Inhibitory Actions of Lupinifolin Isolated from *Derris reticulata* Stem against Carbohydrate-Digesting Enzymes

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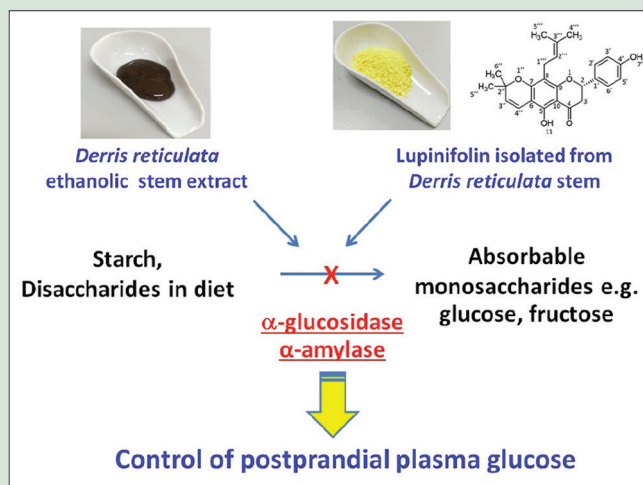
ABSTRACT

Background: Postprandial hyperglycemia is linked with the development of diabetic complications. Inhibition against the carbohydrate-digesting enzymes, specifically pancreatic α-amylase and intestinal α-glucosidase, delays carbohydrate digestion and subsides postprandial plasma glucose (PPG) levels. **Objectives:** This study aimed to examine the effects of lupinifolin, purified from *Derris reticulata* stem, a medicinal plant traditionally used for the treatment of diabetes mellitus, on *in vitro* α-amylase and α-glucosidase enzyme activities and enzyme kinetics. **Subjects and Methods:** The identification of the isolated phytochemical was performed using nuclear magnetic resonance–mass spectrometry (NMR-MS) spectrometry. *In vitro* pancreatic α-amylase and α-glucosidase activities and enzyme kinetics were determined using the enzymatic colorimetric methods. **Results:** The purified phytochemical was identified as lupinifolin from the NMR-MS spectrometry. The isolated lupinifolin produced a significant α-amylase and α-glucosidase inhibitory actions with the median inhibitory concentrations of 3.43 $\pm$ 0.77 and 56.29 $\pm$ 11.64 μg/mL, respectively. The study of enzyme kinetics showed that lupinifolin exhibited the mode of non-competitive and mixed enzyme inhibitions against α-amylase and α-glucosidase, respectively. **Conclusion:** The potent α-amylase and α-glucosidase inhibitory actions of lupinifolin derived from *D. reticulata* stem evidently suggest its potential use as an alternative for the control of PPG.

Key words: α-amylase, α-glucosidase, *Derris reticulate*, diabetes mellitus, lupinifolin

Abbreviations Used: IC_{50}: Median inhibitory concentration; K_m: Substrate concentration which permits the enzyme to achieve the half-maximal rate of reaction; V_{max}: Maximal rate of reaction.

SUMMARY

- This research work is the first report of the potent inhibitory action of lupinifolin, isolated from *Derris reticulata* stem, against pancreatic α-amylase, a crucial enzyme for starch digestion. The inhibitory action of lupinifolin against another essential carbohydrate-digesting enzyme, α-glucosidase, was also demonstrated. The mode of non-competitive and mixed inhibitions against α-amylase and α-glucosidase, respectively, was also firstly described for the isolated lupinifolin in this study. The potent dual carbohydrate-digesting enzyme inhibitions of lupinifolin thus suggest its potential use as an alternative for the control of postprandial plasma glucose.

