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New insights into the biological activities of *Chrysanthemum morifolium*: Natural flavonoids alleviate diabetes by targeting α -glucosidase and the PTP-1B signaling pathway

1 Mingzhu Chen ^a, Kaili Wang ^a, Yinan Zhang ^a, Mengdi Zhang ^a, Yujiao Ma ^a, Haifeng Sun ^a,
2 Zongxin Jin ^a, Hang Zheng ^a, He Jiang ^a, Peng Yu ^a, Yongmin Zhang ^{a, b, *} and Hua Sun ^{a, *}

3
4 ^a Key Laboratory of Industrial Fermentation Microbiology of Ministry of Education, China International Science and Technology
5 Cooperation Base of Food Nutrition/Safety and Medicinal Chemistry, Tianjin University of Science and Technology, Tianjin, 300457, China

6 ^b Sorbonne Université, Institut Parisien de Chimie Moléculaire, UMR CNRS 8232, 4 place Jussieu, 75005 Paris, France

7 ABSTRACT

8 As dual regulators, the PTP-1B signaling pathway and α -glucosidase slow glucose release
9 and increase the degree of insulin sensitivity, representing a promising therapeutic target for
10 type 2 diabetes. In this study, we systematic examined the *in vivo* and *in vitro* anti-diabetic
11 activities of natural flavonoids **1-6** from *Chrysanthemum morifolium*. Flavonoids **1-6**
12 increased glucose consumption-promoting activity and the phosphorylation of GSK-3 β and
13 Akt, and decreased PTP-1B protein level along with slightly inhibitory activity of the PTP1B
14 enzyme. Moreover, flavonoids **1-2** treatment induced insulin secretion in INS-1 cells.
15 Besides, *in vivo* study revealed that flavonoids **2** and **5** demonstrated potent
16 anti-hyperglycemic and anti-hyperlipidemic activity, and improved maltose and glucose
17 tolerance. Although flavonoid **2** exhibited lower inhibitory activity against α -glucosidase *in*
18 *vitro*, it could deglycosylated *in vivo* to diosmetin to function as an α -glucosidase inhibitor.
19 Taken together, these results led to the identification of the natural flavonoids **1-6** from *C.*
20 *morifolium* as dual regulators of α -glucosidase and the PTP-1B signaling pathway, suggesting
21 their potential application as new oral anti-diabetic drugs or functional food ingredients.

22 **Keywords:** Chrysanthemum morifolium; Flavonoid; Diabetes; α -Glucosidase; PTP-1B
23 signaling

1. Introduction

24 Chinese “Hangbaiju” comprises the flowering head of *Chrysanthemum morifolium*, which
25 constitutes an important traditional Chinese medicine used for treatment purposes, e.g.,
26 “scattering cold”, “cleaning heat and toxins”, and “brightening eyes” [1, 2]. *C. morifolium*
27 has been widely used for approximately 2000 years in China as an herbal tea, and is
28 considered a functional food as it contains many types of biologically active components,
29 such as flavonoids [3-5], triterpenoids [6], and volatile oils [7]. Among these, flavonoids are
30 considered to represent the main bioactive components and have been found to exhibit

* Corresponding authors. Tel.: +86-22-6091-2592; e-mail: sunhua@tust.edu.cn. (Hua Sun); yongmin.zhang@upmc.fr (Yongmin Zhang).

31 antioxidant [8, 9], cardiovascular-protective [10, 11], and vasorelaxant activities [2, 10].
32 However, few studies have reported the application of *C. morifolium* as the anti-diabetic
33 functional food and traditional Chinese medicine. The focus of our research is the
34 identification of natural bioactive flavonoids to facilitate their application as functional foods
35 and drug candidates. Our previous studies have identified several series of flavonoids as
36 potent α -glucosidase inhibitors [21, 22]. In the present study, we identified two natural
37 flavonoids **1–2** and their aglycone **3**, along with flavones **4–6** (Fig. 1B) from *C. morifolium*
38 and evaluated their *in vitro* and *in vivo* anti-diabetic activities.

39 **2. Materials and methods**

40 *2.1. Liquid chromatography-mass spectrometry (lc-ms) conditions*

41 For LC-MS, we utilized a DGU-20A3R high-performance LC instrument coupled to a
42 diode array detector and mass spectrometer (Shimadzu Corporation, LC-MS-IT-TOF, Kyoto,
43 Japan). A 250 mm \times 4.6 mm, 5- μ m, Symmetry C18 column (Agela Technologies Inc.,
44 Torrance, CA) with 20 mm \times 3.9 mm i.d, 5- μ m, Sentry guard column was used at a flow rate
45 of 1.0 mL/min. The column oven temperature was set at 25 °C. The mobile phase consisted
46 of a combination of A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in
47 acetonitrile). The gradient was varied linearly from 10% to 26% B (v/v) in 40 min, 65% B at
48 70 min, and finally to 100% B at 71 min and held at 100% B to 75 min. The diode array
49 detection was set at 350, 310, 270 and 520 nm for real-time monitoring of the peak intensity
50 and full spectra (190–650 nm) were continuously recorded for plant component identification.
51 Mass spectra were simultaneously acquired using electrospray ionization in the positive and
52 negative ionization modes at low and high fragmentation voltages (–3.5 and 4.5 kV) over the
53 range of m/z 100–1,000.

