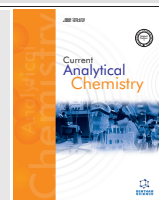




Optimization and Validation of a Microscale *In vitro* Method to Assess α -Glucosidase Inhibition Activity



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Abstract: Background: Microscale *in vitro* assays are fast, simple, and inexpensive, with reduced reagent quantities, waste, and experimental animal use. However, they have low reproducibility and low correlation with the results of *in vivo* models, possibly due to differences in precision and accuracy in methodologies between laboratories.

Objective: The objective was the optimization and validation of an *in vitro* assay, carried out on microscale, to assess the inhibition of α -glucosidase activity, which is indicative of antihyperglycemic activity.

Methods: The optimization was carried out using a fractional factorial design taking into account the best inhibition percentage and the absorbance of the controls. With the optimized experimental conditions in hand, we carried out method validation.

Results: The optimized conditions were as follows: enzyme concentration, 0.55 U/mL; substrate concentration, 111.5 μ M; and 17.5 min incubation at 37°C. A linear range between 100 and 310.2 μ g/mL of acarbose (r^2 0.994) was established. The RSD was <2% and the % error was <3%. The Z factor was >0.96. This method was applied to four plant extracts, one of which was found to be very active.

Conclusion: The method was found to be accurate, precise, selective, linear, and reliable in evaluating the antihyperglycemic activity of natural extracts *in vitro*.

Keywords: Antihyperglycemic activity, method optimization and validation, α -glucosidase activity, fractional factorial design, acarbose, Z factor.

1. INTRODUCTION

Postprandial Hyperglycemia (PPHG) is a condition characterized by an abnormal increase in blood glucose and has been associated with type II diabetes mellitus. Hydrolysis of carbohydrates in the diet is the major source of blood glucose. Several studies suggest that inhibition of carbohydrate-hydrolyzing enzymes slows glucose absorption, thereby decreasing PPHG [1-3].

Glycoside hydrolases are a group of enzymes that have the ability to cleave the glycosidic bond between two sugar residues. These enzymes show a range of substrate preferences. As a result, they are classified into discrete families known as carbohydrate-active enzyme families. Family GH31 is a group of glycoside hydrolases that encompass a range of diverse activities that include α -glucosidase, α -galactosidase, α -mannosidase, α -1,3-glucosidase, sucrose-isomaltase, α -xylosidase, α -blucan lyase, isomaltosyltransferase and oligosaccharide

α -1,4-glucosyltransferase. This family includes α -glucosidases, which are important enzymes in primary metabolism. α -Glucosidases are enzymes located in the membrane of small intestine epithelium [4-7]. They are responsible for the hydrolysis of dietary starch molecules, resulting in the release of free glucose. In humans, two α -glucosidases of the family GH31 are known to play a key role in the final stages of starch digestion: maltase-glucoamylase and sucrase-isomaltase.

One of the methods used to evaluate the α -glucosidase activity is based on the spectrophotometric determination of the p-nitrophenol (pNP) released from p-nitrophenyl- α -D-glucopyranoside (p-NPG) substrate by the action of the enzyme.

α -Glucosidase inhibition assays have been carried out in test tubes [2, 8, 9] and in microdilution plates [3, 10, 11]. However, to our knowledge, optimization and validation of the assay have not been reported. In this paper, the development of a microscale method to assess the inhibition of α -glucosidase activity is presented. Among the advantages of working at microscale levels are that the small quantities of chemicals required reduced material costs, the disposal of

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chemicals after carrying out experiments is easier and safety hazards are often reduced, and many experiments can be carried out quite quickly [12].

A crucial point in performing optimization process and validation of a method is the use of positive and negative controls. Among the compounds considered to have α -glucosidase inhibition activity are acarbose, voglibose, and miglitol. According to a bibliographic review, acarbose is the substance most commonly used as a positive control. Acarbose was the first drug developed for prediabetes treatment; it was approved by the Food and Drug Administration (FDA) in 1996. It is a pseudotetrasaccharide, consisting of a maltose bridged to acarvosine, and has a high specificity for α -glucosidases. As an inhibitor, acarbose reduces PPHG in patients with diabetes mellitus [13, 14].

One of the great problems that are faced to use the information obtained with these assays is the great variability in the activity reported for the reference (positive control) and that is attributed to the "slight modifications" described in the reference methods. However, they do not assess whether these "modifications" affect the final results. This is one of the reasons why it is common to find widely scattered responses for the same compound used as a positive control. This contributes greatly to the difficulty in comparing results between laboratories; and the lack of correlation between *in vitro*-*in vivo* assays.

