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Kinetics and Inhibition of Alpha-Amylase by *Curcuma caesia* for Antihyperglycemic Potential

Muhammad Muaz Junaidi[1], Rasmyie Naszryie Abdul Rahim[1], Nurul Izzati Mohd Ismail[1,*], Nik Nurul Najihah Nik Mat Daud[2], Basirah Fauzi[1]

[1] Department of Science and Mathematics, Centre for Diploma Studies, Universiti Tun Hussein Onn Malaysia, 84600 Pagoh, Johor, Malaysia
[2] Department of Biomedical Science, Faculty of Applied Science, Lincoln University College, 47301 Petaling Jaya, Selangor, Malaysia

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ABSTRACT

Curcuma caesia (*C. caesia*), commonly known as black turmeric, holds a significant place in traditional medicine due to its potent medicinal properties, particularly in managing hyperglycemia associated with diabetes. Despite existing treatments, achieving optimal glycemic control remains a persistent challenge due to their side effects, highlighting the need for additional therapeutic options. Understanding the effects of herbal processing conditions (such as temperature) and the mechanisms of action can lead to the development of more effective treatments with minimal side effects. Knowledge of these mechanisms, using kinetic equations, helps predict and prevent negative interactions with herbal products intended for treatment. This study aims to investigate the antihyperglycemic activity of *C. caesia* extracts (CC50 and CC60) by targeting the alpha-amylase enzyme, a key player in glucose metabolism, with the goal of elucidating its enzymatic kinetics and mode of inhibition. The methodology involves obtaining *C. caesia* extract through reflux and subjecting it to drying at 50°C (CC50) and 60°C (CC60). Antihyperglycemic efficacy was evaluated by employing the extract as an inhibitor of alpha-amylase. The effectiveness of the inhibition was measured using DNS reagent to determine the amount of reduced sugar, and results were analyzed using UV-spectrophotometry at 540 nm. The half minimal inhibitory concentration (IC_{50}) values for CC50 (5094.41 ppm) and CC60 (5083.07 ppm) indicate that drying temperatures between 50°C and 60°C are suitable for processing *C. caesia*. Additionally, the maximum reaction rate (Vmax) for both CC50 and CC60 was 0.0025 mg/min, lower than the uninhibited reaction rate (0.0055 mg/min), suggesting that both CC50 and CC60 cause non-competitive inhibition. Kinetic analysis revealed that both extracts were non-competitive inhibitors, as evidenced by the unchanged Michaelis-Menten constant (K_m). This work enhances the understanding of how to improve the efficacy of *C. caesia* as an antihyperglycemic agent by targeting alpha-amylase. Advanced knowledge of the enzyme-inhibitory activities of *C. caesia* and the conditions for processing it supports the clinical application of blood glucose regulation, addressing the shortcomings of contemporary diabetes care and aiming to minimize the adverse effects of current pharmacotherapy.

* *Corresponding author.*
E-mail address: nizzati@uthm.edu.my

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1. Introduction

Herbaceous plant with a bluish-black rhizome, black turmeric (*Curcuma caesia*), is native to North-East and Central India. Although *C. caesia*) originates from North-East and Central India, the sample plant obtained is from Malaysia, not India. The *C. caesia* is known by a variety of names in different parts of India such as Kali Haldi in Hindi. This study highlights that *C. caesia*'s bioactive curcuminoids confer various medicinal properties, including anti-oxidative, anti-inflammatory, wound healing, hypoglycemic, anti-coagulant, and anti-microbial activities [1]. The plant typically grows upright to a height of 0.5 to 1.0 m and is classified as either underground giant elliptical or aboveground little oval [2]. Flavonoids and phenolic compounds, widely distributed in plants, are known to confer various biological effects such as antioxidant and anti-inflammatory properties. These compounds, including flavonoids and phenolics identified in *C. caesia*, contribute to its medicinal effects [3]. Phenolic compounds, including curcuminoids present in *C. caesia*, are particularly recognized for their antioxidant properties. They effectively scavenge free radicals and reactive oxygen species, mitigating oxidative stress and safeguarding cells from damage. This antioxidative activity is crucial in addressing various health conditions, including diabetes, wherein oxidative stress is implicated [4].

