Antihyperglycemic and Anti-inflammatory Activities of Traditional Philippine Herbal Formula (Pito-pito)

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Abstract: Hyperglycemia causes long-term health complications when left untreated, especially in persons with diabetes. Managing the level of blood sugar is an important aspect of its treatment. Pito-pito is a multi-component herbal formula that may provide bioactivities to prevent elevated blood sugar levels. This study presents Pito-pito extracts' antihyperglycemic and anti-inflammatory activities that may reveal their potential medicinal value in managing blood sugar levels. The extracts of Pito-pito and its plant ingredients water extract were screened for antihyperglycemic activity by inhibition of α-amylase and α-glucosidase enzymes and effect on the glucose uptake in yeast cells. NO inhibition determined the anti-inflammatory activity. The results showed that the ethanolic extracts exhibited the highest inhibition in α-amylase, while the aqueous extract was inactive. Four of the 7 ingredients in the aqueous extract were inactive, and the 3 exhibited low inhibition activity. All extracts exhibited high inhibition in α-glucosidase relative to acarbose by several folds. It was shown that L. speciosa, P. guajava, and M. indica greatly contributed to its activity. The water, methanol, ethanol, acetone, dichloromethane, and hexane extracts of Pito-pito increased glucose uptake in yeast cells. Water, methanol, and hexane extracts inhibited nitric oxide by 88.83 ± 1.40 and 91.10 ± 2.05%, respectively. Pito-pito extracts possess antihyperglycemic and anti-inflammatory activities. The water extract of Pito-pito exhibited no inhibitory activity on α-amylase but exhibited promising activity on inhibiting α-glucosidase, increased glucose uptake in yeast cells, and promising NO inhibition activity.

Keywords: pito-pito; α-amylase inhibition; α-glucosidase inhibition; glucose uptake; nitric oxide inhibition.

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1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by an elevated level of sugar in the bloodstream, which leads over time to serious damage to the eyes, nerves, blood vessels, kidneys, and heart. According to World Health Organization (WHO), about 422 million people worldwide have diabetes, and 1.6 million deaths are directly attributed to diabetes each year. And in the last 2 decades, diabetes has been the leading cause of death and...
disability [1]. The number of cases and prevalence of diabetes are steadily growing and causing increasing concerns in public health.

Diabetes mellitus is a multifactorial disease, and in most cases, a combination of treatments is prescribed to patients. The treatment includes a combination of diet, exercise, administration of insulin, and oral medications, such as sulfonylureas, meglitinides, thiazolidinediones, biguanides, and glycosidase enzyme inhibitors [2]. Diet and exercise are considered the cornerstone of the treatment, and the administration of insulin depends on the type of diabetes and condition of the patient. In general, especially in the case of type II diabetes, oral medication is given in combinations to achieve multiple pharmacological targets that aim to provide a better therapeutic outcome. Ultimately, the objective is to reduce the elevated blood sugar level and maintain glucose homeostasis.

Inhibiting the glycosidase enzyme, such as α-amylase and α-glucosidase, to prevent dietary starch from breaking into monosaccharides is one of the strategies to control blood sugar levels. Alpha-amylase is present in salivary and pancreatic secretions and is responsible for catalyzing the initial step in the hydrolysis of starch to smaller oligosaccharides that are further degraded by α-glucosidase to form glucose [3,4]. Alpha-glucosidase is located in the brush-border surface membrane of the intestinal cell and is the rate-limiting step in converting oligosaccharides and disaccharides into monosaccharides necessary for gastrointestinal absorption [5]. Thus, regulating glycosidase enzymes to retard starch digestion plays a role in glycemic control. Alpha-glucosidase inhibitor, Acarbose (Glucobay) was first marketed in Germany in 1990 to treat diabetes [6]. Years after, the beneficial effects of α-glucosidase inhibitors were demonstrated [7]. However, the benefits of intestinal absorption inhibitors were considered to play a minor to a modest role in the treatment of type 2 diabetes mellitus and have a better role in the prevention of type 2 diabetes in high-risk populations [8].