In addition, a low correlation between *in vitro* and *in vivo* assays is often found and represents a serious problem for the *in vitro* screening methods [15]. It is therefore critical to carefully select the assay target, for both the *in vitro* and *in vivo* models. Furthermore, the method must have low variability and high signal-to-noise to minimize false-positive and/or -negative results [16]. The use of experimental design to establish the optimal conditions for a suitable method is considered the best way to obtain reliable results.

Once optimized, the proposed method should be validated in order to demonstrate that is acceptable for the intended, under specific conditions. In general, the quality of a method is defined by the robustness and precision of the detectable signal that enable a biological process to be quantified, either in the absence of any test compound or in the presence of other compounds [17].

Critical points for *in vitro* tests usually include lack of accuracy and inappropriate use of instrumental techniques. Shortcomings in terms of highly accurate measurements make it difficult to compare the results of two assays and to reproduce results [18]. Furthermore, many assay methods that are based on mechanisms of action, such as the inhibition of α -glucosidase, have been performed with colorimetric techniques, using UV-Vis spectrophotometry as the detection system. However, they do not take the Beer-Lambert Law limitations into consideration, which can subsequently lead to failures in the robustness and precision of the detectable signal.

In this work, the optimization, validation, and application of an *in vitro* method to assess the inhibition of α -glucosidase activity on the microscale level are described.

2. MATERIALS AND METHODS

2.1. Materials

α -Glucosidase (12.4 U/mg) from *Saccharomyces cerevisiae* yeast, bovine serum albumin, Fraction V, 96-99% albumin, potassium phosphate monobasic (KH_2PO_4), p-nitrophenyl- α -D-glucopyranoside (p-NPG, >95%), acarbose, and sodium carbonate (>99%) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Potassium phosphate dibasic (K_2HPO_4 , ACS Reagent), was from Fisher Scientific, Pittsburgh, PA, USA. Polystyrene 96-well, flat-bottomed plates, were obtained from BD Falcon, New York, NY, USA. All experiments were carried out with a Multiskan FC plate reader (Thermo Scientific, Waltham, MA, USA).

2.2. Plant Extracts

Four plant extracts from northern Mexico were used to evaluate the applicability of the proposed method. Extracts were obtained from aerial parts of *Zanthoxylum fagara*, *Teucrium bicolor*, and *Ricinus communis*, and the root of *Jatropha dioica* using an ethanol-water (90:10) mixture. All extracts were kindly provided by researchers from the Analytical Chemistry Department (Facultad de Medicina, UANL, México).

2.3. Optimization

The α -glucosidase inhibition assay is based on the detection of p-nitrophenoxide, derived from nitrophenol in basic medium. The p-nitrophenol is released from p-NPG by the action of the enzyme glucosidase at 405 nm. The inhibition percentage (decrease of the light-absorbption species, p-nitrophenoxide) was calculated in the absence of an inhibitory agent (negative control, 100% of enzyme activity) and in the presence of an inhibitory agent. This inhibition percentage was considered the response of the method. In all cases, the inhibition percentage was calculated using Equation (1):

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

where A is the absorbance of the negative control and B is the absorbance in the presence of an inhibitory agent.

The positive control was acarbose (198 $\mu\text{g/mL}$). All experiments were performed in polystyrene 96-well plates with a final volume of 200 μL . Absorbance readings were carried out with a microplate reader (Multiskan FC, Thermo Scientific) employing the recommended wavelength in the consulted reports (405 nm).

To perform the optimization, a screening was first made to establish the factors with major influence, considering the following parameters: enzyme concentration, substrate (p-NPG) concentration, buffer concentration, pH, incubation temperature, preincubation time, incubation time, albumin concentration, and sodium carbonate concentration. In all cases, the highest or lowest previously reported values were used for the optimization experiments. The experimental design used was a fractional factorial design of a sixteenth fraction 2^{16-4} , without blocks, with a random central point and one replicate. Sixty-four experiments were realized. Ex-

perimental design and data handling were carried out using Statgraphics Centurion XVI software (Statpoint Technologies, Inc., Warrenton, VA, USA).

The results were analyzed and Pareto charts were generated. The most influential variables were optimized following the rules of the basic sequential simplex method [19], looking for the combination of conditions that produced an inhibition percentage close to 50%, while the absorbance of control at 0% inhibition (100% activity) gave the smallest photometric error. Calculations were carried out using Excel 2010 (Microsoft).

