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Inhibitory Effects of Nipa Palm Vinegar on the Carbohydrate Hydrolysing Enzymes

(Kesan Perencatan Cuka Nipah pada Enzim Hidrolisis Karbohidrat)

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ABSTRACT

Nipa palm vinegar has been traditionally used to manage blood glucose levels by diabetic patients in Southeast Asia. This study was designed to evaluate the efficacy of nipa palm vinegar in inhibiting the activity of carbohydrate hydrolyzing enzymes, α-glucosidase, and α-amylase. *In vitro* spectrophotometric assays were used to evaluate the inhibitory activity of nipa palm activity against α-glucosidase and α-amylase. To confirm the *in vitro* findings, an oral starch tolerance test in the normoglycemic Sprague Dawley rat was conducted. Acarbose was used as the positive control for both tests. Nipa palm vinegar at a concentration ranging from 4000 to 62.5 mg/mL inhibited the activity of α-glucosidase and α-amylase in a concentration-dependent manner with the respective IC_{50} values of 144.50 ± 1.1 mg/mL and 90.30 ± 1.7 mg/mL. It also exerted uncompetitive inhibition against α-glucosidase and competitive inhibition towards α-amylase. *In vivo* oral starch tolerance test showed a significant ($p < 0.05$) postprandial glucose-lowering effect of nipa palm vinegar at the doses of 2 mL/kg and 1 mL/kg body weight as compared to the control. In a conclusion, this study demonstrated that nipa palm vinegar suppressed the rise in postprandial glucose levels partly by inhibiting the activity of digestive enzymes.

Keywords: α-amylase; α-glucosidase; Diabetes mellitus; nipa palm vinegar; *Nypa fruticans* Wurmb.

ABSTRAK

Cuka nipah telah digunakan secara tradisi dalam mengawal aras glukosa darah oleh pesakit diabetes di Asia Tenggara. Kajian ini menilai keberkesanan cuka nipah dalam merencat aktiviti enzim-enzim hidrolisis karbohidrat iaitu α-glukosidase dan α-amilase. Ujian spektrofotometrik secara *in vitro* telah digunakan untuk menguji potensi aktiviti perencatan cuka nipah terhadap α-glukosidase dan α-amilase. Bagi mengesahkan penemuan ujian *in vitro*, ujian toleransi kanji oral secara *in vivo* pada tikus normoglisemik dijalankan. Keputusan menunjukkan cuka nipah merencat aktiviti α-glukosidase dan α-amilase secara kebergantungan kepekatan dengan nilai IC_{50} adalah masing-masing adalah 144.50 ± 1.1 mg/mL dan 90.30 ± 1.7 mg/mL. Cuka nipah juga menunjukkan perencatan tidak kompetitif terhadap α-glukosidase dan perencatan kompetitif terhadap α-amilase. Ujian toleransi kanji oral secara *in vivo* menunjukkan cuka nipah pada dos 2 mL/kg dan 1 mL/kg berat badan mampu menurunkan aras glukosa postprandial secara signifikan dibandingkan dengan kawalan ($p<0.05$). Kesimpulannya, kajian ini membuktikan cuka nipah menghalang kenaikan aras glukosa darah postprandial, sebahagiannya dengan merencat aktiviti enzim pencernaan.

Kata kunci: α-amilase; α-glukosidase; cuka nipah; kencing manis; *Nypa fruticans* Wurmb.

