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# *In vitro* and *in vivo* effects of *Zanthoxylum limonella* (Dennst.) Alston. crude extracts and volatile oil on blood glucose reduction

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## ABSTRACT

**Introduction:** Complimentary medicines have been long investigated as new and alternative therapeutic options for treating type 2 diabetes mellitus because the current drugs are associated with adverse drug reactions. The antidiabetic potential of the *Zanthoxylum* genus has been demonstrated previously. However, *Zanthoxylum limonella* (Dennst.) (ZL) has not been evaluated in any clinical trials. **Objectives:** This study aimed to examine the inhibition of α-glucosidases *in vitro* and *in vivo* using ZL fruit extracts, thereby determining the reduction in blood glucose levels using oral glucose and sucrose tolerance tests. **Results and Discussion:** The water extract of ZL fruits showed the greatest inhibition of α-glucosidases ($IC_{50}$, 1.04 ± 0.08 mg/ml) than other extracts, particularly the dichloromethane extract and volatile oil. An acute toxicity test showed that the 50% lethal dose ($LD_{50}$) of the water extract was <5000 mg/kg BW. Regarding oral glucose tolerance test and oral sucrose tolerance test, a single oral dose of ZL water extract (500 mg/kg BW) did not significantly reduce blood glucose compared to the control. **Conclusion:** In conclusion, the water extract of ZL fruits showed an inhibitory effect on α-glucosidase activity *in vitro*. However, it did not decrease blood glucose in mice. Further investigations should be performed to identify the active constituents of ZL fruit extracts.

**Keywords:** α-Glucosidases, antidiabetics, oral glucose tolerance test, oral sucrose tolerance test, *Zanthoxylum limonella* fruits

## INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder, generally characterized into three types: Insulin-dependent DM, non-insulin-dependent DM, and gestational DM. DM results in hyperglycemia, which is characterized by a high level of fasting blood glucose and glycated hemoglobin, and patients with uncontrolled DM tend to suffer from complications such as cardiovascular and renal diseases.[1,2] Glucose homeostasis is controlled by hormones and enzymes, including insulin, glucagon, amylase, α-glucosidase, dipeptidyl peptidase IV, and incretins.[3,4]

α-Glucosidase (EC 3.2.1.20), expressed in the brush border of the small intestine, is the enzyme responsible for the metabolism of oligosaccharides or disaccharides to monosaccharides. This enzyme hydrolyzes glycosidic bonds and is selective for α-1,4-O-glycosidic linkages from the non-reducing end. The final product of the hydrolysis reaction is α-glucose. Inhibition of α-glucosidase reduces postprandial glucose levels. Based on the function of α-glucosidase in carbohydrate metabolism, inhibitors of this enzyme, such as acarbose and voglibose, have been used to treat patients with Type 2 DM in combination with metformin,[5] providing improved effectiveness and reducing cardiovascular risk

in these individuals.[6,7] Potent plant-derived α-glucosidase inhibitors, such as mulberry leaf extract, have been shown to be beneficial as complementary treatments in patients with Type 2 DM.[8,9] In addition, standardized extracts of *Lagerstroemia speciosa* leaves with potent α-glucosidase inhibition have been shown to lower blood glucose levels in patients with Type 2 DM.[10] Therefore, investigating the application of novel antidiabetic agents from natural products may be a viable therapeutic option for DM management and/or treatment.

The genus *Zanthoxylum* belongs to the Rutaceae family. *Zanthoxylum limonella* (Dennst.) Alston. (ZL) is distributed in Thailand and other Southeast Asian countries. In Thailand, ZL is called "Ma-Kwaen." It is a popular spice used for cooking, particularly in North Thailand. Apart from cooking, various parts of this plant species have been used in folk medicine to treat diseases, including certain infections and inflammation, because of their bioactive constituents.[11] The phytochemical constituents of ZL fruits are classified into (1) phenolic compounds (xanthoxyline); (2) monoterpenes (limonene, terpinen-4-ol, sabinene, 3-carene, α-pinene, β-pinene, α-terpinene, and γ-terpinene); (3) monoterpene alcohols (α-terpineol); and (4) alkylbenzenes (*p*-cymene).[12-14] The major constituents that have been determined are sabinene and limonene, which are found in essential oils.[11] Moreover, various fruit extracts have been shown to possess pharmacological activities.

Medicinal plants in the genus *Zanthoxylum* reportedly exhibit antidiabetic activity *in vitro* and *in vivo*. The water extract of *Zanthoxylum armatum* leaves inhibited α-amylase and α- and β-glucosidases. Furthermore, it lowered blood glucose levels in healthy and diabetic mice after overnight fasting, as determined by glucose tolerance tests.[15] The methanolic extract of *Z. armatum* leaves and its hydromethanolic bark extract has been shown to affect α-amylase activity. The methanolic extract showed double potent inhibition of α-amylase activity compared to acarbose. However, the hydromethanolic extract showed a weaker inhibitory effect than glibenclamide (reference compound) in terms of lowering blood glucose in streptozotocin-induced diabetic rats.[16,17] In addition, an *in vitro* study by Alam *et al*.[18] revealed that phytochemical constituents from methanolic extracts of *Z. armatum* leaves, bark, and fruits potentially inhibit α-glucosidase. The results of the same study demonstrated a decrease in blood glucose, following the characterization of all investigated *Z. armatum* extracts, in an alloxan-induced diabetic mouse model. In another study, Kimani *et al*.[19] examined the stem bark extract of *Zanthoxylum chalybeum*. The extract significantly reduced blood glucose levels in streptozotocin-induced diabetic rats. Based on the chemical constituents found in ZL fruits, *R*-(+)-limonene has been shown to inhibit α-glucosidase and stimulates glucose uptake in adipocytes.[20,21] In addition, 1-(*S*)-α-pinene has been shown to inhibit α-glucosidase.[21] Nevertheless, to date, the antidiabetic potential of ZL has not been evaluated in detail.

As previously indicated, α-glucosidase is one of the main target enzymes for carbohydrate breakdown, as it provides glucose to the circulatory system. Therefore, inhibition of this enzyme is important for therapeutic purposes in patients with DM. Consequently, this study aimed to demonstrate the inhibitory potential of ZL extracts on α-glucosidase activity with the ultimate goal of reducing blood glucose levels in mice. Optimization of the α-glucosidase reaction was demonstrated with different protein concentrations and periods of incubation, and the effect of organic solvent on the activity of this enzyme was determined.