2.3.1. Enzyme Kinetics

To establish the optimum substrate concentration, enzyme kinetics was investigated using the proposed method and substrate concentration was changed between 2 and 476 $\mu\text{g/mL}$. From the Lineweaver-Burk plot, maximum velocity (V_{\max}) and the Michaelis-Menten constant (K_m) were calculated. The K_m value was considered as the optimum substrate concentration. All the experiments were carried out in triplicate. Calculations were carried out using Excel 2010 (Microsoft).

2.4. Validation

The proposed method was validated according to international guidelines [17, 20, 21, 22] by testing linearity, precision, accuracy, robustness, Plate uniformity and signal variability.

2.4.1. Linearity

Linearity was evaluated by determination of the inhibition percentage produced by solutions of different concentrations of the positive control acarbose (10, 30, 50, 70, 90, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400 $\mu\text{g/mL}$). The obtained results were subjected to the least-squares method to obtain the plot's equation. The inhibition percentage was calculated, and is represented as a function of the concentration. Linearity was evaluated by visual inspection of the plot of inhibition percentage. The determination coefficient (r^2) and the slope of the regression line were submitted.

Matrix effect was assessed by the construction of a standard curve using the inhibition percentage produced by solutions of different concentrations of acarbose (10, 30, 50, 70, 90, 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400 $\mu\text{g/mL}$). All the solutions were added with 125 $\mu\text{g/mL}$ of an extract from *Jatropha dioica* which does not possess inhibitory activity. The results were subjected to the least-squares method to obtain the plot's equation as well as the determination coefficient. The inhibition percentage was calculated, and is represented as a function of the concentration of acarbose. The matrix effect was evaluated by comparing this regression slope with the obtained from the linearity test using a t-test.

All the experiments were carried out in triplicate.

2.4.2. Precision and Accuracy

The precision and accuracy of the method were tested using the *Jatropha dioica* extract, either with or without the positive control, at three concentration levels: low, medium

and high, in triplicate. Acarbose was used at 99.0, 201.3, and 260.7 $\mu\text{g/mL}$.

The precision was evaluated in the same and in two different days. Intraday and interday precision values were determined from the percentage of the relative standard deviation (%RSD), according to Equation (2):

$$\%RSD = \frac{s}{x} \times 100 \quad (2)$$

where s is the standard deviation of each measurement and x is the average of such measurements.

Accuracy was determined as the percentage of the relative error (% error) of the solutions of *J. dioica* extract spiked with acarbose at the different concentration levels, according to Equation (3):

$$\% \text{ Error} = \frac{c_x^0 - c_x}{c_x^0} \times 100 \quad (3)$$

where c_x^0 is the added concentration of acarbose and c_x is the experimentally determined concentration of acarbose.

2.4.3. Robustness

Robustness was evaluated by performing various experiments, under the following conditions: substrate concentration (101.5 and 121.5 μM), enzyme concentration (0.45 and 0.65 U/mL), incubation temperature (35 and 39 $^\circ\text{C}$), and incubation time (15 and 20 min). In addition, the following solvents were also considered: ethanol (25%) and DMSO (0.15 and 0.25% of the final volume). All experiments were carried out in triplicate. The robustness of each method was evaluated using the Tukey test ($\alpha = 0.05$).

2.4.4. Plate Uniformity, Signal Variability, and Z Factor

Plate uniformity and signal variability were evaluated by measuring signal intensities of the following solutions [17]:

- “Max” signal: Maximum absorbance with the minimal inhibition (IC_{30}),
- “Mid” signal: Mean absorbance with 50% of inhibition (IC_{50}),
- “Min” signal: Minimum absorbance with the maximum inhibition (IC_{62}).

To obtain the desired signals, acarbose was used at concentrations of 100, 180, and 310 $\mu\text{g/mL}$ for the maximum, mean, and minimum inhibitory concentrations (IC), respectively.

Each solution was measured in 32 wells of the same 96-well plate and with three different plates. The position of each solution was changed in each plate (rows or columns of wells). All assays were carried out with independent reagents and evaluated on three different days. The %RSD was calculated for each solution.

The Z factor was quantified in each plate according to Equation (4):

$$Z = \frac{(\text{Average}_{\max} - 3s_{\max} / \sqrt{n}) - (\text{Average}_{\min} + 3s_{\min} / \sqrt{n})}{\text{Average}_{\max} - \text{Average}_{\min}} \quad (4)$$

where n is the number of tests performed.

