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Note

Apple Leaf Extract as a Potential Candidate for Suppressing Postprandial Elevation of the Blood Glucose Level

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Summary While the industrial value of fruits has long been recognized, only recently have the leaves of fruit trees been considered to have immense and mostly-untapped potential. In the present study, the physiological effects of apple leaf extract in mice were investigated. In addition, we sought to elucidate the active principle(s) and examined its potential for application. Apple leaf extract suppressed postprandial elevation of the blood glucose level and increased the residual amount of glucose in the small intestine in glucose-loaded mice compared with those in control mice. Bioassay-guided fractionation led to an active component that was identified as phloridzin, a known SGLT inhibitor, based on an analysis of its spectral data. With regard to an anti-hyperglycemic effect, extraction with ethanol from leaves of apple tree gave the best results. These effects decreased with heating during the extraction procedure. Since bolus ingestion of the extract did not affect blood glucose levels in normal mice with or without an overnight fast, the inhibitory effects on glucose absorption were not considered to be associated with unspecific gastrointestinal impairment and the extract did not cause hypoglycemia at a normally effective dose. Therefore, the leaf parts of apple tree may be a promising candidate as an industrial resource for maintaining good health in the future.

Key Words *Malus pumila*, apple leaf extract, glucose absorption, phloridzin, anti-hyperglycemic effects

Various functional foods have been developed and distributed in the market. To provide the public with low-cost, high-quality health products, a steady supply of inexpensive and evidence-based materials is needed. In previous studies, we recognized that the leaves of fruit trees were a tremendous untapped resource, and elucidated the mechanism of action and active principle in crude extracts (*1*, *2*). In the present study, we found that apple leaf extract showed anti-hyperglycemic activity in mice. We also clarified the mechanism of action and the active principle of apple leaf extract. Apple leaf extract had remarkable suppressive effects on postprandial elevation of the blood glucose level in mice and may be suitable for use as a resource for making functional foods.

Malus pumila is a species of *Malus* that is native to China. It bears an edible juicy fruit called an "apple." The proverb "*An apple a day keeps the doctor away*," which reflects the health effects of the fruit, dates from the 19th century in Wales (*3*). Compared to many other fruits and vegetables, apples contain relatively low amounts of vitamin C, but are a rich source of other antioxidant compounds (*4*). The fiber content, while less than that in most other fruits, helps regulate bowel movements. Apple polyphenols that are contained in apple fruit are effective for slowing the accumulation of fat (*5*) and for protecting liver function (*6*). However, the biological effects of apple leaf in vivo have not been reported previously. Accordingly, in this study, we elucidated the anti-hyperglycemic action of apple leaf in mice and examined its utility as a source for making functional foods for regulating the blood glucose level.

Apple leaf was collected from a field in Okayama, Japan. Fresh leaves (180 g) were extracted with 10 volumes of ethanol (EtOH) for 1 wk at room temperature. The EtOH extract was concentrated under reduced pressure to give a crude extract as a green powder. The recovery rate from the EtOH extract was 10.0% (w/w). This powder was stored at −20°C and used in subsequent experiments as the crude extract of apple leaf. The EtOH extract of apple leaf (hereinafter called "Apple Leaf") was used in all experiments for consideration as a food material.

Animal studies were conducted according to the 2006 guidelines entitled Notification No. 88 of the Ministry of the Environment in Japan and the Guidelines for Animal Experimentation of Tokyo University of Marine Science and Technology with the approval of the Animal Care and Use Committee of Tokyo University of