54 *2.2. Materials and in vitro protocols*

55 *2.2.1. Materials*

56 Dried *C. morifolium* flower was purchased from Tianfu Tea Co. Ltd. (Zhejiang, China).
57 Baker's yeast α -glucosidase, p-nitrophenyl α -D-glucopyranoside (pNGP), 4-nitrophenyl
58 phosphate disodium salt (pNPP), NaVO₄, acarbose, glibenclamide, and streptozocin (STZ)
59 were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human PTP1B protein
60 was purchased from Abcam (Cambridge, UK). The BCA Protein Assay Kit was obtained
61 from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). PTP-1B antibody,
62 Akt (pan) (11E7) rabbit mAb, phospho-Akt (Ser473) (193H12) rabbit mAb, GSK-3 β (D5C5Z)
63 XP rabbit mAb, phospho-GSK-3 β (Ser9) (D3A4) rabbit mAb, α / β -tubulin antibody, and goat
64 anti-rabbit IgG(H+L)-HRP were purchased from Cell Signaling Technology (Danvers, MA,
65 USA). HepG2 (human liver cancer), CHO-K1 (Chinese hamster ovary), and INS-1 (rat
66 insulin-secreting beta) cells were acquired from Shanghai Institutes for Biological Sciences,
67 Chinese Academy of Sciences (Shanghai, China).

68 *2.2.2. Glucose consumption (GC) assay*

69 The GC assay was performed as previously described [22]. Briefly, HepG2 cells were

70 cultured in Dulbecco's-modified Eagle's medium (DMEM) containing 25 mM D-glucose, 10%
71 heat-inactivated fetal bovine serum (FBS), 10 U/mL penicillin, and 10 mg/mL streptomycin
72 at 37 °C under a 5% CO₂ atmosphere. Cells were deprived of serum for 24 h. The cells were
73 washed twice with phosphate buffered saline (PBS), and the medium was replaced by
74 RPMI-1640 containing glucose (11.1 mM) supplemented with 0.2% bovine serum albumin.
75 Then, metformin (0.5 mM) or test flavonoids were added to the medium at different
76 concentrations. Dimethyl sulfoxide (DMSO) was used as the blank control. The glucose
77 concentration in the medium was determined via the glucose oxidase method after 24 h
78 treatment.

79 2.2.3. MTT method

80 The MTT assay was performed before the GC experiment. MTT (20 µL, 5 mg/mL
81 dissolved in PBS) was added to each well. After 4 h, DMSO (100 µL) was added to dissolve
82 the crystals, and the plate was placed on a shaker for 10 min. The OD value was measured
83 using a microplate reader at a wavelength of 492 nm. The MTT results were used to
84 normalize the GC results. GC owing to cell proliferation can be deducted by calculating the
85 ratio of the GC and MTT (GC/MTT = mM/OD).

86 2.2.4. Western blotting

87 CHO-K1 cells were cultured in DMEM/F-12 Ham's medium supplemented with 10% FBS,
88 penicillin (50 U/mL), and streptomycin (100 µg/mL) at 37 °C in a humidified atmosphere
89 with 5% CO₂ [23]. Cells (5×10^4 /mL) were seeded in a 96-well cell plate for 24 h and then
90 treated with compounds 1–6 (20 µM) or DMSO for 48 h. NaVO₄ was used as a positive
91 control. Then, the cells were lysed with ice-cold RIPA buffer containing freshly added
92 aprotinin and phenylmethanesulfonyl fluoride. The BCA Protein Assay Kit was used to
93 determine protein concentration. Cell lysates were separated by sodium dodecyl
94 sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene
95 fluoride membrane (Millipore, Billerica, MA). The membranes were blocked with 5%
96 non-fat milk in 1× PBS containing 0.5% Tween 20 (PBST) for 1 h at room temperature and
97 were then incubated with a primary antibody overnight at 4 °C. The following day, the
98 membranes were washed three times with PBST and probed with a secondary antibody.
99 Pierce™ ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA) was used to
100 detect the bands [24].

101 2.2.5. Glucose-stimulated insulin secretion and basal insulin secretion

102 INS-1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented
103 with 10% FBS, streptomycin (100 µg/mL), and penicillin (50 U/mL) at 37 °C in a humidified
104 atmosphere containing 5% CO₂. Cells (2×10^5 cells/mL) were seeded in a 48-well cell plate
105 for 24 h. The cell culture medium was replaced by IMDM containing 40 mM glucose then
106 cells were incubated with compounds or DMSO for 48 h. Glibenclamide was used as a
107 positive control. Thereafter, the cell culture medium was carefully removed, the cells were
108 washed with PBS, and 300 µL Krebs-Ringer's bicarbonate (KRB) buffer containing 2% FBS
109 was added. After 30 min, the cells were stimulated with KRB buffer containing 5 or 30 mM
110 (BIS or GSIS) glucose for 60 min at 37 °C. The cell medium was then collected for analysis
111 of insulin secretion, which was determined using a rat insulin enzyme-linked immunosorbent

112 assay kit (Nanjingjiancheng, Nanjing, China), whereas 100 μ L RIPA Lysis buffer was added
113 to the cells, which were saved for determination of the total protein content [25].

114 2.2.6. Inhibition studies of PTP-1B

115 pNPP was used as a substrate for the measurement of PTP1B activity. Compounds **1-6**
116 were dissolved in DMSO. PTP-1B, pNPP, and NaVO_4 were dissolved in assay buffer (pH
117 6.0), which contains 150 mM NaCl, 50 mM 2-(N-morpholino) ethanesulfonic acid, 1 mM
118 ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 0.05 % NP-40. Enzymatic reaction
119 mixtures, composed of PTP-1B (20 μ g/mL, 5 μ L), test compounds (10 μ L), and buffer (75
120 μ L), were incubated at 30 $^\circ\text{C}$ for 5 min, then the reactions were initiated by adding pNPP
121 (100 mM, 10 μ L) and incubated at 30 $^\circ\text{C}$ for 10 min. Enzymatic activity was detected by
122 spectrophotometry at 405 nm [27].