2.5. Application

The proposed method was used for the determination of α -glucosidase inhibitory activity of four plant extracts. Each extract was weighed and the extract solutions were prepared at the following concentrations: 660, 330, 165, 82.5, 41.3 and 20.6 $\mu\text{g/mL}$. The optimized protocol and validated method were very carefully followed, throughout.

2.6. Statistical Analysis

The data were analyzed using the Kolmogorov-Smirnov test and the Tukey test where appropriate. The Minitab 17 software package (Minitab, Inc., USA) was used for these analyses and $P < 0.05$ was considered significant.

Statistical analysis and validation were carried out using the program Excel 2010.

3. RESULTS AND DISCUSSION

As noted, this study addresses the optimization and validation of an *in vitro* method, carried out on the microscale, to assess the inhibition of α -glucosidase activity. First, a literature survey of assays for *in vitro* antihyperglycemic activity measurements that include both full procedures and the positive controls employed in the study was carried out. This survey revealed that minor modifications of assay conditions lead to large variations in the mean inhibition concentration (IC_{50}) values (0.00037-6.2 mg/mL) are obtained from acarbose, used in all cases as a positive control. This problem illustrates why results obtained by different methods (using different experimental conditions) should not be compared, particularly if the effect on the response of the test was not assessed. Otherwise, this leads to a perception of low reliability in terms of the results of *in vitro* methods.

In particular one modification, that is frequently found is the use or not of sodium carbonate to stop the enzymatic reaction. According to the method, the ability of a compound to inhibit α -glucosidase activity is based on the generation of p-nitrophenol (pK_a 7.2), which is released from the substrate p-NPG. When carbonate is added to stop the reaction, an alkaline medium (pH 10) will be obtained, and hence p-nitrophenoxide would be the predominant specie. Without carbonate, reaction media present a pH around 6.8 and both p-nitrophenol and p-nitrophenoxide are present. Because the

wavelength of 405 nm, reportedly used in most cases, corresponds to the wavelength of maximum absorbance of p-nitrophenoxide, it is necessary to control the pH to avoid false positives.

Furthermore, specifications and limitations of instrumental techniques used during the optimization process were considered, in this case UV-Vis spectrophotometry. For example, the photometric error was considered; it is known that absorbance measurements are most accurate in the range of 0.2-0.8 units of absorbance [23].

3.1. Optimization

Before optimization, acarbose IC_{50} was determined following the method described by Apostolidis *et al.* [24]. First, the inhibition of α -glucosidase potency was quantified through the decrease of the amount of light-absorbing species (p-nitrophenoxide using a different concentration of inhibitor agent). The IC_{50} was established by measuring the inhibitor concentration required to reduce to 50% of the light-absorbing species. An IC_{50} value of 198 $\mu\text{g/mL}$ was determined.

Optimization of the method of inhibition of α -glucosidase was performed using a 2^{9-4} fractional factorial design as a preliminary screening test to determine the parameters with significant effect over the response. The inhibition percentage and the absorbance values were evaluated as the response. The variables evaluated were: enzyme concentration, substrate (p-NPG) concentration, buffer concentration, pH , incubation temperature, preincubation time, incubation time, albumin concentration, and sodium carbonate concentration.

Fig. (1) shows the Pareto diagram that was obtained. Variables that had a significant effect on the inhibition percentage of acarbose were enzyme concentration and incubation temperature, in addition to interactions of enzyme concentration with substrate concentration and incubation time.

Subsequently, variables with significant influence over the response were optimized using a basic sequential simplex method. During the experiments, an inhibition percentage close to 50% was chosen as the best response, while the absorbance of control at 0% inhibition (100% activity) gave the

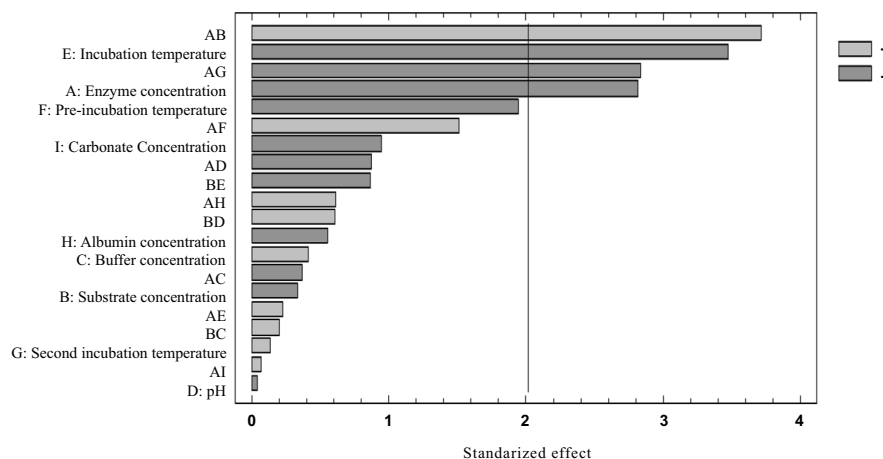


Fig. (1). Pareto chart obtained from the screening of inhibition assay of α -glucosidase.

