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Comparison of 1-Deoxynojirimycin and Aqueous Mulberry Leaf Extract with Emphasis on Postprandial Hypoglycemic Effects: *In Vivo* and *in Vitro* Studies

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ABSTRACT: Carbohydrate digestion by α-glucosidase and subsequent glucose uptake at the brush border are critical for postprandial blood glucose control. Any specific inhibitors are useful as hyperglycemia modulating agents. In this study, it was postulated that an array of active components in mulberry leaf extract (MLE) may provide higher potency in inhibiting intestinal glucose absorption compared to the single component 1-deoxynojirimycin (DNJ), which is recognized as a promising inhibitor of intestinal glucose absorption. Both MLE and DNJ were active in inhibiting α-glucosidase. However, in Caco-2 cells, only MLE showed significant inhibition of 2-deoxyglucose uptake, whereas DNJ was ineffective. For glucose loading, co-administration of MLE resulted in potent inhibitions of glucose responses compared to those by DNJ in Sprague Dawley (SD) rats, but this was not found for maltose loading. These novel findings add evidence that the unabsorbed phytochemicals in MLE compete with glucose for intestinal glucose transporters, but DNJ itself does not. We also evaluated the timing of MLE consumption. By administering MLE for 30 min before glucose loading, the incremental area under the curve (IAUC) was significantly lowered in the rats, as compared to a simultaneously administered group. Similarly, cellular glucose uptake was significantly reduced in Caco-2 cells following pretreatment.

KEYWORDS: 1-deoxynojirimycin, glucose absorption, maltose digestion, mulberry leaf extract, pretreatment

INTRODUCTION

1-Deoxynojirimycin (DNJ) is a 5-amino-1,5-deoxy-D-glucopyranose and, thus, a D-glucose analogue (Figure 1). This analogue has been receiving special attention as the most promising competitive inhibitor of intestinal α-glucosidases known thus far.[1−5] It affects the final step of carbohydrate digestion in the intestinal lumen and retards the absorption of dietary carbohydrates to suppress postprandial hyperglycemia. In addition, DNJ has been postulated to inhibit D-glucose uptake at the intestinal brush border membrane because of its similar size and, to some extent, structure to D-glucose.[6] However, some investigators have suggested that DNJ has very little or no effect on small intestinal D-glucose uptake, on the basis of experiments using brush border membrane vesicles from small intestines or using preparations of intact small intestines.[2]

Despite potent α-glucosidase inhibitory activity *in vitro*, its efficacy *in vivo* was reported to be rather moderate. Some drugs, such as acabose, voglibose, and miglitol, have been developed to improve postprandial hyperglycemic activity. However, because of the undesirable side effects of chemical drugs,[7−9] culinary medicinal plants are gaining attention as alternatives for providing potent but safe hypoglycemic effects. Mulberry leaf, which has been used as an antidiabetic remedy in oriental medicine, contains 18 sugar-mimic alkaloids. DNJ is present in high concentrations, accounting for 50% of the iminosugars.[10] It also contains other bioactive components, such as dietary fiber (8.15−11.32%),[11] rutin (573 mg/100 g), isoquercitrin (194 mg/100 g), quercetin 3-(6-malonylglucoside) (900 mg/100 g), and astragalin (31 mg/100 g), which are known to have limited bioavailability.[12] Moreover, in a previous study, we identified that the bioavailability of DNJ was significantly lower in mulberry leaf extract (MLE) compared to single compound DNJ.[13]

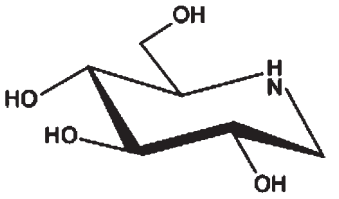

Figure 1. Chemical structure of DNJ.