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INTRODUCTION

The prevalence of diabetes mellitus, a major non-communicable disease, has been continuously increasing worldwide, especially in low- and middle-income countries.[1] Poorly controlled plasma glucose can lead to many serious diabetic complications including myocardial infarction, cerebrovascular disease, renal failure, loss of vision, lower limb amputation, and nerve damage.[2] These complications result in an increased risk of death and disability in patients with diabetes. Apart from its crucial impact on public health system, diabetes also brings substantial economic and social consequences.[1] It has been documented that postprandial plasma glucose (PPG) is linked with the development of cardiovascular complications in diabetes.[3,4] Postprandial hyperglycemia exerts its noxious effect on vascular endothelium via an induction of oxidative stress, which leads to endothelial dysfunction and pathogenesis of atherosclerosis.[5] The importance of PPG control was evidenced in the Study to Prevent Non-Insulin-Dependent Diabetes

Mellitus (STOP-NIDDM) trial, in which patients with impaired glucose tolerance receiving acarbose, an α-amylase and α-glucosidase inhibitor, had significantly fewer cardiovascular events than those given placebo.[6] Carbohydrate-digesting enzymes, specifically pancreatic α-amylase and intestinal α-glucosidase, play an important role in the regulation of PPG levels. Inhibition against the enzymes with acarbose, a clinically used

antidiabetic drug, delays carbohydrate digestion and subsides glucose excursion after a meal.[7] The present treatment of diabetes includes both lifestyle modification (e.g., exercise and dietary control) and pharmacotherapy with antidiabetic agents. Although many antidiabetic agents with various mechanisms of action are currently available for the treatment of diabetes, the medicine accessibility is still limited in many diabetic patients living in developing countries due to their poor economic status.[8] Several traditionally used medicinal plants are thus still valuable alternatives for diabetic treatment. A search for medicinal plants and their phytochemicals with effective carbohydrate-digesting enzyme inhibitory action is an ongoing field to provide alternatives for the control of PPG, particularly for underprivileged diabetic patients.

Derris reticulata Craib is a medicinal plant in family Leguminosae (subfamily Papilionoideae), which has been applied for several purposes in Thai traditional medicines. The extracts of *D. reticulata* stem were found to have several pharmacological actions such as antiinflammation, antibacterials, antioxidant as well as antidiabetes.[9-12] Its stem has been included as one of ingredients in a Thai traditional herbal formula, named "Mathurameha," which is used for the treatment of diabetes mellitus.[13] Local traditional medicine practitioners in some parts of Thailand have also employed the sole plant as an alternative diabetes treatment. The aqueous extract of *D. reticulata* stem had a significant blood glucose-lowering action in diabetic rats when given at the dose of 250 mg/kg/day for 15 days.[10] The extract also exhibited various antidiabetic-related actions such as suppression against *in vitro* glucose absorption, pancreatic β-cell protection as well as moderate antioxidant activity.[10,11] The α-glucosidase inhibitory action of *D. reticulata* stem extracts prepared with various types of solvent, including hexane, chloroform, ethanol, and water was also documented in some studies.[11,14,15] Lupinifolin is a prenylated flavanone, which can be purified from some medicinal plants in family Leguminosae including *D. reticulata* stem.[16,17] This flavanone exhibited many pharmacological actions including antidiarrheal, cytotoxic, and antimicrobial activities against bacteria, virus, and mycobacteria.[18-22] It was recently reported that lupinifolin had an inhibitory action against α-glucosidase enzyme.[15,23] However, the inhibitory action of lupinifolin against pancreatic α-amylase, a crucial enzyme for starch digestion, has not been evidenced. In addition, the mode of carbohydrate-digesting enzyme inhibitions of lupinifolin has not been established. This study thus aimed to investigate the actions of lupinifolin isolated from *D. reticulata* stem on α-amylase and α-glucosidase enzyme activities and enzyme kinetics.

SUBJECTS AND METHODS

Isolation of lupinifolin from *Derris reticulata* stem

The isolation of lupinifolin from *D. reticulata* stem was performed according to the method described by Chivapat *et al.*[24] and Yusook *et al.*[22] with some modifications. The stems of *D. reticulata* were obtained from the local herb store in Bangkok, Thailand. The authentication of the sample was conducted as described previously.[12] The ground sample (60 g) was Soxhlet extracted with hexane (400 mL). The hot extract was filtered and subsequently heated at 65°C until the clear yellow extract was obtained. The extract was then partitioned with deionized water at 65°C and the hexane layer was collected. The hexane extract was evaporated using a rotary evaporator (Heidolph®, Germany) until the turbid suspension was observed. The extract was left at room temperature for crystallization. The obtained lupinifolin crystals were kept at −20°C until use. The % yield of the isolated lupinifolin was 0.9119%.

