In Vitro Investigation of 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone for Glycemic Control

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ABSTRACT: 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC), a compound isolated and purified from the dried flower buds of *Cleistocalyx operculatus* (Roxb.) Merr. et Perry (Myrtaceae), was investigated for its glucose control benefits using in vitro methods. DMC showed strong noncompetitive (IC$_{50}$ of 43 μM) inhibition of pancreatic α-amylase; it was, however, ineffective against intestinal α-glucosidase. In addition, DMC exhibited remarkable glucose transport inhibition effects in both simulated fasting and fed states in Caco-2 cell monolayers ($P < 0.05$). Besides, exposure of MIN6 cells to 250 μM H$_2$O$_2$ for 1 h caused a significant viability loss and insulin secretion reduction. Pretreatment of MIN6 cells with DMC for 2 h protected against the H$_2$O$_2$-induced decrease in glucose-stimulated insulin secretion in a dose-dependent manner and also enhanced the impaired basal insulin secretion. Such effects highlight the therapeutic potential of DMC in the management of hyperglycemia.

KEYWORDS: *2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone, pancreatic α-amylase, intestinal α-glucosidase, intestinal glucose transporters, oxidative stress, insulin secretion*

■ INTRODUCTION

Diabetes is characterized by hyperglycemia and ineffective usage or secretion of insulin, with alterations in carbohydrate, protein, and lipid metabolism. It is known that the onset of diabetes is related to many classic risk factors including oxidative stress.[1−3] Reactive oxygen species (ROS) are involved from the onset and enhance progressively to run throughout the course of diabetes and its complications.[4] The ROS contributes to oxidative stress, resulting in β-cells damage in pancreatic islets and a reduced insulin secretion,[5] in turn resulting in hyperglycemic conditions and regeneration of ROS. Growing evidence shows that β-cells are central to the development of type 2 diabetes.[6] Antioxidant therapy[5] and recent studies of supplementation with antioxidant natural products have shown the effects of alleviation of hyperglycemic status by reducing the oxidative stress.[7−9] Glucose generation and absorption in the intestine also play a vital role in hyperglycemia management. Pancreatic α-amylase and intestinal α-glucosidase are two key enzymes for generating absorbable monosaccharides from complex dietary carbohydrates,[10] leading to glucose generation, and glucose transporters are responsible for glucose transport from intestinal to blood circulation. Inhibition of these two enzymes[11,12] and glucose transporters[13] has been shown to reduce blood glucose levels.

Cleistocalyx operculatus (Roxb.) Merr. et Perry (Myrtaceae) is a well-known medicinal plant widely distributed in China, Vietnam, and some other tropical countries.[14] Its leaves and flower buds have been used as an herbal tea to treat gastrointestinal and respiratory disturbances and as an anti-inflammatory medicine in Vietnam,[15] and its flower buds have been commonly consumed in tonic preparations in southern China for thousands of years.[16] Extracts from *C. operculatus* flower buds exhibit antihyperlipidemic,[17] anti-Alzheimer,[18]

cardiotonic,[19] and antihyperglycemic activities[20,21] and preservation of pancreatic islets[15] activity. *C. operculatus* flower buds have been shown to contain the major constituents of flavonoids (flavanone and chalcone), triterpene acids, sterols, and essential oils.[14,16] 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC) (Figure 1), one of the main flavonoids
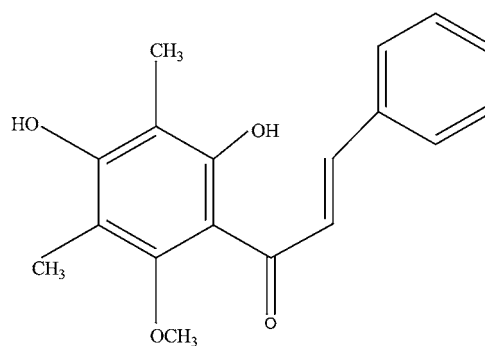

Figure 1. Chemical structure of DMC.