## MATERIALS AND METHODS

### Chemicals and ZL Fruits

Acarbose (≥95%), intestinal acetone powder from rats, and *p*-nitrophenyl α-D-glucopyranoside (PNGP, ≥99%) were purchased from Millipore Sigma (Ascent, Singapore). Glipizide tablets (5 mg/tablet) were obtained from Siam Bheasach (Bangkok, Thailand). All chemicals and organic solvents were of analytical grade. ZL fruits were identified and kindly provided by Asst. Prof. Dr. Maleeruk Utsintong, Department of Pharmaceutical Care, School of Pharmaceutical Sciences, University of Phayao, Thailand.

### Plant and Extraction Procedure

ZL fruits were obtained from Phayao Province in the northern part of Thailand. A voucher specimen (No. 039023) was deposited at the CMU Herbarium, Faculty of Science, Chiang Mai University, Thailand. The samples were successfully extracted by solvents ranging in polarity. Fresh fruits (200 g) were extracted in a thimble with hexane (300 ml) to obtain crude extracts. The extract was collected, and the plant residue was extracted with dichloromethane (300 ml). After collecting the dichloromethane extract, the remaining plant residue was extracted with ethanol (300 ml). Extraction with each solvent was performed for 8 h. The same sample was subjected to the Soxhlet method for the water extract, and the extract was subsequently freeze-dried. Volatile oil extraction was performed using the hydrodistillation method. In this process, clean fruits were blended with water and placed in a round-bottom flask. An appropriate volume of water was added to the flask, and the sample was distilled for 6 h to collect the volatile oil. Finally, anhydrous sodium sulfate was added to the oil extract to remove the residual water. The extraction temperature was not precisely controlled, but instead was set to a gentle boiling point to ensure proper condensation at the condenser.

### Phytochemical Screening

The viscous extracts extracted using hexane, dichloromethane, ethanol, or volatile oil as solvents were dissolved in dimethyl sulfoxide (DMSO), and the water extract was dissolved in water. The extract solutions were tested for the presence of alkaloids, flavonoids, and tannins using the methods outlined in Table 1. The applied phytochemical screening methods followed published procedures.[22-25]

### Determination of Total Phenolic Content (TPC)

The Folin–Ciocalteu colorimetric method was used to determine the TPC, which was the same method used in our previous publication.[26] Gallic acid (GA) solution (15.6–500 μg/ml)

**Table 1:** Screening of chemical compounds in the water extract of *Zanthoxylum limonella*

| Indicative compounds | Test | Result |
|---|---|---|
| Flavonoids | Color presented in 5% ferric chloride | Positive (dark brown precipitate) |
| | Shinoda's test | Negative |
| Alkaloids | Dragendorff's reagent | Negative |
| | Marme's reagent | Negative |
| | Mayer's reagent | Negative |
| | Wagner's reagent | Negative |
| Tannins | Addition of vanillin HCl | Negative |
| | Addition of lead subacetate | Negative |
| | Color presented in 1% ferric chloride | Positive (yellow solution) |

was used as the reference standard. The ZL extract (hexane, dichloromethane, and ethanol) was dissolved in ethanol at a concentration range of 0.31–10 mg/ml, whereas the ZL water extract was dissolved in water as a working solution. In each well of a 96-well plate ($n$ = 3), 20 µl of the sample solution or GA was added and then mixed with Folin–Ciocalteu reagent (100 µl) and 7% (w/v) $Na_2CO_3$ (80 µl). After a 30 min incubation period at 25°C, the absorbance was measured at 760 nm. The TPC values were determined using the GA calibration curve and are represented as mg GA equivalence (eq.)/mg of extract.

## Preparation of Intestinal Acetone Powders from Rats

The preparation of α-glucosidase extracted from rat intestines was performed as described by Jo *et al*.[27] and Nanasombat *et al*.[28] with some modifications. Briefly, 1 g of rat intestinal acetone powder was dissolved in 10 ml of 0.1 M phosphate buffer (pH 6.8). The suspension was then vortexed and ultrasonicated (37 kHz; Elma E300H) at 30 s bursts and 10 s cooling cycles on ice to fully activate the enzyme. The sonication process was performed 15 times and the suspension was subsequently centrifuged at 12,000× g at 4°C for 30 min, and the supernatant was pipetted into Eppendorf tubes. The prepared rat intestinal α-glucosidase was stored at 80°C until use. All preparation processes were performed on ice.

## Optimization of α-Glucosidase Activity

*Linearity of p-nitrophenol formed by α-glucosidase with respect to protein concentration*

The α-glucosidase activity was measured based on absorbance following the formation of *p*-nitrophenol using 4-nitrophenyl-α-D-glucopyranoside as a substrate.[28,29] The formation of *p*-nitrophenol was validated using α-glucosidase isolated from rat intestines to confirm linearity with respect to the enzyme concentration. Concentrations of α-glucosidase were 10, 15, 20, and 25 mg/ml/reaction. Reactions were performed in triplicates in 96-well plates. The total volume of incubations was 125 µl. Mixtures containing α-glucosidase and phosphate buffer (0.1 M, pH 6.8) were pre-incubated at 37°C for 10 min. The reactions were then initiated by adding PNGP dissolved in 0.1 M phosphate buffer (1 mM) and incubated at 37°C for 15 min. Reactions were terminated by adding 0.2 M $Na_2CO_3$ (225 µl). Detection of *p*-nitrophenol was performed at a wavelength of 405 nm using a microplate reader (Biotek®, Vermont, United States).

*Linearity of p-nitrophenol formed by α-glucosidase with respect to incubation time*

To validate the time needed for incubation, the linearity of *p*-nitrophenol was determined using an incubation time between 15 and 30 min for rat intestinal α-glucosidase. Reactions were performed in triplicates in 96-well plates. Mixtures of α-glucosidase (10 mg/ml/reaction) and phosphate buffer (0.1 M, pH 6.8) were pre-incubated at 37°C for 10 min. The reaction was initiated by adding PNGP (1 mM) and terminated as described above.