Another key strategy to lower blood sugar levels is to enhance cells' glucose uptake by stimulation to activate glucose transporter. There are several types of glucose transporter in the human body that function in distinctive processes and mechanisms to facilitate glucose uptake. One of which is Glucose Transporter Type 4 (GLUT-4), the only glucose transporter that is insulin-responsive and is present in skeletal muscles, cardiac muscles, and adipose tissue. GLUT-4 is sequestered intracellularly and is translocated in the plasma membrane by insulin stimulation. Studies on plant materials that enhance glucose uptake have been reported., *Amomum xanthioides*, *Cinnamomum zeylanicum*, *Cecropia obtusifolia*, and *Lagerstroemia speciosa* are among plants that have been shown to enhance glucose uptake in adipose cells, either by potentiating insulin action or by similar signaling mechanisms to the insulin pathway [9-12].

One of the potential strategies to avert and prevent the progression of diabetes is to control the production of nitric oxide inflammatory mediators in the β-cells. The overproduction of nitric oxide can eventually cause further β-cell dysfunction and reduce insulin secretion [13]. Nitric oxide plays a vital role as a mediator in immune response, not only in β-cells but in many other biological processes of the immune system.

The aforementioned strategies are important in screening bioactive materials for diabetes treatment. Previous studies have shown the viability of plant material as bioactive agents, and several active pharmaceutical ingredients have been derived from bioactive plant research. Pito-pito is one of the Philippines' traditional medicines made from decocted leaves of *Lagerstroemia speciosa* Pers., *Mangifera indica* Linn., *Pandanus amaryllifolius* Roxb., *Premna odorata* Blanco., and *Psidium guajava* Linn., and seeds of *Coriandrum sativum* Linn.
and *Pimpinella anisum* Linn. Some of the biological activities of these individual plants as antihyperglycemic and anti-inflammatory, have been reported. However, no single study has been conducted on their combination as Pito-pito herbal medicine formula. The combination of plant materials may offer different bioactivity characteristics with respect to their components, which can justify its medicinal claims as herbal preparation that possess antihyperglycemic and anti-inflammatory properties.

This study determines the antihyperglycemic activity of Pito-pito herbal preparation (extracted from 7 solvents) on the inhibition of α-amylase and α-glucosidase enzymes and the enhancement of glucose uptake in the yeast cell. The water extracts of individual plant ingredients of Pito-pito were investigated to account for the plant material responsible for the activity and determine if additive property enhancement emerges as an effect of their combination. The anti-inflammatory property of Pito-pito was investigated by nitric oxide inhibition.

2. Materials and Methods

2.1. Materials

The leaves of *L. speciosa*, *M. indica*, *P. guavaja*, and *P. amaryllifolius*, *P. odorata*, and seeds of *C. sativum* and *P. anisum* were obtained from Quiapo Church vendor located in Manila City, Philippines. The plant materials were collected in Calumpit Bulacan, Philippines, on the 6th of October 2019. The authenticity of the plants was identified and certified by the Philippine Bureau of Plant Industry.

2.2. Sample preparation and extraction

The plant materials were air-dried for seven days in an enclosed air-conditioned room maintained at 25°C ± 2°C, 35-55% relative humidity. Then, the size of the leaves was reduced by mechanical means. The plant materials were subsequently dried for another 24 hrs in an oven maintained at 40°C. After which, the plant materials were mechanically crushed using a blender. The Pito-pito herbal powder blends were prepared by adding 8.0 g of leaf ingredients and 4.0 g of seed ingredients. A total of 48 g of powder blends were derived from the formula. The powder blends were prepared in seven replicates and then extracted using water, methanol, ethanol, acetone, ethyl acetate, dichloromethane, and hexane. The extraction was performed in a 1:20 ratio under reflux at 60°C for organic solvent for 2 hrs. The decoction with water was made in the traditional Chinese decoction pot until the volume of the water was reduced to approximately 200 mL. The crude extracts were collected by vacuum filtration, and the solvents were removed using a rotary evaporator. Then, the crude extract was freeze-dried and then weighed to determine the percentage yield. The decoction of individual plant ingredients was made in the same manner.

