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# In Vitro Evaluation of Hesperidin's α-Glucosidase Inhibitory Kinetics and Its Potential Role in Type 2 Diabetes Management

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## **ABSTRACT**

**Background:** Type 2 diabetes mellitus is driven in part by postprandial hyperglycemia (PPHG), for which inhibition of intestinal  $\alpha$ -glucosidase is a validated strategy. Hesperidin, a citrus-derived flavonoid, has been reported to modulate carbohydrate metabolism, but its kinetic interaction with  $\alpha$ -glucosidase has not been fully clarified.

**Objective:** To characterize the inhibitory kinetics of hesperidin against Saccharomyces cerevisiae  $\alpha$ -glucosidase and evaluate its potential as a candidate for managing PPHG.

**Methods:**  $\alpha$ -Glucosidase activity was assayed using p-nitrophenyl- $\alpha$ -D-glucopyranoside (pNPGP) in 0.1 M phosphate buffer (pH 6.8). Enzyme was pre-incubated with hesperidin (100  $\mu$ M) at 37 °C for 10 min; reactions were initiated with substrate (0.125–4.000 mM), incubated for 20 min, and quenched with 100 mM Na<sub>2</sub>CO<sub>3</sub>. Product formation (p-nitrophenol) was monitored at 405 nm. Controls lacked inhibitor; acarbose served as a positive control. Initial velocities were fitted by Lineweaver–Burk plots to estimate kinetic parameters; the inhibition constant (K\_i) was derived from secondary plots. Experiments were performed in triplicate and analyzed by linear regression.

**Results:** Hesperidin produced concentration-dependent inhibition consistent with a competitive mechanism: double-reciprocal plots intersected on the y-axis,  $V_{max}$  was effectively unchanged, and  $K_{m}$  increased from 0.046 mM (control) to 2.511 mM (+hesperidin). The calculated  $K_{i} = 0.0019$  mM indicates high affinity for the free enzyme. Acarbose exhibited the expected inhibitory profile, corroborating assay validity.

Conclusions: Hesperidin is a potent competitive inhibitor of  $\alpha$ -glucosidase in vitro, markedly reducing apparent substrate affinity without impacting maximal catalytic rate. These kinetics support hesperidin's promise as a natural adjunct for PPHG control.

**Keywords:** hesperidin;  $\alpha$ -glucosidase; competitive inhibition; enzyme kinetics; postprandial hyperglycemia; type 2 diabetes





## INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia due to impaired insulin secretion, defective insulin action, or both. Type 2 diabetes mellitus (T2DM) accounts for nearly 90–95% of all cases globally and continues to rise as a major public health concern (American Diabetes Association [ADA], 2023; Rogers et al., 2020). Insulin, secreted by pancreatic  $\beta$ -cells, regulates glucose uptake in peripheral tissues, including the liver, adipose tissue, and skeletal muscle, while also controlling lipid and protein metabolism. Impaired insulin secretion or reduced receptor sensitivity plays a pivotal role in the pathophysiology of T2DM (Sanchez et al., 2021).

Postprandial hyperglycemia (PPHG), a hallmark of T2DM, is strongly linked to macrovascular complications such as atherosclerosis, cardiovascular diseases, and stroke (Eringa et al., 2013; Lee et al., 2022). Managing PPHG remains critical for preventing long-term complications associated with diabetes. Carbohydrate digestion, mediated by  $\alpha$ -amylase and  $\alpha$ -glucosidase, leads to rapid glucose absorption and subsequent spikes in blood sugar (Sim et al., 2008; Taslimi et al., 2018). Thus, inhibition of  $\alpha$ -glucosidase, a key enzyme located on the brush border of the small intestine, has emerged as an attractive therapeutic strategy for managing PPHG (Ye et al., 2019).

Commercial  $\alpha$ -glucosidase inhibitors such as acarbose, voglibose, and miglitol are widely used as first-line adjunct therapies. These agents delay carbohydrate digestion and absorption, thereby lowering PPHG (Chiasson, 2006; Kashtoh & Baek, 2022). However, their long-term use is frequently limited by adverse gastrointestinal side effects, including bloating, flatulence, diarrhea, and abdominal discomfort (Krentz & Bailey, 2005; Patil et al., 2015). Consequently, there is a growing demand for alternative inhibitors derived from natural products with minimal side effects and better tolerability.

