Nota Técnica

ASSESSMENT OF α-AMYLASE INHIBITION ACTIVITY BY AN OPTIMIZED AND VALIDATED *IN VITRO* MICROSCALE METHOD

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Metabolic disorders, including hyperglycemia, characterize type-2 diabetes. One of the treatment methods used for postprandial hyperglycemia includes using potential therapeutic agents to inhibit α-amylase activity. This study utilized fractional design and the simplex method to optimize in vitro microscale assay inhibition conditions using Miller's reaction. In addition, the effect of substrate concentration on enzyme activity was analyzed. Enzyme concentration of 0.15 U mL^{-1} and pre- and post-incubation times of 7.2 and 5.5 min, respectively, in water bath (15.6 min) equipment, were set up for optimized condition for the enzyme activity. Analytical validation was performed based on different international guidelines. K_m was found to be 0.38 mg mL^{-1}. Linearity was obtained at the acarbose concentration of 1.5 µg mL^{-1} and 5 µg mL^{-1}. The IC$_{50}$ for the positive control was found to be 0.6 µg mL^{-1}. The relative standard deviation and Z value were found to be <4% and >0.93, respectively. Additionally, the optimized assay was applied to extracts from five different plants. Two plant extracts (*Zanthoxylum fagara* and *Chrysactinia mexicana*) inhibited α-amylase activity. The optimized and validated method was accurate, precise, and linear.

Keywords: optimization of enzyme method; validation of enzyme method; α-amylase activity; design of experiments.

INTRODUCTION

Glycosidase enzymes breakdown carbohydrates into simple sugars they enter the bloodstream, producing postprandial hyperglycemia. A state of sustained hyperglycemia caused by insulin deficiency can lead to type 2 diabetes.[1] This state of hyperglycemia can cause complications such as nephropathy, retinopathy, atherosclerosis, and memory impairment.[2,3]

Postprandial hyperglycemia can be controlled by inhibitors, such as those directed at inhibiting α-amylase, which reduces the flow of glucose by digesting carbohydrates. α-Amylase is an endoglycosidase that catalyzes the cleavage of internal α-D-(1-4) glycosidic bonds in starch to produce oligosaccharides, dextrins, and maltose.[4-6]

Acarbose and miglitol are conventional inhibitors used to inhibit the activity of enzymes. These inhibitors delay the rate of absorption of glucose, thereby maintaining glucose levels in hyperglycemic individuals. However, the use of inhibitors is associated with certain side effects, such as diarrhea and other intestinal disturbances.[1,7] Hence, it is necessary to look for inhibitors of α-amylase from natural sources with fewer side effects.

Selected plant products could provide better alternatives due to their low cost and safer use. Plant extracts contain various phytochemicals, such as phenols, saponins, and alkaloids, that contribute to many observed pharmacologically relevant effects. To search for new compounds, it is necessary to carry out pharmacological screening for a large number of potential α-amylase inhibitors in a short period, for which high-throughput screening can be successfully used.[8]

The use of a microscale method offers several advantages such as 1) fewer chemicals needed, 2) reduction in material cost, 3) ease of disposal of the reaction mixture after experimentation, 4) reduction of the safety risk, and 5) multiple experiments can be performed at the same time. Because the determination of sugars is performed by reducing the aldehyde group of the sugar with 3,5-dinitrosalicylic acid

(DNS) to form 3-amino-5-nitrosalicylic acid, the reaction involves high temperatures, making the determination on microplates difficult.[9] Although other protocols are based either on the glucose oxidase reaction or on the determination by fluorescence, they are commonly used in microplates.