smallest photometric error. It was necessary to carry out seven experiments. The combination of conditions that produced the best inhibition percentage was the following: enzyme concentration 0.55 U/mL, incubation time 17.5 min, and incubation temperature 37°C.

Measurement of enzyme kinetics was carried out to determine the substrate (p-NPG) concentration at which the enzyme works optimally (Fig. 2). Eli Lilly [17] recommends using substrate concentrations around or below the K_m value because, using substrate concentrations higher than K_m will make the identification of extract with competitive inhibition activity more difficult. In the α -glucosidase inhibition assay, a final substrate concentration of 33.6 $\mu\text{g/mL}$ (0.111 μM) was established. It is important to note that none of the references consulted mention use made of the measurement of enzyme kinetics to determine the optimal concentration of the substrate.

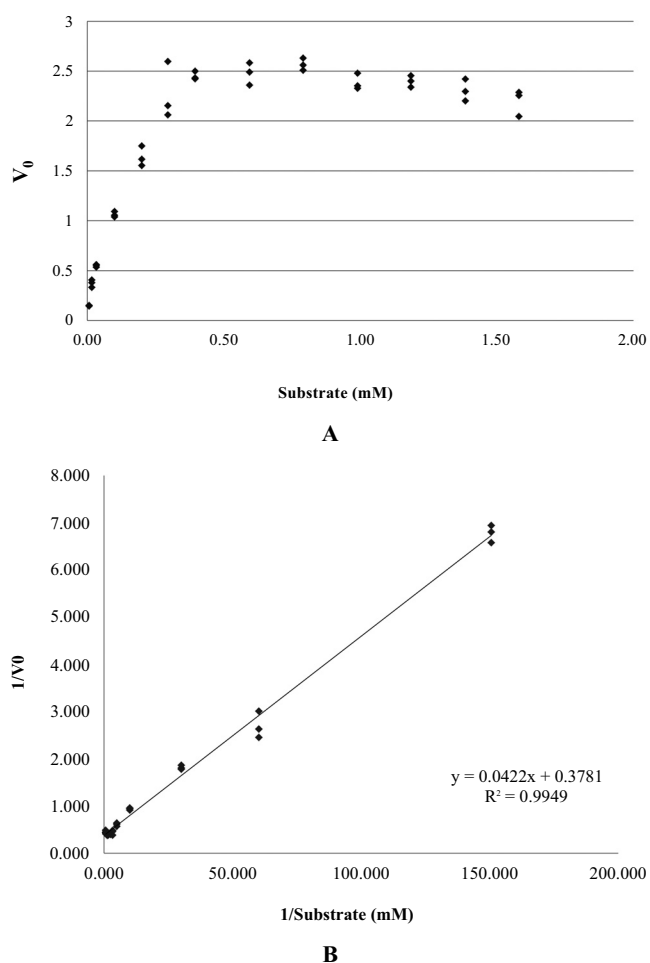


Fig. (2). Kinetics enzyme to determine K_m value. **A)** Kinetic enzyme of α -glucosidase on p-NPG, ambient temperature, $\lambda=405$ nm. **B)** Graphics of Lineweaver-Burke of α -glucosidase.

3.2. Validation

The optimized method was validated following the recommendations and criteria set out in several international guidelines [17-20].

In biological activity assays, in which the potency of a compound is evaluated, linearity must be considered as the

ability of the procedure (within a given range) to obtain test results (inhibition percentages) which are directly proportional to the concentration (or amount) of the test compound [19].

The analysis of acarbose (positive control solutions) at five concentration levels was evaluated by visual inspection of the plot signals and by the calculation of a regression line by the method of least squares. The results were linear range 100-310.2 $\mu\text{g/mL}$, determination coefficient 0.9943, slope value 0.147, and IC_{50} 180.2 (± 2.48) $\mu\text{g/mL}$ (Fig. 3).