*Effect of organic solvents on α-glucosidase activity*

The effects of DMSO, ethanol, methanol, hexane, and dichloromethane on α-glucosidase reactions were investigated. The concentrations of all organic solvents used in the reactions ranged from 0.5 to 4% (v/v). An incubation volume of 125 µl, containing α-glucosidase (10 mg/ml/reaction), phosphate buffer (0.1 M, pH 6.8), and organic solvent, was pre-incubated at 37°C for 10 min. Reactions were initiated by adding PNGP (1 mM) and subsequently terminated as described above. Deionized water was added to the control reaction instead of an organic solvent. Acarbose (300 µg/ml) was used as the positive control, and the remaining activity of α-glucosidase was compared with that of the control. Data are presented as percentages (mean ± SD) following the application of Equation 1.

$$\text{Enzyme activity remaining} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{treatment}}}{\text{Abs}_{\text{control}}}\right) \times 100$$

Equation 1

Where, $Abs_{control}$ is the absorbance of the reaction mixture without an organic solvent or extract and $Abs_{treatment}$ is the absorbance of the reaction mixture with an organic solvent or extract.

## Inhibition of Rat Intestinal α-Glucosidase

The applied method was a modification of that described by Pyner *et al*.[30] and Nanasombat *et al*.[28] In a total volume of 125 µl, the reaction mixture contained phosphate buffer (0.1 M, pH 6.8), inhibitor (5 µl), and α-glucosidase from rat intestine (10 mg/ml). ZL extracts were prepared by dissolving in DMSO, whereas acarbose was dissolved in deionized water. The mixture was pre-incubated for 10 min in an incubator at 37°C, and the reaction was initiated by adding PNGP (1 mM). The reaction mixture was then incubated for 20 min and subsequently terminated by the addition of $Na_2CO_3$. Three replicates were used. The concentration of acarbose (positive control) ranged from 1 to 2000 µg/ml. Deionized water was added to control samples. The concentrations of ZL extracts used for the evaluation of the applied concentration for 50% inhibition ($IC_{50}$) ranged from 10 to 5000 µg/ml. In these studies, a 4% (v/v) final concentration of DMSO was used as the control.

## Animals

ICR mice (8–12 weeks old), weighing 20–30 g, were housed at 23 ± 2°C, 40–60% relative humidity, and under 12 h dark/light cycle conditions before the experiments. The mice were fed standard rodent food and distilled water *ad libitum*. The protocol and procedures for animal handling and experiments of this project were approved by the Animal Ethics Committee of Walailak University, Thailand (approval number, WU-AICUC-024-62; granted on, October 15, 2019).

## Acute Toxicity Test

The OECD guideline 425 was applied for the acute toxicity test of the water extract dissolved in distilled water in both sexes of mice. After 4 h of fasting, a single dose of 5000 mg/kg BW was orally administered. The study was first performed on a single animal from each sex. If the animal did not die, two additional animals of each sex were introduced. In contrast, the main test was required if the first animal died. The experiment was terminated following the survival of all the animals in each group. In addition, the 50% lethal dose ($LD_{50}$) value was considered higher than 5000 mg/kg if fewer than three animals died. All treated mice were closely observed after 30 min, 4 h, and 24 h, and daily for 14 days for delayed death or behavioral changes.

## Oral Glucose Tolerance Test (OGTT) and Oral Sucrose Tolerance Test (OSTT)

Four treatment groups were used: Control, acarbose (40 mg/kg BW), glipizide (2.5 mg/kg BW), and water extract (500 mg/kg BW). Acarbose and water extracts were dissolved in water, and glipizide was prepared in 10% gum acacia. The mice in the control group were administered distilled water. Each group contained seven mice of mixed sex. Experimental mice were fasted overnight to reduce baseline variability of blood glucose. The test compounds were administered to each group before administering glucose or sucrose solution (2 g/kg) for 30 min. Blood glucose levels were measured at 0, 15, 30, 60, 120, and 180 min using a glucometer (Terumo®, Tokyo, Japan). The area under the curve (AUC) in mg·min/dl was estimated using Equation 2:[31]

$$AUC_{t_{0-n}} = \left(\frac{1}{2} \times (Blood\ glucose\ level_{t_0} + Blood\ glucose\ level_{t_1}\right) \times (t_1 - t_0)) + \left(\frac{1}{2} \times (Blood\ glucose\ level_{t_1} + Blood\ glucose\ level_{t_2}\right) \times (t_2 - t_1)) + \left(\frac{1}{2} \times (Blood\ glucose\ level_{t_{n-1}} + Blood\ glucose\ level_{t_n}\right) \times (t_n - t_{n-1}))$$

Equation 2

Where, $n$ is the time point.

## Data Analysis

All results are expressed as the mean ± SD. $IC_{50}$ values were generated using GraphPad Prism ver. 8 (trial version; GraphPad Software, CA, USA). Statistical comparisons were performed using a one-way analysis of variance. *Post hoc* analysis using the least significant difference was performed to test for significant differences between the groups. Statistical significance was set at $P < 0.05$.

# RESULTS

## Chemical Analysis of ZL Extracts

Quantitative measurements of TPC revealed that the ZL extracts contained a wide range of phenolic moieties. In contrast, volatile oil lacked any phenolic components. In extracts obtained using ethanol, dichloromethane, hexane, and water, the TPCs were 15.3 ± 0.60, 12.0 ± 0.98, 8.87 ± 0.35, and 11.7 ± 0.68 mg GA eq./mg of the extract, respectively. The water extract was found to have the strongest inhibitory effect on α-glucosidase activity. The results of the phytochemical screening of ZL water extract are shown in Table 1. The water extract showed the presence of phenolic compounds. However, only after reacting the extract with ferric chloride did the test produce positive results. Flavonoids were not detected following the Shinoda test. In addition, testing for tannin constituents with vanillin hydrochloride and lead subacetate was negative. These results confirmed the presence of phenolics alone. Furthermore, no alkaloids were detected using screening methods.

## Effect of Organic Solvents on α-Glucosidase Activity

The effect of organic solvents on α-glucosidase activity was also investigated. The concentration of the solvents used ranged from 0.5% to 4%. The remaining activity of α-glucosidase was compared with the enzyme activity after incubation with only potassium phosphate buffer (0.1 M, pH 6.8) and no organic solvent. Static alteration of α-glucosidase activity by the addition of organic solvents was not observed [Figure 1]. The coefficient of variation, expressed as a percentage, was <10%. None of the organic solvents at the investigated concentrations could reduce the activity of α-glucosidase by more than 10%.