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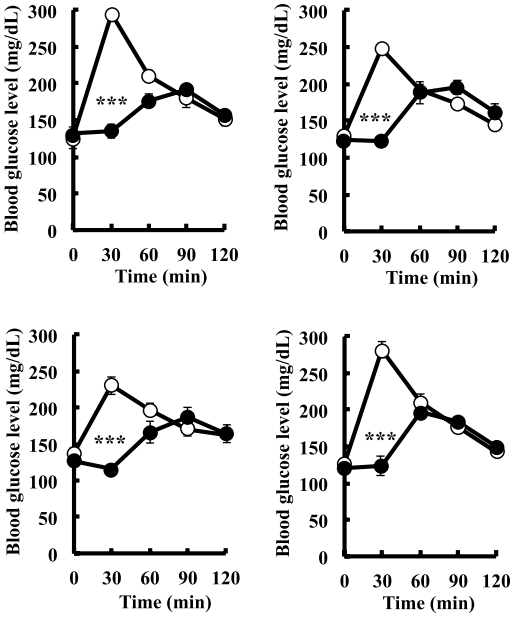

Fig. 1. Effects of Apple Leaf on postprandial elevation of the blood glucose level in orally carbohydrate-loaded mice. Six-week-old male Slc:ddY mice were orally administered starch at 1,000 mg/kg (A), maltose at 1,000 mg/kg (B), sucrose at 2,000 mg/kg (C), or glucose at 1,000 mg/kg (D), either alone (control: open circles) or with Apple Leaf at 1,000 mg/kg (filled circles) after an overnight fast (24 h). Blood samples were taken at 0, 30, 60, 90, and 120 min after loading. Each point represents the mean±SE (n=6). Significant difference in the glucose level vs. that in the corresponding control: *** $p<0.005$ (Student's t-test).

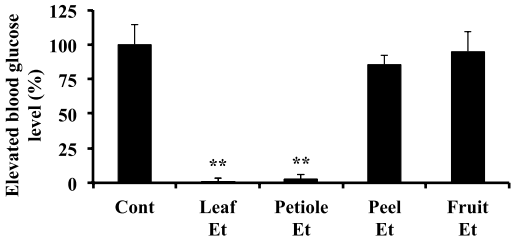

Fig. 2. Effects of different parts of the apple tree on postprandial elevation of the blood glucose level in oral glucose-loaded mice. Six-week-old male Slc:ddY mice were orally administered glucose at 1,000 mg/kg, either alone (control) or with an ethanol extract of the leaf, petiole, peel, or fruit of apple after an overnight fast (24 h). Each increase in the blood glucose level was calculated by subtracting the blood glucose level at 0 min from that at 30 min after loading. Each bar represents the mean±SE (n=6). Significant difference in the glucose level vs. that in the corresponding control: ** $p<0.01$ (ANOVA followed by a Tukey post-hoc analysis).

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The effects of Apple Leaf on the postprandial blood glucose level were examined in the oral carbohydrate-loading test in mice. For the oral carbohydrate-loading test, 6-wk-old male Slc:ddY mice (Japan SLC, Inc., Shizuoka, Japan) were deprived of food for 24 h and then orally administrated starch, maltose, or glucose at 1,000 mg/kg, or sucrose at 2,000 mg/kg, either alone or with Apple Leaf at 1,000 mg/kg, dissolved in 1 mL of distilled water. The blood glucose level was determined with a Glucose C-II Test Wako Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), based on the mutarotase-glucose oxidase method. As shown in Fig. 1, the effects of Apple Leaf on the postprandial blood glucose level were examined in carbohydrate-loaded mice. In the control group (carbohydrates alone without Apple Leaf), the blood glucose level reached a maximum value at 30 min after loading. When Apple Leaf was orally administered simultaneously with starch (A), maltose (B), sucrose (C) or glucose (D), the blood glucose level at 30 min after administration was significantly suppressed ($p<0.005$). Data are expressed as the mean± SE, and the statistical significance was evaluated by the Student t-test or ANOVA followed by a Tukey post-hoc analysis, where $p<0.05$ was considered to be statistically significant.