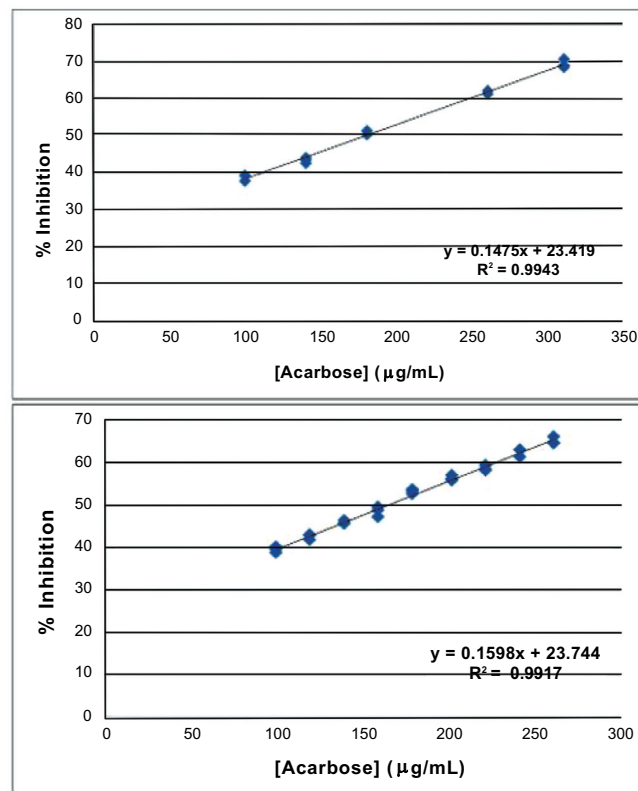


Fig. (3). Linearity at $\lambda=405$ nm. **A)** Inhibition percentage of α -glucosidase by acarbose. **B)** Inhibition percentage of α -glucosidase by a plant extract added with acarbose.

A common problem encountered working with natural products is the matrix effect, which in analytical chemistry is defined as the combined effect of all components of the sample other than the analyte on the measurement of the quantity [25]. When an analytical method is applied, the matrix effect results in a difference in sensitivity, which is evident when a calibration curve for a solvent is compared against a sample curve (extract in this case). The sensitivity is the variation of the analytical signal vs concentration, *i.e.*, the slope of the calibration curve [26]. Therefore, the difference between the slopes of a calibration curve of solvent and of sample matrix indicates the presence of a matrix effect, which is an indeterminate effect in the sample that affects sensitivity.

To evaluate the matrix effect, was carried out the analysis of positive control solutions at five concentration levels added to an extract from *Jatropha dioica*. The results were linear range 99-260.7 $\mu\text{g/mL}$, determination coefficient 0.995, slope value 0.1598, and IC_{50} 178.6 (± 1.65) $\mu\text{g/mL}$.

Table 1. Application of the optimized and validated α -Glucosidase inhibition method to plant extracts.

Sample	IC ₅₀ (μ g/mL)	SD
<i>Zanthoxylum fagara</i>	107.9	2.01
<i>Teucrium bicolor</i>	>660	-
<i>Ricinus communis</i>	>660	-
<i>Jatropha dioica</i>	>660	-
Acarbose	164.31	1.65

IC₅₀: Inhibition concentration medium; SD: Standard deviation.

The matrix effect was evaluated by comparing this regression slope with the obtained from the linearity test using a t-test. We found a significant difference between the slopes of the two curves; hence, the matrix effect is considered. In cases where there is matrix interference, it is advisable to include a sample blank.

It was experimentally established; using diluted acarbose solutions, that the minimum concentration of acarbose that can be determined is 99 μ g/mL (equivalent to 38.5% inhibition) and the maximum is 310.2 μ g/mL (equivalent to 69.2% inhibition).

The methods showed interday and intraday precision, as the %RSD were <2%. The error percentage was calculated to be -1.53%, *i.e.*, the method is accurate. The optimized method was accurate and precise, both intraday and interday, for the three concentration levels tested [26].

To establish the robustness of both methods, slight changes to variables that showed a significant effect on the response during the screening process were evaluated.

Here, it was found that the parameters that had the greatest effect on the inhibition percentage results were temperature, incubation time, substrate concentration, and enzyme concentration. Results were analyzed using the Tukey test ($\alpha = 0.05$). It was found that the method was robust only to temperature and incubation time, therefore making it important to use exact substrate concentrations (>0.111 mM), exact enzyme concentrations (0.55 U/mL), incubation temperatures between 37 and 39°C, and incubation times between 17.5 and 20 min. The response will be affected if these conditions are not satisfied. Furthermore, it was found that, should the test samples not be soluble in phosphate buffer, it is possible to use ethanol (up to 25%) and DMSO (up to 0.25%) as solvent.

