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# Evaluation of *α*-Amylase Inhibitory Activity of Saponins from *Panax bipinnatifidus* Seem. Growing in Vietnam

Hieu N. Ngoc[1], Nguyen T.V. Anh[2], Nguyen L. Nhi[2], Dang T. Yen[1], Vu V. Tuan[1], Nguyen H. Tung[1], Huong T.X Hoang[3], Luan L. Chu[2,4]*

[1]*Faculty of Pharmacy, Phenikaa University, Hanoi 12116, Vietnam*
[2]*Faculty of Biotechnology, Chemistry and Environmental Engineering, Phenikaa University, Hanoi 12116 Vietnam*
[3]*Faculty of Nursing, Phenikaa University, Hanoi 12116, Vietnam*
[4]*Faculty of Biomedical Sciences, Phenikaa University, Hanoi 12116, Vietnam*

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ABSTRACT

*α*-Amylase inhibitors represent a drug target for lowering postprandial hyperglycemia in diabetic patients. According to traditional medicines, *Panax* plants have been utilised to treat diabetes and considered as a valuable source to develop anti-diabetic supplementary products. The current study aimed to evaluate the α-amylase inhibitory activities of *Panax bipinnatifidus* root cultivated in Vietnam and its main chemical constituents. The two major saponins, Araloside A and Stipuleanoside R2 were confirmed by ultra performance liquid chromatography quadruple-time of flight-mass spectrometry (UPLCQ-TOF/MS) and nuclear magnetic resonance (NMR). Then, the results indicated that Araloside A and Stipuleanoside R2, were shown to moderately inhibit the α-amylase activity in an enzymatic assay with $IC_{50}$ values of 781.46 and 2746.90 µg/mL, respectively. Moreover, Araloside A (*Vmax* ~ 0.0635 µM/min, Km ~ 4.691 µM) was displayed as a completive inhibitor of α-amylase in the kinetic study whereas Acarbose (*Vmax* ~ 0.053 µM/min, Km ~ 6.521 µM) showed a mixed inhibition. This study suggested the posibility of Araloside A acting as an α-amylase inhibitor. Also, it supports the traditional use of *P. bipinnatifidus* in antidiabetic dietary.

***Keywords***: *Panax bipinnatifidus*, saponins, Araloside A, Stipuleanoside R2, α-amylase, antidiabetic

## Introduction

Diabetes mellitus (DM) is considered the most challenging metabolic disorder in the world. It is estimated 537 million people worldwide have diabetes currently and it's predicted to reach 700 million by 2045.[1] DM affects up to 5.76 million Vietnamese people in 2020 and is projected to reach 6.1 million people by 2040.[2] DM is characterized by the lack of insulin hormone secretion or a decrease in glucose consumption in the human body. While type 1 diabetes was caused by the insulin secretion of the pancreas, type 2 diabetes was largely related to insulin resistance. The consequences of diabetes lead to serious complications such as anemia, kidney failure, liver failure, multiple organ failure, or diseases related to the nervous system.[3,4] Various treatments have been employed for DM. Conventional therapy, such as glipizide and metformin, which activate the secretion and enhancement of the insulin action at the target tissues, and the inhibition of polysaccharide hydrolysis by α-amylase and α-glucosidase have been widely studied in recent years.[5] Enzyme inhibitors is known as an efficient and safe method for the treatment of type 2 diabetes mellitus.[5] The α-amylase (*α*-1,4 glucan-4-glucanohydrolase; EC 3.2.1.1) is known as a hydrolase that cleaves alpha-linked starch to produce smaller oligosaccharides.[6]

*Corresponding author. E mail: luan.chuluong@phenikaa-uni.edu.vn
Tel: 8402462918118

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Human α-amylase was found in saliva and pancreatic juice that can rapidly degrade dietary starch to raise glucose level in the blood.[6] Therefore, one of the most important therapies for diabetic patients is controlling the catalytic activities of alpha-amylase, which could reduce glucose production in the postprandial stage.[5,6]