The conditions for extraction from materials of the apple tree were investigated in in vivo experiments. While hot-water extraction of fresh apple leaf gave 14.5% (w/w) of crude extract, the suppressive effect at 1,000 mg/kg on the elevated blood glucose level for 30 min in glucose-loaded mice was only 50.1±4.0% of that in control mice. The effects of different parts of the apple tree on postprandial elevation of the blood glucose level were examined in glucose-loaded mice, as shown in Fig. 2. When EtOH extracts of apple leaf and petiole were administrated orally simultaneously with glucose, the elevation of the blood glucose level at 30 min after administration was significantly suppressed ($p<0.05$). On the other hand, extracts of other parts (apple peel and fruit) had no effects on the elevated blood glucose level. Therefore, the extracts of apple leaf and petiole were suggested to be potential sources for materials to suppress postprandial elevation of the blood glucose level in vivo.

To elucidate the mechanism of the suppressive effects of Apple Leaf in glucose-loaded mice, the amounts of glucose in the small intestine were confirmed in fasted mice (deprived of food for 24 h). These mice were first anesthetized with propofol (10 mL/kg i.p., Mylan, Tokyo, Japan), and then an incision was made in the abdomen (*2*). Next, 1,000 mg/kg of glucose with or without Apple Leaf (1,000 mg/kg) was injected by catheter from the pyloric region to the duodenal region. The small intestine was then ligated at two points, 15 cm apart, and returned to its original position. After 30 min, the contents of the intestine segment were recovered and washed with 5 mL of saline and the remaining glucose was measured. The residual amount of glucose in the intestine in the control group was 4.2±1.3%, as shown in Fig. 3A. The residual amount of glucose (i.e., not absorbed) in the recovered intestine contents at 30 min reflected the absorption of glucose from the intestinal mucous membrane. On the other hand, the residual amount of glucose in the intestine of

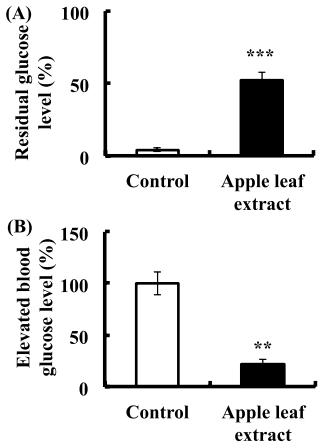

Fig. 3. Effects of Apple Leaf on glucose absorption in enterally glucose-loaded mice. Six-week-old male Slc:ddY mice were enterally administered glucose at 1,000 mg/kg either alone or with Apple Leaf at 1,000 mg/kg after an overnight fast (24 h). The residual amount of glucose (i.e., not absorbed) in the recovered intestine contents (A) and the elevated blood glucose level (B) at 30 min reflected the absorption of glucose from the intestinal mucous membrane into blood vessels. Each point represents the mean±SE (n=8). Significant difference in the glucose level vs. that in the corresponding control: $^{**}p<0.01$, $^{***}p<0.005$ (Student's t-test).

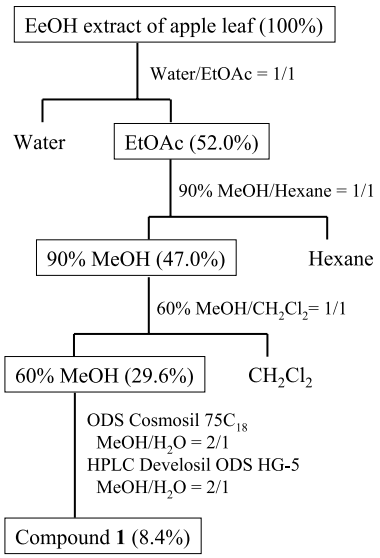

Fig. 4. Scheme for the separation of the active compound (**1**) from apple leaf extract.

mice treated with Apple Leaf at 1,000 mg/kg (52.2±5.4%) was significantly higher than that in the control group. Thus, at 30 min after glucose-loading in mice, Apple Leaf significantly suppressed the elevation of the blood glucose level compared with that in the control group, as shown in Fig. 3B. Apple Leaf did not show inhibitory activity against carbohydrate digestive enzymes (α-amylase and α-glycosidase) in vitro. Therefore, it was considered that Apple Leaf acts by suppressing glucose uptake in the intestine. These results revealed that Apple Leaf is an interesting natural resource with the potential to regulate the postprandial blood glucose level.