Flavonoids, a group of polyphenolic compounds abundant in fruits, vegetables, and herbs, have attracted attention as promising  $\alpha$ -glucosidase inhibitors with antioxidant and anti-inflammatory benefits (Barber et al., 2021; Proença et al., 2022). Among them, hesperidin, a citrus-derived flavonoid glycoside, has shown potential as a bioactive compound with antidiabetic, cardioprotective, and anti-inflammatory properties (Roohbakhsh et al., 2015; Ullah et al., 2022). Preliminary studies report that hesperidin exhibits  $\alpha$ -glucosidase inhibitory activity (Loizzo et al., 2018; Zhang et al., 2022), yet its inhibitory kinetics and mechanism of enzyme interaction remain poorly understood.

Therefore, this study investigates the inhibitory kinetics of hesperidin against  $\alpha$ -glucosidase to elucidate its mechanism of action and therapeutic potential in managing postprandial hyperglycemia.

#### **Statement of the Problem**

Diabetes mellitus, particularly T2DM, is a global health burden. According to the World Health Organization (WHO, 2023), over 422 million people worldwide live with diabetes, and the prevalence is projected to rise significantly by 2030. Complications arising from poor glycemic control account for over 1.5 million deaths annually, emphasizing the need for improved therapeutic strategies. Postprandial hyperglycemia (PPHG) remains a major challenge in diabetes management. Current α-glucosidase inhibitors, while effective, are associated with gastrointestinal side effects and high treatment costs, limiting compliance and accessibility (Smith et al., 2021; Wehmeier & Piepersberg, 2004). As a result, safer, more effective, and affordable alternatives are urgently needed.

Flavonoids have emerged as promising candidates for enzyme inhibition due to their natural origin, low toxicity, and multifunctional pharmacological properties (Şöhretoğlu & Sari, 2020). Although hesperidin has demonstrated antidiabetic potential, no systematic studies have examined its inhibitory kinetics on  $\alpha$ -glucosidase. This knowledge gap hinders its development as a potential therapeutic agent.





#### Aim of the Study

The aim of this study is to investigate the inhibitory kinetics of hesperidin against  $\alpha$ -glucosidase enzyme activity in order to evaluate its potential as a natural therapeutic agent for the management of postprandial hyperglycemia in type 2 diabetes.

# MATERIALS AND METHODS

# **Chemicals and Reagents**

Saccharomyces cerevisiae α-glucosidase enzyme, p-nitrophenyl-α-D-glucopyranoside (pNPGP, substrate), acarbose, sodium dibasic (Na<sub>2</sub>HPO<sub>4</sub>), and sodium monobasic dihydrate (NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O) used for preparing phosphate buffer (pH 6.8), as well as hesperidin, were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) was obtained from BDH Chemicals Ltd. (Poole, England). All other chemicals and reagents used in this study were of analytical grade.

## **Apparatus and Instrumentation**

The instruments used in this study included a UV-Visible spectrophotometer (Spectrum Lab and 52b New Life Medical Instrument, England), a pH meter, a water bath (OLS200, Grant Instruments, Cambridge, UK), and a weighing balance (Scout Pro Spo402, Ohaus Corporation, Pine Brook, NJ, USA).

# Preparation of Enzyme, Substrate, and Hesperidin Compound

Prior to use, 0.01 mg of  $\alpha$ -glucosidase enzyme was dissolved in 1 mL of 0.1 M phosphate buffer (pH 6.8), followed by dilution at 1:100 to obtain the working enzyme solution. A 10 mM stock solution of the substrate (pNPGP) was prepared in 50 mL of 0.1 M phosphate buffer (pH 6.8). A 5 mM stock solution of hesperidin was prepared in 10% dimethyl sulfoxide (DMSO).

## Evaluation of Inhibitory Kinetics of Hesperidin Against α-Glucosidase

The inhibitory kinetics assay was adapted from Fagbohunka et al. (2024) and Kumaravel et al. (2023), with slight modifications. The assay was performed using varying substrate concentrations (0.125, 0.250, 0.500, 1.000, 2.000, and 4.000 mM) in the presence and absence of a fixed concentration (100 µM) of hesperidin.

For each assay,  $\alpha$ -glucosidase was pre-incubated with hesperidin (100  $\mu$ M) in assay buffer (100 mM sodium dibasic and sodium monobasic dihydrate) at 37 °C for 10 minutes. The enzymatic reaction was initiated by adding varying concentrations of the substrate (pNPGP), and the total reaction volume (1000  $\mu$ L) was incubated at 37 °C for 20 minutes in a water bath. The reaction was terminated by adding 1000  $\mu$ L of Na<sub>2</sub>CO<sub>3</sub> (100 mM).