isolated from the dried flower buds of *C. operculatus*,[16] has been shown to exert antitumor,[22−29] antidrug efflux,[30−32] anti-inflammation,[33,34] antioxidant,[14,35,36] antiacute liver injury,[37] antidiarrheal,[37−39] antimicrobial,[40,41] antivirus,[42] and prolyl endopeptidase inhibitor actions.[43] DMC, at 1 mg/20g by oral administration, has also been shown to lower blood glucose

levels in alloxan-induced diabetic mice, an activity the authors attributed to its structure of C-5′-CH$_3$ and the olefinic moiety next to the −C=O group.[44] DMC has been shown to enhance glucose uptake in adipocytes by stimulating the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ).[45] In a previous study, we demonstrated the antihyperglycemic effect of DMC at 0.18 mg/20g by intraperitoneal injection in alloxan-induced diabetic mice (unpublished data) and protection against H$_2$O$_2$-induced toxicity in PC12 cells.[46,47]

Although *C. operculatus* flower buds aqueous extract and DMC have demonstrated hypoglycemic effects in animal models, the underlying mechanism is still unclear. In this study, the effects of DMC on pancreatic α-amylase, intestinal α-glucosidase, intestinal glucose transporters, and the H$_2$O$_2$-induced insulin secretion impairment of MIN6 cell were investigated.

■ MATERIALS AND METHODS

Reagents. DMC was isolated from the dried flower buds of *C. operculatus* in our lab as described by Ye et al.[16] Acarbose, bovine serum albumin (BSA), porcine pancreatic α-amylase, rat intestinal acetone powder, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), *p*-nitrophenyl-α-glucopyranoside (PNPG), phloridzin dehydrate (PZ), and phloretin (PT) were obtained from Sigma-Aldrich (St. Louis, MO). α-(2-Chloro-4-nitrophenyl)-β-1,4-galactopyranosylmaltosede (Gal-G2-α-CNP) was purchased from Toyobo (Osaka, Japan), and dimethyl sulfoxide (DMSO) was from Merck (Gibbstown, NJ). Amplex Red Glucose/Glucose Oxidase Assay Kit was purchased from Invitrogen (Carlsbad, CA). All other chemicals were analytical reagent grade.

Cell Culture. The Caco-2 human colon epithelial cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). Caco-2 cells were cultured in high-glucose DMEM supplemented with 20% fetal bovine serum (FBS), 1% nonessential amino acids (NEAA), and 1% penicillin/streptomycin. The MIN6 mouse insulinoma cell line was a gift from Shanghai Huayi Bio-Lab Co., Ltd. MIN6 cells were cultured in high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin under conditions of a saturated humidity atmosphere containing 5% CO$_2$ at 37 °C.

Porcine Pancreatic α-Amylase Assay. The pancreatic α-amylase activity was determined by monitoring the release kinetics of a yellow hydrolytic product from the substrate of Gal-G2-α-CNP in 20 mM sodium phosphate buffer containing 6 mM NaCl (pH 6.9). In detail, 90 μL of DMC at various concentrations (6.3, 12.5, 25, 50, 100, and 200 μM), 100 μL of Gal-G2-α-CNP (5 mM), and 10 μL of porcine pancreatic α-amylase solution (0.033 units/mL; one unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol of CNP produced from Gal-G2-α-CNP per minute under assay conditions) were sequentially added to a 96-well microplate (Corning, NY) in triplicate. The optical density (OD) of the reaction mixture was determined by a Safire 2 microplate reader (Tecan, Mannedorf, Switzerland) at 400 nm every 1 min over a span of 15 min at 37 °C. The enzymatic activity was expressed as a slope value (OD/min) over the period of 15 min. In addition to acarbose (0.67 μM) as a positive control, appropriate blank controls were maintained. The remaining enzymatic activity was calculated based on the slope obtained in the enzymatic reactions using formula 1. The inhibition percentage (% inhibition) of DMC on the enzymatic activity was calculated using formula 2.