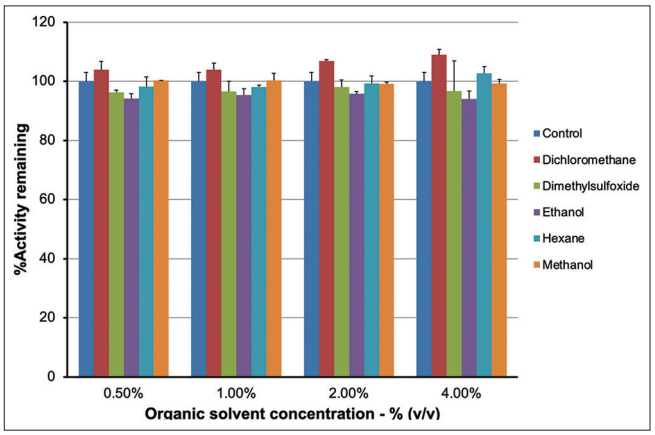


**Figure 1:** Effects of organic solvents on α-glucosidase activity. Effect of dichloromethane, DMSO, ethanol, hexane, and methanol were investigated at various concentrations. Each bar represents the mean percentage of the remaining activity of the control. Each experiment was performed in triplicates. Error bars represent SD. Statistical test ($P = 0.05$) was conducted to compare each organic solvent and its control.

## Linearity of 4-Nitrophenol Formed Using Intestinal Rat α-Glucosidase with Respect to Protein Concentration and Incubation Time

Due to the lack of published reports on the optimization of rat intestinal α-glucosidase activity using PNGP as a substrate, the experiment was also aimed to optimize the reaction conditions. Linearity was demonstrated with respect to protein concentration and the time course of incubation for the reaction. *p*-Nitrophenol was linearly produced from α-glucosidase in the range of 10–20 mg/ml ($R^2$ = 0.9994) [Figure 2a]. The plateau of the reaction was observed at protein concentrations greater than 20 mg/ml. Production of *p*-nitrophenol was linear with respect to incubation time in the range of 15–30 min ($R^2$ = 0.9934) [Figure 2b]. Therefore, the α-glucosidase concentration and incubation time of 10 mg/ml and 20 min, respectively, were used to evaluate the inhibitory effect of ZL extracts.

## Inhibition of α-Glucosidase by ZL Extracts

The effects of ZL extracts (10–5000 μg/ml) and acarbose (1–2000 μg/ml) on the activity of intestinal rat α-glucosidase were investigated. The inhibitory effects of the ethanol, hexane, and water extracts were dose dependent, while those of the dichloromethane extract and volatile oil were not observed [Figure 3]. The $IC_{50}$ values of the ethanol, hexane, and water extracts were 1713 ± 40, 1532 ± 111, and 1044 ± 83 μg/ml, respectively. The positive control, acarbose, showed the greatest inhibitory effect with an $IC_{50}$ value of 54 ± 1 μg/ml. The coefficient of variation for all inhibitions was less than 10%. All $IC_{50}$ values are shown in Table 2.

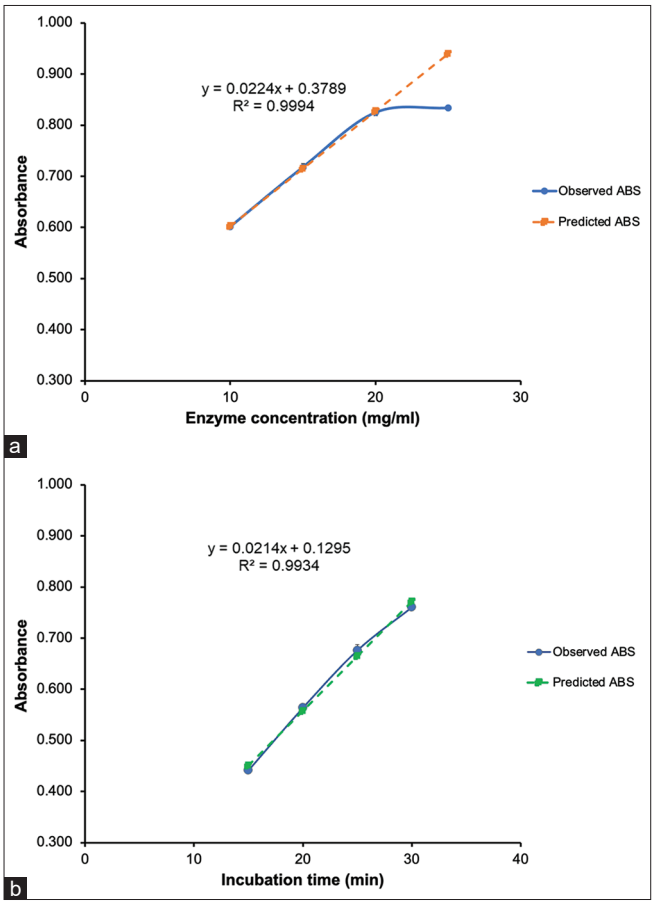


**Figure 2:** Relationship between absorbance and concentration of α-glucosidase (a) and incubation time (b)

## Acute Toxicity Test

According to the results of the $IC_{50}$ determination, an acute oral toxicity test was performed as described above. During intensive observation, no abnormal signs and symptoms of toxicity or death of any animals were observed in any group. All experimental mice were healthy, with normal behavior and regular food and water consumption for 14 days. ZL water extract at 5000 mg/kg BW was not toxic to the mice. Therefore, $LD_{50}$ was found to be more than 5000 mg/kg.

## OGTT and OSTT

Figures 4 and 5 depict the AUC of blood glucose concentration and blood collection times (0–180 min) after glucose and sucrose were administered to the mice ($n$ = 7). The AUC was estimated using Equation 2 based on the trapezoidal rule. In the OGTT experiment, there were no significant differences in the AUC estimated among treatment groups, except for the glipizide-treated group. In the OSTT experiment, the water extract-treated group showed an insignificant difference in AUC compared to the control group ($P > 0.05$), although the AUC value of the water extract-treated group was slightly lower than that of the control group. The AUC of mice treated with glipizide and acarbose was significantly lower than that of the control group ($P < 0.05$).

# DISCUSSION

This study investigated the inhibitory potential of ZL extracts on α-glucosidase derived from rat intestines and the subsequent decrease in postprandial glucose in mice treated with the extracts.

The potency of phytochemical constituents from various extracts of ZL was expressed in the form of $IC_{50}$ values. Postprandial glucose was modeled to investigate the effect of the extracts on carbohydrate metabolism and glucose hemostasis compared to clinically used drugs, glipizide, and acarbose.[4] Although several mechanisms of disrupting carbohydrate metabolism affect postprandial glucose levels, α-glucosidase targeting was the main focus of this study. As shown in the experimental design, an *in vitro* study on the inhibition of α-glucosidase and OSTT was conducted. OGTT was also performed to assess the role of insulin secretion using glipizide as the standard reference.