123 2.2.7. Inhibition studies of α -glucosidase

124 Test compounds **1-6** and acarbose were dissolved in DMSO. α -Glucosidase and pNGP
125 were dissolved in potassium phosphate buffer (0.05 M, pH 6.8). Enzymatic reaction mixtures,
126 composed of α -glucosidase (4 mU, 20 μ L), pNGP (75 μ M, 30 μ L), test compounds (10 μ L),
127 and potassium phosphate buffer (140 μ L), were incubated at 37 $^\circ\text{C}$ for 30 min. Enzymatic
128 activity was detected by spectrophotometry at 405 nm.

129 2.3. Materials and in vivo protocols

130 2.3.1. Animals and diets

131 Four- to six-week-old male Kunming mice (18–22 g) were procured from The Institute for
132 Laboratory Animal Science, Chinese Academy of Medical Sciences and Peking Union
133 Medical College [Beijing, China, License SCXK (Jun) 2012-0004]. Animal procedures were
134 approved by the Tianjin University of Science and Technology Institutional Animal Care
135 Committee and performed in strict compliance with local and national ethical guidelines. The
136 mice were kept in polypropylene cages (five in each cage) and housed under laboratory
137 conditions (18–23 $^\circ\text{C}$, 55–60% humidity, 12 h light/dark cycle). The mice were fed a
138 standard pellet diet for 1 week after arrival and randomly divided into two groups: mice fed a
139 standard pellet diet (normal mice), and mice fed a high-fat, high-fructose diet to induce type 2
140 diabetes. All mice had free access to food and water [21].

141 2.3.2. Induction of experimental type 2 diabetic mice and treatment

142 After ingesting the high-fat, high-fructose food for 3 weeks, the mice were subjected to a
143 12 h fast. Type 2 diabetes was induced by an intraperitoneal injection of STZ [100 mg/kg of
144 body weight, dissolved in 0.05 M citrate buffer (pH 4.5)]. After another 2 weeks of the
145 high-fat, high-fructose diet, fasting blood from the tail vein was used to determine blood GC.
146 The mice were classified as having type 2 diabetes if blood glucose levels were > 11.0 mM.
147 Type 2 diabetic mice were fed the high-fat, high-fructose diet throughout the study. Blood
148 glucose and body weight were checked every week. Type 2 diabetic mice were divided into 6
149 groups with 10 mice per group. Each day, 10 mL of saline/kg, 50 mg of acarbose in saline/kg,
150 50 and 100 mg of diosmin in saline/kg, and 50 and 100 mg of apigenin in saline/kg were

151 orally administered to groups 1 (T2D model), 2 (acarbose), 3 (diosmin, 50 mg/kg), 4
152 (diosmin, 100 mg/kg), 5 (apigenin, 50 mg/kg), and 6 (apigenin, 100 mg/kg), respectively.

153 2.3.3. Determination of blood glucose levels

154 Blood glucose levels were determined as previously described [28]. After receiving
155 treatment for 13 and 14 days and fasting overnight, all mice were orally administered glucose
156 or maltose (2 g/kg), respectively, 60 min after treatment. Blood samples were taken from the
157 tail vein after 0, 30, 60, and 120 min, and blood glucose was measured using a glucometer
158 (Sinocare, Changsha, China). Areas under the curve (AUCs) were calculated using the
159 trapezoidal rule.

160 2.3.4. Determination of blood lipid levels

161 Blood lipid levels were determined as previously described [29]. At the end of the
162 experimental period, blood samples were collected and the serum samples obtained by
163 centrifugation (2000 g for 20 min) were stored at $-20\text{ }^{\circ}\text{C}$ for further analysis. Serum total
164 cholesterol (TC) and triglycerides (TGs) were analyzed using commercial diagnostic kits
165 (Jiancheng, Nanjing, China).

166 3. Results and discussion

167 3.1. Identification of *C. morifolium* flavonoids

168 In order to gain some anti-diabetic insight into the flavonoids of *C. morifolium*, the
169 flavonoids were purified and identified purposely. Dried *C. morifolium* flower was finely
170 powdered. The sample (60 g) was refluxed and extracted with ethanol-water (480 mL, 75:25,
171 v/v) using a Soxhlet extractor 3 times for 1 h. The extract was combined and lyophilized to
172 give crude powder (15.64 g), of which 15 mg was dissolved in methanol (1 mL) and filtered
173 through a 0.22 μm nylon filter. Filtered sample (20 μL) was injected for analysis.
174 Chromatograms of the *C. morifolium* extract are shown in Fig. 1A. The retention times (t_{R}),
175 molecular ions ($[\text{M} + \text{Na}]^{+}/[\text{M} - \text{H}]^{-}$), and major fragment ions of the major peaks are listed
176 in Table 1. Flavonoids in *C. morifolium* flower were identified based on a comparison of
177 retention times and high-resolution mass spectra for authentic standards obtained using the
178 same reported methods [1]. As shown in figure 1, two flavonoids **1-2** and flavones **3-6** in the
179 aqueous ethanol extract of *C. morifolium* were identified as diosmetin 7-glucoside (**1**, peak 1),
180 diosmin (**2**, peak 2), diosmetin (**3**, peak 3), luteolin (**4**, peak 4), apigenin (**5**, peak 5), and
181 acacetin (**6**, peak 6). The assignment of nuclear magnetic resonance and mass spectrometry
182 spectra is described in the supporting information.