To achieve a good assay, there must be good agreement between the *in vivo* and *in vitro* results for the evaluated substance. Additionally, analytical validation of the method it requires using appropriate positive and negative controls. Positive controls are compounds with proven activity in humans, whereas negative controls are those without activity. In addition, the assay method could be applied to a wide variety of molecules, and the obtained results should be reproducible and achieve the same results every time.[10]

Unfortunately, to date, many published studies cannot be reproduced; most of them are nonreplicable or simply erroneous. In general, they have a poor design of experiments and an inadequate statistical significance.[11] Bioassays are challenging in terms of determining accuracy. Hence, the comparison of the two assays becomes difficult. Precision can be compared by replication of the assays, but precision is not directly transferrable to accuracy.[12]

Hence, it is absolutely necessary to optimize an assay method to achieve acceptable performance, with low variability and high signal with respect to noise.[13] Additionally, it is necessary to validate the assay method to demonstrate that it is acceptable for determining the pharmacological activity of a new chemical entity by a detectable signal that allows the biological process to be quantified. Currently, there are different protocols and guidelines to perform the process of bioassay validation, such as Food and Drug Administration, USA (2018), Eli Lilly Guide (2007), and the 1033 Chapter of United States Pharmacopeia (1980).[14-16] These protocols and guidelines are followed in this work.

This work addresses optimization, validation and application of an *in vitro* method for assessment of α-amylase inhibition on a microscale level.

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EXPERIMENTAL

Materials

α-Amylase from the porcine pancreas (15 U mg⁻¹) was used instead of human amylase because of its cost and availability. Starch, acarbose, 3,5-dinitrosalicylic acid (DNS), sodium chloride, dibasic sodium phosphate, monobasic sodium phosphate, sodium, and potassium tartrate were procured from Sigma-Aldrich, USA. Sodium hydroxide was procured from CTR, México.

Plant extracts

Aerial parts of plants *Zanthoxylum fagara, Chrysactinia mexicana, Teucrium bicolor*, and *Ricinus communis* and roots of *Jatropha dioica* were used for the analysis. The extracts were prepared using different solvents: methanol for aerial parts of the plant and a mixture of ethanol and water (90:10) for roots. These plant extracts were offered by researchers from the Analytical Chemistry Department (Facultad de Medicina, UANL, México).

Enzyme assay and enzyme kinetics

The α-amylase activity was assessed by Miller's method by estimating the colored product formation by the action of enzyme and substrate starch (Scheme 1).[9]

In a tube containing 1 mL of phosphate buffer (100 mmol L⁻¹, pH 6.9), 100 μL of α-amylase (final concentration 0.15 U mL⁻¹ in phosphate buffer) was added and incubated at 37 °C for 7.2 min for preincubation. After preincubation, 100 μL of starch was added (final concentration 0.38 mg mL⁻¹) and incubated at 37 °C for 5.5 min for enzymatic action. The reaction was stopped by adding 200 μL of DNS (final concentration 19.2 mmol L⁻¹) and boiling in a water bath for 15.6 min. Later, the mixture was cooled, and 100 μL of this solution was transferred to a 96-well plate and diluted with 100 μL of water. Absorbance was recorded at 540 nm in a microplate reader. Enzyme activity is defined as the amount of product formed in 1 min under defined conditions, and its unit is μmoles min⁻¹.

The kinetic constants for the enzyme were determined by varying the substrate concentration from 0.03 mg mL⁻¹ to 0.6 mg mL⁻¹. The abovementioned protocol was followed with different concentrations of substrates. The absorbance of the product was recorded at 540 nm. The kinetic constant maximum velocity (Vmax) and the Michaelis-Menten constant (K_m) were obtained from the Lineweaver-Burk and Michaelis-Menten plots, respectively. The K_m value is the substrate concentration needed to reach half the maximum velocity. All experiments were performed in triplicate.

Inhibition studies

Acarbose was used as an enzyme inhibitor. Different concentrations of acarbose (0.5 to 12 μg mL⁻¹) were added to the enzymatic solution, and an enzyme assay was performed as mentioned above. One negative control (absence of inhibitor) was set up to obtain 100% enzyme activity. The inhibition percentage was calculated using Equation 1:

$$\% \text{ Inhibition} = 100\left(1 - \frac{B}{A}\right) \qquad (1)$$

where "*A*" is the absorbance of the negative control, and "*B*" is the absorbance of the solution containing an inhibitory agent. The positive control used was acarbose (0.6 μg mL⁻¹).

