In Vitro Evaluation of the Anti-Diabetic Potential of Fruit Extracts: A Comparative Analysis of Enzyme Inhibition and Phytochemical Content

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Abstract

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder marked by hyperglycemia. Inhibiting carbohydrate-hydrolyzing enzymes, such as α-glucosidase and α-amylase, is a well-established therapeutic strategy to manage postprandial glucose levels. While pharmaceutical drugs like acarbose are used for this purpose, their use is often limited by side effects. This study investigates the in vitro anti-diabetic potential of ten fruit-based samples, including grapes, blueberries, and blackcurrants. We quantified the total polyphenol content (TPC) using the Folin-Ciocalteu and Fast Blue BB assays and determined anthocyanin content using the pH differential method. The inhibitory effects on α-glucosidase and α-amylase were assessed by determining the half-maximal inhibitory concentration (IC_{50}) values, with acarbose serving as a positive control. The results demonstrate that all tested fruit extracts possess a potent inhibitory effect on both enzymes, with several samples showing significantly lower IC_{50} values than acarbose. For instance, grape and blueberry extracts were found to be more than 100 times more effective than acarbose at inhibiting α-glucosidase, while grape extracts were over 150 times more effective against α-amylase. The data supports a strong correlation between high polyphenol and anthocyanin content and powerful enzyme inhibitory activity. These findings indicate that these fruits are rich sources of natural inhibitors and could be developed into functional foods or natural therapeutics for the management of T2DM.

1.0 Introduction

1.1 The Global Burden of Diabetes and Postprandial Hyperglycemia

Diabetes mellitus represents one of the most significant health challenges of the 21st century, with its prevalence escalating worldwide. The World Health Organization estimates that the number of people living with diabetes has quadrupled since 1980, affecting hundreds of millions of individuals. The disease is characterized by chronic hyperglycemia, which, if left unmanaged, can lead to severe long-term complications, including cardiovascular disease, neuropathy, nephropathy, and retinopathy. A central aspect of diabetes management is the control of postprandial hyperglycemia (PPHG)—the sharp spike in blood glucose levels that occurs after a meal. This is because repeated and prolonged exposure to high glucose levels is a primary driver of diabetic complications.[1]

1.2 Therapeutic Strategies: Enzyme Inhibition

Dietary carbohydrates, such as starches and complex sugars, are broken down into absorbable monosaccharides, primarily glucose, by enzymes in the small intestine. The two key enzymes responsible for this process are α-amylase and α-glucosidase.[2]

A well-established and effective strategy to manage PPHG is the use of inhibitors for these carbohydrate-digesting enzymes. By delaying the digestion and subsequent absorption of glucose, these inhibitors flatten the postprandial glucose curve, helping to maintain blood glucose within a healthier range. Synthetic drugs like acarbose, miglitol, and voglibose are clinically used for this purpose. However, these drugs are often associated with gastrointestinal side effects, such as flatulence and bloating, which can affect patient compliance.[3] This has led

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[1] Vlachos, D., Malisova, S., Lindberg, F. A., & Karaniki, G. (2020). Glycemic Index (GI) or Glycemic Load (GL) and Dietary Interventions for Optimizing Postprandial Hyperglycemia in Patients with T2 Diabetes: A Review. *Nutrients*, *12*(6), 1561. https://doi.org/10.3390/nu12061561

[2] Proença, C., Ribeiro, D., Freitas, M., & Fernandes, E. (2022). Flavonoids as potential agents in the management of type 2 diabetes through the modulation of α-amylase and α-glucosidase activity: a review. *Critical reviews in food science and nutrition*, *62*(12), 3137–3207. https://doi.org/10.1080/10408398.2020.1862755

[3] Hanefeld M. (2007). Cardiovascular benefits and safety profile of acarbose therapy in prediabetes and established type 2 diabetes. *Cardiovascular diabetology*, *6*, 20. https://doi.org/10.1186/1475-2840-6-20

to a growing interest in exploring natural, plant-based compounds with similar inhibitory properties but fewer side effects.