The active principle in Apple Leaf was obtained by bioassay-guided separation in glucose-loaded mice. The separation scheme is shown in Fig. 4. The crude powder was resolved in water and partitioned with ethyl acetate (EtOAc), and the EtOAc layer was dried and further partitioned between 90% methanol (MeOH) and n-hexane (Hexane). The activity was found in the 60% MeOH layer, which was obtained from the 90% MeOH layer after partitioning with CH_2Cl_2. Some (5.3 g, 29.6%) of the recovered 60% MeOH layer was applied to an ODS gel column. The fraction that showed specific activity was subjected to further purification. Final purification was achieved by repeated separation by reversed-phase HPLC (Develosil HG-5), and gave 1.52 mg of compound **1** (8.4% of the crude extract). For the spectroscopic analysis of compound **1**, NMR data were acquired in CD_3OD on a Bruker Avance 400 MHz instrument. The ^{13}C NMR spectrum (in MeOD) showed signals for 21 carbons, including two overlapping signals. Based on a detailed analysis of 2D NMR (COSY, HMQC, and HMBC) data, the structure of **1** was estimated to be phloridzin. The ^{1}H and ^{13}C NMR spectral data for **1** were consistent with the chemical shifts of commercially available phloridzin isolated from apple tree bark (*7*, *8*), as shown in Table 1. Finally, compound **1** from Apple Leaf was identified as phloridzin (*9*).

Phloridzin is known to be an SGLT inhibitor isolated from the bark of the apple tree (*10*–*13*). The effects of phloridzin, i.e., both isolated compound **1** and that obtained commercially from Sigma-Aldrich Chemical Co. (St. Louis, MO), on the postprandial blood glucose level were then examined with glucose-loaded mice. In the control group (glucose alone without phloridzin), the elevation of the blood glucose level was calculated to be 133.6±10.5 mg/dL at 30 min after glucose-loading. Oral administration of purified compound **1** (31.25, 62.5, and 125 mg/kg) suppressed the elevation of the blood glucose level in a dose-dependent manner, with IC_{50}=109.5 mg/kg. The IC_{50} value was corresponded to that of authentic phloridzin under the same experimental conditions. These results revealed that the active principal in Apple Leaf should be phloridzin, and that the apple leaf extract did not contain any other active components to suppress postprandial elevation of the blood glucose level in mice.

The 60% MeOH fraction, which inhibited glucose absorption, did not contain any active compounds other than phloridzin. Therefore, apple leaf contains phloridzin as the sole active compound. The mechanism of action has been reported to involve competitive inhibition by phloridzin (*14*). These results demonstrated that apple leaf extract strongly suppressed the postprandial elevation of the blood glucose level and has potential as a resource for functional foods. Based on reports on the extraction of phloridzin from apple leaf, phloridzin generally represents 8–20% of the crude extract (*15*, *16*). Since the leaves of fruit trees have not been

Table 1. NMR spectral data for compound 1 and phloridzin (CD_3OD).