The Miller's reactions were performed in 13×100 test tubes. The absorbance was measured using 96-well polystyrene plates with a flat bottom and a final volume of 200 μL. Absorbance was recorded using a microplate reader (Multiskan FC, Thermo Scientific).

Optimization studies

The design of experiments was performed using the fractional factorial of half fraction (2^6-1) method without blocks, with a random central point and one replicate. Six factors (i.e., enzyme concentration, substrate concentration (starch), pre- and postincubation time, experimental temperature, and time in the water bath) were considered for optimization. A total of 64 experiments were performed. Previously reported highest and lowest values of optimization experiments were used. The experimental design and data handling were performed using Statgraphics Centurion XVI software (Statpoint Technologies, Inc., Warrenton, VA, USA). Factors
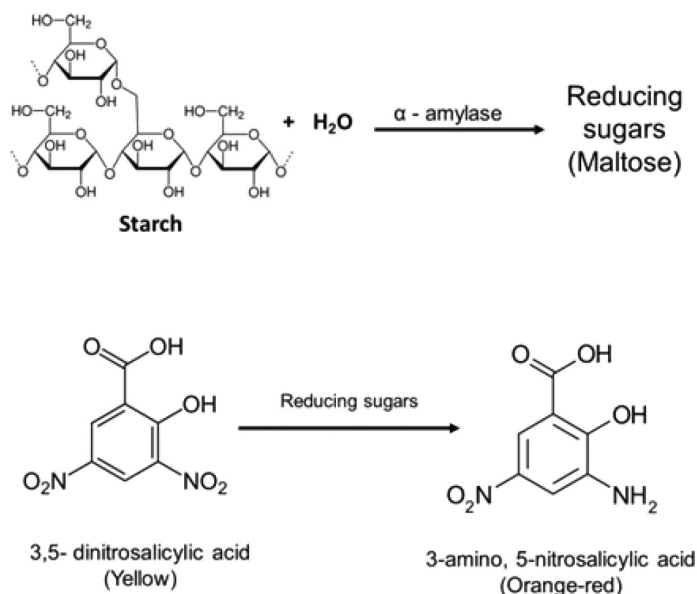
Scheme 1. *Reaction of the α-amylase inhibition assay. First, the reaction is performed in which the starch is reduced and, by the action of α-amylase, reducing sugars are generated. Second, Miller's reaction between DNS and reducing sugars produces ANSA*

significantly contributing to the response were selected by the Pareto graph. Additionally, the significant factors were optimized using the rules of the basic sequential simplex method and the combination of conditions that maximized the inhibition percentage with the lowest photometric error.

Analytical validation

Validation of the method was performed according to international guidelines by analyzing linearity, precision, accuracy, robustness, plate uniformity, and signal variability.[14-17]

Linearity

To evaluate linearity, amylase inhibition studies were performed using different concentrations of the inhibitor acarbose (0.5 to 12 µg mL^{-1}). The inhibition percentage was calculated. The graph of inhibition percentage *vs.* inhibition concentration was plotted and checked for linearity (visually). The results were subjected to regression analysis to determine R^2 and the regression equation for the plot.

The matrix effect was evaluated by plotting the inhibition percentage as a function of different concentrations of acarbose inhibitor (0.5 to 12 µg mL^{-1}). These solutions were added to an extract of *J. dioica* possessing zero inhibitory activity. The results were subjected to regression analysis to determine R^2 and the regression equation for the plot. The slopes of these two were compared to determine linearity using the t-test.

Precision and accuracy

Evaluation of precision and accuracy was performed using the extracted sample of *J. dioica* in the presence or absence of acarbose (positive control). Three different concentrations (1, 2.5, and 5 µg mL^{-1}) were used, and the experiment was conducted in triplicate.