2.3. Determination of α-amylase inhibition activity

A 1.0% (w/v) potato starch solution was prepared by stirring potato starch in 20 mM saline phosphate buffer (pH 6.9, 6.7 mM NaCl) at 65°C for 15 minutes. The enzyme solution was prepared by mixing α-amylase in ice-cold saline phosphate buffer to give a concentration of 1.5 U/mL. The extracts were dissolved in ethanol and saline phosphate buffer solution (40:80) to give various concentrations by serial dilution. Acarbose was used as a reference drug.
and was prepared similarly to the extracts. Dinitrosalicylic acid (DNS) reagent was prepared by dissolving 5 g of dinitrosalicylic acid in 250 mL of double distilled (D.D.) water at 80°C and then cooled to room temperature. One hundred fifty grams (150 g) of potassium sodium tartrate was dissolved into 2N NaOH and then added to the dissolved DNS solution. The solution was diluted to 500 mL using D.D. water.

The inhibition experiment was conducted by mixing 100 µL of extract solution and 100 µL of α-amylase solution and incubated at 25°C for 10 min. Then, 100 µL of the starch solution was added and incubated at 25°C for 30 min. The reaction was terminated by adding 400 µL of DNS reagent and 300 µL of D.D. water and heated to 95°C for 5 min. The solution was allowed to cool at room temperature and centrifuged at 1300 rpm for 5 minutes. Two hundred fifty microliter (250 µL) of the solution was transferred in microliter plates and was analyzed in an ELISA microplate reader at 540 nm. The inhibition of the reference drug was performed similarly to the extract samples. Negative control was conducted by replacing the extract with ethanol – saline phosphate buffer (40:80) and then treated in the same manner. Method blanks were prepared and analyzed, containing the extract or the reference drug (in various concentrations similar to the samples) and α-amylase solution. The starch solution was replaced by the saline phosphate buffer solution.

The % inhibition was computed in all of the concentrations of the extracts and reference drug, as shown below. The 50% inhibitory concentration (IC_{50}) was estimated by regression, which was determined from the plot of % inhibition against the log of inhibitor concentration.

\[
\% \text{Inhibition} = \left( \frac{A_C - A_{C,MB}}{(A_C - A_{C,MB})} - \frac{A_S - A_{S,MB}}{(A_S - A_{S,MB})} \right) \times 100\%
\]

- \(A_C\): Absorbance of control
- \(A_{C,MB}\): Absorbance of the control method blank
- \(A_S\): Absorbance of extract sample or reference drug
- \(A_{S,MB}\): Absorbance of the extract or reference drug method blank

2.4. Determination of \(\alpha\)-glucosidase inhibition activity.

The enzyme solution was prepared by mixing \(\alpha\)-glucosidase in ice-cold 20 mM saline phosphate buffer (pH6.9, 6.7 mM NaCl) to give a concentration of 0.25 U/mL. The extracts were dissolved in ethanol and saline phosphate buffer solution (40:80) to give various concentrations. Acarbose was used as a reference drug and was prepared similarly. Fifty microliters (50 µL) of extracts or reference drug were added with 50 µL of \(\alpha\)-glucosidase solution in microliter plates and incubated for 10 min at 37°C. To initiate the reaction, the reaction was added fifty microliters (50 µL) of substrate, 1.5 mM p-nitrophenyl \(\alpha\)-D-glucopyranoside (pNGP) dissolved in saline phosphate buffer. The reaction was incubated for another 10 min at 37°C. The reaction was terminated by adding 100 µL of 0.5 M Na2CO3 solution. The method blanks of extract samples were also prepared to account for the background absorbance. The activity was determined by measuring the release of p-nitrophenol from pNGP in the ELISA microplate reader at 405 nm, maintained at 37°C. The % inhibition and IC50 were determined similar to the \(\alpha\)-amylase inhibition experiment.