INTRODUCTION

Diabetes mellitus is a chronic non-communicable disease that contributes to serious public health issues globally. According to the National Diabetes Registry Report 2013-2019, Ministry of Health Malaysia, it is estimated that 3.9 million (18.3%) of the adult population in Malaysia live with diabetes, of which 99.3% are diagnosed with type 2 diabetes in 2019 (Chandran, Abdullah & Abdul

2020). Clinical studies have shown that postprandial hyperglycemia is an independent factor contributing to the development of diabetes-related complications (Hershon, Hirsch & Odugbesan 2019; Hiyoshi, Fujiwara & Yao 2019). Postprandial hyperglycaemia is characterised by a rapid and significant hyperglycaemic spike following meal consumption. Hence, by improving postprandial glucose levels, the manifestation of diabetic complications could be reduced. Several therapies focusing on lowering postprandial hyperglycemia are available, including prescribing glucosidase inhibitors such as acarbose, voglibose, and miglitol (Tang, Zhang & Song 2017). These glucosidase inhibitors suppress the activity of carbohydrate hydrolyzing enzymes namely α-amylase and α-glucosidase, slowing intestinal glucose absorption and eventually, controlling postprandial hyperglycemia. In current clinical practice, glucosidase inhibitors are rarely used due to their adverse side effects. Evidence from randomized clinical trials has associated them with gastrointestinal disturbances, including flatulence, diarrhoea, and abdominal pain (Chen et al. 2020; Davies et al. 2022). The side effects of conventional drugs contribute to the growing number of patients who choose complementary and alternative medicines such as functional foods to manage their diseases (Tangkiatkumjai, Boardman & Walker 2020).

Functional foods provide a prospective opportunity for drug development. Apart from being cost-effective, functional foods are promising options for those who have experienced adverse reactions to conventional hypoglycaemic medications (Bumrungpert et al. 2020). Recent studies have investigated the potential of functional foods in managing postprandial hyperglycaemia (Ganesan & Xu 2019). Vinegar is one of the most widely consumed functional foods throughout Asia countries (Perumpuli & Dilrukshi 2022). The positive effects of vinegar intake on the modulation of postprandial blood glucose levels have been documented. Ostman et al. (2005) reported that wine vinegar could reduce postprandial hyperglycaemia in healthy individuals following carbohydrate meals. Similarly, Johnston et al. (2010) showed that intake of apple cider vinegar two minutes before a meal reduced postprandial glucose. Interestingly, it was also discovered that wine vinegar ingestion could improve postprandial glucose levels in patients with type 2 diabetes mellitus when added to a high rather than a low-glycaemic-index meal (Liatis et al. 2010). Furthermore, Shishehbor, Mansoori and Shirani (2017) demonstrated that vinegar intake could improve the postprandial glucose response in both healthy individuals and subjects with glucose metabolism disorders.

Nipa palm (*Nypa fruticans* Wurmb.) vinegar is one of the traditional kinds of vinegar produced by the fermentation of 'Nira', nipa palm sap. Generally, it is used as a food condiment and preservative agents throughout Southeast Asia countries, such as the Philippines, Malaysia, and Thailand. It has been reported to possess several pharmacological effects including antilipidemic (Chatatikun & Kwanhian 2020), anti-obesity, and anti-inflammatory (Beh et al. 2017). In this study, we aimed to determine the potential effects of nipa palm vinegar intake on controlling postprandial hyperglycaemia via α-amylase and α-glucosidase enzyme inhibition. The findings gained from this study provided a better understanding of the physiological actions of nipa vinegar in pathophysiological diabetes mellitus conditions, enabling the design of clinical trials on drug combinations incorporating nipa vinegar for clinical use.

Materials and Methods

Sample Collection and Preparation

Nipa palm vinegar was supplied by a local supplier from Titi Bakong, Yan, Kedah, Malaysia (5°48′9.42″ N, 100°22′35.32″ E). The pH of the nipa palm vinegar was 3.04. Dr. Rahmad Zakaria identified and verified the specimens of a nipa palm tree. These specimens were displayed at the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia, under the voucher number of USM Herbarium 11541.