183 (Figure 1)

184 3.2. Promotion of GC by flavonoids **1-6** in HepG2 cells

185 To assess the anti-diabetic activity of flavonoids **1-6** in *C. morifolium* flower, the GC in
186 HepG2 cells was evaluated. The GC results were normalized using their MTT results. As
187 shown in Fig. 2A, all flavonoids **1-6** significantly promote glucose-consumption at doses of
188 0.4–10 μM . Of these, flavone **4** was found to have the highest activity, being similar potent at
189 dose of 4 μM with metformine at dose of 500 μM . Besides, Flavonoid **2** was slightly more

190 potent than **1**.

191 (Figure 2)

192 3.3. Enhanced insulin signaling by flavonoids **1–6**

193 Insulin signaling is dependent on activation of the insulin receptor, which phosphorylates
194 and recruits different downstream signaling molecules. Thus, phosphorylation of the
195 signaling molecules serves as an indicator of pathway activation. Insulin-stimulated
196 phosphorylation of GSK-3 β (Ser9) was significantly increased by flavonoids **1–6** treatment in
197 the cell cultures (Fig. 3A and B). Phosphorylation of Akt (Ser473) was also obviously
198 enhanced (Fig. 3A and C). Moreover, the protein levels of PTP-1B were evidently decreased
199 by **1–6** (Fig. 3A and D). To further determine the effect on PTP-1B, the inhibitory activity of
200 hPTP-1B was performed. The results showed that flavonoids **1–6** exhibited weak inhibitory
201 activity against hPTP-1B at 1, 5, and 20 μ M (Table 2). These results suggested that
202 flavonoids **1–6** directly enhanced the insulin signaling activity and decreased the protein level
203 of PTP-1B along with slightly inhibiting the activity of the PTP1B enzyme.

204 (Figure 3)

205 (Table 2)

206 3.4. Effects of flavonoids **1–2** on insulin secretion in high glucose-treated INS-1 cells

207 To further determine the effect of insulin signaling pathway by representative flavonoids
208 **1–2**, we performed an insulin secretion test in the high glucose-treated INS-1 cellular model.
209 Glibenclamide used as a positive control. As shown in Fig. 2B and C, after treatment with 30
210 and 5.5 mM glucose, insulin secretion of cells significantly decreased (normal vs. high
211 glucose-treated model). Treatment with 0.1, 1, 10, and 100 μ M flavonoids **1** and **2** increased
212 dose-dependently the level of insulin secretion, which previously decreased by glucose in
213 both groups. These results suggested that flavonoids **1–2** treatment induced insulin secretion
214 in INS-1 cells.

215 3.5. α -Glucosidase inhibition of *C. morifolium* flavonoids

216 To discover the targets of anti-diabetic, α -glucosidase inhibitory activity of flavonoids **1–6**
217 was investigated. Acarbose, a clinical α -glucosidase inhibitor, was selected as a reference
218 compound. As shown in Table 3, flavone **5** (IC₅₀ = 9.04 μ M) was found to have the highest
219 activity, being even more active than acarbose (IC₅₀ = 236.5 μ M). Moreover, no apparent
220 inhibitory activity against α -glucosidase was observed for flavonoids **1** and **2**, with IC₅₀
221 values >100. However, diosmetin, the aglycone (**3**) of flavonoids **1** and **2**, exhibited
222 comparable α -glucosidase inhibitory activity with acarbose. As it has been reported that
223 deglycosylation of flavonoids constitutes a major metabolic pathway for these compounds
224 [30, 31]. We considered that although flavonoids **1** and **2** exhibit no α -glucosidase inhibitory
225 activity *in vitro*, they might be deglycosylated to diosmetin *in vivo*, which is positive for
226 anti-diabetic activity.

227 3.6. Flavonoids ameliorates hyperglycemia *in vivo*

228 To further evaluate the *in vivo* anti-diabetic activity and validate our hypotheses of
229 deglycosylation, the hypoglycemic activity of representative flavonoid **2** and **5** was

230 determined using STZ-induced diabetic mice. Compared with normal mice (health group),
231 diabetic mice (T2D model group) exhibited hyperglycemia with blood glucose levels of
232 approximately 23 mM (Fig. 4A). After 4-week treatment with flavonoids **2** and **5**, blood
233 glucose levels significantly decreased compared with those of the vehicle group (Fig. 4A and
234 B).

(Figure 4)

236 3.7. Flavonoids improves maltose and glucose tolerance *in vivo*

237 To assess glucose homeostasis and insulin sensitivity in diabetic mice treated with
238 flavonoid **2** and **5**, we evaluated the amelioration of glucose tolerance. Flavonoids **2** and **5**
239 administration resulted in a significant improvement in glucose tolerance (Fig. 4C and D). To
240 understand the basis of flavonoid **2** and **5** activity with regard to α -glucosidase suppression,
241 we further investigated the influence of flavonoid **2** and **5** on blood glucose levels using an *in*
242 *vivo* maltose tolerance test. Blood glucose levels after oral administration of maltose
243 indirectly reflect the activity of α -glucosidase, which can hydrolyze maltose into glucose. As
244 shown in Fig. 4E, after treatment with flavonoid **2** and **5** for 2 h, postprandial blood glucose
245 level obviously improved compared with that of the diabetic model group. AUCs of flavonoid
246 **2** were reduced by 11.2% and 13.5% at a dosage of 50 and 100 mg/kg, respectively (Fig. 4F).
247 Thus, despite the lack of *in vitro* inhibitory activity against α -glucosidase, flavonoid **2**
248 appeared capable of significant decreasing fasting blood glucose levels and improving
249 maltose and glucose tolerance *in vivo*. Consequently, flavonoid **2** exhibited comparable *in*
250 *vivo* anti-diabetic activity with acarbose. The results strongly supported our conjecture that
251 flavonoid **2** might be deglycosylated *in vivo* to diosmetin to function as an α -glucosidase
252 inhibitor.