$$\text{remaining enzyme activity (\%)} = \frac{\text{slope}_{(E+I)} - \text{slope}_I}{\text{slope}_E - \text{slope}_{\text{blank}}} \times 100 \tag{1}$$

where slope$_{(E+I)}$ is the slope of the mixture of DMC solution, enzyme, and substrate, slope$_I$ is the slope of the mixture with assay buffer replacing enzyme, slope$_E$ is the slope of assay buffer, enzyme, and

substrate, and slope$_{\text{blank}}$ is the slope of assay buffer and substrate without enzyme.

$$\text{\% inhibition} = 100 - \text{remaining enzyme activity (\%)} \tag{2}$$

Rat Intestinal α-Glucosidase Assay. The α-glucosidase solution was prepared from rat intestinal acetone extract powder. Briefly, 1 g of rat intestinal acetone powder was suspended in 15 mL of 0.1 M potassium phosphate buffer (pH 6.8). The suspension was sonicated in an ice bath for 30 min and then centrifuged. The clear supernatant was used for the assay. The intestinal α-glucosidase activity was determined by monitoring the release kinetics of a yellow hydrolytic product from the substrate of PNPG. In brief, different concentrations of DMC at 3.1, 6.3, 12.5, 25, 50, 100, and 200 μM (90 μL), 8 mM PNPG (100 μL), and 66 mg/mL rat intestinal enzyme extract (10 μL; 0.00366 units/mg protein; one unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol of PNP produced from PNPG per minute under assay conditions) were added in such order to a 96-well microplate in triplicate. After the mixture was mixed for 10 s, OD values were measured by a Safire 2 microplate reader at 37 °C, 400 nm, every 1 min for 15 times. Acarbose (45 μM) was the positive control. The remaining α-glucosidase activity (formula 1) and the inhibition percentage (formula 2) of DMC were calculated the same way as stated above.

Caco-2 Cell Monolayer Glucose Transport Assay. Caco-2 cells (from 14 to 16 passages) were seeded into 24-multiwell inserts (BD Bioscience, Franklin Lakes, NJ) at a density of 3×10^5 cells/well, with the culture medium refreshed every other day. After 21 days, the differentiated Caco-2 cell monolayers were used for glucose transport experiment. Trans-epithelial electrical resistance (TEER) values were measured before and after the transport experiment to ensure the integrity of the cell monolayer. Only if the TEER data were above the criteria (280 ohms cm^2 in our lab), the monolayer integrity of Caco-2 cells could be thought of as qualified and reliable for the experiments. On the experiment day, Caco-2 monolayers were gently washed twice with 0.5 mL of prewarmed DPBS containing calcium and magnesium (pH 7.4) and equilibrated for 30 min at 37 °C. Subsequently, the inserts were transferred to a 24-well high recovery plate. Then, 0.5 mL of various concentrations of DMC with 5 (simulated fasting state) or 25 mM (simulated fed state) glucose was added into the apical side of the inserts, whereas 1 mL of glucose-free DPBS was added to the basolateral side. After 1 h of incubation at 37 °C, the liquid from both apical and basolateral compartments was collected for glucose quantification. PT (150 μM) and PZ (300 μM) were used as positive controls of simulated fasting and fed states, respectively.

The glucose concentration was quantified using an Amplex Red Glucose/Glucose Oxidase Assay Kit according to the manufacturer's manual with minor modifications. Briefly, the reaction buffer consisting of 0.5 μL of 10 mM Ampliflu Red, 1 μL of 10 U/mL horseradish peroxidase (HRP), 1 μL of 100 U/mL glucose oxidase, and 97.5 μL of PBS was prepared before experiment. Subsequently, 50 μL of glucose standards or test samples and 100 μL of the reaction buffer were added to a black 96-well plate with a clear bottom. After incubation at room temperature for 10 min, the relative fluorescence intensity was measured by a Safire 2 microplate reader (λ_{ex} = 530 nm, λ_{em} = 590 nm).