In Vitro Enzyme Inhibition Study of α-Glucosidase

The α-glucosidase inhibition activity of nipa palm vinegar was evaluated as demonstrated by Laaroussi et al. (2021). In brief, 50-μL aliquots of nipa palm vinegar with a concentration ranging from 62.5 to 4000 mg/mL) were mixed with 100 μL of a 0.1 M phosphate buffer (pH 7.0) containing 0.5 U/mL of α-glucosidase. The mixture was incubated in a 96-well plate at 37 °C for 10 min. The control contained a similar aliquot of the 0.1 M phosphate buffer solution instead of the extract. After incubation, 50 μL of a substrate solution, 5 mM *p*-nitrophenyl-α-D-glucopyranoside in a 0.1 M phosphate buffer (pH 7.0) were added to each well. The reaction mixture was further incubated at 37 °C for 5 min. The release of *p*-nitrophenol was calculated by reading the absorbance at λ_{max} 405 nm using a microplate reader

(Power Wave × 340, BioTek® Instruments Inc., Winooski, VT, USA). Acarbose served as the standard reference and was treated similarly to the sample. The sample and control were analysed in triplicates. The percentage of inhibition of the activity of α-glucosidase was calculated using the following equation:

$$\alpha\text{ -glucosidase inhibitory activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ represented the absorbance of the control. A_{sample} represented the absorbance of the sample. The concentration of the sample/acarbose required to inhibit 50% of the α-glucosidase activity (IC_{50}) was determined by the linear regression analysis.

In Vitro ENZYME INHIBITION STUDY OF α-Amylase

The α-amylase inhibitory activity of nipa palm vinegar was assessed as described by Wickramaratne, Punchihewa and Wickramaratne (2016). In a 96-well plate, 50 µL of vinegar was mixed with 150 µL of starch solution and 10 µL of α-amylase solution (50 unit/1 mL). At 37 °C, the plate was incubated for 30 min. Then, 20 µL of NaOH and 20 µL of colour reagent were added. The plate was incubated in the boiling water bath for 20 min. After that, the mixture was removed and cooled. The absorbance value of the reaction mixture was calculated using a microplate reader (Power Wave × 340, BioTek® Instruments Inc., Winooski, VT, USA) at a wavelength of 570 nm. Acarbose was used as a standard reference. The sample and control were analysed in triplicates. The percentage of inhibition of α-amylase was determined using the following equation:

$$\alpha\text{-amylase inhibitory activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance value of the control solution, and A_{sample} is the absorbance value of the sample. The half-maximal inhibitory concentration (IC_{50}) values which indicated the concentration of nipa palm vinegar required to inhibit 50% of the enzymatic activity, were also calculated.

KINETIC OF α-Glucosidase AND α-Amylase INHIBITION

A constant amount of α-glucosidase was incubated with increasing concentrations of substrate, 4-nitrophenyl-α-D-glucopyranoside (20 mM, 10 mM, 5 mM, 2.5 mM, and 1.25 mM) with (inhibited) and without (uninhibited) the samples. For the α-amylase kinetic study, the enzyme in the presence and absence of the nipa palm vinegar was incubated with starch at varying concentrations of 4%, 2%, 1%, 0.5%, and 0.25%. The samples concentrations were equivalent to the calculated IC_{50} value (144.50 ± 1.1 mg/mL for α-glucosidase and 90.30 ± 1.7 mg/mL for α-amylase). The V_{max} and K_m values were determined using the Michaelis-Menten equation, while Lineweaver-Burk plots were employed to determine the type of inhibition. All the reactions were performed in triplicates.