253 3.8. Flavonoids reduces serum levels of TG and TC

254 After 4 weeks of treatment with flavonoid **2** and **5**, a significant decrease in serum levels of
255 TG and TC was observed (Table 4). TG levels were decreased by 33.8% (50 mg/kg) and 27.3%
256 (100 mg/kg) for flavonoid **2**, whereas TC levels were decreased by 8.3% (100 mg/kg)
257 compared to those of diabetic model mice. Especially, TC levels were decreased by 22.1%
258 (50 mg/kg) and 15.7% (100 mg/kg) for flavonoid **5** compared to those of diabetic model mice.
259 However, acarbose at a dosage of 50 mg/kg exhibited no anti- hyperlipidemia activity.

(Table 4)

261 4. Conclusion

262 The PTP-1B signaling pathway plays an important in the regulation of insulin signaling
263 and the development of type 2 diabetes and obesity. α -Glucosidase catalyzes the final step of
264 carbohydrate digestion and releases glucose. As dual regulators, the PTP-1B signaling
265 pathway and α -glucosidase slow the release of glucose and increase the degree of insulin
266 sensitivity, resulting in alleviation of disorders of glucose and lipid metabolism and insulin
267 resistance, thereby representing a promising potential therapeutic strategy for the treatment of
268 type 2 diabetes. To identify natural compounds that may influence this process, we performed
269 *in vitro* cell-based and enzyme-based experiments to determine the effect on proteins related
270 to the PTP-1B signaling pathway and α -glucosidase. The results showed that flavonoids **1–6**

271 significantly promote glucose consumption (Fig. 2A). The phosphorylation of GSK-3 β and
272 Akt was increased and the protein level of PTP1B was decreased by flavonoid **1–6** treatment
273 (Fig. 3). However, flavonoids **1–6** showed only weak inhibitory activity against PTP-1B at 1,
274 5, and 20 μ M (Table 2). Moreover, flavonoids **1–2** were capable of increasing insulin
275 secretion in INS-1 cells (Fig. 2B and C).

276 Given that the *in vitro* potencies of many PTP-1B regulators or α -glucosidase inhibitors
277 are not consistent with their *in vivo* activities, a series of *in vivo* anti-diabetic activities were
278 evaluated in STZ-induced type 2 diabetic mice. Moreover, as the oral availability of PTP-1B
279 represents a challenge for the development of PTP-1B inhibitors [24], we examined the
280 effects of 4-week oral administration of flavonoid **2** and **5** in diabetic mice. Flavonoid **2**
281 administration resulted in a significant reduction in fasting blood glucose levels (Fig. 4A and
282 B) combined with a significant improvement in glucose tolerance (Fig. 4C and D).
283 Furthermore, flavonoid **2** and **5** ameliorated maltose tolerance comparing with that in the
284 diabetic model group (Fig. 4E and F), which indirectly reflected the *in vivo* activity of
285 α -glucosidase.

286 Dyslipidemia, characterized by hypertriglyceridemia including TG and TC levels,
287 constitutes a major risk factor contributing to the burden of macrovascular disease in type 2
288 diabetes. In the present study, flavonoid **2** and **5** showed potent lipid lowering capability in
289 the diabetic mice, eliciting reduced serum TC and TG levels (Table 4). It is considered that
290 the alleviation of insulin resistance may have contributed to the lower TC and TG levels.

291 In conclusion, natural flavonoids **1–6** from *C. morifolium* were identified as α -glucosidase
292 and PTP-1B signaling pathway dual regulators, which could serve as a new starting point for
293 the discovery of novel oral anti-diabetic agents and potential functional food ingredients.

294 **Conflict of interest statement**

295 The authors declare no conflicts of interest.

296 **Ethics statement**

297 I have read and adhere to the Publishing Ethics.

298 **Acknowledgements**

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301 **Supporting information**

302 Supplementary data related to this article can be found at <http://>.
303 These data include NMR and mass spectra signals of compounds **1–6** described in this article.
304

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393 **Figure legends**

394 **Fig. 1.** Chromatograms of the aqueous ethanol extract and structures of natural flavonoids **1–6** of *C. morifolium*.
395 (A) Chromatograms (350 nm) of the aqueous ethanol extract. Chromatograms of the *C. morifolium* extract (a),
396 diosmetin 7-glucoside (b), diosmin (c), diosmetin (d), luteolin (e), apigenin (f), and acacetin (g). (B) Structures
397 of identified flavonoids **1–6** from *C. morifolium*.

398 **Fig. 2.** Effects of flavonoids **1–6** on promote glucose consumption (GC) and flavonoids **1–2** on insulin secretion.
399 (A) Flavonoids **1–6** promote GC in HepG2 cells. Metformin (Met) was used as a reference compound. * $P < 0.05$,
400 ** $P < 0.01$, and *** $P < 0.001$ vs control. Effects of flavonoids **1–2** on insulin secretion in high glucose-treated
401 INS-1 cells. INS-1 cells were preincubated with 40 mM glucose and compounds or DMSO for 48 h. Cells were
402 incubated with 5.5 mM (B) and 30 mM (C) glucose for 60 min, respectively. * $P < 0.05$, ** $P < 0.01$, and *** $P <$
403 0.001 vs model. Data represent the average of three independent experiments, each performed in duplicate.

404 **Fig. 3.** Effects of flavonoid **1–6** and NaVO_4 on insulin signaling. (A) Akt, GSK3 β , and PTP-1B phosphorylation
405 status was determined via western blotting. Relative ratio of p-Akt (B), p-GSK3 β (C), and PTP-1B (D). Data are
406 expressed as the means \pm SD (n = 3). ** $P < 0.01$ and *** $P < 0.001$ vs control.