Insulin Secretion Assay and Cell Viability of MIN6 Cells. MIN6 cells seeded onto 96-well plates at a density of 5×10^5 cells/mL were incubated for 48 h at 37 °C in 100 μL of culture medium. Then, MIN6 cells were preincubated with or without different concentrations of DMC (6.25, 12.5, and 25 μM) for 2 h. Subsequently, a final concentration of 250 μM H$_2$O$_2$ was added to each well except the control and incubated for 1 h. After a washout of the cell layers with prewarmed PBS, 100 μL of Krebs-ringer bicarbonate buffer (KRBB, 129 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 5.0 mM NaHCO$_3$, 10 mM HEPES, and 0.1% BSA, pH 7.4) was added to each well and incubated for another 1 h. Followed by another round of cells washout, KRBB containing 3 (simulated basal insulin secretion state) or 27 mM (simulated glucose-stimulated insulin secretion state) glucose was added to the cells and incubated for 1 h. At the end of the treatment, the supernatant was collected, and

the insulin level in the supernatant was measured using a mouse insulin ELISA kit (Linco Research Inc., MO) according to the manufacturer's manual; at the same time, the viability of the treated MIN6 cells was checked using the MTT method.

Statistics. Results were expressed as means ± standard errors of the mean (SEMs). The IC_{50} of DMC was calculated by a four-parameter logistic nonlinear regression analysis of log concentration versus % inhibition curve using GraphPad Prism (GraphPad Software Inc., CA). The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's posthoc comparisons to determine the significant differences between groups. Differences with $P < 0.05$ were considered statistically significant.

■ RESULTS

Effect of DMC on Pancreatic α-Amylase and Intestinal α-Glucosidase. DMC inhibited pancreatic α-amylase in a dose-dependent manner, and the IC_{50} value, which is defined as the concentration of DMC required to inhibit 50% of the enzyme activity, was found to be 43 μM (Figure 2A). The IC_{50}
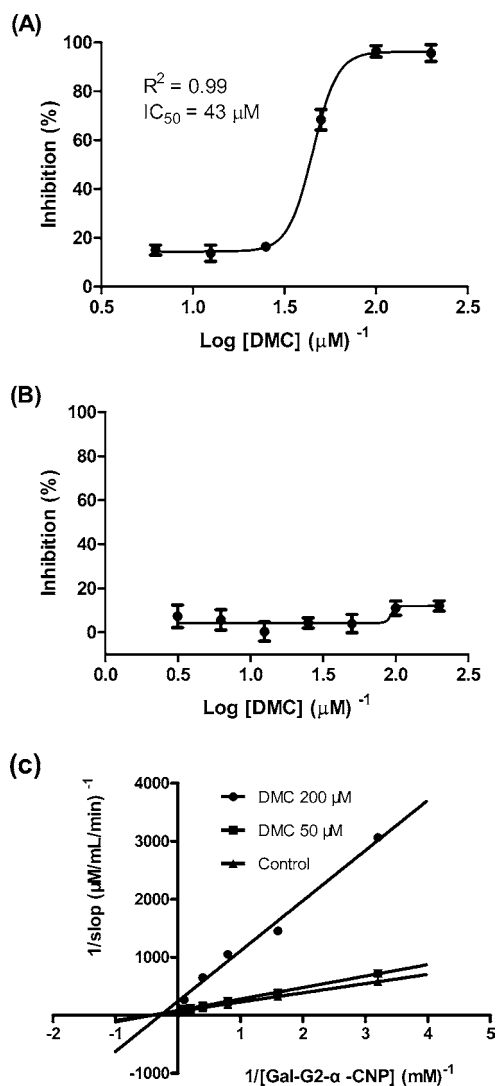

Figure 2. Inhibitory effect of different levels of DMC on (A) pancreatic α-amylase (0.03 units/mL), (B) intestinal α-glucosidase (0.24 units/mL), and (C) Lineweaver−Burk plots of DMC. Values are means ± SEMs ($n = 3$) in A and B. In C, the employed Gal-G2-α-CNP concentrations were 0.31, 0.63, 1.25, 2.5, 5.0, and 10 mM; each data point represents the average of three independent experiments.

value of acarbose (positive control) in this assay was 1.64 μM (dose−effect curve not shown), whereas DMC only showed a marginal effect on α-glucosidase [∼20% inhibition at the concentration ranges from 3.1 to 200 μM (Figure 2B)]. DMC inhibited pancreatic α-amylase activity in a noncompetitive manner (Figure 2C).