1.3 Natural Bioactive Compounds: Polyphenols and Anthocyanins

Fruits and vegetables are rich sources of bioactive compounds, most notably polyphenols and anthocyanins. Polyphenols are a large class of plant compounds characterized by multiple phenolic units, while anthocyanins are a specific subclass of flavonoids responsible for the red, purple, and blue colors in many fruits. A wealth of scientific literature demonstrates that these compounds can inhibit α-glucosidase and α-amylase. Their inhibitory mechanisms often involve non-covalent interactions with the enzyme's active site, altering its conformation and reducing its catalytic efficiency.[4]

1.4 Study Objective

This study aims to investigate the anti-diabetic potential of a variety of fruit samples through a series of in vitro experiments. The samples tested include:

- Grapes (GP)
- Grapes with Beetroot (GP+BR)
- Blueberry (fast dried) (BB_W)
- Blueberry (whole sample) (BB_ZZ)
- Hibiscus (H)
- Blackcurrant (skin) (BC_Skin)
- Blackcurrant (whole) (BC_Whole)
- Blackcurrant (liquid) (BC-Liquid)
- Cherry (Cherry-Liquid)
- Blackcurrant with Beetroot (BC-BR)

[4] Kim, Y., Keogh, J. B., & Clifton, P. M. (2016). Polyphenols and Glycemic Control. *Nutrients*, *8*(1), 17. https://doi.org/10.3390/nu8010017

The study will quantify the total phenolic content (TPC) and total anthocyanin content (TAC) of these samples. Furthermore, it will evaluate their inhibitory effects on α-glucosidase and α-amylase, using acarbose as a positive control. The ultimate goal is to identify which samples possess the strongest anti-diabetic properties, thus highlighting their potential as functional foods or sources for developing new natural inhibitors.

2.0 Methodology

2.1 Sample Preparation

All fruit samples were processed to obtain extracts for the assays. The extracts were prepared following standard laboratory procedures to ensure the preservation of bioactive compounds.

2.2 Total Polyphenol Content (TPC) Assays

The TPC of the samples was determined using two colorimetric methods: the Folin-Ciocalteu assay and the Fast Blue assay. Both methods rely on the ability of phenolic compounds to react with a reagent, producing a measurable color change proportional to the concentration of phenolics.

2.2.1 Folin-Ciocalteu Assay

The assay was performed based on the method described by Singleton et al. (1999) with minor modifications. [5] The Folin-Ciocalteu reagent, a mixture of phosphotungstic and phosphomolybdic acids, reacts with phenols under alkaline conditions. This redox reaction reduces the reagent, forming a blue-colored phosphotungstic-phosphomolybdenum complex.

[5] Singleton, V.L. et al. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods in enzymology 299, 152-178. https://doi.org/10.1016/S0076-6879(99)99017-1

The intensity of the blue color, measured at 765 nm using a Tecan Spark plate reader, is directly proportional to the total phenolic content.

It is crucial to note that this assay is not specific to phenols; other non-phenolic reducing substances, such as ascorbic acid, certain amino acids, and sugars, can also react with the reagent.[6] Therefore, the results from this assay are more accurately described as a measure of the sample's total reducing capacity, which can lead to an overestimation of the true phenolic content.

Procedure

Samples or standards (gallic acid) (10 µL) were pipetted in triplicate into a microtiter plate. To each well, 40 µL of 25% Folin-Ciocalteu reagent was added, followed by 150 µL of 4% sodium carbonate solution. The plate was mixed and incubated in the dark for 30 minutes at room temperature. The absorbance was then read at 765 nm. A standard curve was generated using varying concentrations of gallic acid. The equation for the standard curve was determined to be $y = 0.0029x + 0.0174$ with a coefficient of determination ($R^2 = 0.9972$), where y is absorbance and x is the concentration in µg/mL.

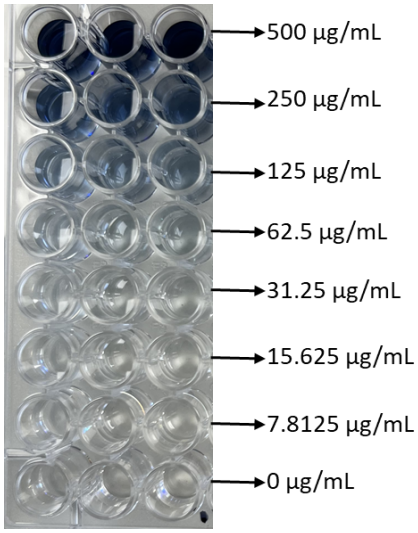

Figure 1. Calibration curve using gallic acid in a 96 well plate.