The precision of the method was analyzed on the day of experimentation and on two different days. Standard deviation was calculated, and the relative standard deviation in terms of percentage was determined (Equation 2) to check the precision values on the experimentation day and other different days.

$$\% \text{ RSD} = \left(\frac{SD}{AVG} \right) \times 100 \qquad (2)$$

where "SD" is the standard deviation of each measurement, and "AVG" is the average of such measurements.

The accuracy of the method was determined by calculating the percentage of relative error (%) (Equation 3) of the extracts of *J. dioica* challenged with the inhibitor at different concentrations.

$$\% \text{ Error} = \left(\frac{X_i - X_t}{X_t} \right) \times 100 \qquad (3)$$

where "X_t" is the added concentration of acarbose (the expected or true value of acarbose) and "X_i" is the experimentally determined concentration of acarbose.

Robustness

Different experiments were performed to evaluate the robustness of the method. Experiments included different concentrations of the enzyme (0.1 and 0.2 U mL^{-1}), preincubation time (5.2 and 9.2 min), incubation time (4.5 and 6.5 min), and time in a water bath (10.6 and 20.6 min). Additionally, different solvents, such as ethanol (25%) and dimethyl sulfoxide (DMSO) (final concentrations of 0.15% and 0.25%), were included. Tukey's test ($\alpha = 0.05$) was used to evaluate the robustness of the individual methods.

Assessment of plate uniformity and signal variability

Plate uniformity assessment is mandatory for all assays. A uniformity assay is performed at maximum and minimum response levels, ensuring that the difference is adequate to detect active compounds or the influence of the inhibitor in the assay system.

The variability test is conducted at three response levels with varied signal intensities.[15]

- Max signal: The maximum absorbance was recorded by the assay procedure. For enzyme inhibition studies, the absorbance was recorded at a lower concentration of inhibitor (IC$_{50}$). In this experiment, acarbose at a concentration of 1.05 µg mL^{-1} was used to record the desired maximum signal.
- Mid signal: Mean absorbance was recorded by the assay procedure. For enzyme inhibition studies, the absorbance was recorded at a concentration with 50% inhibition (IC$_{60}$). In this experiment, acarbose at a concentration of 2.5 µg mL^{-1} was used to record the desired mean or mid signal.
- Min signal: The minimum absorbance was recorded by the assay procedure. For enzyme inhibition studies, the absorbance was recorded at a higher or maximum concentration of inhibitor (IC$_{70}$). In this experiment, acarbose at a concentration of 5 µg mL^{-1} was used to record the desired minimum signal.

The experiments were performed in a 96-well plate. The solution with varying concentrations of acarbose was placed in 32 wells in three different plates. In each plate, the location of each solution was changed with respect to its column or row of wells. For each experiment, fresh and independent reagents were used, and the evaluation was performed on three different days. The % RSD was calculated for each solution. The Z factor was computed for each plate (Equation 4).

$$Z = \frac{\left(AVG_{max} - 3SD_{max} \div \sqrt{n} \right) - \left(AVG_{min} + 3SD_{min} \div \sqrt{n} \right)}{AVG_{max} - AVG_{min}} \qquad (4)$$

where "AVG_{max}" is the average of the maximum absorbance values, "SD_{max}" is the standard deviation of the maximum absorbance values, "AVG_{min}" is the average of the minimum absorbance values, "SD_{min}" is the standard deviation of the minimum absorbance values, "n" is the number of tests performed.

Statistical analysis

Minitab 17 (Minitab, Inc., USA) was used for data analysis. Kolmogorov–Smirnov and Tukey tests were performed, and P < 0.05 was considered significant. Statistical analysis and validation were performed using Microsoft Excel 2010.

Application

Five plant extracts were used to determine α-amylase inhibitory activity using the proposed method. The plant extract solutions were in the concentration range of 6 µg mL^{-1} to 200 µg mL^{-1}. The optimized and validated method was very carefully followed.