2.5. Determination of glucose uptake by yeast cells.

This study was adopted from the method developed by Cirillo (1962) with modification [14]. Several researchers also adopted the method to determine the increase in glucose uptake in yeast cells due to introducing the plant extract in the yeast cells [15-18]. Yeast from \(\text{Saccharomyces cerevisiae}\) was washed and centrifuged at 1000 rpm for 5 min using double-
distilled (D.D.) water repeatedly until the supernatant liquid was clear. The yeast was suspended with D.D. water at 20% (v/v) concentration at 4°C before use. The extract and reference drugs (metronidazole and metformin) were dissolved in DMSO with concentrations 1.0-, 2.0-, and 5.0 µg/mL, corresponding to 100-, 200-, and 500 µg extract/drug load in the experiment. Six hundred twenty-five microliters (625 µL) of yeast suspension and 100 µL of extract or reference drug solution were pipetted to Eppendorf cupules and then incubated at 37°C for 10 min. Two hundred microliter (200 µL) of glucose solution with varying concentrations corresponding to 500-, 1000- and 2500 µg of glucose is loaded in the Eppendorf cupules and then incubated at 37°C for 60 min. After which, the Eppendorf cupules were centrifuged at 1300 rpm for 10 min. A predetermined volume of supernatant liquid (according to the amount of glucose loaded in the reaction vessel) was transferred to 2-mL Eppendorf cupules. A volume of 600-, 300-, and 120 µL (corresponding to 500-, 1000- and 2500 µg) of supernatant liquid was added with 400 µL of DNS reagent and then diluted to 1,000 µL using D.D. water. The solution was then heated to 95°C for 5 min. Two hundred fifty microliter (250 µL) of the solution was then transferred to 96-well microliter plates, and the absorbance was measured at 540 nm using an ELISA microplate reader. Positive and negative control samples were employed in the experiment. The positive control contained yeast suspension, glucose, and D.D. water (in place of extract/drug solution) in the experiment. The negative control contained glucose and extract/drug solution. The yeast suspension was replaced by D.D. water. The positive control accounts for the normal glucose uptake of the yeast cell, whereas the negative control serves as an indicative parameter to distinguish yeast inactivity in the samples. The negative control was performed in all of the extracts and glucose concentrations. Both controls were conducted in every single batch run of the experiments. Method blanks were prepared and analyzed, containing the extract or the reference drug (in various concentrations similar to the samples) to account for the background signals. The % in increase in glucose uptake of the extracts was computed as shown below.

\[
\% \text{Increase in glucose uptake} = \frac{(A_{PC} - A_B) - (A_S - A_{MB})}{(A_{PC} - A_B)} \times 100%
\]

- \(A_{PC}\): Absorbance of positive control
- \(A_B\): Absorbance of the negative control method blank
- \(A_S\): Absorbance of extract sample or reference drug
- \(A_{MB}\): Absorbance of the extract or reference drug method blank


The potential of Pito-pito as anti-inflammatory was performed on the RAW 264.7 murine macrophage cell line cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin in an incubator with 5% CO₂ at 37°C. Different doses of analyte were added into a 96-well plate and treated with 500 ng/mL lipopolysaccharide (LPS) overnight. Five milligram per milliliter (5 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 4 hours. The medium was then removed, and the formazan crystals were dissolved by isopropanol. The absorbance of the samples in the plate was measured at 570 nm, and the nitric oxide (NO) level present in the medium was measured at 530 nm after the addition of the Griess reagent. The percent inhibition of NO was computed as shown below.