Identification of lupinifolin

The identification of the isolated lupinifolin was elucidated by proton nuclear magnetic resonance (¹H-NMR) and ¹³Carbon-NMR. ¹H-NMR and ¹³C-NMR spectra were obtained at 300 MHz using Bruker Avance 300 and Varian Mercury-300. Chemical shift values (δ) were given in ppm by referencing on the chemical shift of the deuterated solvent. Mass spectra were recorded on either a Thermo Finnigan or liquid chromatography MS (LCMS) Bruker micrOTOF.

Alpha-amylase activity assay

The α-amylase activity assay was performed according to the method of Ali *et al.*[25] with slight modifications. Briefly, 20 μL of the sample was preincubated with 200 μL of porcine pancreatic α-amylase (10 unit/mL) at 25°C for 5 min. Four hundred microliter of potato starch solution (0.5% w/v) and 180 μL of deionized water were subsequently added into the mixture and incubated at 25°C for further 3 min. An aliquot of the mixture (200 μL) was then transferred to mix with 100 μL of 3,5-dinitrosalicylic acid (DNS) color reagent (96 mM DNS and 5.31 M sodium potassium tartrate in 2 M NaOH) and then boiled at 85°C for 15 min. The optical density (OD) of the mixture was measured at the wavelength of 540 nm by using an absorbance microplate reader (BMG LABTECH, Ortenberg, Germany). The amount of maltose released from α-glucosidase-catalyzing reaction was used as an index of α-amylase activity. The percentage of inhibition was calculated as the following equation: % inhibition = ([OD of negative control] − [OD of sample]/[OD of negative control]) × 100. Acarbose (1000 μg/mL) was used as a positive control in the experiment. The median inhibitory concentration (IC_{50}) was obtained from the concentration-response curve plotted using GraphPad Prism software version 6.0 (San Diego, California, USA).

Alpha-glucosidase activity assay

The α-glucosidase activity assay was performed following the protocol of Elya *et al.*[26] with some modifications. Briefly, a mixture of the sample (10 μL), 50 mM phosphate buffer, pH 6.9 (460 μL), and 0.15 unit/mL yeast α-glucosidase enzyme (125 μL) was prepared and incubated at 37°C for 5 min. 250 μL of *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG; 5 mM) and 155 μL of phosphate buffer were added into the mixture and incubated for further 15 min at 37°C. Subsequently, an aliquot of the mixture (200 μL) was mixed with 400 μL of Na_2CO_3 (200 mM); the solution was then left at room temperature for further 5 min. The OD of the final mixture was measured at the wavelength of 405 nm. The amount of *p*-nitrophenol released from α-glucosidase-catalyzing reaction was used as an index of α-glucosidase activity. The percentage of inhibition was calculated as the following equation: % inhibition = ([OD of negative control] − [OD of sample]/[OD of negative control]) × 100. Acarbose (500 μg/mL) was used as a positive control in the experiment. The IC_{50} was obtained from the concentration-response curve plotted using GraphPad Prism software version 6.0.

Enzyme kinetics study

The mode of enzyme inhibition was studied using the protocols of α-amylase and α-glucosidase activity assays described above. The reactions were conducted by various concentrations of the substrate, whereas the concentrations of the enzymes and lupinifolin were fixed. The Michaelis–Menten curve was plotted using least squares nonlinear regression in GraphPad Prism software version 6.0 to achieve the maximal rate of reaction (V_{max}) and the Michaelis constant (substrate concentration which permits the enzyme to achieve the half-maximal rate of reaction [K_m]). The changes of V_{max} (ΔV_{max}) and K_m (ΔK_m) in the presence of the enzyme inhibitor were subsequently calculated by comparing the parameters with those obtained in the absence of the enzyme inhibitor. The experiments were performed in triplicate.