407 **Fig. 4.** Effects on blood glucose levels following administration of flavonoid **2** and **5** in diabetic mice. Healthy
408 mice were treated with vehicle, and type 2 diabetes (T2D) mice were treated with vehicle, flavonoids **2**, **5**, and
409 acarbose for 4 weeks. Fasting blood glucose concentrations (A) and corresponding AUC (B). Effects on maltose
410 and glucose tolerance after administration of flavonoid **2** and **5** in diabetic mice. (C) Oral glucose tolerance test
411 (OGTT), (D) AUC in OGTT, (E) Oral maltose tolerance test (OMTT), and (F) AUC in OMTT. Data are
412 expressed as the means \pm SD (n = 10). * $P < 0.05$ and ** $P < 0.01$ vs the T2D model.

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414

415 **Table 1**416 Chemical components identified from the extract of *C. morifolium* flower.

Peak No.	t_R (min)	$[M+Na]^+/[M-H]^-$ (m/z)	Predicted values (m/z)	fragments (m/z)	Identification
1	35.83	485.1047/461.1079	485.1054/461.1089	217.0383, 418.7810, 475.3239 /130.9476, 285.0398	Diosmetin 7-glucoside
2	34.65	/607.1607	/607.1668	/130.9468, 255.2341, 285.0399	Diosmin
3	53.63	323.0518/	323.0526/	217.0446, 324.0552, 419.2826/	Diosmetin
4	47.61	309.0356/	309.0370/	310.0385, 418.7813/	Luteolin
5	52.55	293.0406/	293.0420/	217.0385, 301.1409, 475.8242/	Apigenin
6	61.10	307.0559/	307.0577/	217.0368, 418.7820/	Acacetin

417

418 **Table 2**

419 Human PTP-1B inhibition of flavonoids 1–6.

No.	Name	Inhibition (%) ^a		
		20 μ M	5 μ M	1 μ M
1	Diosmetin 7-glucoside	29.79 \pm 1.03	23.36 \pm 0.52	27.74 \pm 2.10
2	Diosmin	30.53 \pm 3.63	32.82 \pm 2.84	36.26 \pm 1.62
3	Diosmetin	28.27 \pm 3.71	27.10 \pm 2.89	22.81 \pm 2.07
4	Luteolin	39.92 \pm 1.62	40.75 \pm 2.84	19.80 \pm 2.18
5	Apigenin	39.17 \pm 0.87	20.23 \pm 0.10	29.77 \pm 1.48
6	Acacetin	32.05 \pm 2.85	36.26 \pm 2.70	38.17 \pm 1.62
	Sodium orthovanadate ^b	56.58 \pm 4.86		

420 ^a Results represent the average of three independent experiments, each performed in duplicate. ^b Reference
421 compound.

422

423 **Table 3**424 α -Glucosidase inhibition of flavonoids 1–6.

No.	Name	IC ₅₀ (μ M) ^a
1	Diosmetin 7-glucoside	> 100
2	Diosmin	> 100
3	Diosmetin	46.45
4	Luteolin	16.65
5	Apigenin	9.04
6	Acacetin	> 100
	Acarbose ^b	51.30

425 ^a Results represent the average of three independent experiments, each performed in duplicate. ^b Reference
426 compound.

427

428 **Table 4**

429 TC and TG levels of mouse groups.

Group	Dosage (mg/kg)	TG (mM) ^a	TC (mM) ^a
Healthy		1.55 \pm 0.24	5.23 \pm 0.17
T2D model		3.08 \pm 0.84	9.75 \pm 0.11

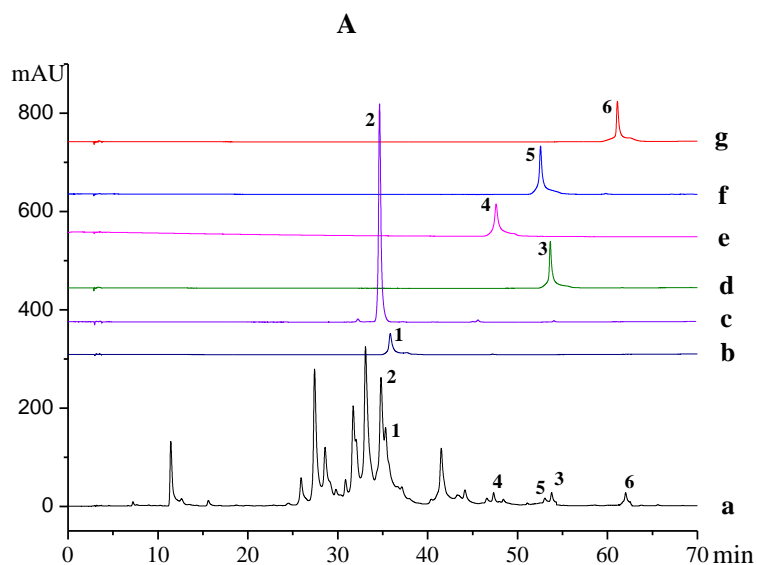
Acarbose	50	3.02 ± 0.79	9.61 ± 0.27
2	50	2.04 ± 0.36 [*]	9.18 ± 0.20
2	100	2.24 ± 0.38 [*]	8.94 ± 0.18 [*]
5	50	3.13 ± 0.35	7.60 ± 0.64 ^{**}
5	100	3.06 ± 0.26	8.22 ± 0.38 ^{**}

430 ^aData are expressed as the means ± SEM (n = 10). ^{**}*P* < 0.01, ^{*}*P* < 0.05 vs the type 2 diabetes (T2D) model.