\[
\% \text{NO Inhibition} = \left(1 - \frac{T}{C}\right) \times 100\%
\]

- \(T\): Optical density of LPS-stimulated RAW 264.7 cells with sample
- \(C\): Optical density of LPS-stimulated RAW 264.7 cells without sample
3. Results and Discussion

3.1. Determination of α-amylase inhibition activity.

The inhibition activities of the Pito-pito extracts and the water extract of individual plant ingredients are shown in Table 1. There was no α-amylase observed in the water extract of Pito-pito (AQ); whereas the most potent extract was derived from the ethanol extract (CQ), and the least was obtained from the acetone extract (DQ) with IC\textsubscript{50} values 1.71 ± 0.09 and 14.08 ± 1.11 mg/mL, respectively. The IC\textsubscript{50} value of the reference drug (acarbose) was determined as 1.27 ± 0.07 mg/mL, which is comparable to CQ extract. The next most potent extract is the methanol extract (BQ) with an IC\textsubscript{50} value of 2.75 ± 0.14 mg/mL, which is relatively half potent to acarbose. Next is hexane extract (GQ), dichloromethane extract (FQ), and ethyl acetate extract (EQ) with IC\textsubscript{50} values of 4.07 ± 0.01, 7.01 ± 0.25 and 12.24 ± 0.68 mg/mL, respectively.

Table 1. Alpha-amylase and α-glucosidase inhibition activity of Pito-pito extracts and their individual plant ingredients.

<table>
<thead>
<tr>
<th>Extract</th>
<th>α-Amylase Inhibition (IC\textsubscript{50}, mg/mL)</th>
<th>α-Glucosidase Inhibition (IC\textsubscript{50}, µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acarbose (RD)</td>
<td>1.27 ± 0.07</td>
<td>4,731 ± 24</td>
</tr>
<tr>
<td>Pito-pito, water extract (AQ)</td>
<td>NI</td>
<td>28.70 ± 0.08</td>
</tr>
<tr>
<td>Pito-pito, methanol extract (BQ)</td>
<td>2.75 ± 0.14</td>
<td>40.71 ± 0.70</td>
</tr>
<tr>
<td>Pito-pito, ethanol extract (CQ)</td>
<td>1.71 ± 0.09</td>
<td>72.26 ± 1.18</td>
</tr>
<tr>
<td>Pito-pito, acetone extract (DQ)</td>
<td>14.08 ± 1.11</td>
<td>77.61 ± 0.66</td>
</tr>
<tr>
<td>Pito-pito, ethyl acetate extract (EQ)</td>
<td>12.24 ± 0.68</td>
<td>225.53 ± 1.35</td>
</tr>
<tr>
<td>Pito-pito, DCM extract (FQ)</td>
<td>7.01 ± 0.25</td>
<td>174.13 ± 2.36</td>
</tr>
<tr>
<td>Pito-pito, hexane (GQ)</td>
<td>4.07 ± 0.01</td>
<td>68.57 ± 3.06</td>
</tr>
<tr>
<td>P. odorata, water extract (AR)</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>P. anisum, water extract (AS)</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>L. speciosa, water extract (AT)</td>
<td>38.11 ± 1.46</td>
<td>10.57 ± 0.28</td>
</tr>
<tr>
<td>C. sativum, water extract (AW)</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>P. guajava, water extract (AX)</td>
<td>19.85 ± 0.09</td>
<td>11.40 ± 0.28</td>
</tr>
<tr>
<td>M. indica, water extract (AY)</td>
<td>34.31 ± 0.94</td>
<td>23.55 ± 0.72</td>
</tr>
<tr>
<td>P. amaryllifolius, water extract (AZ)</td>
<td>NI</td>
<td>179.47 ± 2.25</td>
</tr>
</tbody>
</table>

The water extracts derived from individual plant ingredients P. odorata (AR), P. anisum (AS), C. sativum (AW), and P. amaryllifolius (AZ) were inactive against α-amylase as shown in Table 1. The water extract of P. guajava (AX) exhibited the most potent plant ingredient present in the water extract of Pito-pito. The activity is roughly 15-fold less potent than acarbose. L. speciosa (AT) and M. indica (AY) water extracts were approximately 30-fold less potent than acarbose with IC\textsubscript{50} values of 38.11 ± 1.46 and 34.31 ± 0.94 mg/mL, respectively.