Impact of DMC on Glucose Transport across Caco-2 Cell Monolayer. When the initial glucose concentration on the apical side was 5 mM (the simulated fasting state), treatment with DMC at 2.5, 10, and 40 μM significantly reduced the transport of glucose across the cell monolayer in a dose-dependent manner to 64, 40, and 18%, respectively ($P < 0.05$ vs control group) (Figure 3A). PZ (300 μM) is an
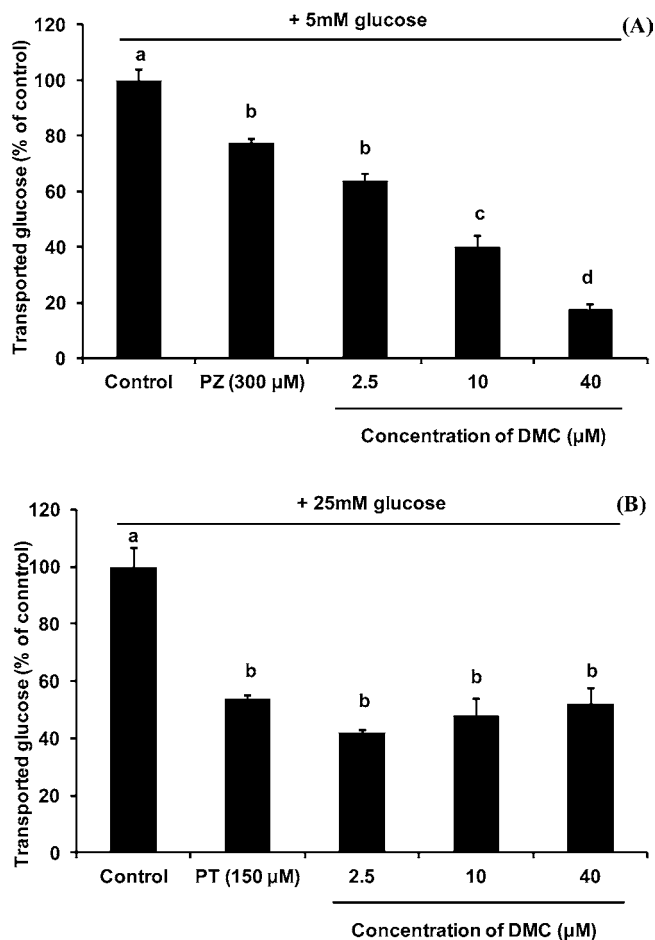

Figure 3. Inhibition of different levels of DMC on glucose transport (A) at the simulated fasting state and (B) at the simulated fed state. Values are means ± SEMs ($n = 8$). The data values were analyzed by one-way ANOVA followed by Tukey's posthoc multiple comparison tests, and significant differences between groups are indicated by different letters a−d at $P < 0.05$.

inhibitor of sodium-glucose cotransporter 1 (SGLT1), which was mainly expressed in a fasting state and the positive control employed in this assay, which reduced the transport of glucose to 77% ($P < 0.05$ vs control group).

Under the simulated fed state, with the initial glucose concentration on the apical side being 25 mM, treatment with DMC at 2.5, 10, and 40 μM remarkably decreased glucose transport to 42, 48, and 52%, respectively ($P < 0.05$ vs control group) but did not show significant difference between concentrations ($P > 0.05$) (Figure 3B). PT (positive control),

which is the inhibitor of glucose transporter 2 (GLUT2), reduced glucose transport to 54% at a concentration of 150 μM as compared with control group.