Therefore, in this study, we proposed that an array of unabsorbed ingredients in MLE may play additional roles in the gut to reduce or inhibit intestinal glucose digestion and absorption, resulting in more potent hypoglycemic effects, when compared to the equivalent amount of single-component DNJ. First, to compare the inhibitory potency of MLE and DNJ on α-glucosidase activity, half maximal inhibitory concentration (IC_{50}) values were calculated in cell-free systems. Second, to determine whether both MLE and DNJ inhibit glucose transport at the intestinal mucosal epithelium, Caco-2 cell monolayers, derived from a human colonic carcinoma, were studied. Then, to test the usefulness of our *in vitro* findings, the postprandial glucose responses of MLE and DNJ were compared using

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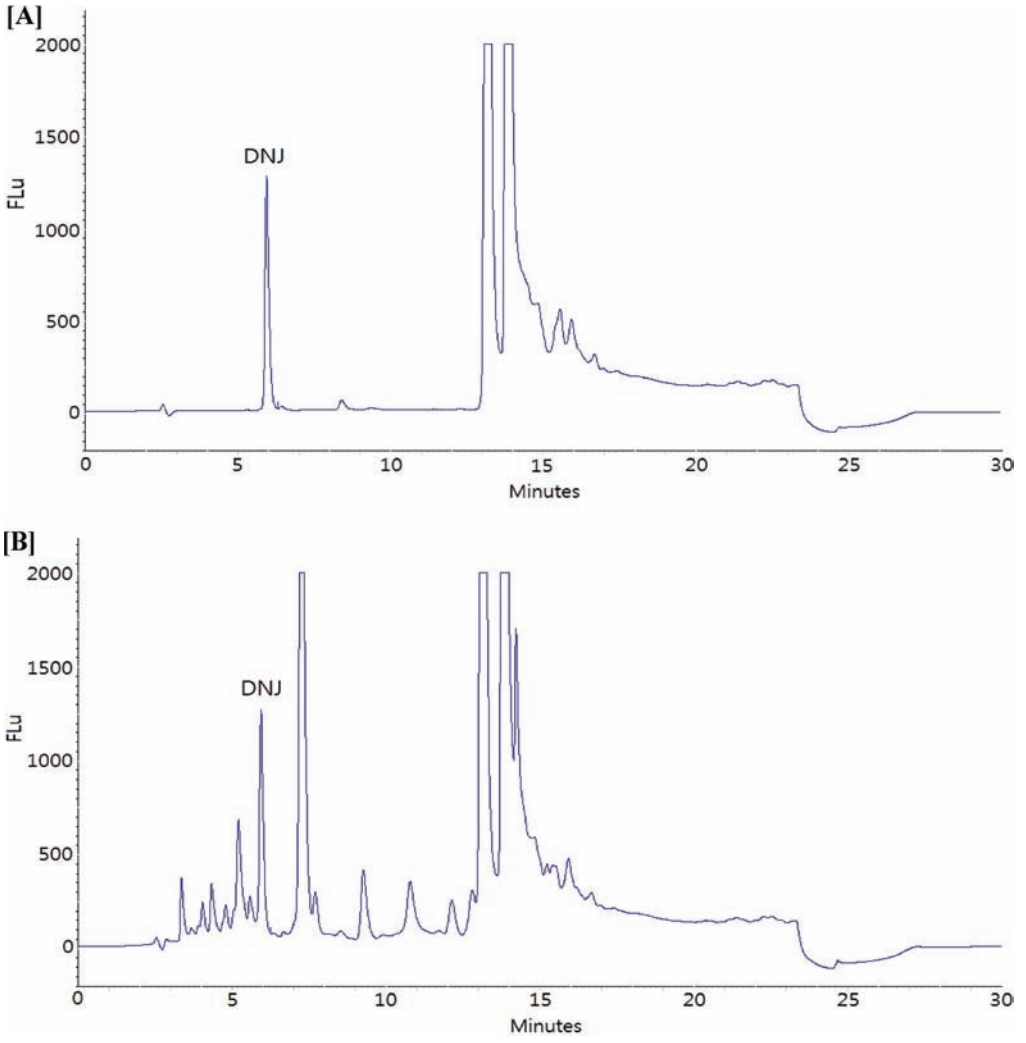

Figure 2. Representative HPLC chromatograms of (A) DNJ and (B) aqueous MLE. HPLC conditions are described in the Materials and Methods.