Antihyperglycemia is well known for inhibiting the formation of an abnormally large amount of sugar in the blood [5]. A popular plant well-known as *C. caesia* can provide some benefits in treating diseases such as hyperglycemia. Understanding the benefits of *C. caesia*, also known as Curcumin, is crucial, especially in cardiovascular health. Curcumin, a compound found in *C. caesia*, shows potential in improving endothelial function, the lining of blood vessels. This is significant because endothelial dysfunction is associated with conditions like blood clotting and hypertension, increasing the risk of heart disease [6]. While *C. caesia*, contains curcumin, most research has focused on the effects of curcumin itself, rather than the whole *C. caesia* plant [7]. Research suggests that curcumin, the most active component of turmeric, may be beneficial for people with diabetes in managing their blood sugar levels [8]. This promising finding aligns with the growing scientific interest in curcumin as a potential therapeutic agent for both diabetes and its associated complications. Therefore, hyperglycemia is a symptom of diabetes mellitus, a metabolic condition caused by relative or absolute insulin insufficiency or resistance [9]. The key to the treatment of hyperglycemia is by inhibiting the activity of alpha-amylase. Inhibition of alpha-amylase may reduce the production of reduced sugar which is the main culprit for the spike of sugar level in blood. Both phenolics and flavonoids in *C. caesia* contribute to its potential health benefits, including its antihyperglycemic activity. These compounds possess various biological activities that may help regulate blood sugar levels, improve insulin sensitivity, and reduce inflammation, all of which are relevant in the context of diabetes management [10].

Antihyperglycemic activity refers to the ability of a substance, such as a medication or natural compound, to reduce elevated blood glucose levels in individuals with hyperglycemia, which is high blood sugar. Hyperglycemia is commonly associated with diabetes mellitus but can also occur due to other factors such as genetic, environmental, immunologic and ingestion of food with high glycemic index [11]. Antihyperglycemic agents work by various mechanisms, including increasing insulin sensitivity, promoting insulin secretion, inhibiting glucose absorption in the intestines, or reducing glucose production in the liver [12]. Controlling hyperglycemia is essential for preventing complications associated with diabetes, such as cardiovascular disease, nerve damage, kidney disease, and vision problems. Therefore, substances with antihyperglycemic activity play a crucial role in the management of diabetes and related conditions.

The limitations of modern medicine, such as high costs and potential side effects of some medications, have fueled the search for new approaches to managing chronic conditions like hyperglycemia. *C. caesia*, is emerging as a potential natural supplement in this regard. Early research suggests that curcumin, a bioactive compound in *C. caesia*, may improve insulin sensitivity or regulate blood sugar levels. While more research is needed, *C. caesia* presents a promising new alternative for individuals with hyperglycemia, potentially alongside conventional medical treatments. While modern medicine has revolutionized healthcare, some medications can have side effects and chronic illnesses can be challenging to manage. Since *C. caesia*, also known presents a potential new approach for managing hyperglycemia, this natural product might offer advantages beyond just cost-effectiveness [10]. For example, early research suggests that curcumin, a compound in *C. caesia*, may improve insulin sensitivity or regulate blood sugar levels, potentially with fewer side effects compared to some medications [13]. Additionally, *C. caesia* could potentially be used alongside existing therapies for a more comprehensive approach. It's important to note that more research is needed to fully understand the safety and efficacy of *C. caesia*, but it holds promise as a new natural alternative for individuals with hyperglycemia.

C. caesia remains a relatively under-researched plant compared to traditional turmeric, and this study delves into its potential as a source of natural antihyperglycemic agents. Furthermore, this research goes beyond simply confirming activity. By investigating the enzyme kinetics of these fractions, the study aims to elucidate the specific mechanisms in future by which they influence blood sugar control. This deeper mechanistic understanding could pave the way for the development of more targeted therapies in the future. Additionally, optimizing the yield of these terpenoid fractions is crucial for their potential application in clinical settings.

This study advances previous research on *C. caesia* by not only identifying the mode of enzyme inhibition but also analyzing the enzyme kinetics of its extracts. The aim is to provide a deeper understanding of how *C. caesia* exerts its antihyperglycemic effects, paving the way for its potential clinical application in managing diabetes [14,15]. By elucidating the mechanistic insights, this study can contribute to developing targeted therapeutic strategies that utilize *C. caesia* as a complementary or alternative approach to conventional treatments, especially for individuals seeking cheaper natural options with fewer adverse effects [16]. Additionally, the study examines the impact of drying temperatures on the efficacy of *C. caesia* extract, a previously unexplored aspect [17]. This comprehensive evaluation contributes to optimizing *C. caesia* for health maintenance and offers new avenues for preventing, diagnosing, and treating both physical and mental health conditions related to diabetes.