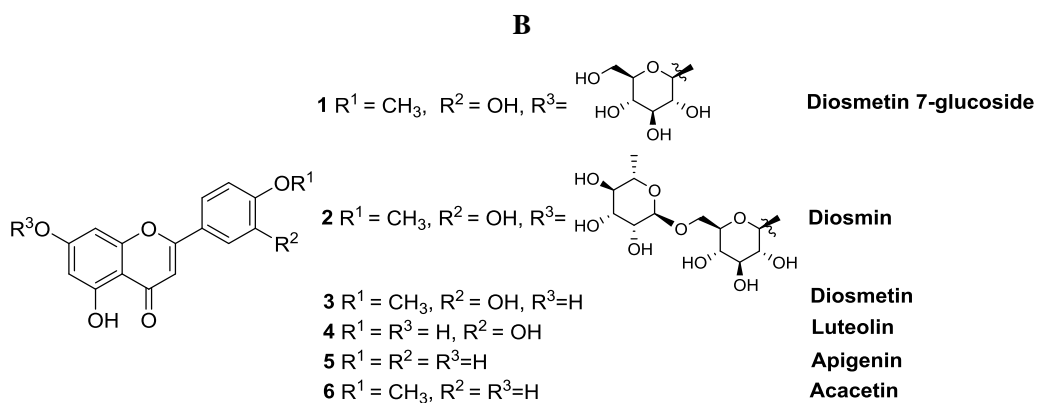
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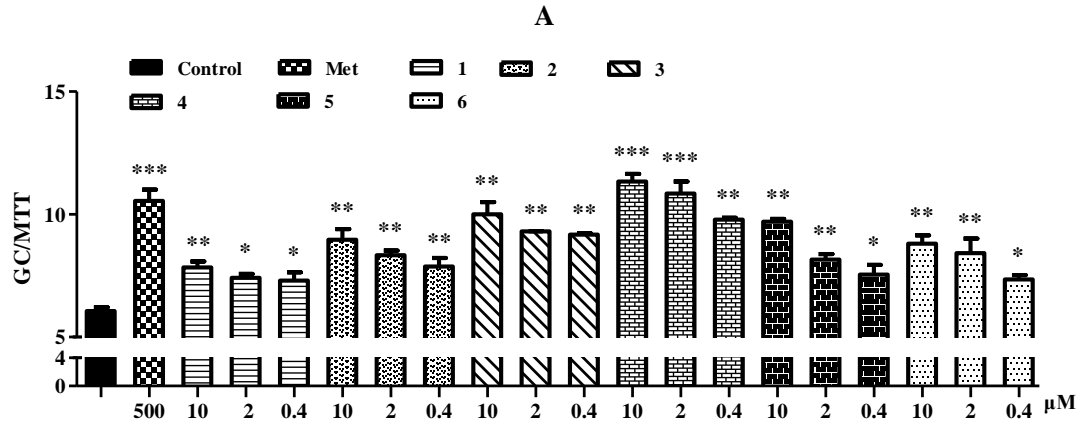
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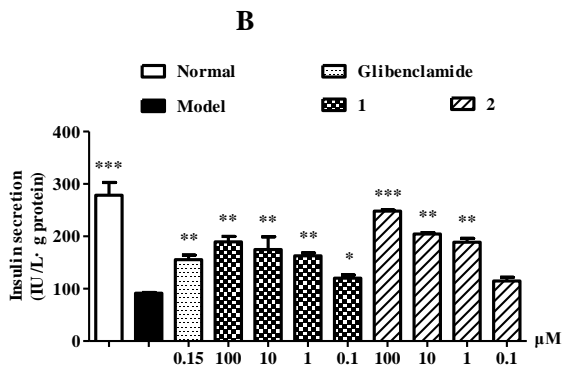