Saponins are known as a major compound found in *Panax* plants. Saponins exhibit various biological and pharmacological activities, such as antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, and anticancer.[7,8] Vietnam is assessed to possess a diverse source of natural medicinal herbs. Several ginseng species can be found in nature in Vietnam, including *P. vietnamensis* Ha et Grushv.[9], *P. bipinnatifidus* Seem.[10], and *P. stipuleanatus* H. T. Tsai and K. M. Feng.[11] *P. bipinnatifidus*, also known as 'Sam Vu Diep' in Vietnamese, is a perennial herb with 10-20 cm height with erect and solitary rhizome. The plant was found and taxonomically authenticated in 1868.[10,12] It is distributed in China, India, Nepal (the sub-Himalayan Range), and Vietnam. In Vietnam, *P. bipinnatifidus* was found naturally in Hoang Lien Son mountainous area.[12] Phytochemical investigations on the plant have revealed that the main chemical compositions were oleanane-type saponins, typically Stipuleanoside R2 and Araloside A methyl ester.[13] Furthermore, Ginsenosides were also detected in the rhizomes of *P. bipinnatifidus* collected in Sikkim Himalayan region, including Ginsenoside Rg1, Rg2, Re, Rd, Rb1, Rb2 with the total content of 18.54 ± 0.68 mg/g dry weight.[14] In terms of pharmacological activities, there have been several initial bioactivity screenings of *P. bipinnatifidus*. It investigated the antithrombotic activities of the crude extract and n-butanolic fraction of *P. bipinnatifidus*, which showed significant antiplatelet aggregation activity in the tested concentration range (0.5-5.0 mg/mL). The *n*-butanolic fraction was also shown to exhibit bioactivity towards prolonged activated partial thromboplastin time and prothrombin time at the high dose.[10] Oleanane-type triterpenoid saponins from *P. bipinnatifidus* also showed strong inhibitory effects on nitric oxide production in lipopolysaccharide

(LPS)-stimulated the macrophage RAW 264.7 cell line with $IC_{50}$ values of 0.21-0.62 µg/mL.[15] In this study, the main saponin composition was isolated and characterized (Figure 1), and their α-amylase inhibitory activity was also evaluated for the first time in an enzymatic assay.

## Materials and Methods

*Chemicals and reagents*

Acarbose, α-amylase from porcine pancreas (Type VI-B, >= 5 units/mg solid), starch, and iodine reagents were purchased from Sigma-Aldrich (Merck Vietnam Ltd.). Organic solvents used in the study were purchased from Samchun (Samchun Chemical Co., Ltd., South Korea). HPLC solvents for analytical procedures were obtained from Macron Fine Chemicals (Avantor, USA). Thin-layer chromatography (TLC) plate, the normal-phase silica gel (40-63 µm), and the reverse-phase silica gel RP-$C_{18}$ were purchased from Merck, Germany. Moreover, D101 resin and Diaion HP20 resin were obtained from Mitshubishi Chemical, Japan. Column chromatography was employed for isolation using normal-phase silica gel. The reverse-phase silica gel RP-$C_{18}$, D101 resin, and Diaion HP20 resin were used as adsorbents.

*Plant materials*

*P. bipinnatifidus* Seem. stems were collected in Sa Pa, Lao Cai, Vietnam (22° 20' 10.8996" N, 103° 50' 37.626" E) in July 2019. The sample was authenticated by Dr. Tung Pham-Ha-Thanh from the Department of Pharmacognosy and Traditional Pharmacy, Faculty of Pharmacy, Phenikaa University, Hanoi, Vietnam. A voucher specimen (2019/07-PB-01) was deposited at the Laboratory of Pharmaceutical Chemistry and Bioactive Compounds, Faculty of Pharmacy, Phenikaa University, Hanoi, Vietnam.

Extraction of isolation saponins from the rhizomes of *P. bipinnatifidus*

The rhizomes of *P. bipinnatifidus* (500 g) (Analytical Balance, WBA-220, Daihan, South Korea) were extracted by ethanol (70 °C, reflux extraction, 1.5 L × 3 times). The plant extracts were then filtered, combined, and evaporated under reduced pressure using the Rotavapor® R-100 (BUCHI Labortechnik AG, Flawil, Switzerland). The concentrated extract was then partitioned with dichloromethane (0.5 L × 3 times) to separate non-polar and polar compounds from the crude extract. The aqueous phase, which contained polar compounds, was applied into a D101 resin column, eluting with ethanol 96°. The eluate was then evaporated *in vacuo* to obtain a brownish residue (21.82 g).