2. Methodology

2.1 Preparation of Curcuma caesia

C. caesia required for drying was collected from a local farmer. The weights of the rhizomes were 10 g. Drying of turmeric was carried out in an oven drying. Samples were spread uniformly on the trays in a single layer. Two separate portions of the rhizome, each weighing 10 grams, were dried in ovens. One portion (CC50) was dried at 50°C, and the other portion (CC60) was dried at 60°C.

2.2 Extraction of Dried Plant Samples using the Reflux Method

10g of *C. caesia* rhizomes were weighed and added to a round-bottom flask containing 100 mL of 50% (v/v) methanol solution. The rhizomes were then submerged in the solution. The ratio of solvent and plant material was 10. After 2 hours of reflux at heat 64.7°C, the heat was turned off and the

sample was concentrated in a rotary evaporator with the rotation speed around 50-70 rpm, temperature around 35-45°C, and pressure around 10-20 mbar. The sample was filtered and only the filtrate was collected. *C. caesia* crude extract was dried in a drying oven at 50°C until the weight was constant around 0.01g (CC50) and 0.02g (CC60). After that, the *C. caesia* crude extract was stored at 4°C. Storing the extract at 4°C helps minimize potential degradation of the active compounds in the extract. Many natural products, including plant extracts, contain heat-sensitive bioactive compounds and heat can accelerate reactions that might break down these compounds [18].

2.3 Antihyperglycemic Activity of Curcuma caesia, Kinetics and Mode of Inhibition

To analyze the antihyperglycemic activity of *C. caesia*, a 10% (w/v) of sample was prepared by dissolving in 200µl pure water. Then add 200µl of 0.5 mg/mL of alpha-amylase. Then incubate for 10 minutes followed by the addition of 200µl of 1% (w/v) starch. After that, DNS reagent was added. Then incubate for 10 minutes at 90°C and add 5mL water. Analyze it with UV-spectrophotometer at 540nm. Glucose was used as standard to construct standard calibration curve producing Eq. (1) as follows:

$$\mathrm{y} = 0.0007\mathrm{x} + 0.062 \tag{1}$$

To study on the enzyme kinetics and the mode of inhibition, the same method was employed by replacing 200µl of *C. caesia* extract with pure water. A graph of product rate versus concentration of substrate was plotted, and the Michaelis constant (K_m) was determined by using the Michaelis Menten enzyme kinetics equation [19] as in Eq. (2) as follows:

$$\mathrm{v}_0 = \frac{\mathrm{V_{max}}[\mathrm{S}]}{\mathrm{K_m}+[\mathrm{S}]} \tag{2}$$

where v_o is velocity of reaction, V_{max} is maximum rate of reaction, K_m is Michaelis constant and [S] is substrate concentration.

3. Results

3.1 Inhibition of Alpha-Amylase by Curcuma caesia Extracts

Through this experiment, *C. caesia* was extracted with a polar solvent which contained 50% (v/v) methanol in water. The solvent was used to extract the polar constituent from *C. caesia* (in the filtrate) as its non-polar constituent remains in the discarded retentate. The purpose of this filtration is to isolate polar compound from its non-polar counterpart. After undergoing reflux extraction, the *C. caesia* extract was dried in two different temperature which are 50˚C (denoted as CC50) and 60˚C (denoted as CC60). It was observed that higher temperature applied reduce the drying duration by 50% as constant weight was achieved after 2 hours of drying. On the other hand, CC50 takes 4 hours to achieve a constant weight after drying.

The aim of this study was to identify the ability of CC50 and CC60 to inhibit alpha-amylase activity. Alpha-amylase is responsible to convert starch forming reduced sugar [19,20]. Inhibition of alpha-amylase will reduce the formation of reduced sugar in a solution. The performance of both extracts will be compared, and its effectiveness will be determined based on its Minimal inhibitory concentration (IC_{50}) value. The IC_{50} is quantitative measure that indicate how much of a particular inhibitory substance is needed to inhibit, in-vitro, a given biological process or biological component by 50%.

DNS method was used to quantify the reduced sugar due to the conversion of starch by alpha-amylase. The colour changes were analysed using a UV-Vis Spectrometer where the function measures the intensity of light before and after it passes through the sample. Higher absorbance indicates higher reduced sugar content present in the sample [21]. The colour of the DNS reagent changed from yellow to orange or red for its reaction with reduced sugar. On heating with reducing sugars, the 3-nitro (NO_2) group of DNS is reduced to an amino (NH_2) group [22].