The preliminary chemical analysis of the ZL extracts can be correlated with a previous report,[11] in which the phenolic compound xanthoxylin was found in the fruits. A previous study showed that (*R*)-(+)-limonene from volatile oils inhibits α-glucosidase activity.[20] However, this study found that the α-glucosidase inhibitory effect of the water extract was superior to that of volatile oil, where the content of (*R*)-(+)-limonene in ZL fruit oil could not be determined. The additional isolation of individual compounds from the water extract may yield a

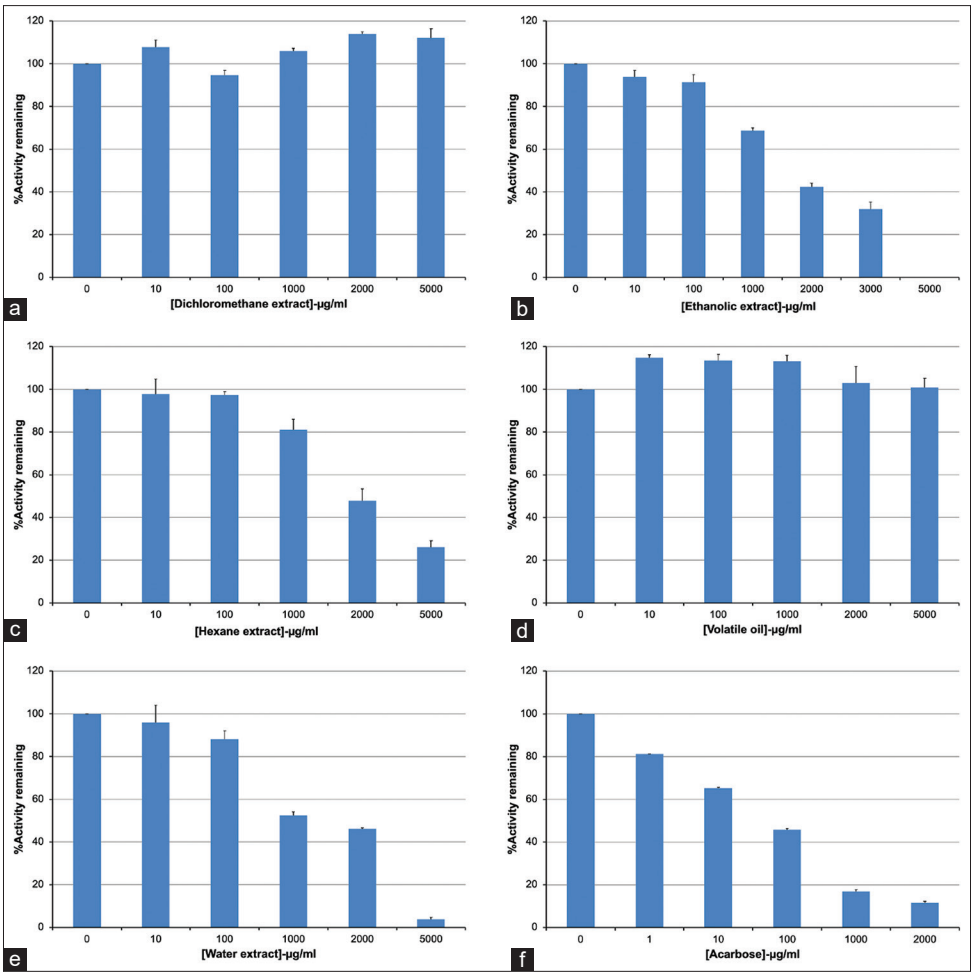


**Figure 3:** Inhibitory effects of *Zanthoxylum limonella* extracts in the concentration range of 10–5000 µg/ml and acarbose on α-glucosidase activity. (a) Dichloromethane extract, (b) ethanolic extract, (c) hexane extract, (d) volatile oil, (e) water extract, and (f) acarbose

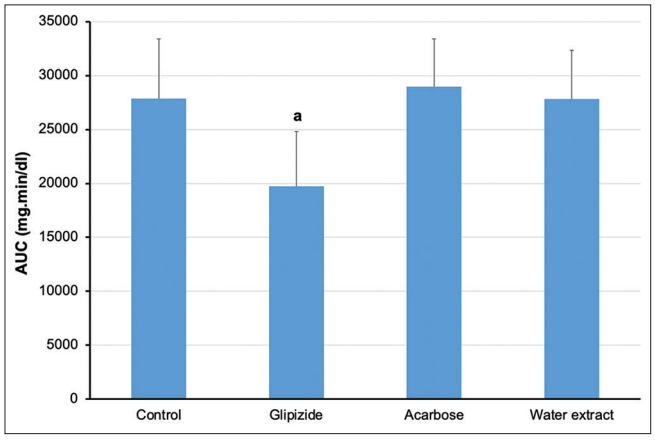


**Figure 4:** Effects of glipizide, acarbose, and the water extract of *Zanthoxylum limonella* on the area under the curve (AUC) in oral glucose tolerance tests. AUC values are between 0 and 180 min and are expressed as mean ± SD (error bars; $n = 7$). (a-c) Statistical tests compared to the control, acarbose-treated, and water extract-treated group, respectively.

new α-glucosidase inhibitor from ZL. Some plants of the genus *Zanthoxylum* were investigated for α-glucosidase inhibition or other pharmacological properties both *in vitro* and *in vivo*, and only the ethanolic extract was shown to possess slight α-glucosidase activity.[28] Alkaloids from *Z. chalybeum* Engl. root bark have been reported to inhibit α-glucosidase.[32] Other studies have also reported the antidiabetic effects of *Zanthoxylum* spp. bark extracts.[17,19] Thus, this study suggests that this fruit is the new potential raw material based on non-destructive harvesting for α-glucosidase inhibition. Water is an appropriate solvent for the recovery of α-glucosidase inhibitors from ZL fruits. However, the active constituents should be further investigated.