The saponin fraction (21.82 g) was dissolved into distilled water (500 mL) and then subjected into a Diaion HP-20 macroporous resin chromatography column (Φ80 mm × 500 mm), eluting with a gradient solvent system consisting of $H_2O$ and ethanol (100:0 → 4:96, v/v), to yield five fractions (Fr.1-Fr.5). According to TLC profile, fraction Fr.3 (1.5 g) was chosen for further purification, specifically a reverse-phase silica gel chromatography column MeOH-$H_2O$ (1:1, v/v) and recrystallization in ethanol 50%, to yield compound 1 (140 mg). Fraction Fr.4 (3.8 g) was also purified by a normal-phase silica gel chromatography column (Φ40 mm x 300 mm) with the solvent system consisting of $CH_2Cl_2$-MeOH-$H_2O$ (50/10/1, v/v/v) as eluent to yield compound 2 (80 mg).

Thin-layer chromatography (TLC) analysis

TLC was carried out on TLC silica gel 60 RP-18 $F_{254S}$ (Merck, Darmstadt, Germany) plates. Spots on TLC plates were visualized by destructive methods.[15] The method was carried out by spraying with 10% aqueous $H_2SO_4$ solution in MeOH and then heating at 100°C for 1-2 min.

*Mass spectrometry (MS) analysis*

High-resolution quadruple time-of-flight electrospray ionization-mass spectrometry (HR–QTOF ESI/MS) analysis was carried out with High Performance Liquid Chromatograph Triple Quadrupole Mass Spectrometer-8405 system (Shimadzu, Japan). LabSolutions LCMS (Shimadzu, Japan) was used for analysis the chromatograms.

*Nuclear magnetic resonance (NMR) analysis*

For NMR analysis of the purified product, compounds 1 and 2 were dissolved in methanol-$d_4$ and subjected to 600 MHz Bruker Biospin NMR for one-dimensional $^1$H-NMR, and $^{13}$C-NMR analyses. Tetramethylsilane (TMS) was used as an internal reference. The data was further analyzed using Mnova software 14.1 (Mestrelab Research, S.L., USA).

*α-Amylase inhibitory assay*

The two identified saponins, Araloside A and Stipuleanoside R2, from *P. bipinnatifidus* for α-amylase inhibitory activity were carried out in a 96-Well Microtiter™ Microplates with the various concentrations (Araloside A: 100, 200, 400, 600, and 800 µg/µL; Stipuleanoside R2: 200, 500, 1000, 2000, and 3000 µg/µL). The starch-Iodine color assay with slight modifications was used to determine α-amylase inhibitory activity.[16] The total assay mixture, including 50 µL of phosphate buffer at pH ~ 6.9 ($(Na_2HPO_4/NaH_2PO_4$, 0.2M containing NaCl 0.006M)), 10 µL of phosphate-buffered α-amylase (2 unit/mL). The mixture was incubated on the incubator IN30 (Memmert GmbH + Co. KG, Schwabach, Germany) for 10 minutes at 37 °C. This step was followed by addition of 10 µL of starch solution 1% (w/v) and equilibrated for 15 min at 37 °C. The reaction mixture was terminated by adding 40 µL of 1M HCl. The presence of starch was visualized with 100 µL of Iodine reagent (5 mM $I_2$ and 5 mM KI). The absorbance was measured at 620 nm using HiPo MPP-96, Microplate Photometer-Biosan (Riga, Latvia). Acarbose was used as a positive control at the concentration range of 10-200 µg/µL (10, 20, 50, 100, 200 µg/µL). The control reaction representing 100% enzyme activity did not contain any saponins from *P. bipinnatifidus*. The absorbance of saponins was eliminated through control reaction without the enzyme. Inhibition of enzyme activity was calculated as follows: $\%\ inhibition = (1\text{-}A_{Sample}/A_{Control}) \times 100\%$ (1).[16] The $IC_{50}$ values were defined as concentration of saponins that inhibited 50% of α-amylase enzyme. The $IC_{50}$ values were determined from by plotting α-amylase inhibition (%) against sample concentration (µg/mL).