Glucose-Stimulated Insulin Secretion and Cell Viability of MIN6 Cells. As shown in Figure 4A, MIN6 cells
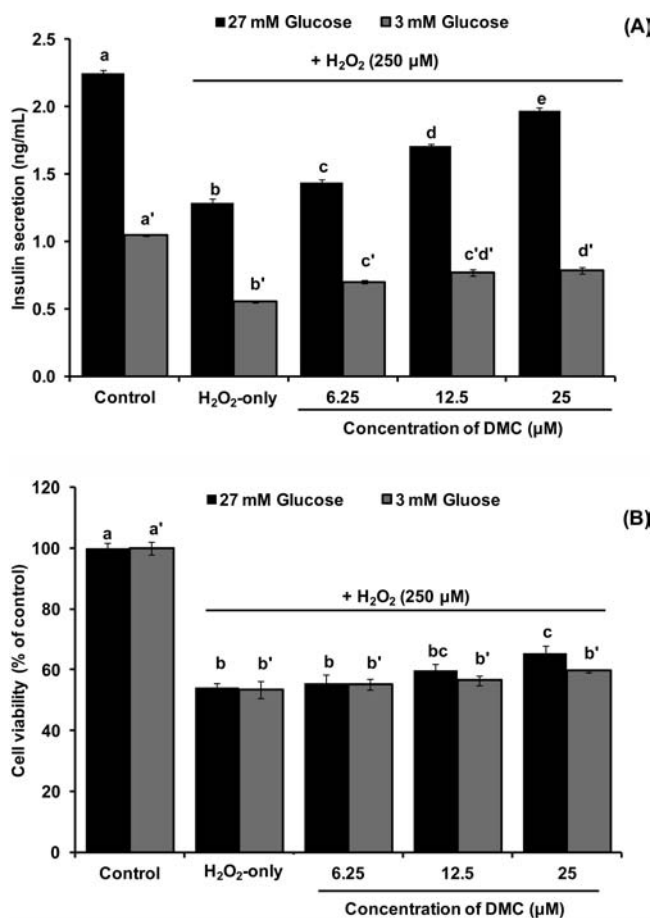

Figure 4. Effect of DMC on H_2O_2-induced basal (3 mM glucose) and glucose-stimulated (27 mM glucose) (A) insulin secretion and (B) cell viability. Values are presented as means $\pm$ SEMs (n = 3). The data values were analyzed by one-way ANOVA followed by Tukey's posthoc multiple comparison tests, and significant differences between groups are indicated by different letters a–e and a′–e′ at $P < 0.05$.

responded to high glucose (27 mM) and secreted 2.2-fold insulin of that at basal level (3 mM glucose). Treatment with 250 μM H_2O_2 for 1 h significantly ($P < 0.05$) reduced glucose-stimulated insulin secretion. Pretreatment with DMC at a concentration of 6.25, 12.5, and 25 μM prevented the H_2O_2-induced decrease in insulin secretions stimulated by both basal (3 mM) and high (27 mM) glucose ($P < 0.05$). DMC improved the impaired insulin secretion at a high glucose level in a concentration-dependent manner ($P < 0.05$ vs only-H_2O_2 treatment group); it increased insulin secretion by 0.15, 0.43, and 0.69 ng/mL (i.e., 12, 33, and 54%) at a concentration of 6.25, 12.5, and 25 μM, respectively, while in the simulated basal insulin secretion mode (3 mM glucose), pretreatment with DMC at a concentration of 6.25, 12.5, and 25 μM remarkably restored insulin secretion ($P < 0.05$ vs only-H_2O_2 treatment group) by 0.14, 0.21, and 0.23 ng/mL (i.e., 26, 38, and 41%), respectively, and statistical difference ($P < 0.05$) among doses was only shown between low (6.25 μM) and high (25 μM) concentrations.

To assess if the insulin secretion increase was due to the proliferative effect of DMC against the injury of H_2O_2 in MIN6 cells, a MTT assay was conducted. As shown in Figure 4B, treatment with 250 μM H_2O_2 for 1 h resulted in a nearly 50% viability loss in MIN6 cells. Pretreatment with DMC for 2 h showed a trend for protection for MIN6 cells against the H_2O_2-induced toxicity in the glucose-simulated insulin secretion condition, which at the high dose (25 μM) attained statistical significance ($P < 0.05$). The viability of DMC pretreated MIN6 cells at basal glucose concentration, however, did not show any significant change as compared with control.

