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

Pawitra Pulbutr, Pichnatcha Nantana, Surabor Suksabai, Chawannuch Mudjupa, Rattazart Denchai, Sakulrat Rattanakiat, Taweesak Dhammaraj

Pharmaceutical Chemistry and Natural Product Research Unit, Faculty of Pharmacy, Mahasarakham University, Maha Sarakham, Thailand

**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 spectrum. The isolated lupinifolin produced a significant α-amylase and α-glucosidase inhibitory actions with the median inhibitory concentrations of 3.43 ± 0.77 and 56.29 ± 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

**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.

**INTRODUCTION**

The prevalence of diabetes mellitus, a major non-communicable disease, has been continuously increasing worldwide, especially in low- and middle-income countries. 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. 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. It has been documented that postprandial plasma glucose (PPG) is linked with the development of cardiovascular complications in diabetes. Postprandial hyperglycemia exerts its noxious effect on vascular endothelium via an induction of oxidative stress, which leads to endothelial dysfunction and pathogenesis of atherosclerosis. 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.

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 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 α-glucosidase inhibitor, had significantly fewer cardiovascular events than those given placebo.
antidiabetic drug, delays carbohydrate digestion and subsides glucose excursion after a meal.\textsuperscript{[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.\textsuperscript{[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. \textit{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 \textit{D. reticulata} stem were found to have several pharmacological actions such as antiinflammation, antibacterials, antioxidant as well as antidiabetics.\textsuperscript{[9,12]} \textit{D. reticulata} stem was included as one of ingredients in a Thai traditional herbal formula, named “Mathurameha,” which is used for the treatment of diabetes mellitus.\textsuperscript{[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 \textit{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.\textsuperscript{[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.\textsuperscript{[10,11]} The α-glucosidase inhibitory action of \textit{D. reticulata} stem extracts prepared with various types of solvent, including hexane, chloroform, ethanol, and water was also documented in some studies.\textsuperscript{[11,14,15]} Lupinifolin is a prenylated flavanone, which can be purified from some medicinal plants in family Leguminosae including \textit{D. reticulata} stem.\textsuperscript{[16,17]} This flavanone exhibited many pharmacological actions including antiinflammatory, antiobesity, and antinociceptive activities against bacteria, virus, and mycobacteria.\textsuperscript{[18–22]} It was recently reported that lupinifolin had an inhibitory action against α-glucosidase enzyme.\textsuperscript{[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 \textit{D. reticulata} stem on α-amylase and α-glucosidase enzyme activities and enzyme kinetics.

**SUBJECTS AND METHODS**

**Isolation of lupinifolin from \textit{Derris reticulata} stem**

The isolation of lupinifolin from \textit{D. reticulata} stem was performed according to the method described by Chivapat et al.\textsuperscript{[24]} and Yusook et al.\textsuperscript{[21]} with some modifications. The stems of \textit{D. reticulata} were obtained from the local herb store in Bangkok, Thailand. The authentication of the sample was conducted as described previously.\textsuperscript{[21]} 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 13C-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.\textsuperscript{[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\textsubscript{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.\textsuperscript{[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 \textit{p}-nitrophenyl-\textit{α}-D-glucopyranoside (\textit{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\textsubscript{2}CO\textsubscript{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 \textit{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\textsubscript{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 \(V_{max}\) (\(\Delta V_{max}\)) and \(K_m\) (\(\Delta K_m\)) 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.
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-pyran-3,2-g-chromen-6-one (C_{16}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. 1H-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), 2.82 (dd, J=3.16, 17.13 Hz, 1H), 1.67 (s, 6H), 1.46 (d, J=4.31, 6H). 13C-NMR (300 MHz, Chloroform-D) δ 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, 126.72, 130.80, 130.16, 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 1H-NMR, 13C-NMR, 1H-13C correlation Heteronuclear Multiple Quantum Coherence (HMQC), Heteronuclear Multiple Bond Coherence (HMBC), Distortionless Enhancement by Polarization Transfer (DEPT) where their spectra were in agreement with the assigned structure.

Alpha-amylose activity assay

The isolated lupinifolin at the concentration of 50, 12.5, 5, 2.5, and 1 µg/mL produced a significant α-amylose 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-amylose activity with % inhibition of 44.10% ±1.15% (n = 5).

Alpha-amylose 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].

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. Since lupinifolin has a remarkably low water solubility, it is critical to use the appropriate vehicle for lupinifolin dilution before adding it.

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

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Percentage inhibition (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>74.97±1.77*</td>
</tr>
<tr>
<td>12.5</td>
<td>75.85±2.27*</td>
</tr>
<tr>
<td>5</td>
<td>69.48±3.47*</td>
</tr>
<tr>
<td>25</td>
<td>44.84±1.93*</td>
</tr>
<tr>
<td>0.5</td>
<td>20.54±3.52*</td>
</tr>
<tr>
<td>0.25</td>
<td>6.99±2.02</td>
</tr>
<tr>
<td>0.25</td>
<td>5.70±2.78</td>
</tr>
<tr>
<td>1</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

*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)

<table>
<thead>
<tr>
<th>Test agents</th>
<th>∆V_{max} (mean±SD)</th>
<th>∆K_{m} (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lupinifolin (4 µg/mL)</td>
<td>−0.2712±0.0444</td>
<td>−0.0065±0.0129</td>
</tr>
<tr>
<td>Acarbose (1000 µg/mL)</td>
<td>−0.3046±0.0178</td>
<td>−0.2041±0.0232</td>
</tr>
</tbody>
</table>

SD: Standard deviation

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

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

*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)

<table>
<thead>
<tr>
<th>Enzyme inhibitors</th>
<th>∆V_{max} (mean±SD)</th>
<th>∆K_{m} (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lupinifolin (60 µg/mL)</td>
<td>−0.2712±0.0520</td>
<td>+0.2950±0.0709</td>
</tr>
<tr>
<td>Acarbose (500 µg/mL)</td>
<td>−0.1422±0.0399</td>
<td>+5.4917±1.3810</td>
</tr>
</tbody>
</table>

SD: Standard deviation

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.
It was reported that acarbose produced an inhibitory action against both α-glucosidase enzymes with IC\textsubscript{50} of 258 ± 17 and 280 ± 19 μg/mL, respectively. 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. Nonetheless, both the studies indicated a potent α-glucosidase inhibition of D. reticulata-derived lupinifolin with relatively low IC\textsubscript{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 IC\textsubscript{50} of 258 ± 17 and 280 ± 19 μg/mL, respectively. 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.

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. A meta-analysis on the use of acarbose in Type 2 diabetic patients also showed a reduced risk of myocardial infarction. 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.

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).

The results from the enzyme kinetics study indicated that lupinifolin acted as a non-competitive inhibitor against α-amylase with a decrease in V\textsubscript{max} and no significant change in K\textsubscript{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. Meanwhile, lupinifolin behaved as a mixed inhibitor against α-glucosidase since it caused a decrease in V\textsubscript{max} and an increase in K\textsubscript{m}. An increase in K\textsubscript{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\textsubscript{m}. Unlike a competitive inhibitor, the inhibitory actions of both non-competitive and mixed inhibitors do not depend on a concentration of available substrate.

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. 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.
CONCLUSION

Lupinifolin, isolated from *D. reticulata* stem, significantly inhibited α-amylase and α-glucosidase activities with the IC\textsubscript{50} 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.

Financial support and sponsorship

This project is financially supported by the Faculty of Pharmacy, Mahasarakham University research grant (Grant number S5/2562).

Conflicts of interest

There are no conflicts of interest.

REFERENCES