Sprague Dawley (SD) rats. Lastly, to determine the appropriate timing of MLE administration, inhibitory effects of pre-administration and simultaneous administration were compared using both Caco-2 cell monolayers and rats.

MATERIALS AND METHODS

Preparation of MLE. MLE was kindly provided by the Jeonnam Biotechnology Center (Naju, Korea). Briefly, dried mulberry (*Morus alba* L.) leaves (ca. 80 g) were powdered and extracted with a 50-fold (w/v) amount of hot water (60 °C) for 1 h by sonication. The extract was centrifuged at 12000*g* for 15 min at 4 °C and freeze-dried for 37 h. The dried extract (yield = 34%) was examined for DNJ content (350 mg/100 g) by high-performance liquid chromatography (HPLC) and used as the MLE. A single compound of DNJ (>98.0%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Determination of the DNJ Content. The DNJ content was determined from the method by Kim et al.[14] A Shiseido HPLC system (Tokyo, Japan), equipped with a fluorescence detector (excitation, 254 nm; emission, 322 nm), was used after derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl). HPLC separation was achieved using an Ultrasphere C18 column (250 × 3.2 mm inner diameter, 5 μm) and a mobile phase consisting of acetonitrile/0.1% aqueous acetic acid (4:6, v/v). The sample injection volume was 10 μL. DNJ was identified by the retention time, and the samples were quantified using standard curves with HPLC peak areas as a function of the concentration (Figure 2).

Assay of α-Glucosidase Activity. The crude rat intestinal α-glucosidase enzyme in 0.9% sodium chloride solution was prepared from rat intestinal acetone powder. Maltase inhibitory activity was measured according to Ohta et al.[15] The reaction mixture for maltase inhibitory activity determination consisted of a 100 mM maleate buffer adjusted to pH 6.0 (0.7 mL), 100 mM maltose (0.1 mL), and DNJ or MLE in distilled water (0.1 mL). The mixture was preincubated for 5 min at 37 °C, and the reaction was initiated by adding a crude α-glucosidase solution (0.1 mL). After 60 min at 37 °C, the reaction was terminated by adding 2.0 M maleate-Tris-NaOH buffer at pH 7.4 (1.0 mL). The glucose released in the reaction mixture was determined by the glucose oxidase−peroxidase method.

Glucose Transport Assay. Caco-2 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), and 1% penicillin−streptomycin (50 units/mL). For the experiment, the cells were seeded on 12-well plates (Nalge Nunc International, Naperville, IL) and grown to confluence. Monolayers were washed twice with phosphate-buffered saline (PBS) and preincubated with Krebs buffer [5 mM glucose, 30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 130 mM NaCl, 4 mM