[6] Raposo, F., Borja, R., & Gutiérrez-González, J. A. (2024). A comprehensive and critical review of the unstandardized Folin-Ciocalteu assay to determine the total content of polyphenols: The conundrum of the experimental factors and method validation. *Talanta*, *272*, 125771. https://doi.org/10.1016/j.talanta.2024.125771

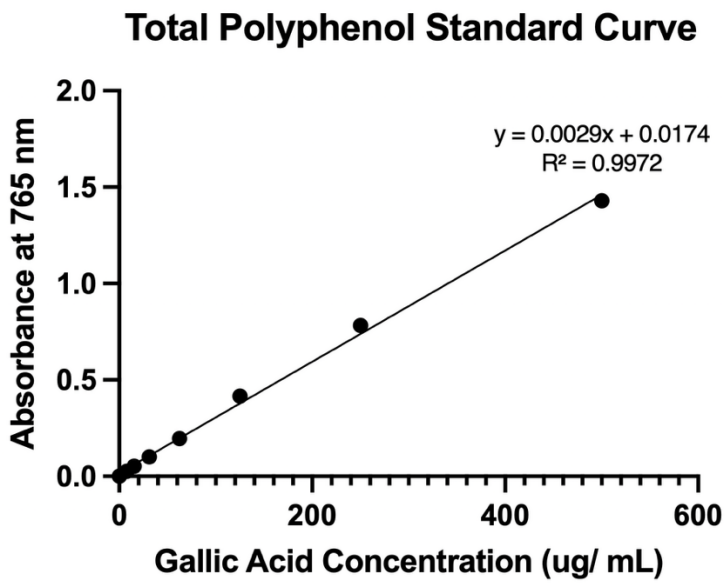

Figure 2. Calibration curve with Gallic acid

2.2.2 Fast Blue Assay

This method, adapted from Medina (2011) [7]and Hinojosa-Nogueira et al. (2017)[8], utilizes the diazonium group in the Fast Blue BB reagent. This group reacts with phenolic hydroxyl groups under alkaline conditions to form stable azo complexes, which are then measured at 420 nm.

The Fast Blue BB assay offers a more specific approach to quantifying phenolics. Unlike the Folin–Ciocalteu assay, the Fast Blue assay is less prone to interference from other non-phenolic reducing agents. It provides a more targeted measure of compounds with free phenolic hydroxyl groups, which are a key structural feature for many bioactive polyphenols.[9] Consequently, the results from this assay can provide a different, and potentially more precise, representation of a sample's phenolic profile compared to the Folin assay.

[7] Medina M. B. (2011). Simple and rapid method for the analysis of phenolic compounds in beverages and grains. *Journal of agricultural and food chemistry*, *59*(5), 1565–1571. https://doi.org/10.1021/jf103711c
[8] Hinojosa-Nogueira, D., Muros, J., Rufián-Henares, J. A., & Pastoriza, S. (2017). New Method To Estimate Total Polyphenol Excretion: Comparison of Fast Blue BB versus Folin-Ciocalteu Performance in Urine. *Journal of agricultural and food chemistry*, *65*(20), 4216–4222. https://doi.org/10.1021/acs.jafc.7b01000
[9] Pico, J., , Pismag, R. Y., , Laudouze, M., , & Martinez, M. M., (2020). Systematic evaluation of the Folin-Ciocalteu and Fast Blue BB reactions during the analysis of total phenolics in legumes, nuts and plant seeds. *Food & function*, *11*(11), 9868–9880. https://doi.org/10.1039/d0fo01857k

Procedure

Standard or sample solution (200 µL) was pipetted into each well. Then, 20 µL of 0.1% Fast Blue BB reagent and 20 µL of 5% NaOH were added. The plate was mixed and incubated in the dark for 2 hours at room temperature. The absorbance was measured at 420 nm. The standard curve for the Fast Blue assay using gallic acid was $y = 0.0029x + 0.0553$ with an $R^2 = 0.9868$, with concentration units in µg/mL.