According to Table 1 the absorbance value of the CC50 decreases as the concentration of CC50 sample increases. The decrement also indicates that less conversion of starch to reduced sugar occur as more concentrated samples were added. This also indicates that CC50 inhibited alpha-amylase thus reducing the conversion of starch to glucose. The glucose concentration was calculated using Eq. (1) and was expressed in glucose equivalent (GE) as tabulated in Table 1.

Table 1
Glucose concentration for CC50

Concentration of CC50 (ppm)	Absorbance	Glucose equivalent (GE) concentration (ppm)
0	0.310	354.29
39.06	0.277	307.14
78.13	0.189	181.43
156.25	0.157	135.71
312.50	0.163	144.29
625.00	0.160	140.00
1250.00	0.158	137.14
2500.00	0.157	135.71
5000.00	0.167	150.00
10000.00	0.153	130

A similar trend was observed on CC60 which increment of CC60 concentration reduces the conversion of starch to sugar by alpha amylase. The alpha- amylase activity was inhibited by CC60. The glucose concentration was calculated using Eq. (1) and was expressed in glucose equivalent (GE) as tabulated in Table 2. The inhibitory activities of both CC50 and CC60 were due to its high antioxidant capacity thus also contributing to its high antioxidant capacity of the methanolic extracts [23]. The presence of phenolic acid interferes the interaction of the enzyme and its substrate (starch) resulting in the inhibition effect on the alpha-amylase [24]. Phenolic acids interact with starch digestive enzymes through non-covalent interactions, which are responsible for their inhibitory activity [25]. Flavonoids, which exhibit strong antioxidant activity, were suggested to be beneficial in managing diabetes mellitus. Antioxidants' capacity to protect against the harmful effects of hyperglycemia and to improve glucose metabolism and uptake positions them as a promising alternative for diabetes treatment [26].

The data in Tables 1 and 2 were then used to determine the percentage (%) of inhibition of the alpha-amylase activity. Table 3 shows the glucose concentration and inhibition of alpha-amylase by CC50. The CC50 sample showed that it can achieve inhibition up to 63% of inhibition only with a concentration of 10000.00 ppm. To determine the IC_{50} value of CC50, a graph of percentage of inhibition versus concentration of CC50 was plotted (Figure 1).

Table 2
Glucose concentration for CC60

Concentration of CC60 (ppm)	Absorbance	Glucose equivalent (GE) concentration (ppm)
0	0.341	398.57
39.06	0.233	244.29
78.13	0.201	198.57
156.25	0.163	144.29
312.50	0.169	152.86
625.00	0.168	151.43
1250.00	0.176	162.86
2500.00	0.157	135.71
5000.00	0.157	135.71
10000.00	0.152	128.57

Table 3
Inhibition versus concentration of CC50

Concentration of CC50 (ppm)	Inhibition (%)
0	0
39.06	13.33
78.13	4.21
156.25	5.21
312.50	7.14
625.00	9.90
1250.00	4.21
2500.00	28.35
5000.00	57.67
10000.00	63.30

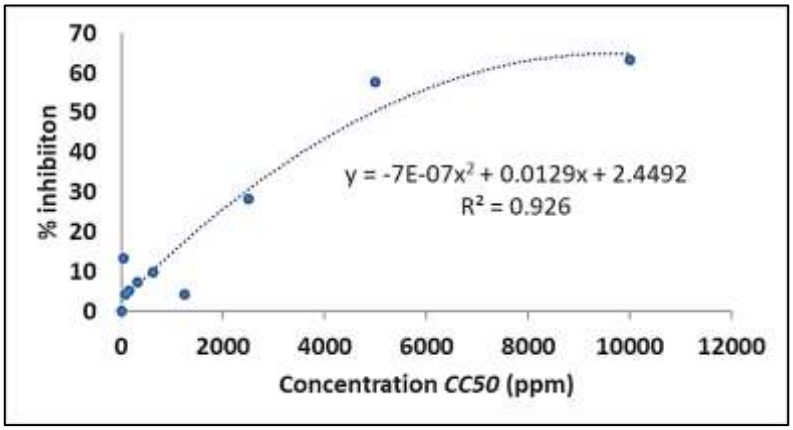

Fig. 1. Graph percentage inhibition versus concentration of CC50

The Eq. (3) generated from the graph was used to calculate the IC_{50} value. It was found that the IC_{50} value for CC50 sample was 5094.41 ppm. The equation is as follows:

$$y = -0.0000007x^2 + 0.0129x + 2.4492 \quad (3)$$

Table 4 shows the glucose concentration and inhibition of alpha-amylase by CC60. The CC60 sample showed that it can achieve inhibition up to 68% of inhibition only with a concentration of 10000 ppm. The graph of percentage of inhibition versus concentration of CC60 was plotted (Figure 2). The Eq. (4) generated from the graph was used to calculate the IC_{50} value for CC60 sample. It was found that the IC_{50} value for CC60 sample was 5083.07 ppm. Eq. (4) is as follows:

$$y = -0.0000004x^2 + 0.0104x + 7.4711 \quad (4)$$

Table 4
Inhibition versus concentration of *C. caesia* for 60 °C

Concentration of *C. caesia* (ppm)	Inhibition (%)
0	0
39.06	5.26
78.13	5.26
156.25	21.05
312.50	15.09
625.00	15.89
1250.00	10.89
2500.00	35.25
5000.00	47.37
10000.00	67.74

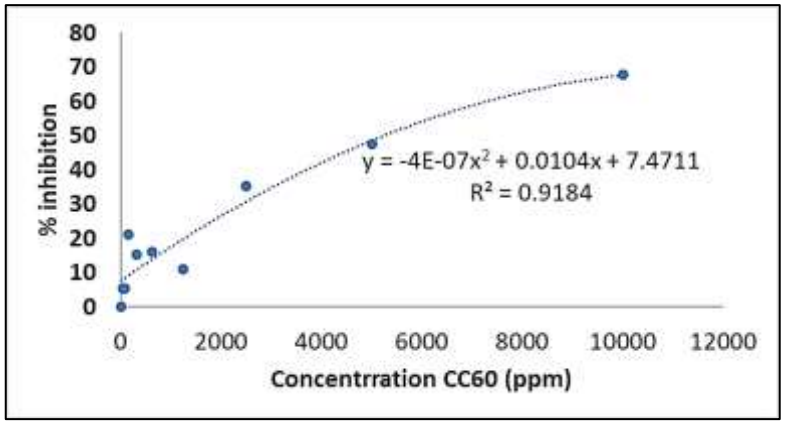

Fig. 2. Graph percentage inhibition versus concentration of CC60

Based on the obtained IC_{50} value, the performance of CC50 and CC60 can be determined. It was found that the performance of CC60 sample better as it possesses smaller IC_{50} value (5083.07 ppm) compared to CC50 sample (5094.41 ppm). This indicates that CC60 sample is more effective in inhibiting the alpha-amylase activity as smaller concentration of sample is required to inhibit the alpha-amylase activity by 50%. The reasons behind the higher IC_{50} value of CC50 was due to longer drying time (50% longer) were required to achieve a constant weight. Longer drying duration caused the degradation of bioactive compounds due to the exposure to free oxygen. Longer exposure to oxygen caused the polar compounds from phenol and flavonoid groups to be easily reducible. Higher drying temperature reduces the exposure to free oxygen thus causing less degradation of these bioactive compounds [17]. The slight difference in the IC_{50} value of both CC50 and CC60 samples also proved that 50°C and 60°C were still suitable to be used in the drying process. This is because, at these temperatures, the *C. caesia*'s bioactive compounds especially its polar constituents were still preserved and still able to inhibit the alpha-amylase activity.

3.2 Enzyme Kinetic and Mode of Alpha-Amylase Inhibition by Curcuma caesia

To determine the mode of inhibition of alpha-amylase by CC50 and CC60, a graph product rate against substrate concentration (Figure 3) was constructed by using data tabulated in Table 5 in which the rate of reaction of alpha-amylase without the presence of *C. caesia* (uninhibited) was included.