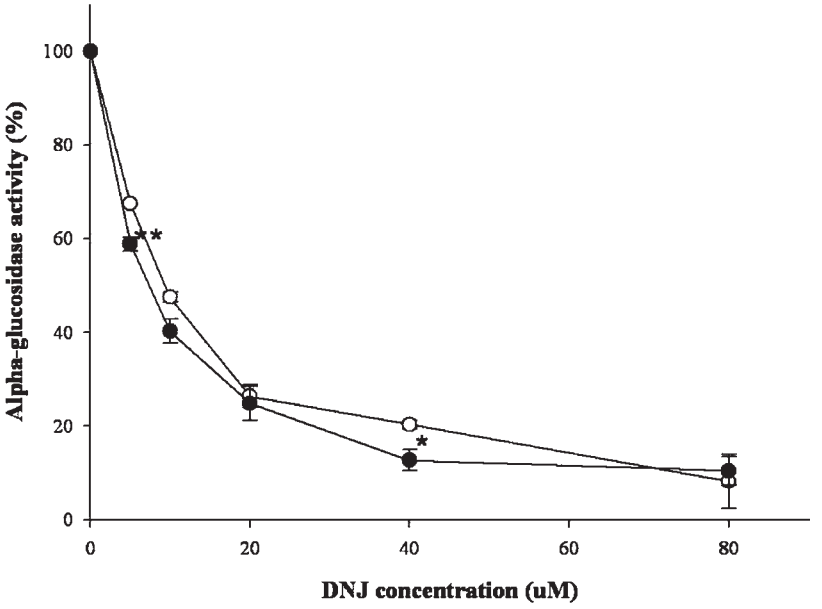

Figure 3. α-Glucosidase activity was dose-dependently inhibited by MLE and DNJ in cell-free systems. A maltose and α-glucosidase mixture was treated with MLE (●) or DNJ (○). Both MLE and DNJ were prepared to provide 0, 5, 10, 20, 40, and 80 μM as DNJ equivalents. After 5 min of incubation, the glucose released was measured. The data shown (mean ± SE; $n = 3$) are typical of more than three experiments with similar results. The presence of an asterisk indicates that the cellular uptake of 2-deoxyglucose was significantly less (*, $p < 0.05$; **, $p < 0.01$) with MLE treatment than with DNJ treatment at each concentration by an independent t test.

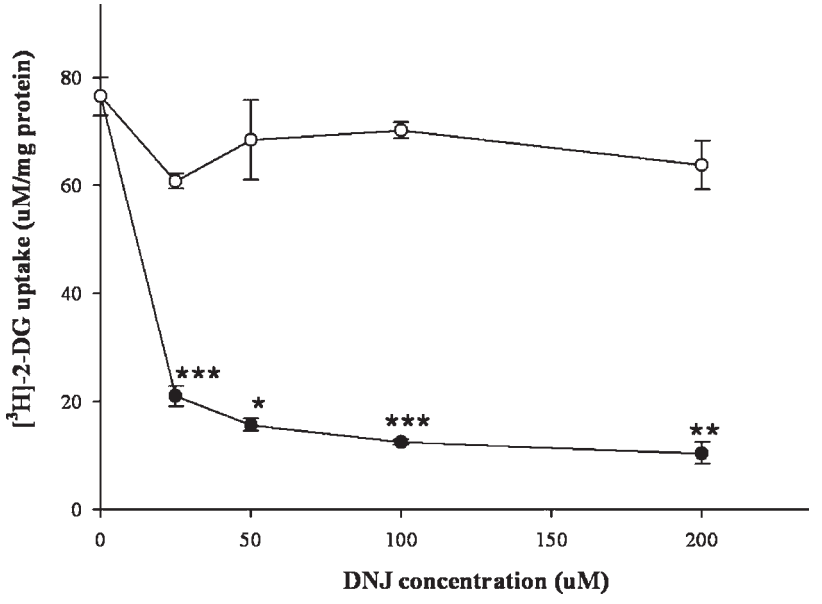

Figure 4. Apparent uptake of [^{3}H]-2-deoxygucose was significantly inhibited by increasing concentrations of MLE (●) but not by DNJ (○) in Caco-2 cells. Caco-2 cells in 12-well plates were incubated in Krebs buffer containing 1 mM [^{3}H]-2-deoxygucose for 5 min with increasing concentrations of MLE or DNJ (0, 25, 50, 100, and 200 μM as DNJ equivalents) and then determined by scintillation spectrometry. The data shown (mean ± SE; $n = 3$) are typical of more than three experiments with similar results. The presence of an asterisk indicates that the cellular uptake of 2-deoxyglucose was significantly less (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$) with MLE treatment than with DNJ treatment at each concentration by an independent t test.