*Kinetic tests for α -amylase*

While enzyme concentration was kept constant (2 U/mL), the amount of hydrolyzed starch in presence of compounds was used in varying concentration (0.1605; 0.227; 0.4487; 0.7363; 1.2555 µM). Michaelis Menten equation was used to calculate the kinetic parameters, and Lineweaver-Burk plots were used to determine the type of inhibition (equation (2)).[17] Lineweaver-Burk plot was plotted for saponins from *P. bipinnatifidus* with $IC_{50}$ of µg/mL. While *Km* is the Michaelis Menten constant, *Vo* is the initial rate of reaction, Moreover, *Km/Vmax* is the slope, and the y-intercept (*1/Vmax*) was calculated using equation (3)[17] as below:

$$Vo = \frac{Vmax\,[S]}{Km+[S]} \quad (2) \qquad \frac{1}{Vo} = \frac{Km}{Vmax}\frac{1}{[S]} + \frac{1}{Vmax} \quad (3)$$

*Statistical analysis*

The experiment for α-amylase inhibitory activity was carried out in triplicate. The data were analyzed and presented as Mean ± SE (standard error) by using Microsoft Excel (Microsoft Corporation, 2023). Comparisons between test samples and control were performed using T-tests. The statistical significance level was set at $p < 0.05$.

## Results and Discussion

*Chemical composition*

Stipulenaoside $R_2$ (1): White powder; ESI-MS: *m/z* 1087.75 $[M\text{-}H]^-$, (Supplementary data, Figure S1.1). $^1$H-NMR (600 MHz, methanol-$d_4$): $\delta_H$ 0.82, 0.85, 0.93, 0.95, 0.96, 1.05, 1.17 (7× $CH_3$, all s, $CH_3$-25, 26, 24, 23, 30, 29, 27), 3.16 (1H, dd, *J*= 4.2, 11.4 Hz, H-3), 4.37 (1H, d, *J* = 7.8 Hz, H-1′), 4.88 (1H, d, *J*= 7.8 Hz, H-1″), 5.20 (1H, brs, H-1‴), 5.27 (1H, brt, *J* = 4.2 Hz, H-12), 5.40 (1H, d, *J*= 7.8 Hz, H-1⁗); $^{13}$C-NMR (150 MHz, methanol-$d_4$): δ 39.8 (C-1), 26.9 (C-2), 90.8 (C-3), 40.2 (C-4), 57.1 (C-5), 19.3 (C-6), 34.0 (C-7), 40.8 (C-8), 49.1 (C-9), 37.9 (C-10), 24.6 (C-11), 123.9 (C-12), 144.8 (C-13), 42.9 (C-14), 28.9 (C-15), 24.0 (C-16), 48.1 (C-17), 42.6 (C-18), 47.2 (C-19), 31.5 (C-20), 34.9 (C-21), 33.2 (C-22), 28.5 (C-23), 17.0 (C-24), 16.0 (C-25), 17.8 (C-26), 26.3 (C-27), 178.1 (C-28), 33.5 (C-29), 24.0 (C-30). GlcA: 106.4 (C-1), 76.5 (C-2), 82.1 (C-3), 79.4 (C-4), 78.2 (C-5),

176.4 (C-6); Glc: 104.4 (C-1), 75.6 (C-2), 78.3 (C-3), 71.1 (C-4), 78.3 (C-5), 63.4 (C-6); 28-Glc: 95.8 (C-1), 74.0 (C-2), 78.0 (C-3), 71.2 (C-4), 78.7 (C-5), 62.3 (C-6). Ara: 108.3 (C-1), 82.1 (C-2), 75.3 (C-3), 87.2 (C-4), 62.5 (C-5).[18] (Supplementary data_Figure S1.2-6).