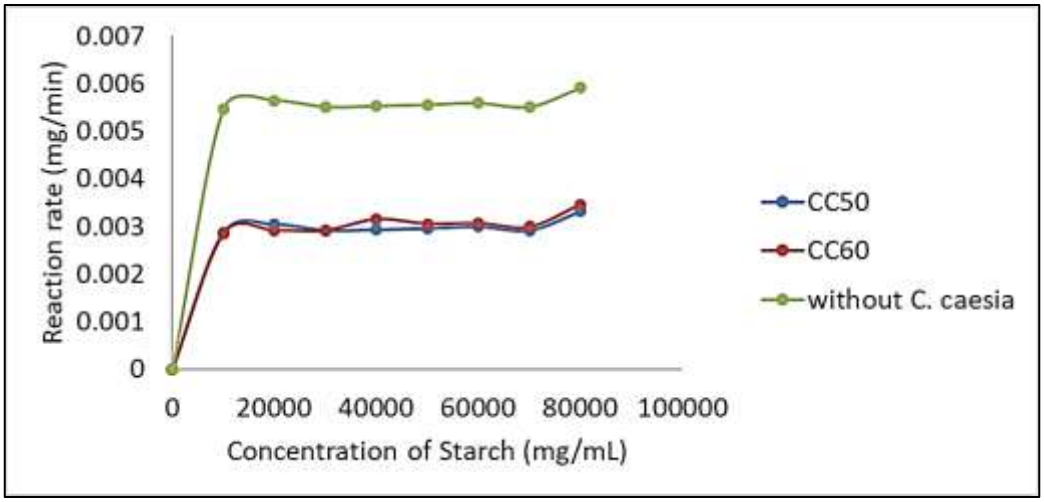

Fig. 3. Graph of product rate (mg/min) versus concentration of starch (mg/ml) for CC50, CC60 and without *C. caesia* extract

Table 5
Product rate versus concentration of starch with *C. caesia*

Concentration of Starch (mg/mL)	Product rate for CC50 (mg/min)	Product rate for CC60 (mg/min)	Product rate without *C. caesia* (mg/min)
0	0	0	0
10000.00	0.0029	0.00285714	0.00547826
20000.00	0.0030	0.00291925	0.00565217
30000.00	0.0029	0.00291925	0.00552174
40000.00	0.0029	0.00315528	0.00553913
50000.00	0.0029	0.0030559	0.00556522
60000.00	0.0030	0.00306832	0.00560000
70000.00	0.0029	0.00299379	0.00552174
80000.00	0.0033	0.00346584	0.00592174

According to Figure 3, it was observed that the V_{max} for both CC50 and CC60 is 0.0025 mg/min. Whereas, the V_{max} for uninhibited reaction is higher which is 0.0055 mg/min. For this it is concluded that the mode of inhibition is a non-competitive inhibition as the V_{max} reduced after treated with CC50 and CC60 [27,28]. To further elucidate on this conclusion, the Michaelis constant (K_m) was calculated using the Michaelis-Menten enzyme kinetics as in Eq. (2). Based on the obtained V_{max} values, the calculated K_m is tabulated in Table 6.

Table 6
Michaelis constant of CC50, CC60 and without *C. caesia*

Sample	K_m
CC50	9041.7638
CC60	9063.5348
Without *C. caesia* sample	8968.9437

Based on Table 6, the obtained K_m values supported that the mode of alpha-amylase inhibition by CC50 and CC60 where a non-competitive type as the K_m values were almost unaltered [29,30]. A non-competitive inhibition is a specific type of enzyme inhibition characterized by an inhibitor binding to an allosteric site resulting in decreased efficacy of the enzyme. A non-competitive inhibition is a reversible inhibition [29]. A reversible inhibition does not cause the change of enzymes

conformation and active site permanently. A non-competitive inhibition will not be affected by the increment of the substrate concentration [20].

4. Conclusions

The inhibitory concentration (IC_{50}) values for CC50 and CC60 were 5083.07ppm and 5094.41ppm, respectively. This indicated that CC60 was slightly more effective in inhibiting alpha-amylase activity. Although the difference is minor, it is noteworthy that higher drying temperatures are preferred to reduce the exposure of free oxygen, which may degrade bioactive compounds in the extract during the drying process. The kinetics and mode of inhibition for both CC50 and CC60 were non-competitive, as indicated by a higher V_{max} compared to the uninhibited counterpart. This was also supported by the Michaelis-Menten kinetics equation, where the calculated K_m remained constant for both inhibited and uninhibited reactions. The constant K_m value further indicates a non-competitive inhibition type. The bioactive compounds responsible for this effect are likely due to the presence of polar constituents such as phenolics and flavonoids, which were extracted using a polar solvent in this study enhancing their efficacy in inhibiting alpha-amylase activity. This comprehensive mechanistic insight is essential for devising targeted therapeutic strategies utilizing *C. caesia* to manage diabetes and emerges as a promising natural supplement for controlling hyperglycemia, potentially providing a complementary approach to conventional treatments.

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