KH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$ at pH 7.4]. Glucose uptake measurements were initiated by replacing the medium with 300 μL of prewarmed Krebs buffer without glucose, supplemented with [^{3}H]-2-deoxyglucose and MLE or DNJ together for 5 min at 37 °C. The MLE and DNJ solutions were prepared fresh. Uptake was terminated by adding 1 mL of ice-cold PBS, and the cells were washed 3 times with the same solution before lysis with 300 μL of NaOH (0.1 M)/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (10 g/L) solution. Aliquots of 100 μL were added to 0.5 mL of scintillation cocktail for radioactivity determination, using a LS 6500 liquid scintillation counter (Beckman Coulter, Brea, CA). Aliquots of 10 μL were used for protein measurement by bichinchonic acid.

Oral Glucose Tolerance Test. Male SD rats at 13 weeks of age (Orient Bio, Inc., Seoul, Korea) were housed individually in standard stainless-steel cages at 23 °C and 45 ± 5% humidity with a 12 h light/dark cycle. All facilities and standards of care were maintained in

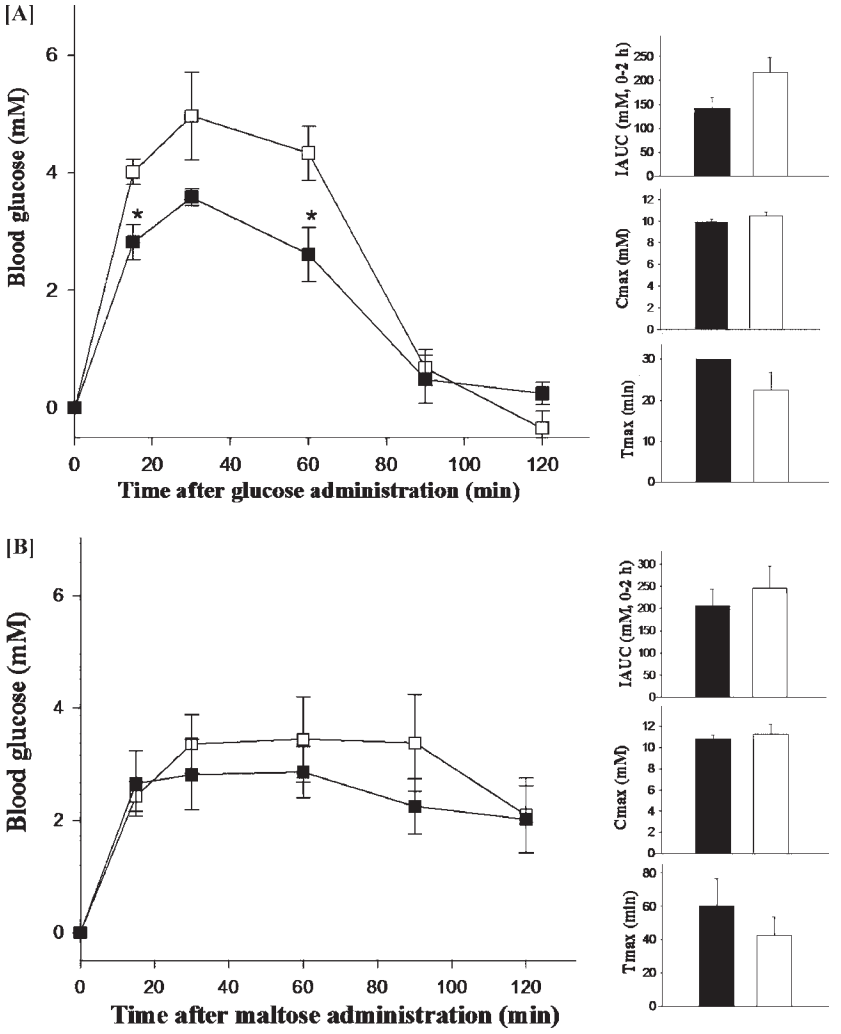

Figure 5. Hypoglycemic effect of MLE was more potent than that of DNJ in SD rats. After overnight fasting for 12 h, the rats (n = 5/group) received 1 mL of sugar [(A) glucose or (B) maltose] solution (2 g/kg of body weight) with MLE (●) or DNJ (○) equivalent to 3 mg/kg of body weight DNJ by gavage. Blood glucose levels were determined from tail blood samples by a glucometer at the indicated times. Comparisons were performed between the MLE- and DNJ-treated groups. (∗) $p < 0.05$.