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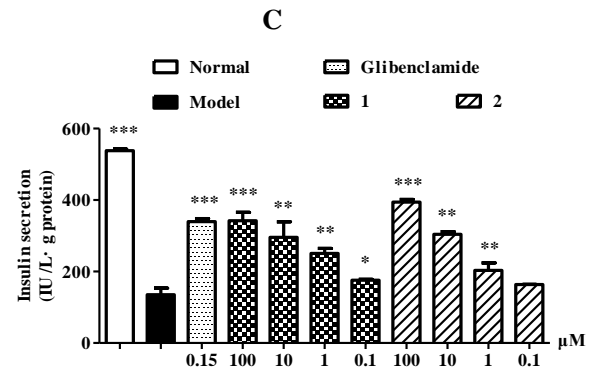
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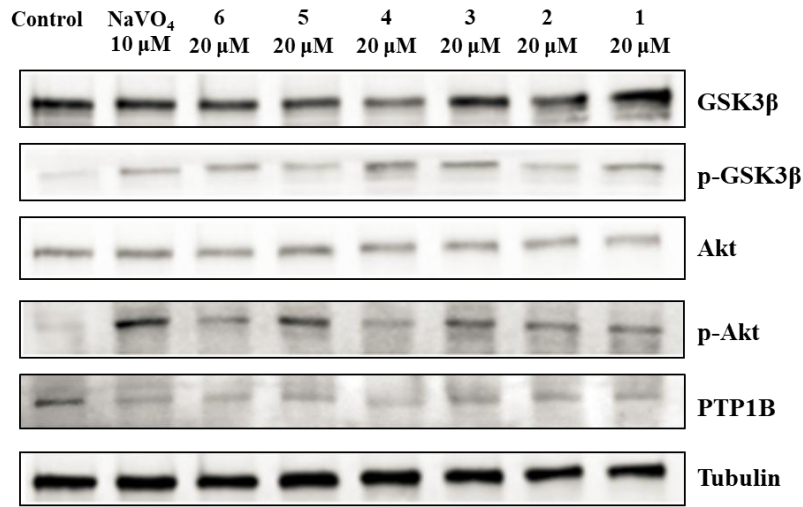
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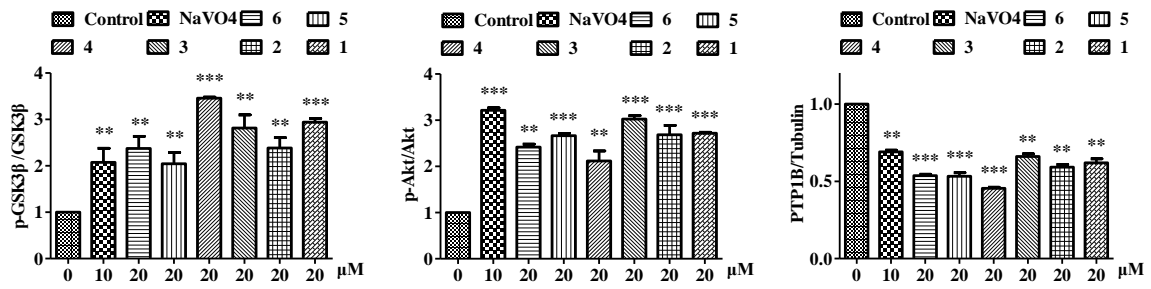
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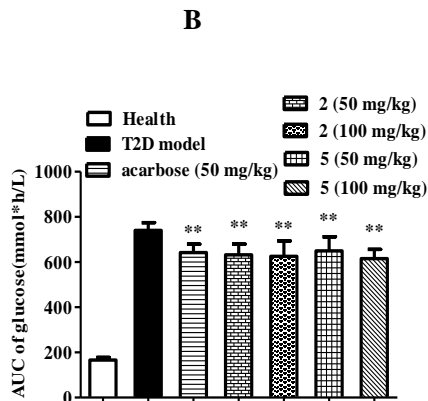
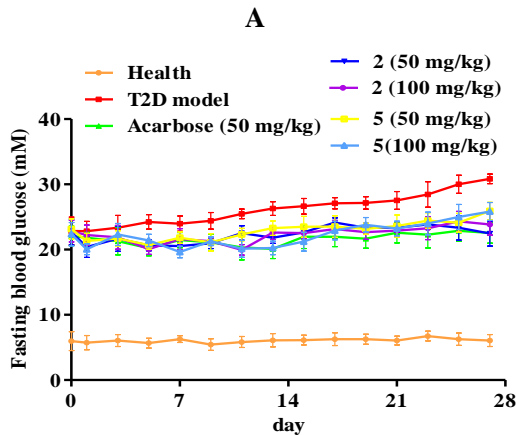
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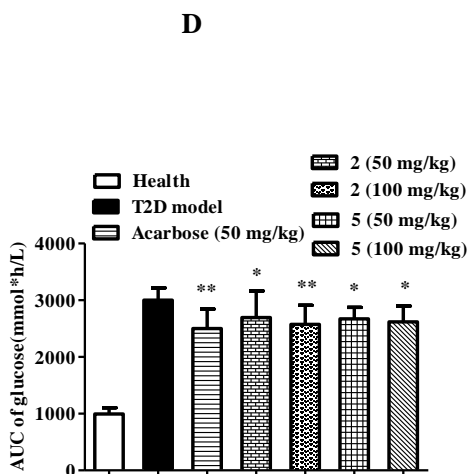
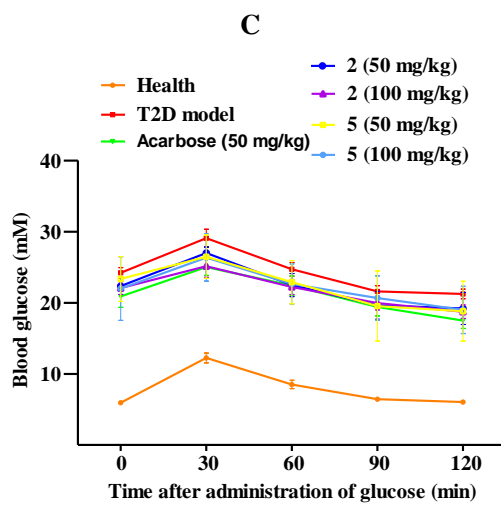
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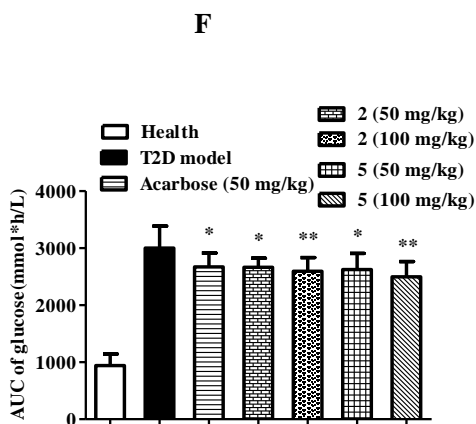
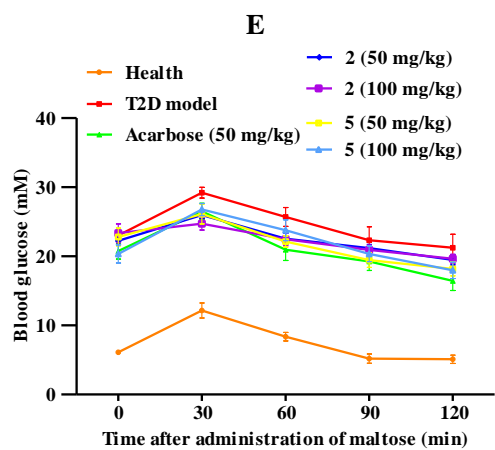


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