One milligram of each extract was weighed and dissolved in 1 mL of phosphate buffer (100 mmol L^{-1}, pH 6.9); some solutions were sonicated to achieve complete dissolution. To each tube, 100 µL of plant extracts with different concentrations (final concentration 6 µg mL^{-1} to 200 µg mL^{-1}) and 100 µL of α-amylase (final concentration 0.15 U mL^{-1} in phosphate buffer) were added and incubated at 37 °C for 7.2 min. Then, 100 µL of starch was added (final concentration 0.38 mg mL^{-1}) and incubated at 37 °C for 5.5 min. The enzymatic reaction was stopped by adding 200 µL of DNS (final

concentration 19.2 mmol L^{-1}) and subsequently placed in a boiling water bath for 15.6 min. Tubes were cooled, and 100 μL of the mixture was transferred to a 96-well plate and diluted with 100 μL of water. Absorbance was recorded at 540 nm using a microplate reader. In all cases, a sample blank was analyzed to demonstrate that there were no matrix interferences caused by the extract.

RESULTS AND DISCUSSION

Previous studies citing the assays of *in vitro* antihyperglycemic activity measurements, including detailed procedures and the use of positive controls, were collected and analyzed (Table 1). This evaluation revealed that "minor modifications/changes" in the assay conditions can lead to a significant variation in IC$_{50}$ values (0.00068 to 47.5 mg mL^{-1}) with acarbose used as a positive control. This discrepancy highlights the reason for not comparing the results obtained by different reports, although they might have used the same method but with different experimental conditions. Comparison of such results will lead to low reliability of these *in vitro* methods.

This study optimizes and validates microscale *in vitro* methods for the assessment of α-amylase inhibition. In the present method of Miller's reaction, the product to be analyzed was obtained by heating it in a boiling water bath. Because it is impossible to perform boiling in a microplate because a significant volume of liquid evaporates and negatively affects the response, the experiment was conducted in the tubes, and the microquantities were transferred to microplates and recorded.

Enzyme kinetics and inhibition studies

The α-amylase assay was performed using Miller's method, i.e., the DNS method. This method is a redox reaction where DNS (yellow color) is reduced by reducing sugars to 3-amino-5-nitrosalicylic acid (red color) in an alkaline medium. Due to

the presence of free carbonyl groups in sugars, they can reduce DNS and are oxidized to carboxyl groups. The reduced 3-amino-5-nitrosalicylic acid is red–brown in color and can be quantified spectrophotometrically with an absorption maximum at 540 nm. The higher is the intensity of color, the higher is the concentration of reducing sugars in the solution.

Acarbose, a positive control, was used as an inhibitor for this experiment. It is a reversible, competitive inhibitor. Because acarbose is structurally similar to the substrate (starch), it competes for the active site on the enzyme. When the substrate concentration is increased, the inhibition by the competitive inhibitor is reversed. The degree of inhibition was evaluated in terms of the concentration of the inhibitor that inhibited half of the enzyme activity (IC$_{50}$). When the enzymatic activity decreases, the formation of the product also decreases; therefore, the intensity of the color is reduced. In this study, the IC$_{50}$ was determined following the method described by Tundis *et al.*, and an IC$_{50}$ value of 0.6 μg mL^{-1} was obtained.[26]

Statistical optimization

Optimization of the α-amylase inhibition assay was performed using fractionalized factorial design (2^6-1). Different parameters, such as enzyme concentration, substrate concentration (starch), preincubation time, incubation time, incubation temperature, and time in the water bath, were evaluated. The Pareto diagram (Figure 1) shows that some factors exhibit a significant effect on the response. The enzyme concentration, incubation time, interaction between enzyme concentration and preincubation time, and the interaction between preincubation and boiling time showed a significant effect on the inhibition percentage. An independent parameter, i.e., enzyme concentration, has a significant effect because as the enzyme concentration increases, the enzyme activity increases up to a certain level and then decreases. However, because the incubation temperature is increased, more reactions between the enzyme and