compliance with the Guide for the Care and Use of Laboratory Animals of Ewha Womans University. For the experiments, the animals were randomly divided into two groups according to fasting blood glucose (n = 5, each). After overnight fasting for 12 h, a glucose solution was administered simultaneously with MLE or DNJ by gavage feeding. Blood samples were collected by tail vein bleeding at 0, 15, 30, 60, 90, and 120 min. Glucose concentrations were immediately determined using an Accu-Check (Roche Diagnostics, Manheim, Germany). To confirm the appropriate timing of MLE administration, MLE equivalent to 3 mg/kg of body weight DNJ was given 15 or 30 min before the administration of glucose. The total incremental area under the curve (IAUC), peak time (T_{max}), and peak concentration (C_{max}) were determined for each condition.

Statistical Analysis. The results are expressed as the mean ± standard error (SE). All *in vitro* data shown are representative of at least three experiments that yielded similar results. For α-glucosidase activity in the cell-free systems as well as [^{3}H]-2-deoxyglucose uptake in Caco-2 cells, IC_{50} values were calculated for MLE and DNJ using nonlinear regression analysis by fitting to a mono-exponential equation (Sigmaplot). Comparisons of the MLE and DNJ groups were achieved by Student's t test using the SAS package program (version 9.1, for Windows). Differences between the means were considered to be significant when $p < 0.05$.

RESULTS AND DISCUSSION

Comparison of Inhibitory Effects on α-Glucosidase. Cell-free *in vitro* systems were used to investigate whether MLE and DNJ would inhibit α-glucosidase in a similar manner. Prior to the experiments, all concentration points from MLE and DNJ were matched using HPLC analysis and presented as DNJ equivalents (5−80 μM). Both MLE and DNJ inhibited α-glucosidase in a dose-dependent manner (Figure 3). Data points were analyzed to calculate the DNJ concentration at which α-glucosidase activity was decreased by half compared to a control without DNJ (IC_{50}). The IC_{50} values for MLE and DNJ were 7.35 and 9.39 μM, respectively. Although MLE showed statistically higher potency at certain concentration levels, the inhibitory patterns of MLE and DNJ were very similar to each other. This result clearly shows that the inhibitory effects of MLE on α-glucosidase activity were essentially attributed to DNJ. There seems to be no other components that have antagonistic effects on them.

Comparison of Inhibitory Effects on Glucose Uptake in the Caco-2 Cell Line. The inhibitory effects of MLE and DNJ on the apparent uptake of glucose from the apical sides were tested using Caco-2 cells grown on plastic. The Caco-2 cell