The inhibitory activities of Pito-pito extracts derived from different solvents showed no evident trend in polarity. Moreover, the trend of the extract activities cannot be correlated to the content of total phenolics, flavonoids, condensed tannins, and polysaccharides determined from the previous study. The previous study revealed the following trends as to the content of total metabolites: phenolics (AQ > BQ > CQ > DQ > EQ > FQ > GQ), flavonoids (DQ > CQ > BQ > AQ > EQ > FQ > GQ), condensed tannin (DQ > CQ > EQ > FQ > BQ > GQ > AQ), and only water extract (AQ) was found to contain polysaccharides [19]. Based on these, it can be inferred that, in high likelihood, a single secondary metabolic compound exclusive in alcoholic extracts are responsible for the activity. This is because the water extract (AQ) that is high in phenolics and polysaccharides was found inactive, and the acetone extract that is high in flavonoid and condensed tannin was found to be least active among other extracts.
The α-amylase inhibition activities of the water extracts of individual plant ingredients justify no inhibition observed in AQ at 40 mg/mL concentration and down below. As presented, only 3 plant ingredients exhibited inhibition activity, and two of them are nearly 40 mg/mL. The presence of activity in these plant ingredients suggests that AQ extract may have inhibition activity above 40 mg/mL concentration. However, the experiment to determine the activity above 40 mg/mL entails difficulty because of analytical method challenges. Accounting for the exact magnitude greater than 40 mg/mL is impractical since the comparator or the reference drug is far lesser in magnitude.

Also, the results corroborated agreeably to available studies conducted on the individual plant ingredients. L. speciosa, P. guajava, and M. indica exhibited antihyperglycaemic properties. The bioactive component extracted from L. speciosa, corosolic acid, exhibited inhibition of α-amylase with an IC\textsubscript{50} value of 100.23 ± 1.09 µg/mL in the study conducted by Hou et al. (2008) [20]. Hung et al. (2007) derived ethyl acetate extract of P. guajava with α-amylase inhibition activity of 48.8 ± 3.6 µg/mL (IC\textsubscript{50}) [21]. The bioactive compound Mangeferin (from M. indica) has been shown to inhibit α-amylase activity. The IC\textsubscript{50} value of 36.84 µg/mL was determined and is relatively comparable to that of the acarbose IC\textsubscript{50} (21.32 µg/mL) [22]. Moreover, antihyperglycemic properties were determined in animal studies conducted on rats [23,24]. The inhibition of the α-amylase activity of P. odorata, P. anisum, and C. sativum was not reported to date. However, P. amaryllifolius was shown to increase insulin sensitivity in high-fat-diet-induced obese mice, and α-glucosidase inhibition activity was demonstrated [25,26]. In addition, the inhibition activity of P. amaryllifolius aqueous extract on α-amylase was reported by Nurdin et al. (2019) [27]. In this study, no inhibition activity was observed for P. amaryllifolius in α-amylase in extract concentrations of 40 mg/mL and below.