Table 1. α-Amylase method references

Reference	Enzyme (U mL^{-1})	Starch (mg mL^{-1})	Buffer (M)	pH	Temperature (°C)	Incubation time (min)	Boiling at 100 °C time (min)	λ (nm)	DNS Mm	IC$_{50}$ (mg mL^{-1}) Acarbose
18	0.52	0.4	0.02	6.9	25	10	5	540	n.i.	0.00068
19	0.2	0.4	0.02	6.9	25	10	5	540	n.i.	0.00156
20	n.i.	n.i.	n.i.	n.i.	37	5	15	540	n.i.	0.00182
21	1	1	0.02	6.9	25	10	5	540	96	14.9
22	0.11	0.22	0.02	6.9	25	10	5	540	96	47.5
23	n.i.	n.i.	0.02	6.9	25	10	5	540	n.i.	< 0.03228
24	0.14	0.29	0.02	6.9	25	10	5	540	n.i.	< 0.15
25	0.03	0.07	0.02	6.9	25	30	5	540	n.i.	> 1.0

(DNS): 3,5-dinitrosalicylic acid; (IC$_{50}$): medium inhibitory concentration; (n.i.): not indicated.
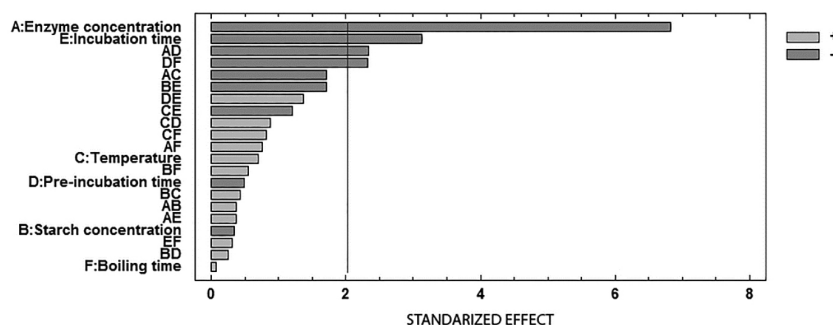

Figure 1. *Pareto chart for different factors affecting the response*

substrate occur. Thus, it is important to optimize these two parameters to obtain a fixed value.

Factors showing a significant effect on the inhibition percentage were selected and optimized using a basic sequential simplex method. An inhibition percentage near 50% was chosen as the best response, while the absorbance of the control at 0% inhibition (100% activity) resulted in the smallest photometric error. Ten different experiments were performed. The optimum conditions used were an enzyme concentration of 0.15 U mL^{-1}, preincubation and incubation times of 7.2 min and 5.5 min, respectively, and a water bath duration of 15.6 min; all abovementioned conditions produced the best inhibition percentage.

Determination of kinetic constants was performed by determining the effect of substrate concentration on enzymatic activity. Lilly (2007) recommended the use of the substrate at or below the K_m value. A higher substrate concentration (more than K_m) will interfere with the competitive inhibitor present in the extract and hinder the identification of the inhibitor in the extract. The optimum substrate concentration for the α-amylase inhibition assay was found to be 0.38 mg mL^{-1} (1.11 mmol L^{-1}). Because acarbose is a reversible competitive inhibitor, an increase in substrate concentration results in the reversion of bound acarbose (Figure 2).
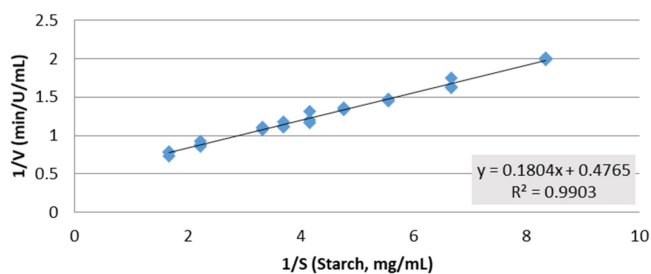