Araloside A (2): White powder; ESI-MS: *m/z* 925.65 $[M-H]^-$ (Supplementary data_Figure S2.1). $^1$H-NMR (600 MHz, methanol-$d_4$): $\delta_H$ 0.80, 0.85, 0.91, 0.93, 0.95, 1.05, 1.16, 1.22 (7× $CH_3$, all s, $CH_3$-25, 26, 24, 23, 30, 29, 27), 3.17 (1H, dd, *J*= 4.8, 12.0 Hz, H-3), 4.40 (1H, d, *J* = 7.8 Hz, H-1′), 5.06 (1H, brs, H-1′′), 5.25 (1H, brt, *J*= 3.6 Hz, H-12), 5.38 (1H, d, *J* = 8.4 Hz, H-1′′′); $^{13}$C-NMR (150 MHz, methanol-$d_4$): δ 39.7 (C-1), 27.0 (C-2), 91.2 (C-3), 40.2 (C-4), 57.0 (C-5), 19.3 (C-6), 34.0 (C-7), 40.7 (C-8), 49.1 (C-9), 37.9 (C-10), 24.6 (C-11), 123.8 (C-12), 144.8 (C-13), 42.9 (C-14), 28.9 (C-15), 24.0 (C-16), 48.0 (C-17), 42.6 (C-18), 47.2 (C-19), 31.5 (C-20), 34.9 (C-21), 33.2 (C-22), 28.5 (C-23), 16.9 (C-24), 16.0 (C-25), 17.8 (C-26), 26.3 (C-27), 178.1 (C-28), 33.5 (C-29), 24.0 (C-30). GlcA: 106.9 (C-1), 74.0 (C-2), 75.4 (C-3), 78.7 (C-4), 76.2 (C-5), 172.0 (C-6); Glc: 95.7 (C-1), 75.2 (C-2), 78.3 (C-3), 71.2 (C-4), 78.2 (C-5), 63.1 (C-6). Ara: 109.2 (C-1), 82.9 (C-2), 78.7 (C-3), 87.0 (C-4), 62.5 (C-5).[18] (Supplementary data_Figure S2.2-6).

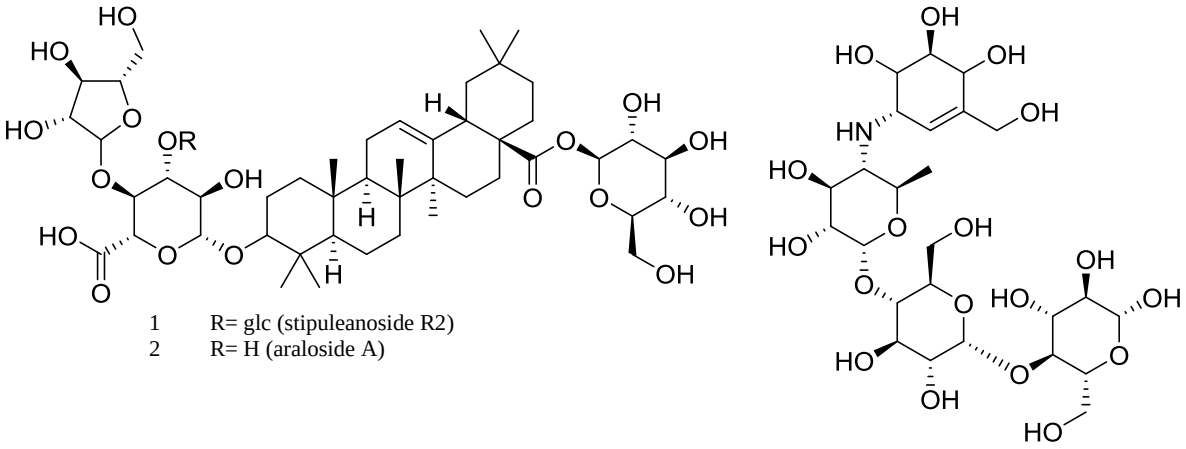
*In vitro α -Amylase Inhibitory Activity*