In Vivo ORAL STARCH TOLERANCE TEST

Healthy adult male *Sprague-Dawley* rats (180 to 200 g) were used, and the experiment was approved by the Institutional Animal Ethics Committee, Universiti Sains Malaysia (Approval number: USM/IACUC/2018/(114)947). Overnight fasted *Sprague-Dawley* normoglycemic rats were divided into five groups of six rats each. The doses of vinegar were determined based on the recommended amount, 0.08 mL/kg body weight (BW) (1 tablespoon of vinegar per day; low concentration), and the amount used in Mohamad et al. (2015) study, 1 mL/kg BW (moderate concentration) and 2 mL/kg BW (high concentration). The rats were treated orally as follows: Group 1 was administered distilled water (10 mL/kg BW) and denoted as a negative control. Group 2 was treated with acarbose (10 mg/kg BW). Groups 3, 4, and 5 were treated with nipa palm vinegar at doses of 0.08 mL/kg BW, 1 mL/kg BW, and 2 mL/kg BW. Starch (3 g/kg BW) was administered orally 10 min post-treatment. The tail prick method was used to collect the blood sample at 0 (before treatment), 30, 60, 90, and 120 min after starch loading. This method was conducted as per The University of British Columbia (UBC) guidelines on the collection of small amounts of blood from tail tip microsampling in rats (UBC Animal Care Guidelines 2014). The acute blood-glucose-lowering effect was monitored for two hours, and the area under the curve (AUC) was calculated using the trapezoidal method. The oral starch tolerance test was summarized in Figure 1.

STATISTICAL ANALYSIS

Data were expressed as the mean ± standard error of the mean (SEM). Statistical significance was assessed using a one-way analysis of variance (ANOVA), followed by Dunnett as the post hoc test. *P* values less than 0.05 indicated statistical significance. GraphPad Prism9 software was used to analyse IC_{50} value and enzyme

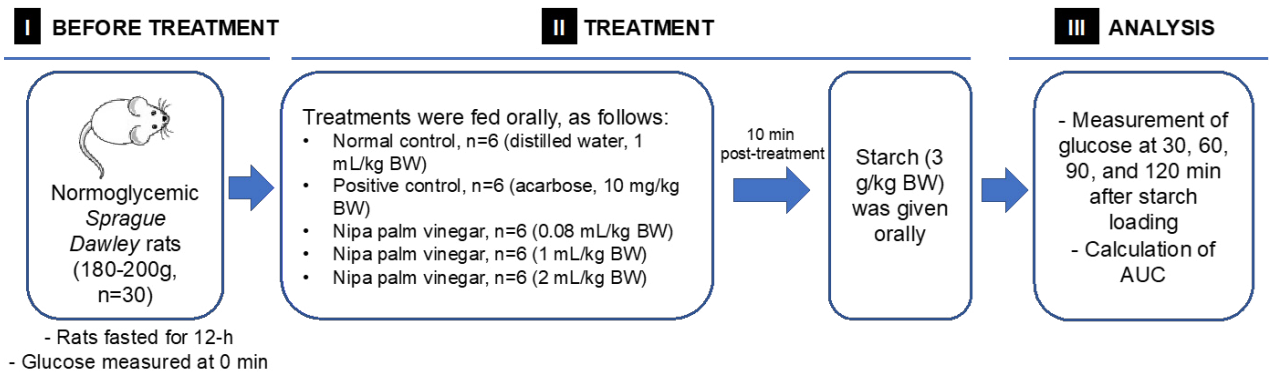


FIGURE 1. Oral starch tolerance test

kinetic data. IC_{50} values were calculated using a dose-response curve of the logarithmic x-axis (concentration). V_{max} and K_m values were calculated using Michaelis-Menten kinetic equation and represented by Lineweaver Burk plot whereby the y-intercept denoted the $1/ V_{max}$ value and the x-intercept represented $-1/ K_m$. The area under the curve (AUC) was calculated using the trapezoidal method.