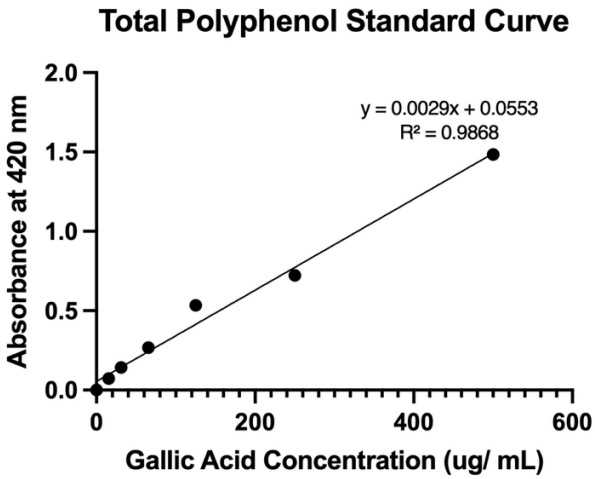

Figure 3. Calibration curve with Gallic acid

2.3 Anthocyanin Assay

The total monomeric anthocyanin content was determined using the pH differential method. This method is based on the structural transformation of anthocyanins with changing pH. At pH 1.0, anthocyanins exist in their red-colored flavylium cation form, whereas at pH 4.5, they convert to a colorless hemiacetal form. The difference in absorbance at 520 nm and 700 nm at these two pH values allows for the calculation of anthocyanin concentration.

Procedure

Each sample was diluted separately with 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5). The dilutions were made by adding 60 µL of sample to 240 µL of the respective buffers in a 96-well plate. After a 15-minute incubation, the absorbance

of all samples was measured at both 520 nm and 700 nm. The concentration was calculated using the formula:

$$\text{Anthocyanins (mg/L)} = (A \times MW \times DF \times 1000) / (\epsilon \times 1)$$

where A = (Abs 520 – Abs 700) pH 1.0 − (Abs 520 – Abs 700) pH 4.5, MW (molecular weight of cyanidin-3-glucoside) = 449.2, DF = dilution factor, and ϵ (molar absorptivity of cyanidin-3-glucoside) = 26900 $L \cdot mol^{-1} \cdot cm^{-1}$.

2.4 Enzyme Inhibition Assays

2.4.1 Alpha-Glucosidase Inhibition Assay

The α-glucosidase inhibitory activity was measured kinetically by monitoring the hydrolysis of p-nitrophenyl-α-D-glucopyranoside (pNPG) into p-nitrophenol, a yellow product with a maximum absorbance at 405 nm.

Procedure

Samples or controls (acarbose) were pre-incubated with α-glucosidase solution (0.5 U/mL) for 10 minutes at 37°C. The reaction was started by adding the pNPG substrate (2.5 mM). The absorbance at 405 nm was then measured every minute for 10 minutes. The percentage inhibition was calculated relative to a 100% activity control (buffer, enzyme, and substrate only). IC_{50} values (the concentration required to inhibit 50% of the enzyme activity) were determined. The IC_{50} for the positive control, acarbose, was found to be 1.287.[10]

[10] Sadia Zulfiqar, Lisa J. Marshall, Christine Boesch, Hibiscus sabdariffa inhibits α-glucosidase activity in vitro and lowers postprandial blood glucose response in humans, Human Nutrition & Metabolism, Volume 30, 2022, 200164, ISSN 2666-1497, https://doi.org/10.1016/j.hnm.2022.200164

2.4.2 Alpha-Amylase Inhibition Assay

The α-amylase inhibitory activity was determined using 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNPG3) as the substrate. This substrate is cleaved by α-amylase to release the chromophore 2-chloro-4-nitrophenol, which can be monitored at 405 nm.

Procedure

Samples or acarbose were pre-incubated with porcine pancreatic α-amylase (1 U/mL) for 10 minutes at 37°C. The reaction was initiated by adding the CNPG3 substrate (2 mM). The absorbance was measured at 405 nm after a 10-minute incubation. The percentage inhibition was calculated relative to a 100% activity control. The IC_{50} value for the positive control, acarbose, was found to be 4.853.