Absorbance was measured at 405 nm using a UV-Visible spectrophotometer. Control reactions (without inhibitor) were included, while acarbose served as a positive control. Enzyme activity was calculated from absorbance values corresponding to the concentration of p-nitrophenol (pNP) released.

The mode of inhibition was determined from the Lineweaver–Burk plot of the reciprocal of enzyme velocity (1/V) versus substrate concentration (1/[S]). The Michaelis–Menten constant (Km) and maximum velocity (Vmax) were determined from the slope and intercept of the plot (Equation 1). The inhibition constant (Ki) was calculated according to Equation 2.

$$\frac{1}{V} = \frac{Km}{Vmax} \cdot \frac{1}{[S]} + \frac{1}{Vmax} \dots \tag{1}$$

$$Ki = \frac{Km}{KmApp-Km} \cdot [I] \dots (2)$$

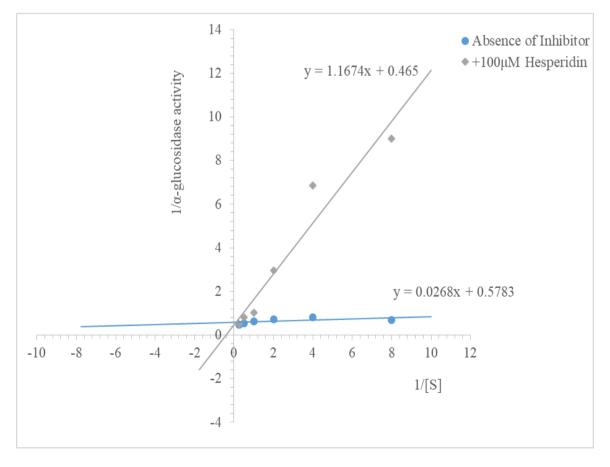


## **Statistical Analysis**

Experimental data were analyzed using Microsoft Excel 2016. Linear regression was performed using the LINEST function and the Regression tool in the Data Analysis ToolPak to determine the slope and intercept values, which were subsequently used to calculate Km and Vmax. All experiments were conducted in triplicate, and results were expressed as mean ± standard deviation (SD).

## RESULTS AND DISCUSSION

This study investigated the inhibitory kinetics of hesperidin on alpha-glucosidase, and the results are presented in Figure 1 and Table 1. The mode of inhibition was determined using the Lineweaver-Burk plot (Figure 4.1) in the absence and presence of hesperidin. The Lineweaver-Burk plots showed intersecting lines on the y-axis in the first quadrant, indicating a competitive type of inhibition. Table 1 shows the calculated values of the kinetic parameters (Vmax and Km) from the Lineweaver-Burk plot using Equation (1). In the absence of hesperidin, the Michaelis-Menten constant (Km) was 0.046 mM, and the maximal reaction rate (Vmax) was 2.020 mM/min. In the presence of hesperidin, Km increased to 2.511 mM while Vmax was 2.150 mM/min. A significant increase in Km in the presence of hesperidin suggests that hesperidin competes with the substrate (para-nitrophenyl-α-D-glucopyranoside, pNPGP) for binding to the active site of alpha-glucosidase. This increase in Km reflects a decreased affinity of the enzyme for the substrate because hesperidin's binding to the active site reduces the enzyme's ability to bind to pNPGP. There was no significant difference in the Vmax values in the absence and presence of the inhibitor (Table 1) because the inhibitor does not affect the enzyme's catalytic activity when the substrate concentration is high enough. Instead, it only affects the enzyme's ability to bind to the substrate. The observed stability in Vmax indicates that hesperidin does not alter the maximum catalytic efficiency of alpha-glucosidase; it only competes with the substrate. The inhibition constant (Ki), calculated using Equation (2), was 0.0019 mM, indicating a strong binding affinity of hesperidin to alphaglucosidase, which is consistent with competitive inhibition, where the inhibitor binds to the active site of the enzyme.



**Figure 1:** Lineweaver-burk plot depicting the mode of inhibition of alpha-glucosidase enzyme by hesperidin (inhibitor).