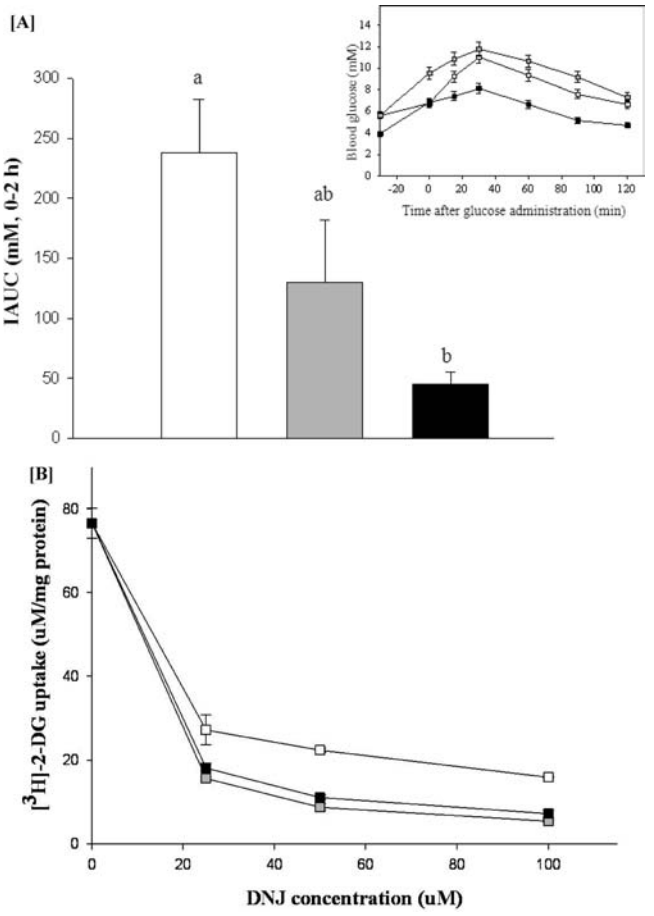

Figure 6. Inhibition of cellular [^{3}H]-2-deoxyglucose uptake and postprandial glucose response were enhanced by MLE pretreatment. (A) IAUC of plasma glucose levels from 0 to 120 min. (B) Cellular uptake of [^{3}H]-2-deoxyglucose in Caco-2 monolayers. (White) Control, (gray) 15 min pretreatment, and (black) 30 min pretreatment. Data are expressed as the mean ± SE. (a and b) Mean values among bars without a common letter differ ($p < 0.05$).

system derived from human adenocarcinoma is a straightforward model system useful for evaluating uptake inhibition because the cells will differentiate into polarized enterocyte-like monolayers with a well-defined brush border on the apical surface, acting similarly to intestinal epithelial cells.[16] The tested sugar substrate was 2-deoxyglucose at 1 mM. Glucose and 2-deoxyglucose are phosphorylated by hexokinases in cells. Glucose-6-phosphate does not accumulate at a high level because it rapidly enters the glycolytic and pentose phosphate pathways. In contrast, 2-deoxyglucose-6-phosphate cannot be metabolized further but accumulates; therefore, it is useful for cellular absorption assays of glucose.[17] Levels of MLE were determined in the range of 1.05−8.41 mg/mL (25−200 μM, if expressed as DNJ) by considering the maximum concentration used for rats in a previous experiment[18] and the estimated volume of gastrointestinal fluid in the rats (12.6 mL).[19] HPLC analyses were always performed prior to each experiment.

Without MLE, the apparent uptake of 2-deoxyglucose was 76.6 μM/mg of cell protein. With MLE, 2-deoxyglucose uptake was significantly suppressed by increasing the concentration of MLE and its IC_{50} was observed at 0.49 mg/mL (11.75 μM if expressed as DNJ). At the maximum concentration tested, 2-deoxyglucose uptake was 10.7 μM/mg of cell protein, showing a decrease of 13.6%. In contrast, 2-deoxyglucose uptake was unchanged (Figure 4). To our knowledge, this is the first study to support that DNJ itself does not compete with glucose for glucose transporters using Caco-2 human intestinal cells. In particular, components that are inefficiently absorbed, such as quercetin[17] and soluble dietary fiber,[20] might inhibit intestinal luminal transport of glucose. Soluble dietary fiber has the function of inhibiting glucose uptake by binding sugar groups.[20] Moreover, quercetin has been well-known as a potent luminal inhibitor of sugar absorption.[17] Because MLE is multi-compounds mixed with soluble dietary fiber and quercetin including DNJ, although single-compound DNJ did not show the inhibition of glucose uptake, the MLE could inhibit glucose uptake. According to our lab data, it was identified that MLE contained 73.5 mg/100 g of quercetin (55.3 mg/100 g as aglycone) and 24% of soluble dietary fiber.