Statistical analysis

The data of % inhibition against enzyme activity were expressed as mean ± standard error of the mean, while the data of the IC_{50} and the changes of V_{max} and K_m were shown as mean ± standard deviation. The statistical analysis was performed using one-way analysis of variance followed by the Bonferroni *post hoc* test or Kruskal–Wallis test followed by Mann–Whitney U-test. The difference between the data was considered statistically significant when $P < 0.05$.

RESULTS

Identification of lupinifolin

The obtained chemical was identified as lupinifolin (*S*)-5-hydroxy-8-(4-hydroxyphenyl)-2,2-dimethyl-10-(3-methylbut-2-en-1-yl)-7,8-dihydro-2H,6H-pyrano[3,2-g]chromen-6-one) ($C_{25}H_{26}O_5$) with light yellow crystal (%yield = 0.9119%). The characteristics of the isolated compound are described as follows: mp 113.7-115.5°C. ^{1}H-NMR (300 MHz, Chloroform-D) d 12.25 (s, 1H), 7.33 (d, J=8.51 Hz, 2H), 6.90 (d, J=8.59, 2H), 6.65 (d, J=9.99 Hz, 1H), 5.52 (d, J=10.00 Hz, 1H), 5.35 (dd, J=3.06, 12.72 Hz, 1H), 5.16 (t, J=7.38 Hz, 1H), 3.22 (d, J=7.30 Hz, 2H), 3.07 (dd, J=12.77, 17.13 Hz, 1H), 2.82 (dd, J=3.16, 17.13 Hz, 1H), 1.67 (s, 6H), 1.46 (d, J=4.31, 6H); ^{13}C-NMR (300 MHz, Chloroform-D) d 21.48, 25.83, 28.31, 28.41, 43.15, 76.63, 77.05, 77.48, 78.20, 78.54, 102.67, 102.83, 108.71, 115.56, 115.62, 122.47, 126.05, 127.72, 130.80, 131.16, 156.08, 156.56, 159.44, 160.03, 196.70; LC-MS (electrospray ionization) 407.1853 [M+Na]. The structures of lupinifolin were fully characterized by ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{13}C correlation Heteronuclear Multiple Quantum Coherence (HMCQ), Heteronuclear Multiple Bond Coherence (HMBC), Distortionless Enhancement by Polarization Transfer (DEPT) where their spectra were in agreement with the assigned structure.

Alpha-amylase activity assay

The isolated lupinifolin at the concentration of 50, 12.5, 5, 2.5, and 1 μg/mL produced a significant α-amylase inhibitory action with the IC_{50} of 3.43 ± 0.77 μg/mL (*n* = 5) [Table 1]. Lupinifolin at concentration of 12.5 μg/mL caused a maximal inhibition of 75.85% ±2.27%.

Acarbose (positive control) at the concentration of 1000 μg/mL significantly inhibited α-amylase activity with % inhibition of 44.10% ±1.15% (*n* = 5).

Alpha-amylase enzyme kinetics study

From the Michaelis–Menten plot, lupinifolin at its approximate IC_{50} of 4 μg/mL caused a decrease in V_{max} with no significant change in K_m [Table 2 and Figure 1]. On the contrary, acarbose (1000 μg/mL) decreased both V_{max} and K_m [Table 2 and Figure 1].

Alpha-glucosidase activity assay

The isolated lupinifolin had a concentration-dependent inhibitory action against α-glucosidase with the IC_{50} of 56.29 ± 11.64 μg/mL (*n* = 3) [Table 3]. Lupinifolin at the highest concentration tested (250 μg/mL) caused a maximal inhibition of 98.58% ±2.44%.

Acarbose (1000 μg/mL), positive control, produced a significant α-glucosidase inhibition with the % inhibition of 68.18 ± 0.50 (*n* = 9).

Alpha-glucosidase enzyme kinetics study

The isolated lupinifolin at its IC_{50} (60 μg/mL) caused changes of both V_{max} and K_m with a decrease in V_{max} and an increase in K_m from the Michaelis–Menten plot [Table 4 and Figure 2]. Acarbose (500 μg/mL) also changed the α-glucosidase enzyme kinetics with an increase in K_m and a slight decrease in V_{max} [Table 4 and Figure 2].