3.0 Summary of Results

3.1 Total Polyphenol Content and Anthocyanins

The two TPC assays yielded valuable data on the polyphenol content of the samples, while the anthocyanin assay provided specific insights into this class of flavonoids.

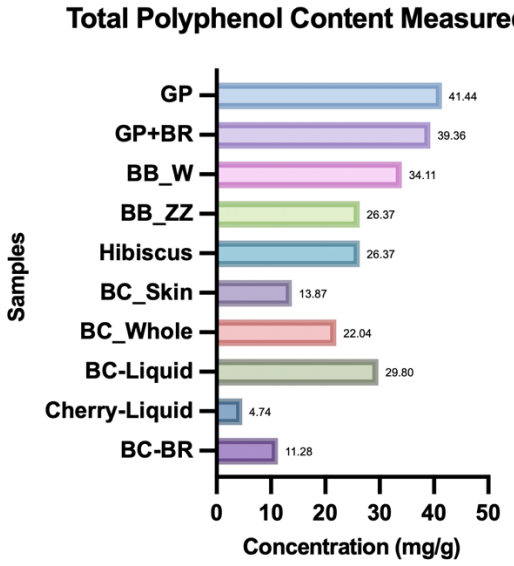

Figure 4. Total Polyphenol Content Measured by Folin

The Folin-Ciocalteu assay results indicate that the Grapes (GP) sample possesses the highest total phenolic content (TPC), at 41.44 µg/mL, followed closely by Grapes with Beetroot (GP+BR) at 39.36 µg/mL. This finding is consistent with existing research, which identifies grapes, particularly their pomace, as a rich source of phenolic compounds. The Blueberry (whole sample) (BB_W) also demonstrated a high TPC of 34.11 µg/mL. The blackcurrant samples showed a wide range of TPC values, with the Blackcurrant (liquid) (BC-Liquid) having a significantly higher content (29.80µg/mL) compared to the Blackcurrant (skin) (BC_Skin) (13.87 µg/mL). The Cherry-Liquid sample had the lowest TPC at 4.74 µg/mL. These results highlight the varying concentrations of total phenolic compounds among the fruit samples, providing a preliminary measure of their potential antioxidant and bioactive properties.

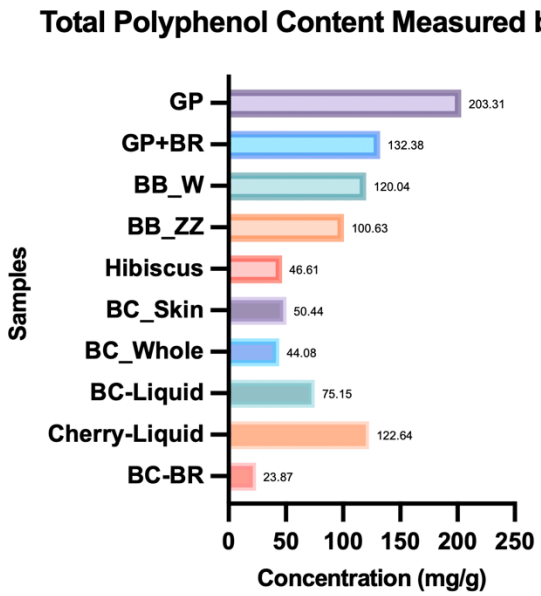

Figure 5. Total Polyphenol Content Measured by Fast Blue

The Fast Blue assay results provide a more specific measure of total phenolic content (TPC), as this method is less prone to interference from non-phenolic reducing agents. Consistent with the Folin-Ciocalteu assay, the Grapes (GP) sample showed the highest TPC at 203.31 µg/mL. This reinforces the conclusion that grapes are a rich source of phenolics. Interestingly, the Cherry-Liquid sample, which had the lowest TPC in the Folin assay, showed a relatively high TPC of 122.64 µg/mL in the Fast Blue assay. This discrepancy

suggests that its phenolic profile contains compounds that are more reactive with the diazonium coupling reagent used in this assay. The Blackcurrant with Beetroot (BC-BR) sample had the lowest TPC at 23.87 µg/mL. These results highlight the varying phenolic compositions among the samples and demonstrate the value of using a more specific assay to complement the broader Folin-Ciocalteu method.