Results and Discussion

Inhibition of carbohydrate hydrolysing enzymes, α-amylases and α-glucosidase has been proven to be one of the effective strategies to suppress the rise of postprandial blood glucose following consumption of a carbohydrate meal. Alpha-amylase is an enzyme that catalyses the breakdown of complex carbohydrates by hydrolyzing α-1,4 glycosidic linkages in starch. In humans, α-amylases are found primarily in the saliva and pancreas. These oligosaccharides are further catalysed by alpha-glucosidase. Alpha-glucosidase, an exohydrolases is a membrane-bound enzyme of the intestinal epithelium. It assists intestinal glucose absorption by hydrolysing the hydrolytic cleavage of oligosaccharides into absorbable monosaccharides (Dirir et al. 2022). By inhibiting α-glucosidase in the intestine, the rate of oligosaccharide breakdown into absorbable monosaccharides; is decreased - as is the rate of intestinal glucose absorption. As a result, the rise in postprandial glucose is suppressed. In this study, the potential inhibitory effects of nipa palm vinegar on α-amylase and α-glucosidase were investigated using *in vitro* and *in vivo* techniques. For comparison purposes, acarbose, a glucosidase inhibitor was utilised as a positive control. The dose of vinegar used in the study was selected based on the reported daily intake of vinegar (0.08 mL/kg body weight equal to 1 tablespoon of vinegar per day (Mohamad et al. 2015) and the highest dose of 2 mL/kg body weight, as suggested by the previous study (Beh et al. 2017). The highest dose of nipa palm vinegar has been regarded as safe considering the oral LD_{50} value of acetic acid, the main compound of vinegar has been reported to be 3310 mg/kg body weight (APS 2015).

Figure 2 shows the inhibitory activities of nipa palm vinegar on α-glucosidase and α-amylase enzymes, as tested via *in vitro* enzymatic assays. As shown in Figure 2(A), acarbose exhibited the lowest IC_{50} value (9.31 ± 7.3 mg/mL), denoting its potential as a glucosidase inhibitor. Nipa palm vinegar inhibited the activity of α-glucosidase in a dose-dependent manner with the IC_{50} value of 144.50 ± 1.1 mg/mL. The inhibitory potency of nipa palm vinegar, however, was minimal when compared with that of acarbose with a calculated IC_{50} value 15.5 times higher. A similar observation was seen in the α-amylase inhibitory test. Nipa palm vinegar exerted a dose-dependent inhibitory pattern against α-amylase. At the concentration of 4000 mg/mL, nipa palm vinegar caused the maximum inhibition as indicated by a plateau. The IC_{50} value of nipa palm vinegar was 90.30 ± 1.7 mg/mL (Figure 2(B)). Overall, as indicated by the IC_{50} values, nipa palm vinegar showed a stronger inhibition against α-amylase than α-glucosidase. A similar observation was seen in acarbose. The findings further suggested that the enzyme inhibitory effects of nipa palm vinegar were weaker as compared to the acarbose. It could be due to its acidic property. Noh et al. (2020) have reported that the enzyme-inhibitory activity of various types of vinegar is positively correlated with