Table 1: The percentage of inhibition of lupinifolin against pancreatic α-amylase activity

Concentration (μg/mL)	Percentage inhibition (mean±SEM)
50	74.97±1.77*
12.5	75.85±2.27*
5	69.48±3.47*
2.5	44.84±1.93*
1	20.54±3.52*
0. 5	6.99±2.02
0.25	5.70±2.78
0	0.00±0.00

*$P<0.05$ when compared with negative control; One-way ANOVA followed by the Bonferroni *post hoc* test; *n*=5. ANOVA: Analysis of variance; SEM: Standard error of mean

Table 2: Enzyme kinetics study of lupinifolin and acarbose against pancreatic α-amylase (*n*=4)

Test agents	ΔV_{max} (mean±SD)	ΔK_m (mean±SD)
Lupinifolin (4 μg/mL)	−0.2712±0.0444	−0.0065±0.0129
Acarbose (1000 μg/mL)	−0.3046±0.0178	−0.2041±0.0232

SD: Standard deviation

Table 3: The percentage of inhibition of lupinifolin against α-glucosidase activity

Concentration (μg/mL)	Percentage inhibition (mean±SEM)
250	98.58±2.44*
100	58.51±3.74*
20	20.59±1.32*
10	15.06±1.46*
1	11.31±1.38*
0.1	7.60±0.69
0	0.00±0.00

*$P<0.05$ when compared with negative control; Kruskal-Wallis test followed by Mann-Whitney U-test; *n*=9. SEM: Standard error of mean

Table 4: Enzyme kinetics study of lupinifolin and acarbose against α-glucosidase (*n*=3)

Enzyme inhibitors	ΔV_{max} (mean±SD)	ΔK_m (mean±SD)
Lupinifolin (60 μg/mL)	−0.2716±0.0520	+0.2950±0.0709
Acarbose (500 μg/mL)	−0.1422±0.0399	+5.4917±1.3810

SD: Standard deviation

DISCUSSION

Pancreatic α-amylase, located in the intestinal lumen, is a crucial enzyme for starch digestion into oligosaccharides. The obtained oligosaccharides are subsequently digested into absorbable monosaccharides by α-glucosidase. Since starch is the main source of carbohydrate in the diet, both α-amylase and α-glucosidase enzymes are essential for dietary carbohydrate digestion. The potent inhibitory action of lupinifolin against pancreatic α-amylase was firstly demonstrated here in this study with the IC_{50} of 3.43 ± 0.77 μg/mL. The isolated lupinifolin also produced a concentration-dependent inhibition against α-glucosidase with the IC_{50} of 56.29 ± 11.64 μg/mL. The α-glucosidase inhibiting potency of lupinifolin isolated in the current study was somewhat lower than that reported earlier by Issarachote and Joycharat (2015) (IC_{50} of 11.22 μg/mL). Lupinifolin is a prenylated flavanone with a good solubility in organic solvent, but it has a very low water solubility of 0.009 mg/L, estimated from its octanol–water partition coefficient at 25°C.[22] Since lupinifolin has a remarkably low water solubility, it is critical to use the appropriate vehicle for lupinifolin dilution before adding it
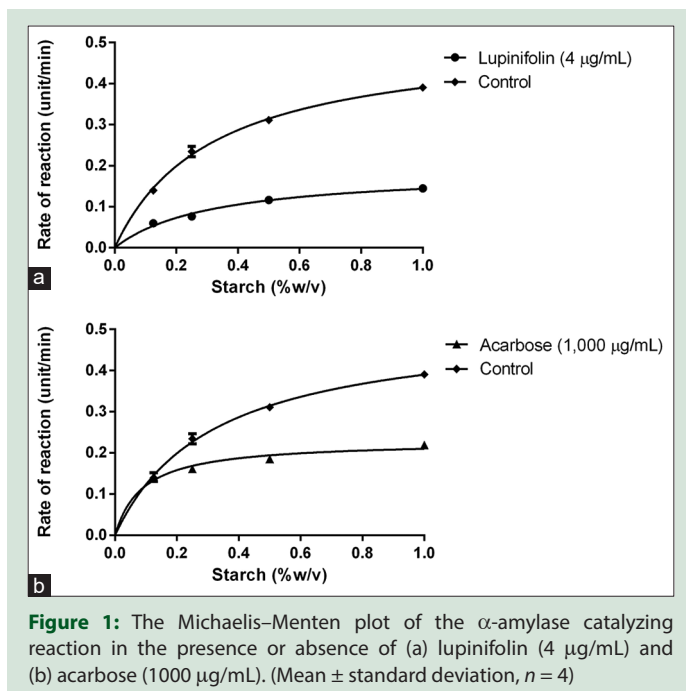
Figure 1: The Michaelis–Menten plot of the α-amylase catalyzing reaction in the presence or absence of (a) lupinifolin (4 μg/mL) and (b) acarbose (1000 μg/mL). (Mean ± standard deviation, $n = 4$)