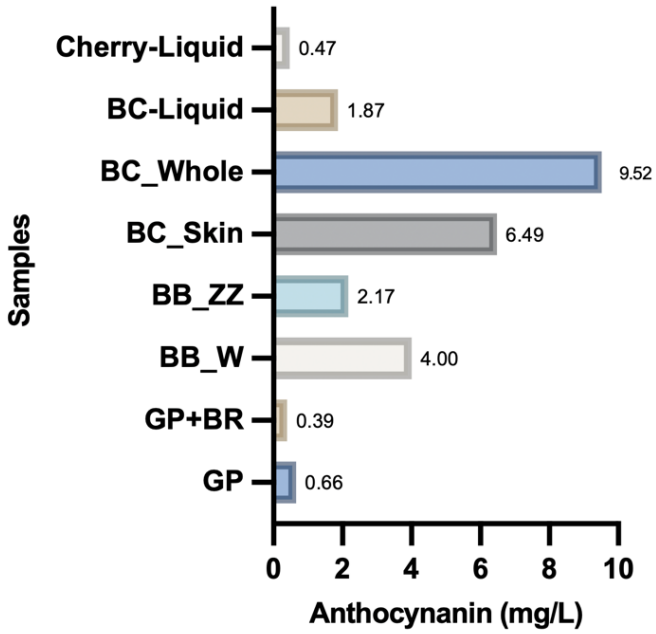

Figure 6. Anthocyanin Content

The BC_Whole sample contained the highest concentration of anthocyanins at 9.52 mg/L, followed closely by BC_Skin at 6.49 mg/L. This is expected, as anthocyanins are responsible for the dark color of blackcurrants and are highly concentrated in the skin.[11]

3.2 Enzyme Inhibition Activity

[11] Krga, I., & Milenkovic, D. (2019). Anthocyanins: From Sources and Bioavailability to Cardiovascular-Health Benefits and Molecular Mechanisms of Action. *Journal of agricultural and food chemistry*, *67*(7), 1771–1783. https://doi.org/10.1021/acs.jafc.8b06737

All fruit samples tested showed significantly greater inhibitory potency against both α-glucosidase and α-amylase than the pharmaceutical drug acarbose, as demonstrated by their much lower IC_{50} values.