Comparison of Suppressive Effects on Plasma Glucose Increases *in Vivo*. Postprandial hypoglycemic effects are frequently expressed by the area under the plasma concentration−time curve (IAUC) and maximum concentration (C_{max}). In a previous experiment, we confirmed that co-administration of MLE (3.75 g/kg of body weight, equivalent to 6 mg/kg of body weight DNJ) suppressed postprandial hyperglycemia by both glucose loading ($p < 0.01$) and maltose loading ($p < 0.05$), as compared to their respective controls.[18] In this study, to test the physiological relevance of our *in vitro* findings in animals, we compared the hypoglycemic effects of MLE and DNJ. For glucose loading (Figure 5A), MLE showed significantly higher potency toward plasma glucose suppression at 15 and 60 min compared to DNJ ($p < 0.05$). Accordingly, the IAUC from the basal level for 120 min decreased by 34.3% ($p = 0.1076$). Although not statistically significant, the peak time was delayed from 22.5 to 30.0 min ($p = 0.1817$) and the peak height was reduced from 10.4 to 9.9 mM ($p = 0.2226$). For maltose loading (Figure 5B), MLE tended to have an overall more potent inhibitory effect compared to DNJ: T_{max} (60 versus 42.5 min; $p = 0.4084$), C_{max} (10.8 versus 11.3 mM; $p = 0.6498$), and IAUC (205.9 versus 245.9 mM; $p = 0.5363$). However, these differences were not statistically significant. Taken together, the *in vivo* data were consistent with the *in vitro* findings.

Timing of MLE Administration on Postprandial Hyperglycemia. Miyahara et al.[21] reported that the ethanol extract of mulberry leaf suppressed postprandial increases in blood glucose when given 30 min before carbohydrate administration. However, some others have reported the inhibitory effects of MLE when it is given simultaneously with sugars.[1,22] On the basis of the assumption that MLE should be taken before meals because its inhibitions of α-glucosidase and glucose uptake primarily modify postprandial plasma glucose levels, we chose to compare the pre-administration versus simultaneous administration of MLE *in vitro* as well as *in vivo*. The IAUC of the pre-administration group decreased significantly compared to the simultaneous-administration group (Figure 6A). Similarly, cellular uptake of 2-DG was lowered by pretreatment at higher concentrations, although the differences did not reach statistical significance (Figure 6B).

Conclusion. The health benefits of herbal extracts are based on their chemical composition and bioavailability. MLE contains an array of phytochemicals that have limited bioavailability, and their occurrence at the systemic level is relatively low.[13,23] In this study, we postulated that unabsorbed phytochemicals may be

active in the gut to reduce or inhibit intestinal glucose absorption. The novel finding of our study was that DNJ itself did not compete with glucose for glucose transporters. We used Caco-2 cell monolayers *in vitro* to test the hypothesis, and postprandial glucose responses were examined *in vivo* for physiological relevance. While more research is needed, the results obtained from this study support the hypothesis, and therefore, MLE could potentially be used as a dietary supplement to reduce or suppress postprandial glucose absorption. Furthermore, the data presented in this paper support that MLE is more effective when ingested 30 min before a meal.

The number of patients with diabetes or prediabetic conditions is currently increasing. Therefore, it is crucial to develop functional foods that have suppressive effects on both blood glucose response and insulin secretion. Although the majority of DNJ is excreted in the feces, DNJ can also be found in plasma in the intact form.[13] Additional evaluations of the systemic effects of MLE on glucose metabolism are currently in progress.

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ABBREVIATIONS USED

DNJ, 1-deoxynojirimycin; FMOC-Cl, 9-fluorenylmethyl chloroformate; HPLC, high-performance liquid chromatography; IAUC, incremental area under the curve; IC_{50}, half maximal inhibitory concentration; MLE, mulberry leaf extract; SD, Sprague Dawley.

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