The porcine pancreatic α-amylase inhibitory activity of two saponins from *P. bipinnatifidus* was assayed using the starch-iodine method. α-amylase hydrolysed starch molecules into small-chain dextrins by acting upon the α-1,4 glycosidic bonds.[16,19] While a yellow color indicates the starch is completely hydrolyzed by α-amylase, a brownish color indicates partially degraded starch in the enzyme assay mixture. If the inhibitor of α-amylase is added to the reaction mixture, starch is not degraded, and a dark blue color will be visually observed. Moreover, the degradation of starch by α-amylase activity is also measured at 620 nm by spectrometry.[20] The higher absorbance (darker color) indicates the stronger suppression of α-amylase inhibitory activity. As shown in Table 1, the α-amylase inhibitory activity of the two saponins were recorded and compared to the standard Acarbose. While the inhibitory activity at 50 µg/mL of Araloside A represented 9.63% with $IC_{50}$= 781.46 µg/mL, the inhibitory activity at 50 µg/mL of Stipuleanoside R2 showed with $IC_{50}$ = 2746.90 µg/mL. Two saponins exhibited inhibitory activity lower than the activity shown by positive control (Acarbose) with 60.88% inhibition with $IC_{50}$ = 15.47 µg/mL.

Acarbose is a potent α-amylase inhibitor and has been used in the treatment of type 2 diabetes. In this study, Acarbose was used as the positive control with the $IC_{50}$ value of 15.47 µg/mL. The result was found to be in good agreement with that of previous research. In previous studies, the α-amylase inhibitory effects of Acarbose were 83.33 µg/mL using starch as a substrate[19], 57.6 µg/mL[21], 14.1 µg/mL[22], 6.5 µg/mL.[23] In our study, for the first time, the α-amylase inhibitory effects of Araloside A and Stipuleanoside R2 isolated from *P. bipinnatifidus* were investigated. The two saponins displayed significantly lower inhibitory effects when compared to the Acarbose (Table 1). The $IC_{50}$ values of Araloside A and Stipuleanoside R2 were up to 50.52-folds and 177.56-folds higher than Acarbose in terms of the α-amylase inhibitory activity. The difference in the addition of a sugar moiety to oleanolic acid backbone led to the different levels on the α-amylase inhibitory activity.[21,24] There, both Araloside A and Stipuleanoside R2 have a glucose moiety at C-28 of the aglycone. However, the inhibitory activity of Stipuleanoside R2 might be abolished due to the addition and replacement of glucose by another sugar unit. Therefore, we have only used Araloside A in the kinetic study.

Table 1: The α-amylase inhibitory activities of saponins from *P. bipinnatifidus* Seem

| Sample No. | Araloside A | | Stipuleanoside R2 | | Acarbose | |
|---|---|---|---|---|---|---|
| | Concentration (µg/mL) | Inhibition (%) | Concentration (µg/mL) | Inhibition (%) | Concentration (µg/mL) | Inhibition (%) |
| 1 | 100 | 11.51 ± 0.14 | 200 | 13.80 ± 0.22 | 10 | 45.98 ± 1.64 |
| 2 | 200 | 17.65 ± 0.78 | 500 | 18.19 ± 0.14 | 20 | 51.24 ± 2.12 |
| 3 | 400 | 32.59± 0.54 | 1000 | 25.19 ± 0.24 | 50 | 60.89 ± 2.87 |
| 4 | 600 | 38.02 ± 0.24 | 2000 | 39.39 ± 0.28 | 100 | 76.58 ± 3.36 |
| 5 | 800 | 51.00 ± 0.98 | 3000 | 53.71 ± 0.46 | 200 | 99.96 ± 4.24 |
| Linear regression equation | y = 0.0549x + 7.0975 | | y = 0.0142x + 10.994. | | y = 0.2797x + 45.673. | |
| | $R^2$ = 0.9822 | | $R^2$ = 0.9984 | | $R^2$ = 0.99 | |
| $IC_{50}$ | 781.46 ± 18.5 | | 2746.90 ± 48.2 | | 15.47 ± 0.72 | |