The inhibitory effects of ZL extracts and acarbose on α-glucosidase were assessed. Limited data on the $IC_{50}$ values have been reported. There are significant variations in the $IC_{50}$ values of acarbose reported by the previous studies. Nonetheless, the $IC_{50}$ values of acarbose in this study were similar to those reported by Sahu *et al.*[33] and Ochieng *et al.*[32] The $IC_{50}$ values reported by Vongsak *et al.*[34] and Wongnawa *et al.*[35] were 3- and 18-fold higher, respectively, than those reported in this study, showing significant interlaboratory variations. This may be because of different sources of acarbose, tablets, standard powder, or different analytical software. Phytochemical constituents have been reported to inhibit α-glucosidase activity. *Coriandrum sativum* contains various phytochemical constituents, including flavonoids, alkaloids, and tannins.[36]

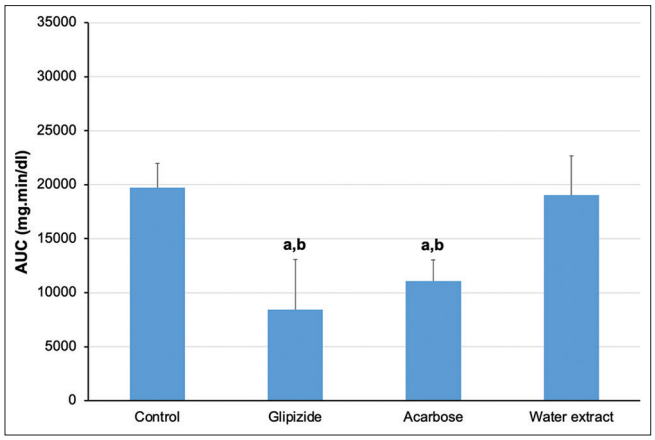


**Figure 5:** Effects of glipizide, acarbose, and the water extract of *Zanthoxylum limonella* on the area under the curve (AUC) in oral sucrose tolerance tests. AUC values are between 0 and 180 min and are expressed as mean ± SD (error bars; $n = 7$). (a and b) Statistical tests compared to the control and water extract-treated group, respectively.

**Table 2:** $IC_{50}$ values for α-glucosidase activity

| Inhibitor | $IC_{50}$ (μg/ml) [a] |
|---|---|
| Dichloromethane | >5000 |
| Ethanolic extract | 1713±40 |
| Hexane extract | 1532±111 |
| Volatile oil | >5000 |
| Water extract | 1044±83 |
| Acarbose | 54±1 |

[a]$IC_{50}$ values were expressed as mean±SD. Data were from triplication

An ethanolic extract of coriander leaves exhibited a 2.54-fold higher inhibition of α-glucosidase than the standard acarbose. The estimated $IC_{50}$ of the extract was 32.38 mg/ml,[37] which was about 30-fold higher than that obtained in this study. Rynjah *et al*.[15] showed that the water extract of *Z. armatum* leaves contains tannins, but not flavonoids, following phytochemical screening. The group examined the inhibitory potential of the extract on α-glucosidase; however, the $IC_{50}$ value was not determined. Furthermore, water extracts of *Eugenia jambolana* seeds, as well as extracts from the fruits, bark, and leaves of *Z. armatum* effectively inhibit α-glucosidase.[18,38]

Optimization of the enzymatic reaction is necessary to study enzyme kinetics. Unfortunately, the α-glucosidase reaction has not been optimized in previously published studies. This study thus investigated the optimal conditions with respect to protein concentration and time ranges for performing the α-glucosidase assay using the enzyme extracted from rat intestines and PNGP as the substrate.[28,37,39,40] In addition, organic solvents, which are normally used for dissolving crude extracts, or any chemicals added to incubations, have been reported to reduce the activity of some enzymes.[41,42] Hence, the optimal enzyme concentration, incubation time range, and effects of organic solvents on enzyme activity are reported herein. This is advantageous for evaluating and characterizing molecules for inhibition studies. Previously, the optimal assay conditions for several enzyme reactions, such as cytochromes P450 and UDP-glucuronosyltransferases, have been revealed before using them in inhibition studies.[41,42] Various organic solvents were also tested, and the results indicated that the type and final concentration of the organic solvent used in the incubations should be determined. The five organic solvents used in this study, as previously shown, did not reduce α-glucosidase activity. Therefore, sensitivity of the inhibition studies was not interfered using those organic solvents. In addition to the optimization of the amount of α-glucosidase extracted from the rat intestine, Pyner *et al*.[30] optimized the assay conditions using maltose and sucrose as substrates rather than PNGP. Furthermore, an inhibition study using rat intestinal α-glucosidase was conducted by Nanasombat *et al*.[28] The group used 1 mM PNGP as a substrate and 20 mg/ml of rat intestinal α-glucosidase as the enzyme; however, the assay was not optimized. Another study used intestinal α-glucosidase at 12.5 mg/ml concentration.[43] Consistent with the present study, the concentration of PNGP generally used in the previous reports was 1 mM for α-glucosidase assays using yeast or rat intestinal α-glucosidase.[28,35] The range of incubation times used in the previous studies was 10–15 min. In this study, the concentration of α-glucosidase and incubation time was varied, although the concentration of PNGP was kept constant. The optimal concentration of rat intestinal α-glucosidase used in this study was only 10 mg/ml, and a slightly longer incubation time was applied than that in the previous studies. These conditions resulted in suitable absorbance readings. Hence, the concentration of rat intestinal α-glucosidase and incubation time should be considered depending on the required amount of *p*-nitrophenol in the inhibition study.

As previously indicated, the control of blood glucose levels in the body involves several mechanisms. This study demonstrated that both the OGTT and the OSTT could be used to evaluate the body's response to high blood glucose levels or limitations in breaking down disaccharides. Insulin secretion from β-pancreatic cells and inhibition of α-amylase and DPP-IV may be included. Different kinds of plants in the genus *Zanthoxylum* have been shown to reduce blood glucose levels in animal models. The water extract of *Z. armatum* leaves decreased glucose levels in healthy and diabetes-induced mice after glucose administration. A dose of 250 mg/kg BW was the most effective. However, researchers have not performed an OSTT to determine the disruption of sucrose catabolism.[15] Stem bark, fruit, and leaf extracts of *Z. armatum* exhibited hypoglycemic effects on fasting, normal, and streptozotocin- or alloxan-induced diabetic rats.[17,18] Investigation of effects of tambulin isolated from *Z. armatum* fruits on the pathway of blood glucose attenuation showed that the compound stimulates insulin secretion through ATP-dependent $K^+$ channels.[44] Kyei-Barffour *et al*.[45] demonstrated that the leaf extracts of *Z. zanthoxyloides* activated insulin release. These results indicate that insulin secretion, which is caused by the inhibition of ATP-dependent $K^+$ channels in pancreatic β-cells, or others (e.g., DPP-IV and glucose transport), was the mechanism of action of the extract, except for the digestion of carbohydrates. Furthermore, transient receptor potential vanilloid subtype 1 or capsaicin receptor, which is responsible for many physiological functions, such as the regulation of insulin secretion, was mainly inhibited by *Z. alkylamides*. This caused a statistically significant reduction in blood glucose

levels in streptozotocin-induced rats.[46] Unfortunately, the water extraction of ZL did not affect the decrease in plasma glucose levels in the animal model. This may be due to the use of a low dose of ZL extract for animal treatment. Further investigation using higher dose of the extract or their active compound is recommended to improve the antidiabetic effect of ZL in animal models. Furthermore, the phytochemical constituents following water extraction were presumably degraded by the acidic environment in the stomachs of the experimental animals in this study.