Position	Compound 1		Phloridzin dihydrate	
	δ_C (ppm)[a]	δ_H (ppm)[b]	δ_C (ppm)[a]	δ_H (ppm)[b]
2	31.4 t	2.88 (2H, t, J=7.4)	31.3 t	2.88 (2H, t, J=7.6)
3	47.5 t	3.43 (2H, m)	47.4 t	3.43 (2H, m)
4	207.1 s	—	207.0 s	—
4a	107.4 s	—	107.3 s	—
5	162.8 s	—	162.8 s	—
6	96.0 d	6.18 (1H, d, J=1.9)	95.9 d	6.18 (1H, d, J=2.2)
7	166.4 s	—	166.4 s	—
8	98.9 d	5.96 (1H, d, J=1.9)	98.8 d	5.96 (1H, d, J=2.2)
8a	168.1 s	—	168.0 s	—
1′	134.4 s	—	134.4 s	—
2′, 6′	116.6 d	6.68 (2H, d, J=8.4)	116.6 d	6.69 (2H, d, J=8.5)
3′, 5′	130.9 d	7.06 (2H, d, J=8.4)	130.9 d	7.06 (2H, d, J=8.5)
4′	156.9 s	—	156.8 s	—
Glc 1″	102.6 d	5.04 (1H, d, J=7.0)	102.5 d	5.04 (1H, d, J=7.2)
2″	75.2 d	3.45* (1H, m)	75.2 d	3.48* (1H, m)
3″	79.0 d	3.47* (1H, m)	78.9 d	3.48* (1H, m)
4″	71.7 d	3.39* (1H, m)	71.5 d	3.39* (1H, m)
5″	79.0 d	3.41* (1H, m)	78.8 d	3.40* (1H, m)
6″	63.0 t	3.91 (1H, d, J=11.9)	62.9 t	3.91 (1H, d, J=12.0)
		3.72 (1H, dd, J=5.1, 11.9)		3.72 (1H, dd, J=5.3, 12.0)

[a] ^{13}C chemical shifts (100 MHz) referenced to CD_3OD (47.5 ppm) followed by multiplicity based on the HMQC spectrum.
[b] ^{1}H chemical shifts (400 MHz) referenced to residual CD_3OD (3.31 ppm) followed bymultiplicity and coupling constants (J/Hz) in parentheses.
* Assignment based on HMQC and COSY spectra.

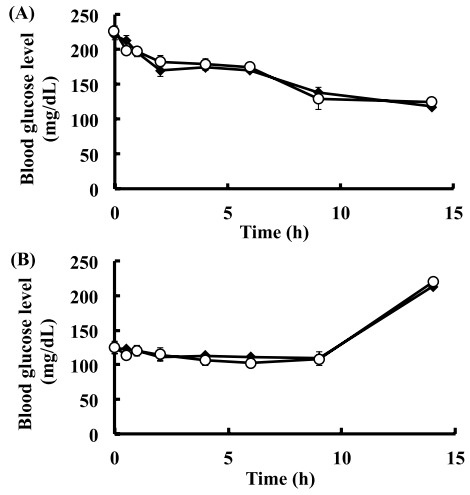

Fig. 5. Effects of Apple Leaf on the time-course of the change in the blood glucose level in mice without (A) or with a 24-h fast (B). In each experiment, 6-wk-old male Slc:ddY mice were separated into two groups. Control mice (closed diamonds) and treated mice given 2,000 mg/kg of Apple Leaf (open circles) were drunk water freely. The time-course of changes in the blood glucose level in unfasted mice (A) and fasted mice (B). In Panel B, fasting was discontinued at 9 h after ingestion. There was no significant differences between the two groups in either experiment (n=8).

used for industrial application, apple leaves are expected to be an important potential resource for functional foods.

The effects of Apple Leaf on the blood glucose level were investigated by experiments with the ingestion of Apple Leaf (2,000 mg/kg) by unfasted and fasted mice. As shown in Fig. 5, there was no significant difference between the control and apple leaf extract-injected groups under either unfasted (Fig. 5A) or 24-h fasted conditions (Fig. 5B). Apple Leaf did not induce hypoglycemia or impair the absorption of glucose in vivo. These findings suggested that Apple Leaf does not have an irreversible harmful effect on normal nutrition in mice. In addition, a bolus oral administration of Apple Leaf (5,000 mg/kg) did not alter the behavior of mice as observed for 24 h. These findings suggest that Apple Leaf should be safe as a food material under these conditions in mice. Future studies will be needed to examine the pharmacological actions of Apple Leaf compared to those of other anti-diabetic agents after long-term administration with the use of animal models of diabetes mellitus.

In conclusion, the present results revealed that Apple Leaf suppressed the postprandial blood glucose level through the inhibition of glucose absorption by the active principle phloridzin. These findings suggest that Apple Leaf may be useful as a natural source for improving postprandial hyperglycemia.

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