1 R= glc (stipuleanoside R2)
2 R= H (araloside A)

Acarbose

**Figure 1:** The chemical structures of two saponins from *P. bipinnatifidus* Seem. and Acarbose.

**Table 2:** The kinetic parameters of α-amylase in absence and presence of Araloside A compared to Acarbose

| Sample No. | Name of sample | Vmax (µM/min) | Km (µM) | Variation of Km and Vmax | Type of Inhibition |
|---|---|---|---|---|---|
| 1 | No inhibitor | 0.0635 ± 0.005 | 2.428 ± 0.08 | | |
| 2 | Araloside A | 0.0635 ± 0.005 | 4.691 ± 0.22 | = Vmax<br>↑ Km | Competitive |
| 3 | Acarbose | 0.053 ± 0.004 | 6.521 ± 0.32 | ↓ Vmax | Mixed |

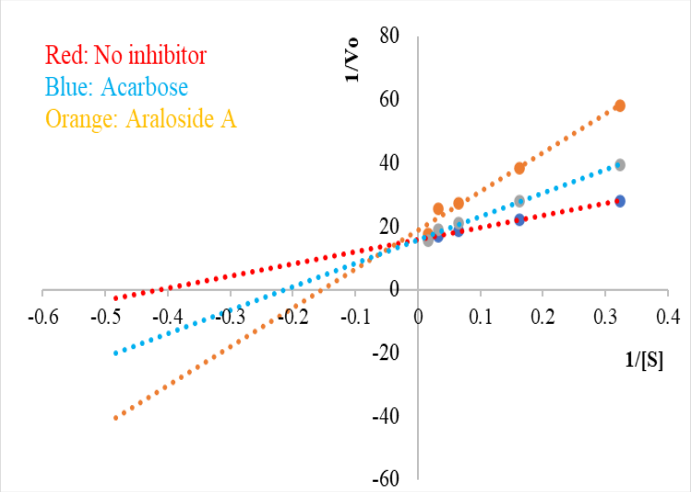



**Figure 2:** Lineweaver-Burk plot of saponin inhibitory activity. $V_o$ represents the initial rate of the enzymatic reaction and [S] the concentration of starch in the reaction. Red indicates no inhibitor, blue and orange indicate presence of Acarbose and Araloside A at $IC_{50}$ (µg/mL).

*Kinetic study*

For elucidating the kinetic mechanism of α-amylase inhibitory activity, a series of substrate concentrations were adopted at $IC_{50}$ of Acarbose and Araloside A. A Lineweaver-Burk plot of the initial velocities versus variable concentration of substrate is shown in Figure 2. In the case of Acarbose, *Vmax* decreased whereas the *Km* values increased compared to the uninhibited control (Table 2). Therefore, the Acarbose showed mixed inhibition mechanisms for α-amylase from porcine pancreas. Similar to the previous results, Acarbose showed its ability to inhibit all four α-amylase from *Aspergillus oryzae*, *Baccillus amyloliquefaciens*, human salivary, and porcine pancreatic.[25] Acarbose was displayed as a mixed-type inhibitor of porcine pancreatic α-amylase.[26,27] In the sample test, no changes in *Vmax* were observed for Araloside A while the Km values increased 1.93-folds compared to the uninhibited control indicating a competitive inhibition mechanism (Table 2). Competitive inhibitor is effective due to Araloside A prosses structural analog of substrate, and thus can bind to the active site of α-amylase. When Araloside A binds to enzyme, it forms an Araloside A-amylase complex instead of Amylase-Starch complex, which prevents the enzyme from acting on its substrate. Finally, Araloside A could prevent starch breakdown to smaller molecules and glucose.[22] The *Km* value in the presence of Araloside A was significantly lower than the *Km* value in the case of Acarbose showing again a lower α-amylase inhibitory activity effect of saponin from *P. bipinnatifidus*.

## Conclusion

The α-amylase inhibitory activity of Araloside A and Stipuleanoside R2 was tested for the first time. While Araloside A showed moderate α-amylase inhibitory activity with $IC_{50}$ values of 781.46 µg/mL, Stipuleanoside R2 exhibited low inhibitory activity with $IC_{50}$ values of 2746.90 µg/mL. The kinetic study indicated that Araloside A can prevent porcine pancreatic α-amylase as a competitive inhibitor, and Acarbose was displayed as a mixed-type inhibitor of porcine pancreatic α-amylase. Results of the current study suggested that saponins from *P. bipinnatifidus* can be used as a potential α-amylase inhibitor in antidiabetic dietary.

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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