DISCUSSION

Pancreatic α-amylase breaks down complex carbohydrates to oligosaccharides and disaccharides, and intestinal α-glucosidases further digest diet-derived oligosaccharides and disaccharides into monosaccharides, mainly glucose. Previous studies have shown that an aqueous extract of *C. operculatus* flower buds could inhibit the activity of α-glucosidase to lower the blood glucose level in diabetic rats.[20,21] In the present study, we showed that DMC could inhibit the activity of pancreatic α-amylase in a noncompetitive manner but had no effect against intestinal α-glucosidase. DMC is poorly soluble in water (estimated 5 mg/L at 25 °C) and thus may not have been the major compound in aqueous extract (data not shown); previous studies may have not observed the effect of DMC because of its limiting concentration in aqueous phase.

Glucose transporters are responsible for glucose absorption from the intestine to the blood; SGLT1 and GLUT2 are two major transporters responsible for glucose uptake in the small intestine at fasting and fed states, respectively.[48,49] The results in the present study indicated that DMC inhibited glucose transport at both the simulated fasting and fed state. In terms of intestinal glucose transporters, a well-accepted model proposed by Kellett and his colleagues postulated that SGLT1, in the presence of luminal glucose, promotes the rapid recruitment of GLUT2 from the basolateral membrane and subsequent insertion into the brush border membrane via protein kinase C (PKC) β II and the mitogen-activated protein kinase (MAPK)-dependent signal transduction pathways.[48,49] The characteristic of SGLT1 only expressed in brush membrane and GLUT2 shifted between two membranes may explain that DMC inhibited glucose transport with a dose-dependent effect at the fasting state and without a significant dose effect at the fed state. To the best of our knowledge, this is the first report on the inhibition of glucose absorption by DMC in the small intestine, and its interaction with SGLT1 and GLUT2 needs further exploration.

Enzymes digestion of foods and the transport of glucose happen in the intestine, and will DMC work beyond intestine? DMC could lower blood glucose levels by intraperitoneal injection in our previous animal study, which indicated that DMC may work beyond the intestine; one of the possible targets is pancreatic islets. Pancreatic islet is the most vulnerable organ for attack after ROS generation in human as it has less antioxidant enzymes as compared to other organs,[5] and the damage of pancreatic islets leads to insulin secretion decrease, resulting in hyperglycemic conditions and regeneration of ROS again in a bad circle. In the present study, H_2O_2 was chosen as model biological ROS as it has been extensively used in this kind of study. Our studies showed that 250 μM H_2O_2 significantly impaired the basal and glucose responded insulin secretion of MIN6 cells. Pretreatment with DMC (6.25–25

μM) for 2 h caused a significant increase of glucose-induced insulin secretion in a dose-dependent manner and also resulted in a high basal insulin secretion; this may be mainly attributed to the effect of DMC on the insulin secretion enhancement capacity of living MIN6 cells. Pretreatment of aqueous extract of *C. operculatus* flower buds has been shown to restore the islets insulin secretion injury of streptozotocin-induced diabetic rats.[15] It was confirmed that DMC could protect MIN6 cells by antioxidant and antiapoptosis by the improvement of mitochondrial function in our previous work.[50] Therefore, we postulate that DMC increases insulin secretion by antioxidant and antiapoptosis.

In conclusion, the present study provided the evidence that DMC could inhibit the activity of pancreatic α-amylase and intestinal glucose transport and ameliorate impaired insulin secretion by increasing the insulin secretion capacity of live MIN6 cells. The underlying molecular mechanism, however, remains to be understood. Besides the direct activity against pancreatic α-amylase and glucose transporters in the gastrointestinal tract, it has also been reported that DMC improved glucose uptake in adipocytes by stimulating PPAR-γ.[45] Therefore, the mechanisms of hypoglycemic benefits of DMC need further investigation.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

DMC, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone; PZ, phloridzin dehydrate; PT, phloretin; SGLT1, sodium-glucose cotransporter 1; GLUT2, glucose transporter 2; PPAR-γ, peroxisome proliferator-activated receptor-γ

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