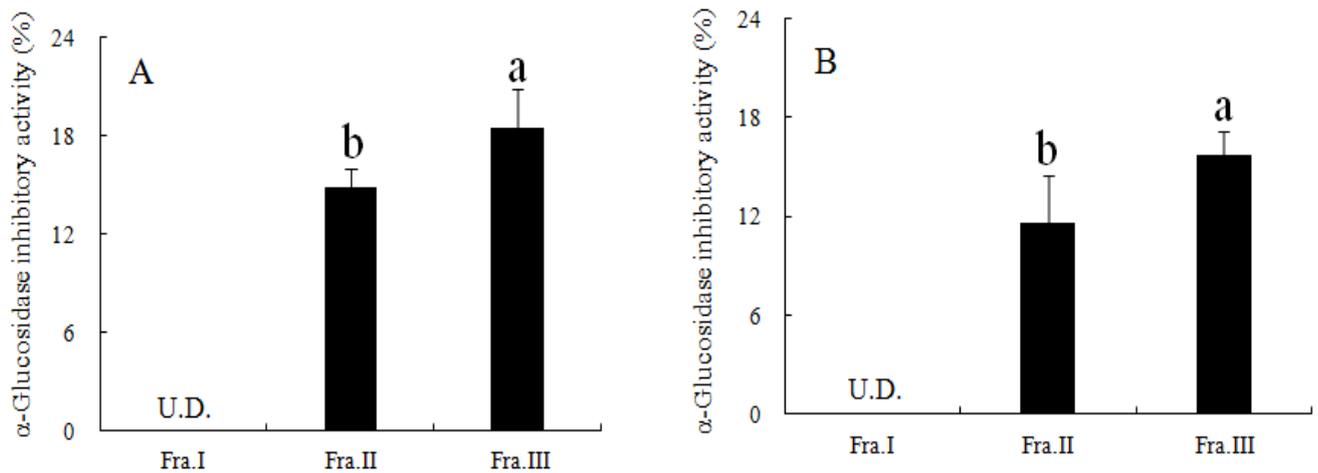


Figure 2. α -Glucosidase inhibitory activity of 20 μ g/mL of Fra.I, Fra.II, or Fra.III polyphenols from immature Hosui and Kosui pear fruits. (A) Immature Hosui pear fruits. (B) Immature Kosui pear fruits. U.D., undetected. Values represent mean \pm S.E.M. Different superscript letters indicate significant differences ($p < 0.05$)

3.3. Inhibitory Effects of Polyphenols on Blood Glucose Levels after Oral Administration of Starch

Compared with those in immature Kosui fruits, the polyphenols of immature Hosui fruits resulted in more effective inhibition of α -glucosidase *in vitro*. Therefore, the polyphenols from immature Hosui fruits were tested for their inhibitory effects on blood glucose levels by conducting an oral starch tolerance test in mice. After the administration of starch, the maximum increase in blood glucose levels was observed at 30 min in all mice, but mice treated with 250 mg/kg or 500 mg/kg mouse exhibited significantly lower blood glucose concentrations than those in the control group ($p < 0.05$; Figure 3A). At 60 and 120 min, the 500 mg/kg mouse group still showed significantly lower glucose levels than the control group ($p < 0.05$), indicating that Hosui fruit polyphenols suppress the breakdown of starch into glucose. As immature Kosui

fruits shared the same polyphenol distribution as immature Hosui fruits and also exhibited α -glucosidase inhibitory activity, we speculate that immature Kosui fruits would also effectively reduce blood glucose levels *in vivo*, though much higher concentrations of polyphenols would be needed due to their less potent activity. The area under the curve (AUC) of blood glucose levels over the 120-min period is shown in Figure 3B. The AUC was significantly lower for the 500 mg/kg mouse group compared with that of the control group, confirming that an increase in the glucose level was significantly suppressed by the polyphenols from immature Hosui fruits. This is consistent with results from Wang et al. 2015 [18], who reported that Yaguang pear (*Pyrus ussuriensis*) fruits exhibited potential α -glucosidase inhibitory activity *in vitro* and exerted anti-diabetic effects in streptozocin-induced type 2 diabetic mice. Moreover, the polyphenols from the fruits of grape, apple, cacao, and persimmon have also been found to suppress increases in blood glucose [19,20,21,22].