Lineweaver–Burk Plot
α-amylase 0.15 U/mL

$y = 0.1804x + 0.4765$
$R^2 = 0.9903$

*Figure 2. Lineweaver-Burk plot to determine K_m*

Analytical validation

Recommendations and criteria presented by various international guidelines were followed to validate the optimized media.[14-16,27] To evaluate the potentiality of any compound in the biological assay, linearity must be measured by the capability of the test procedure (within a given range) to produce a reproducible result (percentage of inhibition), which is directly related to the concentration (or amount) of the test compound.

Acarbose, an inhibitor of the amylase enzyme, was evaluated at six different concentration levels by regression analysis. Linearity was determined using the graph of concentration and percent inhibition. The linear range of amylase inhibition by acarbose was 1.5-5 µg mL^{-1}, with a regression equation of y = 7.5784x + 37.80, a determination coefficient of 0.9906, and an IC$_{50}$ of 1.61 ± 0.06 µg mL^{-1} (Figure 3).

A frequent problem associated with plant product analysis is the matrix effect. It is defined as the combinatorial effect of all components of the analysis other than the analyte that is to be measured.[28] When an analytical method is considered, the matrix effect results in variation in the sensitivity, which is manifested by comparing the calibration curve of the standard (positive control) with that of the sample (plant extract). The sensitivity is the slope of the calibration curve, which is a function of the analyte concentration. Hence, the disparity between the slopes of the standard and sample highlights the presence of a matrix effect, which affects sensitivity.

Percentage of Amylase activity in the presence of acarbose

$y = -7.5784x + 62.198$
$R^2 = 0.9906$
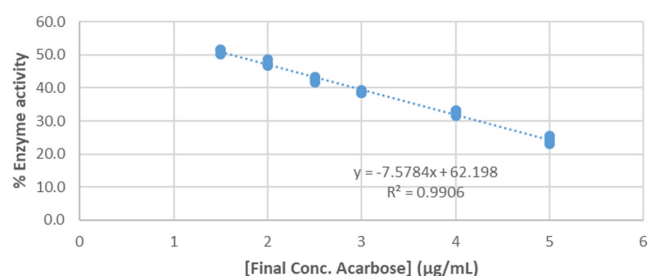
Percentage of inhibition of acarbose on amylase activity
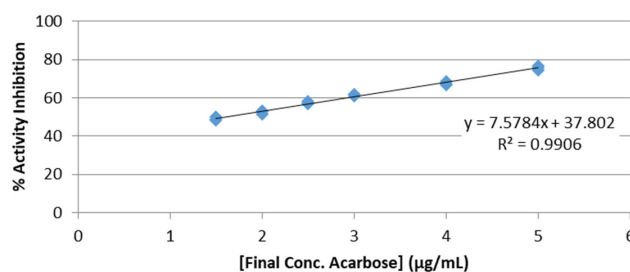
$y = 7.5784x + 37.802$
$R^2 = 0.9906$

Figure 3. Inhibition percentage of α-amylase by acarbose

The evaluation of the matrix effect showed linearity at 1-5 µg mL^{-1}, a determination coefficient of 0.9947, a slope of 11.493, and an IC$_{50}$ value of 2.9 ± 0.03 µg mL^{-1} (data not shown). Slopes of the curves were compared using the t-test, and a significant difference was obtained, indicating the existence of the matrix effect. It is sensible to include a blank sample for analysis, especially when matrix inference is obtained.

It was experimentally established that the minimum concentration of acarbose that can be determined is 1 µg mL^{-1}. The percentage RSD on the day of experimentation (intraday) and on different days (interday) was found to be <4%, indicating the precision of the method. The error percentage was calculated to be 5.46%, which indicates that the method is accurate. Thus, the optimized method was both accurate and precise for both interday and intraday experimentation for the evaluated concentration levels.