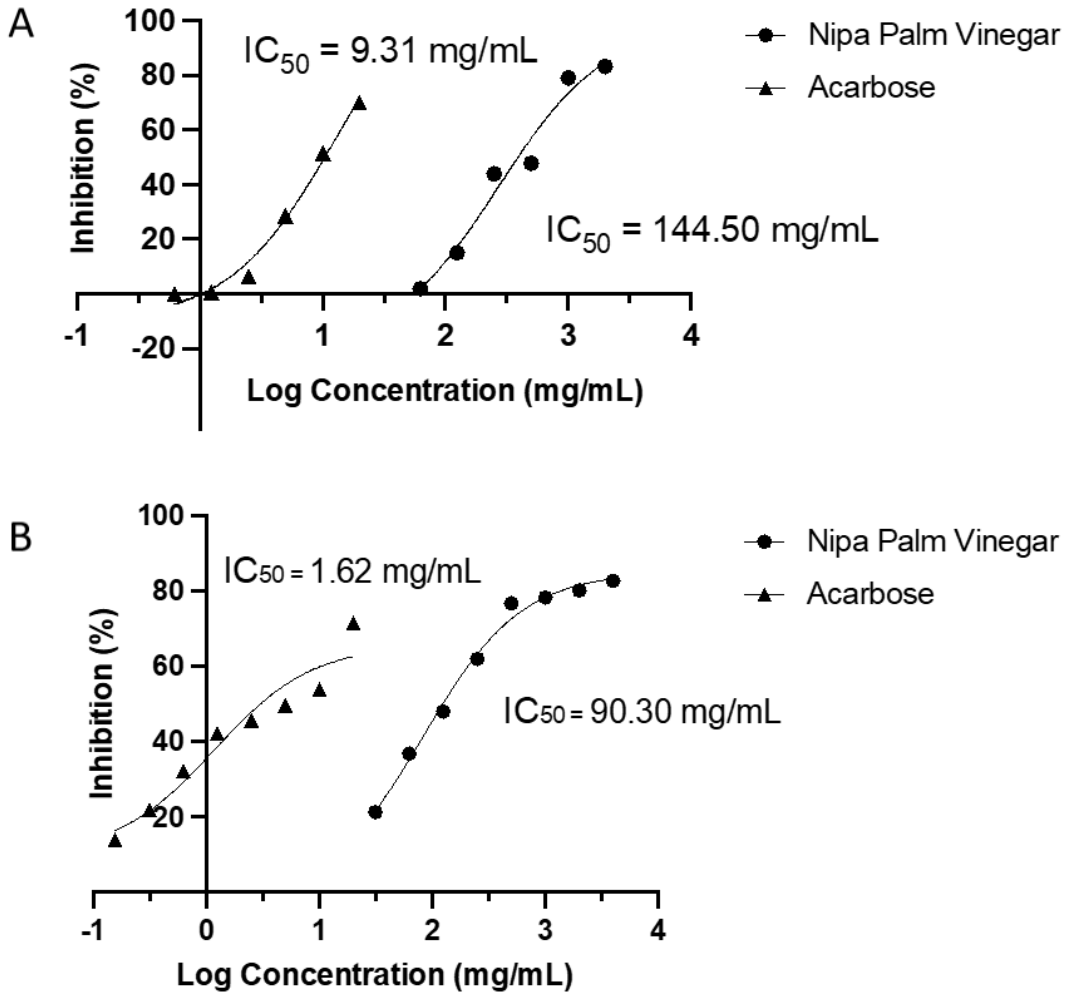


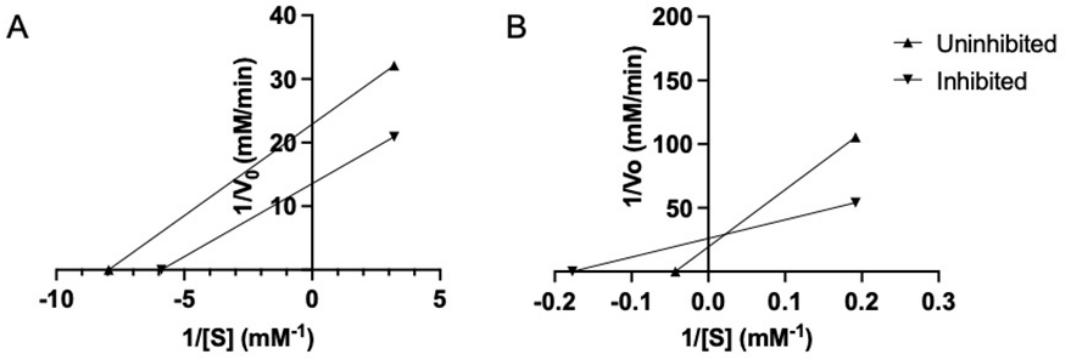
FIGURE 2. Dose-response curve of nipa palm vinegar against (A) α-glucosidase and (B) α-amylase enzymes in comparison to acarbose. Values are expressed as means ± standard error means (S.E.M)

the total organic acid content. In this study, mulberry fruit vinegar which had the highest organic acid content exhibited the strongest inhibitory effect towards digestive enzymes, while brown rice vinegar with the lowest organic acid content showed the weakest inhibitory effect. In addition to that, the weak acid group (organic acids) has proven to induce mild inhibitory effects against digestive enzymes (Marunaka 2018). This might explain the moderate enzyme inhibitory effect of nipa palm vinegar. The present findings are consistent with the reports by Wihansah, Arief and Batubara (2018), where yoghurt (weak acid-containing food) also exhibited low enzyme inhibition than those of acarbose.