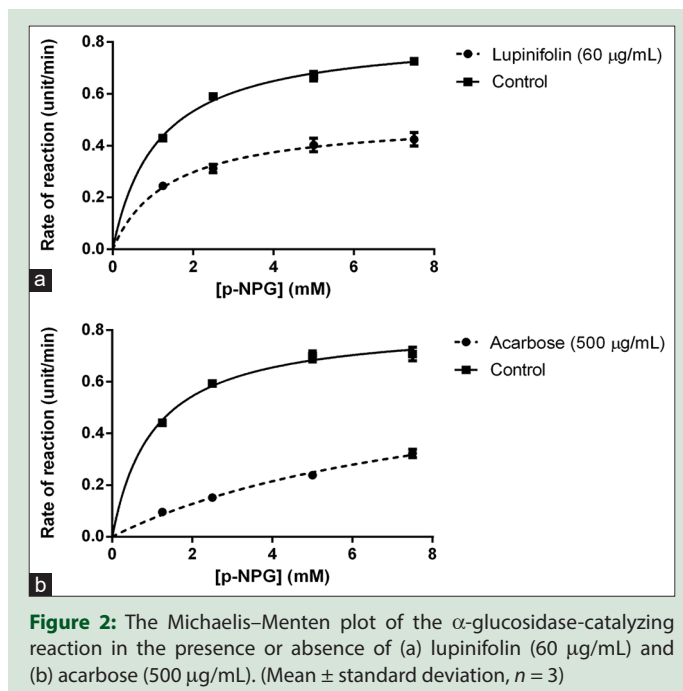**Figure 2:** The Michaelis–Menten plot of the α-glucosidase-catalyzing reaction in the presence or absence of (a) lupinifolin (60 μg/mL) and (b) acarbose (500 μg/mL). (Mean ± standard deviation, $n = 3$)

into the aqueous buffer. To avoid precipitation in aqueous media, Yusook *et al.* (2017) suggested that lupinifolin should be prepared with 0.1 M NaOH. This basic solution was thus used as a vehicle for lupinifolin in the present work, whereas dimethyl sulfoxide was indicated as a vehicle for lupinifolin in the study of Issarachote and Joycharat.[15] A difference in the vehicle used for lupinifolin dilution may possibly cause a variation in its inhibitory action against the enzyme. In addition, dissimilarity in enzyme activity between studies can also lead to a slight difference in the inhibitory action of lupinifolin. Nonetheless, both the studies indicated a potent α-glucosidase inhibition of *D. reticulata*-derived lupinifolin with relatively low IC$_{50}$ in ranges of double-digit μg/mL.