The robustness of the method was analyzed by estimating the response obtained by deliberately changing the conditions. It was observed that slight variation in the experimental conditions had a significant effect on the response. Slight changes in enzyme concentration, time duration prior to and during the incubation, and time in the water bath resulted in significant changes in the inhibition percentage. Analysis of results using the Tukey test (α = 0.05) indicated that the method is robust for two conditions, i.e., incubation temperature and preincubation time. Changes in these parameters do not have any effect on the inhibition percentage. Changes in conditions/parameters, such as enzyme concentrations (< 0.15 U mL^{-1}) and the time in the water bath (< 15.6 min), had a large influence on the outcome.

The use of phosphate buffer also affected the response. Among solvents, ethanol (up to 25%) and DMSO (up to 0.5%) at lower concentrations did not have a significant out-turn, indicating that the method is robust for the abovementioned conditions.

Experiments conducted to evaluate plate uniformity and signal variability indicated that the response is not influenced by its location on the plate. This experiment also counteracts problems associated with drift patterns, edge effects, and other systematic sources of variability. The results of the experiment include RSD values of <3.13

(maximum signal), <3.19 (mid signal), and <5.02 (minimum signal), which are in the acceptable range (RSD < 20%).

The Z factor that measures both the variability in the assay and the dynamic range between maximum and minimum controls should be in the acceptable value of Z > 0.4.[29] In this study, the values of the Z factor were 0.93 and 0.96.

Application

The proposed optimized and validated method was applied to extracts of five plants. The obtained results are shown in Table 2. Extract from *Zanthoxylum fagara* showed maximum inhibitory activity with an IC$_{50}$ value of 4.75 μg mL^{-1}. This extract was the closest to the acarbose positive control. This indicates that the plant extracts contain a strong inhibitor of the amylase enzyme. Species of the genus *Zanthoxylum* contain phytoconstituents, which are used to treat many diseases.[30]

Table 2. *In vitro* α-amylase inhibitory activity of plant extracts

	IC$_{50}$ (μg/mL)	SD
Zanthoxylum fagara	4.75	0.04
Chrysactinia mexicana	9.09	0.60
Teucrium bicolor	>50	-
Ricinus communis	>50	-
Jatropha dioica	>50	-
Acarbose	1.61	0.06

(IC$_{50}$): Medium Inhibitory Concentration. (SD): Standard deviation.

The phytochemical analysis indicated the presence of various metabolites such as alkaloids, flavonoids, terpenes, steroids, and phenols. *In vivo* studies of species of *Zanthoxylum* revealed that the phenolic content of the plant had a potential hypoglycemic effect.[31]

Otherwise, the extract from *Chrysactinia mexicana* had an IC$_{50}$ value of 9.09 μg mL^{-1}, indicating that the extract from this plant also contained bioactive metabolites, which have an inhibitory effect on the enzyme α-amylase. This is the first report of this plant showing α-amylase inhibition activity. However, extracts from other plants had no effect on the enzyme activity, suggesting that plant extracts do not contain inhibitors of the enzyme α-amylase.

Phytochemical constituent analysis from the *Zanthoxylum fagara* and *Chrysactinia mexicana* plants using different solvents for extraction can be performed. Because crude extract contains a variety of bioactive metabolites, it is necessary to characterize the bioactive compound showing inhibition activity by purifying it to determine its IC$_{50}$ value.

These findings demonstrated that our optimized and validated method is highly selective, enabling the identification of samples with or without potent activity.

CONCLUSIONS

A microscale *in vitro* method for the assessment of α-amylase inhibition activity was optimized, validated and used for the analysis of enzyme inhibition using plant extracts *in vitro*. The method was ideal with respect to linearity, accuracy, precision, selectivity, and reliability. It is also crucial to accurately use well-defined conditions for laboratory transfer.

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