To characterize the type of enzyme inhibition of nipa palm vinegar, Lineweaver-Burk kinetic analysis was conducted. Enzyme kinetic analysis provides information on the binding affinities between substrate and inhibitors, as well as the maximum attainable catalytic rates from the reaction. Figure 3(A) represents the Lineweaver-Burk plot of nipa palm vinegar against different concentrations of *p*-nitrophenyl-α-D-glucopyranoside in the α-glucosidase inhibitory test. In this plot, lines of uninhibited (without nipa palm vinegar as an inhibitor) and inhibited (with nipa palm vinegar as an inhibitor) were not intersected, which indicated that nipa palm vinegar exhibited

uncompetitive inhibition against the α-glucosidase enzyme. Uncompetitive inhibitors reduced substrate maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) values as compared with the uninhibited (Figure 3(C)). This behaviour points to the fact that the active compounds of nipa palm vinegar bind to the enzyme-substrate complex rather than the free enzyme to prevent the breakdown of disaccharides into monosaccharides. This inhibition mode is ideal for drug design as the inhibitors bind to the enzyme's active site only when the site is active with the presence of substrate (Ouertani et al. 2019). On the other hand, a competitive inhibition was observed for the α-amylase enzyme (Figure 3(B)). Lines of uninhibited and inhibited intersected at the y-axis. As compared with the uninhibited, the K_m value increased in the presence of nipa palm vinegar, whereas the V_{max} value remained constant (Figure 3(C)). A competitive inhibition suggests that the active compounds of nipa palm vinegar competed with the substrate to bind at the enzyme's active site, thereby preventing the hydrolysis of polysaccharides to disaccharides. A similar mode of inhibition was exerted by acarbose (Assefa et al. 2019).

To confirm the *in vitro* enzyme inhibitory test results, an oral starch tolerance test was performed. The effectiveness of nipa palm vinegar in eliminating loaded glucose from the blood circulation system, as well as its potential antihyperglycemic impact, were determined during the oral starch tolerance test. The *in vivo* finding agreed with the *in vitro* result. As shown in Figure 4(A), the blood glucose levels of all treated groups were not statistically different at 0 minute (before starch administration). For the negative control group, the blood glucose levels rose to 9.55 mmol/L, 30 min after starch loading. Later, at minutes 60, 90, and 120, the glucose levels decreased, respectively, to 8.05 mmol/L, 7.55 mmol/L, and 6.945 mmol/L. Acarbose significantly ($p < 0.05$) inhibited the rise in blood glucose level up to minute 90. However, at minute 120, the glucose started raising. Nipa palm vinegar at the doses of 2 mL/kg BW and 1 mL/kg BW significantly ($p < 0.05$) reduced the blood glucose level during the 2-h observation period when compared with the negative control. Interestingly, the findings also indicated that nipa palm vinegar has a longer duration of action as compared to acarbose. The