Plate uniformity and signal variability assessment indicated that the results would not be affected by the position of the well in the plate. This study can reveal problems as patterns of drift, edge effects and other systematic sources of variability. Plate uniformity and signal variability were acceptable according with the criteria established by Eli Lilly (RSD <20%) [17]. The following RSD values were recorded for maximum signal (<1.99), average (<2.10), and low (<4.32).

The Z factor is used to measure the separation between maximum and minimum controls in an assay that accounts

for the amount of variability in the assay. Is a dimensionless value that represents both the variability and the dynamic range between two sets of controls (maximum and minimum) [27], with the recommended acceptance criterion being $Z \geq 0.4$ [28]. In this study, calculation of the Z factor gave values of 0.96 and 0.97.

3.3. Application

Finally, the proposed method was applied to four plant extracts. Results are shown in Table 1. *Zanthoxylum fagara* was found to have the highest inhibitory activity on α -glucosidase, with an IC₅₀ value lower than for the positive control acarbose, and hence it is considered a strong inhibitor. Other extracts showed no effect on the enzyme.

These findings demonstrated that the optimized and validated method is also selective; it enables the identification of samples with potent activity and inactivity.

CONCLUSION

A microscale *in vitro* method to assess inhibition of α -glucosidase activity was optimized and validated. The method is linear, accurate, precise, selective, and reliable in evaluating the antihyperglycemic activity of natural extracts *in vitro*. It is nonetheless crucial to emphasize the importance of accurately using the established conditions for testing and the need for validation testing, upon interlaboratory transfer, with acarbose as a positive control.

AUTHOR CONTRIBUTIONS

GGG performed the study and analysed data; RCR analysed data, wrote paper; NWT contributed important reagents, wrote paper; RSA designed research, wrote paper.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