It was reported that acarbose produced an inhibitory action against both α-amylase and α-glucosidase enzymes with the IC$_{50s}$ of 258 ± 17 and 280 ± 19 μg/mL, respectively.[27] The potency of lupinifolin against both α-amylase and α-glucosidase was thus apparently higher than those of acarbose, which is clinically used for a control of PPG in Type 2 diabetic patients. Postprandial hyperglycemia has been found to be linked with chronic complications, especially cardiovascular diseases in patients with diabetes.[3-5,28] From the STOP-NIDDM trial, it was shown that the risk of cardiovascular events was significantly lower in patients with impaired glucose tolerance taking acarbose.[6] A meta-analysis on the use of acarbose in Type 2 diabetic patients also showed a reduced risk of myocardial infarction.[29] However, a clinical use of α-glucosidase inhibitor for controlling PPG is still limited in some areas due to its relatively high cost comparing to other anti-diabetic agents such as metformin and sulfonylureas.[30] The current findings of potent α-amylase and α-glucosidase inhibitory actions of lupinifolin isolated from *D. reticulata* stem may potentially improve antidiabetic drug accessibility regarding α-amylase and α-glucosidase inhibitors. Since various types of carbohydrates (e.g., starch, oligosaccharides, and disaccharides) can be found in a normal diet, a digestion of dietary carbohydrate would be suppressed more effectively by an agent inhibiting both α-amylase and α-glucosidase enzymes. Thus, the dual enzyme inhibitory actions of lupinifolin potentially imply its advantage in the regulation of PPG. Nonetheless, further *in vivo* study, specifically oral starch-tolerance and oral sucrose-tolerance tests, is still necessarily required to verify their actions in the body. In addition, a chronic toxicity test is also needed

since long-term treatment is committed to the control of PPG in patients with diabetes. Nonetheless, it was reported from the animal study that lupinifolin at the highest dose tested of 5 g/kg did not produce any acute toxic effects and no subacute toxic effect was detected at the dose of 60 mg/kg/day (given for 28 days).[24]

The results from the enzyme kinetics study indicated that lupinifolin acted as a non-competitive inhibitor against α-amylase with a decrease in V$_{max}$ and no significant change in K$_m$. Non-competitive inhibitor binds to allosteric site of the enzyme and impedes the binding of substrate to the active site by inducing conformational change.[31] Meanwhile, lupinifolin behaved as a mixed inhibitor against α-glucosidase since it caused a decrease in V$_{max}$ and an increase in K$_m$. An increase in K$_m$ indicates that a mixed enzyme inhibitor could decrease the substrate binding affinity to the enzyme. This is dissimilar to the action of a non-competitive inhibitor, which does not cause any change in K$_m$. Unlike a competitive inhibitor, the inhibitory actions of both non-competitive and mixed inhibitors do not depend on a concentration of available substrate.[31] Both non-competitive and mixed inhibitors restrain a maximal rate of reaction to a lower level even in the presence of higher substrate concentration. A sustainably low level of carbohydrate digestion would be considerably expected at any larger extent of carbohydrate consumed. This advantage of non-competitive and mixed enzyme inhibitors may be crucial in clinical practice since dietary control is one of the main obstacles frequently found in the management of Type 2 diabetes.[32] However, a higher risk of gastrointestinal adverse events, as a consequence of undigested carbohydrate accumulation, may be observed during the use of these types of enzyme inhibitor.

In addition to its inhibition against α-glucosidase, it was documented that the crude *D. reticulata* aqueous extract further possessed other antidiabetic actions, including pancreatic β-cell protection and suppression of glucose absorption.[10,11] It is still not known whether the isolated lupinifolin can exhibit those antidiabetic mechanisms of action. Nevertheless, the potent α-amylase and α-glucosidase inhibitory actions of lupinifolin derived from *D. reticulata* stem were demonstrated in this study thus firmly support its potential use as an alternative for the treatment of diabetes mellitus, particularly for the control of PPG.

CONCLUSION

Lupinifolin, isolated from *D. reticulata* stem, significantly inhibited α-amylase and α-glucosidase activities with the IC_{50s} of 3.43 ± 0.77 and 56.29 ± 11.64 μg/mL, respectively. The enzyme kinetics study showed that lupinifolin exhibited the mode of non-competitive and mixed inhibitions against α-amylase and α-glucosidase, respectively.

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Conflicts of interest

There are no conflicts of interest.

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