3.2.1 Alpha-Glucosidase Inhibition

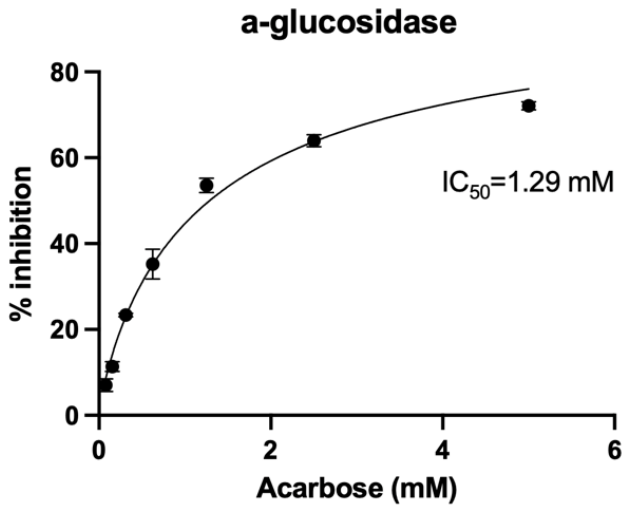

Figure 7. Percentage Inhibition of a-glucosidase by Acarbose

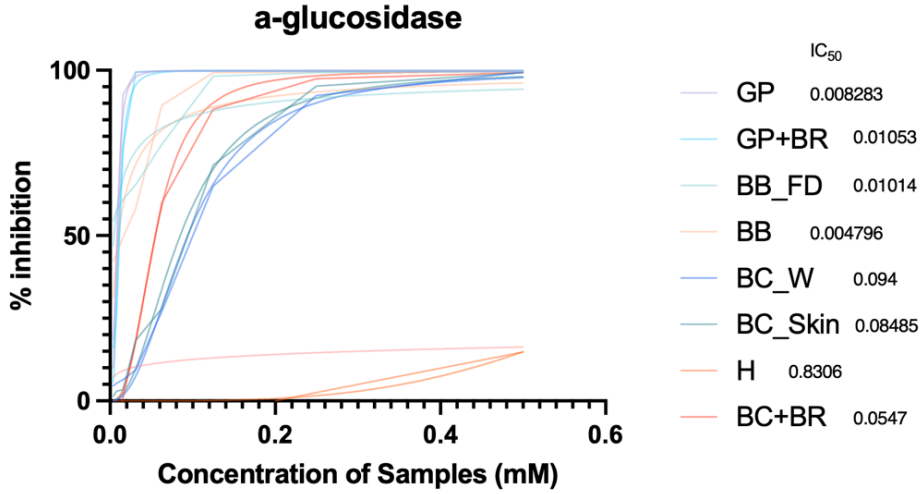

Figure 8. Percentage Inhibition of a-glucosidase by Different Samples

For α-glucosidase, the data reveals that the IC_{50} values for all tested fruit extracts are substantially lower than the positive control, acarbose (IC_{50} = 1.29 mM), highlighting their superior inhibitory efficacy in an in vitro setting.

Blueberry (fast dried) sample was the most potent, with an IC_{50} of 0.0048 mM, approximately 270 times lower than acarbose (1.29 mM). The Grapes and Blueberry (whole) samples were also highly effective, with IC_{50} values of 0.0083 mM and 0.0101 mM, respectively. Even the less potent extracts like Hibiscus (0.8306 mM) and the various blackcurrant samples (IC_{50} range 0.0547−0.0940 mM) proved to be more effective than acarbose. This superior efficacy suggests that the complex mixture of compounds in these natural extracts may provide synergistic inhibition.