## CONCLUSION

This study demonstrated a higher *in vitro* inhibitory effect of the water extract of ZL fruits on α-glucosidase compared to the other extracts. In contrast, the water extract did not affect blood glucose in normal mice following both OGTT and OSTT studies, although phenolic compounds found in ZL fruits have previously been reported to decrease blood glucose levels. However, further studies on active ingredient purification for α-glucosidase inhibition should be performed.

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## REFERENCES

1. Cole JB, Florez JC. Genetics of diabetes mellitus and diabetes complications. Nat Rev Nephrol 2020;16:377-90.
2. American Diabetes Association. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2021. Diabetes Care 2021;44 Supp 1:S15-33.
3. Chandel NS. Carbohydrate metabolism. J Diabetes Investig 2021;13:1-15.
4. Dimitriadis GD, Maratou E, Kountouri A, Board M, Lambadiari V. Regulation of postabsorptive and postprandial glucose metabolism by insulin-dependent and insulin-independent mechanisms: An integrative approach. Nutrients 2021;13:159.
5. Fang H, Xu F, Du J, Liang L, Li W, Shen L, *et al*. Impact of baseline characteristics on glycemic effects of add-on saxagliptin or acarbose to metformin therapy: Subgroup analysis of the SMART study in Chinese patients with Type 2 diabetes mellitus. J Diabetes Investig 2020;11:896-905.
6. Chan CW, Yu CL, Lin JC, Hsieh YC, Lin CC, Hung CY, *et al*. Glitazones and alpha-glucosidase inhibitors as the second-line oral anti-diabetic agents added to metformin reduce cardiovascular risk in Type 2 diabetes patients: A nationwide cohort observational study. Cardiovasc Diabetol 2018;17:20.
7. Liu Z, Zhao X, Sun W, Wang Y, Liu S, Kang L. Metformin combined with acarbose vs. single medicine in the treatment of Type 2 diabetes: A meta-analysis. Exp Ther Med 2017;13:3137-45.
8. Riche DM, Riche KD, East HE, Barrett EK, May WL. Impact of mulberry leaf extract on Type 2 diabetes (Mul-DM): A randomized, placebo-controlled pilot study. Complement Ther Med 2017;32:105-8.
9. Hwang SH, Li HM, Lim SS, Wang Z, Hong JS, Huang B. Evaluation of a standardized extract from *Morus alba* against α-glucosidase inhibitory effect and postprandial antihyperglycemic in patients with impaired glucose tolerance: A randomized double-blind clinical trial. Evid Based Complement Alternat Med 2016;2016:8983232.
10. Judy WV, Hari SP, Stogsdill WW, Judy JS, Naguib YM, Passwater R. Antidiabetic activity of a standardized extract (Glucosol™) from *Lagerstroemia speciosa* leaves in Type II diabetics. A dose-dependence study. J Ethnopharmacol 2003;87:115-7.
11. Supabphol R, Tangjitjareonkun J. Chemical constituents and biological activities of *Zanthoxylum limonella* (Rutaceae): A review. Trop J Pharm Res 2014;13:2119-30.
12. Itthipanichpong C, Ruangrungsi N, Pattanaautsahakit C. Chemical compositions and pharmacological effects of essential oil from the fruit of *Zanthoxylum limonella*. J Med Assoc Thai 2002;85 Suppl 1:S344-54.
13. Tangjitjaroenkun J, Chavasiri W, Thunyaharn S, Yompakdee C. Bactericidal effects and time–kill studies of the essential oil from the fruits of *Zanthoxylum limonella* on multi-drug resistant bacteria. J Essent Oil Res 2012;24:363-70.
14. Jirovetz L, Buchbauer G, Shafi M, Saidutty A. Analysis of the aroma compounds of the essential oil of seeds of the spice plant *Zanthoxylum rhetsa* from Southern India. Z Lebensm Unters Forsch 1998;206:228-9.
15. Rynjah CV, Devi NN, Khongthaw N, Syiem D, Majaw S. Evaluation of the antidiabetic property of aqueous leaves extract of *Zanthoxylum armatum* DC. using *in vivo* and *in vitro* approaches. J Tradit Complement Med 2018;8:134-40.
16. Jhamta R, Kaur H. Antidiabetic and antioxidant potential of *Zanthoxylum armatum* DC. leaves (Rutaceae): An endangered medicinal plant. Plant Sci Today 2020;7:93-100.
17. Karki H, Upadhayay K, Pal H, Singh R. Antidiabetic potential of *Zanthoxylum armatum* bark extract on streptozotocin-induced diabetic rats. Int J Green Pharm 2014;8:77-83.
18. Alam F, Us Saqib QN, Ashraf M. *Zanthoxylum armatum* DC extracts from fruit, bark and leaf induce hypolipidemic and hypoglycemic effects in mice-*in vivo* and *in vitro* study. BMC Complement Altern Med 2018;18:68.
19. Kimani C, Mbaria J, Suleiman M, Gakuya D, Kiama SG. Antihyperglycemic activity of *Zanthoxylum chalybeum* stem bark extracts in diabetic rats. J Phytopharmacol 2015;4:183-9.
20. Tan XC, Chua KH, Ram MR, Kuppusamy UR. Monoterpenes: Novel insights into their biological effects and roles on glucose uptake and lipid metabolism in 3T3-L1 adipocytes. Food Chem 2016;196:242-50.
21. Basak SS, Candan F. Effect of *Laurus nobilis* L. essential oil and its main components on α-glucosidase and reactive oxygen species scavenging activity. Iran J Pharm Sci 2013;12:367-79.
22. Saptarini N, Herawati I, Permatasari U. Total flavonoids content in acidified extract of flowers and leaves of gardenia (*Gardenia jasminoides* Ellis). Asian J Pharm Clin Res 2016;9:213-5.
23. Cai LY, Shi FX, Gao X. Preliminary phytochemical analysis of *Canthopanan trifoliatus* (L.) Merr. J Med Plant Res 2011;5:4059-64.
24. Suphiratwanich P, Lomarat P, Julsrigival J. Assessment of total phenolic and flavonoid contents, antioxidant activity, and anti-acetylcholinesterase activity from *Codiaeum variegatum* (L.) Blume leaves found in Thailand. Khon Kaen Agric J 2021;49:517-23.
25. Ukoha PO, Cemaluk EA, Nnamdi OL, Ejikeme PM. Tannins and other phytochemical of the *Samanaea saman* pods and their antimicrobial activities. Afr J Pure Appl Chem 2011;5:237-44.
26. Phaisan S, Yusakul G, Sakdamas A, Taluengjit N, Sakamoto S, Putalun W. A green and effective method using oils to remove chlorophyll from *Chromolaena odorata* (L.) R.M. King and H. Rob. Songklanakarin J Sci Technol 2020;42:1084-90.
27. Jo SH, Cho CY, Lee JY, Ha KS, Kwon YI, Apostolidis E. *In vitro* and *in vivo* reduction of post-prandial blood glucose levels by ethyl