- [1] Kim, J.-S.; Kwon, C.-S.; Son, K.H. Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Biosci. Biotechnol. Biochem.*, **2000**, *64*, 2458-2461.
- [2] Hou, W.; Li, Y.; Zhang, Q.; Wei, X.; Peng, A.; Chen, L.; Wei, Y. Triterpene acids isolated from *Lagersstroemia speciosa* leaves as α -glucosidase inhibitors. *Phytother. Res.*, **2009**, *23*, 614-618.
- [3] Apostolidis, E.; Lee, C.M. *In vitro* potential of *Ascophyllum nodosum* phenolic antioxidant-mediated α -glucosidase and α -amylase inhibition. *J. Food Sci.*, **2010**, *75*, H97-H102.
- [4] Quezada-Calvillo, R.; Sim, L.; Ao, Z.; Hamaker, B.R.; Quaroni, A.; Brayer G.D.; Sterchi E.E.; Robayo-Torres, C.C.; Rose, D.R.; Nichols B.L. Luminal starch substrate "brake" on maltase-glucoamylase activity is located within the glucoamylase subunit¹⁻³. *J. Nutr.*, **2008**, *138*, 685-692.
- [5] Chaudet, M.M.; Rose, D.R. Divergent evolution for diverse substrate recognition by family 31 glycoside hydrolases. *Biochem. Cell Biol.*, **2016**, *94*, 323-330.
- [6] Matsui, T.; Yoshimoto, C.; Osajima, K.; Oki, T.; Osajima, Y. *In vitro* survey of α -glucosidase inhibitory food components. *Biosci. Biotechnol. Biochem.*, **1996**, *60*, 2019-2022.
- [7] Champe, P.C. *Bioquímica*, 4th ed., Lippincott Williams and Wilkins, Wolters Kluwer Health: Barcelona, **2007**.
- [8] Choudhary, M.I.; Shah, S.A.; Atta-ur-Rahman; Khan, S.N.; Khan, M.T. Alpha-glucosidase and tyrosinase inhibitors from fungal hydroxylation of tibolone and hydroxytibolones. *Steroids*, **2010**, *75*, 956-966.
- [9] Shai, L.J.; Masoko, P.; Mokgotho, M.P.; Magano, S.R.; Mogale, A.M.; Boaduo, N.; Eloff, J.N. Yeast alpha glucosidase inhibitory and antioxidant activities of six medicinal plants collected in Phalaborwa, South Africa. *S. Afr. J. Bot.*, **2010**, *76*, 465-470.
- [10] Ren, S.; Xu, D.; Pan, Z.; Gao, Y.; Jiang, Z.; Gao, Q. Two flavanone compounds from litchi (*Litchi chinensis* Sonn.) seeds, one previously unreported, and appraisal of their α -glucosidase inhibitory activities. *Food Chem.*, **2011**, *127*, 1760-1763.
- [11] Lee, S.-S.; Lin, H.-C.; Chen, C.-K. Acylated flavonol monorhamnosides, α -glucosidase inhibitors, from *Machilus Philippinensis*. *Phytochem.*, **2008**, *69*, 2347-2353.
- [12] Skinner, J.; *Microscale Chemistry: Experiments in miniature*. Royal Society of Chemistry, London, England. **1998**.
- [13] Dash, R.P.; Babu, R.J.; Srinivas, N.R. Reappraisal and perspectives of clinical drug-drug interaction potential of α -glucosidase inhibitors such as acarbose, voglibose and miglitol in the treatment of type 2 diabetes mellitus. *Xenobiotica*, **2017**, *1*, 1-20.
- [14] Sun, W.; Sang, Y.; Zhang, B.; Yu, X.; Xu, Q.; Xiu, Z.; Dong, Y. Synergistic effects of acarbose and an *Oroxylum indicum* seed extract in streptozotocin and high-fat-diet induced prediabetic mice. *Biomed. Pharmacother.*, **2017**, *87*, 160-170.
- [15] White, R.E. High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. *Annu. Rev. Pharmacol. Toxicol.*, **2000**, *40*, 133-157.
- [16] Macarrón, R.; Hertzberg, R.P. Design and implementation of high throughput screening assays. *Mol. Biotechnol.*, **2011**, *47*, 270-285.
- [17] Eli Lilly. Guidance for Assay Development & HTS. Available from: https://hts.ku.edu/sites/hts.drupal.ku.edu/files/docs/NCGC_Assay_Guidance_Manual.pdf. (Accessed on March 7, 2017).
- [18] Stevenson, R.L. Bioassays 2011. Available from: <http://www.americanlaboratory.com/913-Technical-Articles/19109-Bioassays-2011/>. (Accessed on March 7, 2017).
- [19] Granados-Guzmán, G.; Garza-Tapia, M.; Castro-Ríos, R.; Waksman de Torres, N.; Salazar-Aranda, R. Optimization and validation of two high-throughput methods indicating antiradical activity. *Curr. Anal. Chem.*, **2017**, *13*(6), 499-507.
- [20] ICH Expert Working Group. *Q2 (R1) Validation of Analytical Procedures: Text and Methodology*. ICH Harmonised Tripartite Guideline. International conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use: Chigago, **2005**.
- [21] United States Pharmacopeial Convention, Inc. Biological Assay Validation (Chapter <1033>). *U.S. Pharmacopeia & National Formulary*; United States Pharmacopeial Convention, Inc.: Rockwell, **2010**; Vol. 2010, pp. 1-25.
- [22] US Department of Health and Human Services. Food and Drug Administration. Guidance for Industry. Bioanalytical Method Validation. Available from: <http://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf>. (Accessed on March 7, 2017).
- [23] Skoog, D.A.; Crouch, S.R.; Holler, F.J. *Principios de Análisis Instrumental [Principles of Instrumental Analysis]*. Cengage Learning Latin America: Distrito Federal, México, **2008**.
- [24] Apostolidis, E.; Li, L.; Lee, C.; Seeram, N.P. *In vitro* evaluation of phenolic-enriched maple syrup extracts for inhibition of carbohydrate hydrolyzing enzymes relevant to type 2 diabetes management. *J. Funct. Foods*, **2011**, *3*, 100-106.
- [25] Tiwari, G.; Tiwari, R. Bioanalytical method validation: An updated review. *Pharm. Methods*, **2010**, *2*, 25-38.
- [26] IUPAC, *Compendium of Chemical Terminology*, 2nd ed. (the Gold Book), compiled by McNaught, A.D. and Wilkinson, A., Blackwell Scientific Publications: Oxford, **1997**.
- [27] Iversen, P.W.; Beck, B.; Chen, Y.F.; Dere, W.; Devanarayan, V.; Eastwood, B.J.; Farnen, M.W.; Iturria, S.J.; Montrose C.; Moore, R.A.; Weidner, J.R.; Sittampalam, G.S. HTS Assay Validation. In: *Assay Guidance Manual*. Eds. Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda, MD, US, **2012**, pp. 1-14.
- [28] Zhang, J.H.; Chung, T.D.; Oldenburg, K.R. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.*, **1999**, *4*, 67-73.