3.2.2 Alpha-Amylase Inhibition

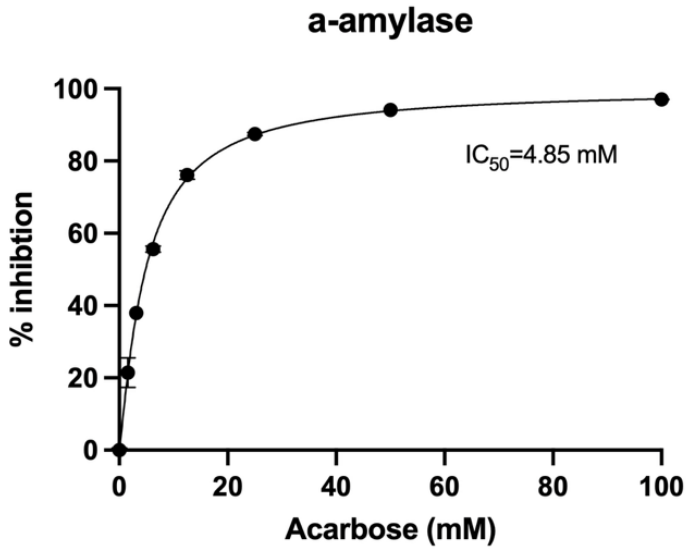

Figure 9. Percentage Inhibition of a-amylase by Acarbose

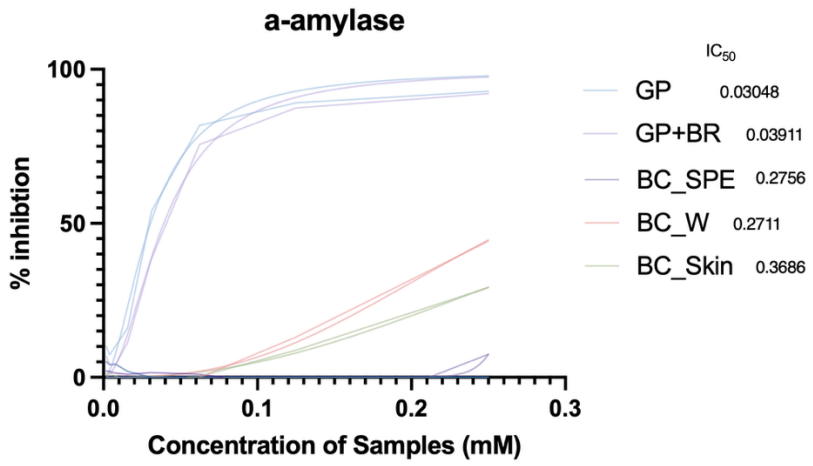

Figure 10. Percentage Inhibition of a-aymlase by Different Samples

The data reveals that the IC_{50} values for all tested fruit extracts are significantly lower than the IC_{50} of acarbose (4.853 mM), confirming their potential as natural anti-diabetic agents.

For α-amylase, the Grapes sample was the most potent inhibitor, with an IC_{50} of 0.0305 mM, which is about 159 times lower than that of acarbose (4.85 mM). The Grapes with Beetroot sample also demonstrated high potency (0.0391mM). Blackcurrant and blueberry samples were also significantly more effective than acarbose, with the Blackcurrant (whole) sample having an IC_{50} of 0.0940 mM. These findings provide strong evidence that these fruit extracts are potent natural anti-diabetic agents, and their differential potency highlights the importance of specific phenolic compounds in the inhibitory process.

4.0 Discussion

The results of this study provide a strong case for the anti-diabetic potential of the tested fruit samples, demonstrating a clear correlation between the presence of bioactive compounds and significant enzyme inhibitory activity. The use of two distinct total phenolic content (TPC) assays, Folin-Ciocalteu and Fast Blue, was critical for a comprehensive analysis of the samples' phytochemical profiles. While the Folin-Ciocalteu assay provides a broad measure of a sample's total reducing capacity, the Fast Blue assay is more specific to phenolic hydroxyl groups, offering a more precise estimate of bioactive phenolic compounds. The consistent

finding that grape samples had the highest TPC in both assays adds confidence to the results, supporting the notion that grapes, particularly their pomace, are a powerhouse of phenolic compounds. This aligns with their potent inhibitory effects on both α-glucosidase and α-amylase, likely due to a high concentration of non-anthocyanin polyphenols such as proanthocyanidins.

The exceptionally low IC_{50} values of the fruit extracts compared to acarbose are a key finding. This suggests that the complex mixture of bioactive compounds in these natural extracts may act synergistically, leading to a more potent inhibitory effect than the single-compound mechanism of acarbose. While acarbose is a known competitive inhibitor, the multi-compound nature of the fruit extracts may allow for a variety of inhibitory mechanisms, including competitive, non-competitive, or mixed inhibition, to occur simultaneously.

This multi-target approach could explain their superior efficacy and highlights the potential of whole-food matrices as a therapeutic strategy. Furthermore, the high anthocyanin content of the blackcurrant and blueberry samples likely contributed significantly to their bioactivity, consistent with existing literature. The observation that the fast-dried blueberry sample exhibited the most potent α-glucosidase inhibition is particularly interesting, as it suggests that specific processing methods can be optimized to enhance the anti-diabetic properties of food products. The slightly lower potency of the Grapes with Beetroot sample compared to pure Grapes also suggests a potential dilution or minor antagonistic effect from the added beetroot compounds. These findings provide a robust foundation for the future development of functional foods and natural inhibitors for the dietary management of postprandial hyperglycemia.

5.0 Conclusion

In summary, this study provides strong evidence that a variety of fruit samples, particularly grapes, blueberries, and blackcurrants, are rich in bioactive compounds with significant anti-diabetic potential. The experiments successfully quantified the total phenolic and anthocyanin

content and, most importantly, demonstrated a potent inhibitory effect on the carbohydrate-digesting enzymes, α-glucosidase and α-amylase. The IC_{50} values of several fruit extracts were found to be remarkably lower than that of the positive control, acarbose, suggesting that these natural sources possess a powerful capacity to manage postprandial hyperglycemia. The findings underscore the importance of dietary interventions and the potential of functional foods in the management of diabetes. Future research should focus on isolating the specific compounds responsible for the observed activities and conducting in vivo studies to confirm their efficacy and safety in a living system.