alcohol and water *Zingiber mioga* extracts through the inhibition of carbohydrate hydrolyzing enzymes. BMC Complement Altern Med 2016;16:111.
28. Nanasombat S, Yansodthee K, Jongjaited I. Evaluation of antidiabetic, antioxidant and other phytochemical properties of Thai fruits, vegetables and some local food plants. Walailak J Sci Technol 2019;16:851-66.
29. Ashiq U, Jamal RA, Saleem M, Mahroof-Tahircd M. Alpha-glucosidase and carbonic anhydrase inhibition studies of Pd (II)-hydrazide complexes. Arab J Chem 2017;10:488-99.
30. Pyner A, Nyambe-Silavwe H, Williamson G. Inhibition of human and rat sucrase and maltase activities to assess antiglycemic potential: Optimization of the assay using acarbose and polyphenols. J Agric Food Chem 2017;65:8643-51.
31. Ye F, Shen Z, Xie M. Alpha-glucosidase inhibition from a Chinese medical herb (*Ramulus mori*) in normal and diabetic rats and mice. Phytomedicine 2002;9:161-6.
32. Ochieng CO, Nyongesa DW, Yamo KO, Onyango JO, Langat MK, Manguro LA. α-Amylase and α-glucosidase inhibitors from *Zanthoxylum chalybeum* Engl. root bark. Fitoterapia 2020;146:104719.
33. Sahu PK, Prasad P, Roy A. Screening of *in vitro* antidiabetic activity of herbal formulation Meshashringi. UK J Pharm Biosci 2016;4:75-9.
34. Vongsak B, Kongkiatpaiboon S, Jaisamut S, Machana S, Pattarapanich C. *In vitro* alpha glucosidase inhibition and free-radical scavenging activity of propolis from Thai stingless bees in mangosteen orchard. Rev Bras Farmacogn 2015;25:445-50.
35. Wongnawa M, Tohkayomatee R, Bumrungwong N, Wongnawa S. Alpha-glucosidase inhibitory effect and inorganic constituents of *Phyllanthus amarus* Schum. and Thonn. ash. Songklanakarin J Sci Technol 2014;36:541-6.
36. Patel K, Vakilwala M. Phytochemical study and bioactivity of solvent extracts on *Coriandrum sativum*. Int J Adv Res Biol Sci 2016;3:193-9.
37. Aligita W, Susilawati E, Septiani H, Atsil R. Antidiabetic activity of Coriander (*Coriandrum sativum* L.) leaves' ethanolic extract. Int J Pharm Phytopharmacol Res 2018;8:59-63.
38. Ahmed F, Chandra J, Timmaiah N. An *in vitro* study on the inhibitory activities of *Eugenia jambolana* seeds against carbohydrate hydrolyzing enzymes. J Young Pharma 2009;1:317.
39. Hu X, Li S, Wang L, Zhu D, Wang Y, Li Y, *et al*. Anti-diabetic activities of aqueous extract from *Actinidia kolomikta* root against [alpha]-glucosidase. J Pharmacogn Phytochem 2013;2:53-7.
40. Kim GN, Shin JG, Jang HD. Antioxidant and antidiabetic activity of Dangyuja (*Citrus grandis* Osbeck) extract treated with *Aspergillus saitoi*. Food Chem 2009;117:35-41.
41. Uchaipichat V, Mackenzie PI, Guo XH, Gardner-Stephen D, Galetin A, Houston JB, *et al*. Human UDP-glucuronosyltransferases: Isoform selectivity and kinetics of 4-methylumbelliferone and 1-naphthol glucuronidation, effects of organic solvents, and inhibition by diclofenac and probenecid. Drug Metab Dispos 2004;32:413-23.
42. Zhang J, Ha PT, Lou Y, Hoogmartens J, van Schepdael A. Kinetic study of cytochrome P450 3A4 activity on warfarin by capillary electrophoresis with fluorescence detection. J Chromatogr A 2005;1082:235-9.
43. Malunga LN, Eck P. Inhibition of intestinal α-glucosidase and glucose absorption by feruloylated arabinoxylan mono-and oligosaccharides from corn bran and wheat aleurone. J Nutr Metab 2016;2016:1932532.
44. Hameed A, Raza SA, Khan MI, Baral J, Adhikari A, Nur-E-Alam M, *et al*. Tambulin from *Zanthoxylum armatum* acutely potentiates the glucose-induced insulin secretion via KATP-independent $Ca^{2+}$-dependent amplifying pathway. Biomed Pharmacother 2019;120:109348.
45. Kyei-Barffour I, Kwarkoh RK, Arthur OD, Akwetey SA, Acheampong DO, Aboagye B, *et al*. Alkaloidal extract from *Zanthoxylum zanthoxyloides* stimulates insulin secretion in normoglycemic and nicotinamide/streptozotocin-induced diabetic rats. Heliyon 2021;7:e07452.
46. Wang R, You YM, Liu X. Effect of *Zanthoxylum alkylamides* on glucose metabolism in streptozotocin-induced diabetic Sprague-Dawley rats. Appl Biol Chem 2021;64:e07452.