**Table 1:** Effect of hesperidin on the kinetic parameters (Km and Vmax) of alpha-glucosidase enzyme

| Enzyme +Inhibitor          | Km (mM) | Vmax (mM/minutes) |
|----------------------------|---------|-------------------|
| α-glucosidase              | 0.046   | 2.020             |
| α-glucosidase + Hesperidin | 2.511   | 2.150             |

**NOTE:** Km is Michaelis-Menten constant Vmax is maximal reaction velocity

The management of diabetes mellitus, particularly postprandial hyperglycemia, has increasingly focused on  $\alpha$ -glucosidase inhibition as a therapeutic strategy. Inhibiting  $\alpha$ -glucosidase reduces carbohydrate digestion and glucose absorption, thereby lowering postprandial blood glucose levels (Chen et al., 2006). Beyond glycemic control, this approach minimizes the formation of advanced glycation end-products (AGEs), which are closely associated with cardiovascular complications (Ceriello et al., 2006). Importantly,  $\alpha$ -glucosidase inhibition is especially beneficial for patients with impaired insulin responses and is often used in combination with other hypoglycemic agents (Mahomoodally et al., 2012).

Although synthetic inhibitors such as acarbose and metformin have demonstrated clinical efficacy, their long-term use is often limited by gastrointestinal side effects and cost, which has prompted exploration of safer, naturally derived alternatives (Sudha et al., 2011; Mohamed et al., 2012). In this regard, flavonoids, particularly hesperidin—a citrus-derived glycoside—have attracted significant attention due to their  $\alpha$ -glucosidase inhibitory activity and additional pharmacological benefits (Li et al., 2004; Fu et al., 2021).

The present study revealed that hesperidin acts as a competitive inhibitor of  $\alpha$ -glucosidase, as demonstrated by the Lineweaver–Burk plots (Figure 4.1). The presence of hesperidin significantly increased the Km value (from 0.046 mM to 2.511 mM) while leaving Vmax relatively unchanged, consistent with the mechanism of competitive inhibition. This suggests that hesperidin directly competes with the substrate p-nitrophenyl- $\alpha$ -D-glucopyranoside (pNPGP) for the enzyme's active site, thereby reducing substrate affinity without altering the maximum catalytic rate. Such findings are in line with earlier reports of competitive inhibition for flavonoids, including quercetin and catechin (Kim et al., 2018; Shen et al., 2022). Similarly, Chi et al. (2019) reported competitive inhibition by hypericin, while Fu et al. (2021) also demonstrated flavonoid-mediated competitive inhibition of  $\alpha$ -glucosidase.

Interestingly, not all studies agree with this inhibition mechanism. For example, Song et al. (2016) reported an uncompetitive mode of inhibition for hesperidin, highlighting how structural variations in the enzyme—inhibitor complex or differences in assay conditions may yield different inhibition patterns. These discrepancies emphasize the need for further mechanistic studies, possibly involving molecular docking and crystallographic approaches, to better understand hesperidin—enzyme interactions.

The present study also calculated an inhibition constant (Ki) of 0.0019 mM, suggesting that hesperidin exhibits a strong binding affinity to α-glucosidase. A lower Ki value is indicative of more potent inhibitory activity, and in this case, hesperidin displayed stronger affinity compared to some synthetic inhibitors and other natural compounds (Haguet et al., 2023). For example, Chi et al. (2019) reported Ki values of 9.4 mg/L for hypericin and 40.6 mg/L for acarbose, suggesting that hesperidin is potentially more effective. This finding underscores hesperidin's promise as a natural candidate for therapeutic development against postprandial hyperglycemia.

Overall, the study provides strong evidence that hesperidin is a potent competitive inhibitor of  $\alpha$ -glucosidase with significant potential for the management of postprandial hyperglycemia. Its favorable inhibitory kinetics, natural origin, and lower risk of side effects compared to conventional synthetic inhibitors position hesperidin as a valuable adjunct or alternative therapeutic option in diabetes management.

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## **CONCLUSION**

This research studied on the inhibitory kinetics of hesperidin against alpha-glucosidase has demonstrated the mode of inhibition of hesperidin as well as the effect hesperidin has on the kinetic parameters of the enzyme which are the core objectives of this research. The kinetic analysis showed that hesperidin inhibited alpha-glucosidase enzyme activity in a competitive mode indicating that hesperidin only binds to the active site of the enzyme (alpha-glucosidase). The effect of hesperidin on the kinetic parameters of alpha-glucosidase showed that the Michaelis-Menten (Km) increased in the presence of inhibitor (hesperidin) while the maximal reaction rate (Vmax) was relatively constant both in the presence and absence of the inhibitor (hesperidin). Hesperidin's high binding affinity for alpha-glucosidase is indicated by its calculated inhibition constant (Ki), further indicates a strong binding affinity of hesperidin for the enzyme. These findings suggest that hesperidin effectively reduces enzyme-substrate interaction, which could be beneficial for managing postprandial glucose levels.

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