C

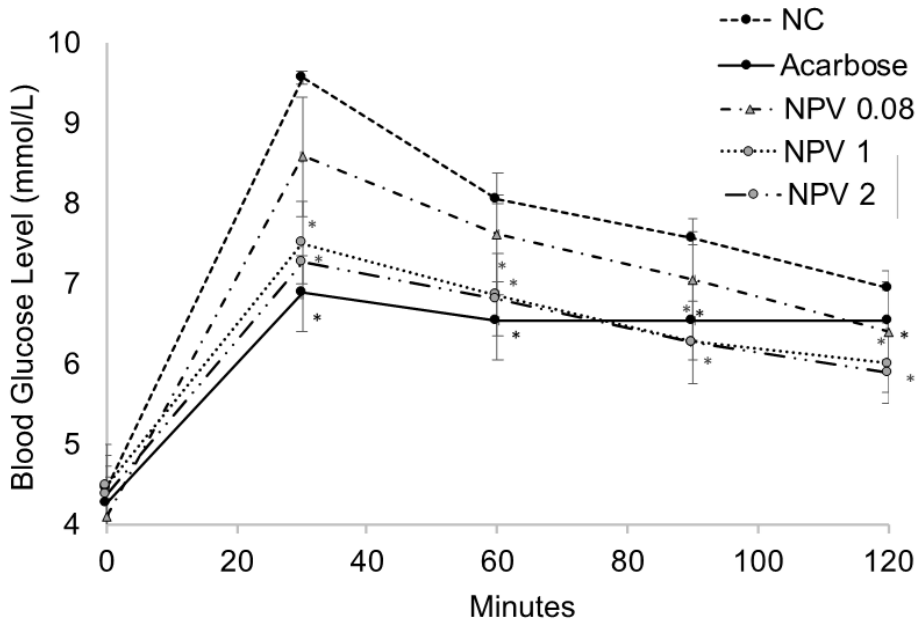
	α - Glucosidase		α - Amylase	
	V_{max}	K_m	V_{max}	K_m
Uninhibited	0.07379	0.1695	0.04	5.65
Inhibited	0.04368	0.1257	0.05	23.3

FIGURE 3. Lineweaver-Burk plot of nipa palm vinegar showed (A) uncompetitive inhibition on alpha-glucosidase and (B) competitive inhibition on alpha-amylase. (C) Maximum velocity, V_{max} and Michaelis-Menten constant, K_m values for nipa palm vinegar

blood glucose level of the nipa palm vinegar-treated group remained significantly inhibited until minute 120, whilst the glucose level of the acarbose-treated group started to rise after minute 60. Although due to the limitations of the study, pharmacokinetic profiling was not conducted, the aforementioned observation seems promising considering the possibility of reduced daily intake frequency, which would be advantageous to avoid the known adverse effect of frequent vinegar used on the gastrointestinal tract. Due to the caustic property of acetic acid in vinegar, there have been reports of oropharynx and oesophagal injury caused by the intake of commercial vinegar beverages and rice vinegar (Chang et al. 2020). Thus, further long-term study to determine the safe concentration of nipa palm vinegar for daily intake is warranted.

The glucose area under the curve (AUC) is a measure of the whole glucose excursion following glucose loading. It has been applied comprehensively to assess the efficiency of the treatments. Lower AUC values reflect lower glucose concentration in the blood, suggesting that the treatment was effective in inhibiting intestinal glucose absorption. Based on the AUC values (Figure 4(B)), the inhibitory effects of tested samples

A



B

Samples	AUC (mmol· min/L)
Negative Control	225.71± 11.98[a]
Acarbose	196.05± 7.82[d]
NPV 0.08	210.82 ± 11.45[b]
NPV 1	190.35 ± 7.99[c]
NPV 2	189.07 ± 8.02[c]

Data were expressed as mean ± SEM of six rats.

FIGURE 4. Nipa palm vinegar (NPV) at the dose of 1 mg/mL BW and 2 mg/mL B W significantly suppressed postprandial blood glucose levels compared to the negative control (NC) in the oral starch tolerance test. All the data were statistically significant at *p < 0.05, as analysed using Dunnett's post-hoc test. (B) Area under the curve (AUC) values were expressed as means ± standard error means (SEM). Values with different superscripted letters were statistically different, as analysed using the Tukey HSD test

from strongest to lowest were as follows: NPV 2 > NPV 1 > Acarbose > NPV 0.08 > NC. The AUC values of NPV 2 and NPV 1 were not statistically different ($p>0.05$), indicating the comparable glucose-lowering effect of these two doses.

Conclusions

The study suggested that nipa palm vinegar controlled postprandial glucose level partly by inhibiting the activity of carbohydrate digestive enzymes, α-glucosidase, and α-amylase. The present findings could offer a scientific provision of nipa palm vinegar for further development as an alternative therapy for managing postprandial glucose levels.

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