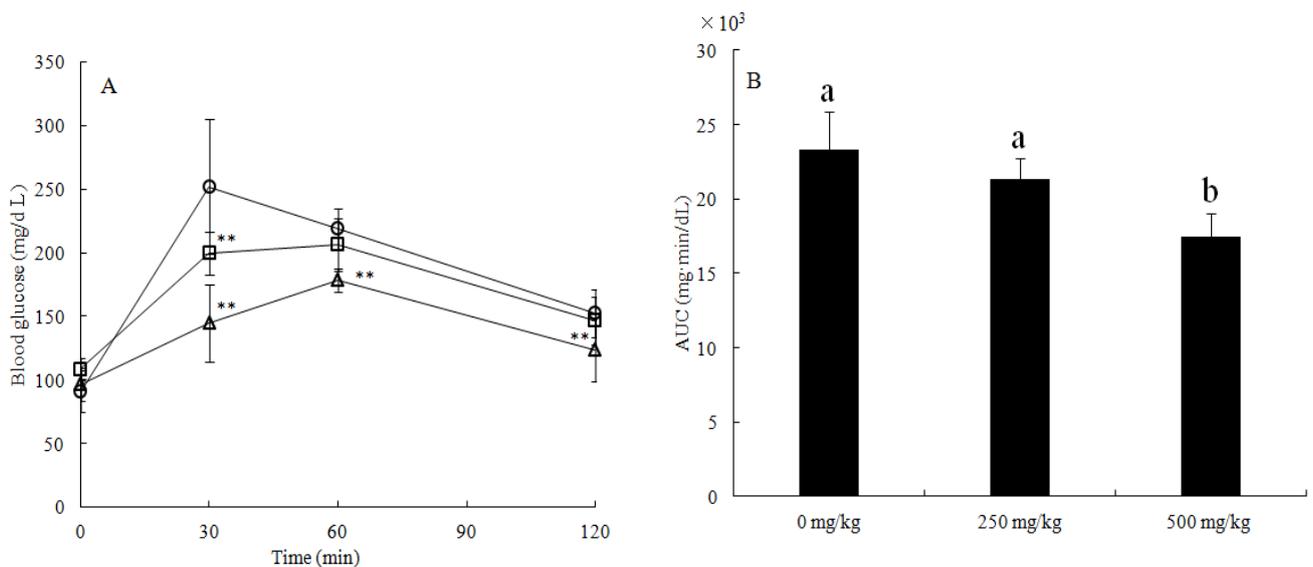


Figure 3. Inhibitory effect of immature Hosui pear fruit polyphenols on blood glucose levels *in vivo*. (A) Blood glucose levels after oral starch tolerance test in mice to monitor the inhibitory effect of polyphenols from immature Hosui pear fruits. Circles, 0 mg/kg mouse; squares, 250 mg/kg mouse; triangles, 500 mg/kg mouse. Data represent the mean \pm standard error of 5 mice in each group. ** $p < 0.05$ vs. starch (control). (B) AUC values of data shown in panel A. Values represent mean \pm S.E.M. Different superscript letters indicate significant differences ($p < 0.05$)

4. Conclusions

In this study, we found that immature Hosui fruits possess a higher polyphenol content and DPPH radical scavenging activity than immature Kosui fruits. The immature pear fruits of the Hosui and Kosui cultivars showed high ratios of oligomeric and polymeric polyphenols, which inhibited the activity of α -glucosidase in a dose-dependent manner. Moreover, the polyphenols from immature Hosui fruits effectively suppressed elevations in blood glucose levels *in vivo*, as shown by experiments involving the oral administration of starch in mice. These observations indicate that Hosui and Kosui immature pear fruits, which are usually treated as agricultural waste, may serve as a source of nutraceuticals with anti-diabetic activity.

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Statement of Competing Interests

The authors have no competing interests.

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