### 3.2. Determination of α-glucosidase inhibition activity

As opposed to the activity observed in α-amylase inhibition, AQ extract exhibited the highest α-glucosidase inhibition activity among Pito-pito extracts. The trend is decreasing as polarity decreases but somehow increases again in non-polar solvents (activity trend: AQ > BQ > GQ > CQ > DQ > FQ > EQ). The following α-glucosidase inhibition activities were determined, arranged in order of decreasing polarity as shown in Table 1: 28.70 ± 0.08 (AQ), 40.71 ± 0.70 (BQ), 72.26 ± 1.18 (CQ), 77.61 ± 0.66 (DQ), 225.53 ± 1.35 (EQ), 174.13 ± 2.36 (FQ) and 68.57 ± 3.06 µg/mL (GQ). The inhibition activity of acarbose was found to be significantly lower than all of Pito-pito extracts 4,731 ± 24 µg/mL).

Similar to the inhibition activity of α-amylase, the absence of α-glucosidase inhibition was also observed in extracts of AR, AS, and AW. The AZ extract, however, showed inhibitory activity with an IC\textsubscript{50} value of 179.47 ± 2.25 µg/mL. The extracts, AT, AX and AY likewise exhibited inhibition activity on α-glucosidase with IC\textsubscript{50} values of 10.57 ± 0.43, 11.40 ± 0.28, and 23.55 ± 0.72 µg/mL, respectively.

The results indicated that Pito-pito is a highly potent α-glucosidase inhibitor, as depicted in all extracts (from most polar to non-polar). The trend is interesting because the extracts derived from non-polar solvents deviate from the trend. From the water, the most polar, to ethyl acetate, the potency of the extract as inhibitor decreases (AQ > BQ > CQ > DQ > EQ); and then increases again in the extracts obtained from dichloromethane and hexane. Remarkably, the hexane extract has shown promising activities in two glycosidase enzymes, comparable to aqueous and alcoholic extracts.
The result showed that AT, AX, and AY extracts are strongly potent α-glucosidase inhibitors. In the previous study, several researchers have shown these plant ingredients to possess inhibitory activity on α-glucosidase in the previous study. The bioactive compounds from L. speciosa (extracted from ethyl acetate) were isolated and tested for α-glucosidase and α-amylase inhibitory activity by Hou et al. (2009) [20]. Here, the ethyl acetate extract of L. speciosa was found to inhibit the activity of α-glucosidase with an IC50 value of 88.72 ± 1.02 µg/mL. The isolated bioactive compounds were determined to inhibit the activity of α-glucosidase with IC50’s (in µg/mL) as follows: oleanolic acid (6.29 ± 0.37), arjunolic acid (18.63 ± 0.32), Asiatic acid (30.03 ± 0.41), maslinic acid (5.52 ± 0.19), corosolic acid (3.53 ± 0.27) and 23-hydrousolic acid (8.14 ± 0.18). Previous studies have reported the beneficial effects and human consumption safety of corosolic acid in managing diabetes mellitus [28-30]. Dihydro-3,3',4',5,7-pentahydroxyflavone glycoside was isolated from the water extract of P. guajava leaves and was found responsible for the α-glucosidase inhibitory activity [31]. An IC50 of 10 ± 0.04 mg/mL was determined for the bioactive flavone glucoside, a class of compounds belonging to the flavonoid. The bioactive compound, mangiferin from M. indica was reported to inhibit α-glucosidase with IC50 comparable to acarbose (mangiferin = 36.84 µg/mL; acarbose = 21.32 µg/mL) in the study conducted by Sekar et al. (2019) [22]. Mangiferin is a phenolic compound belonging to xanthone glycoside. Likewise, the water extracts of P. amaryllifolius have been shown to possess inhibitory activity [25,27]. The bioactive flavonoid compound quercetin has been shown to inhibit α-glucosidase activity [32].

In the previous study in Pito-pito extract, phenolics and flavonoids were determined as abundant chemical constituents; and the water extracts of L. speciosa, P. guajava, and M. indica have shown that they contributed significantly to Pito-pito herbal formula [19]. Moreover, flavonoid bioactive plant materials were frequently associated with glycosidase inhibition activity [32,33]. Hence, a bioactive compound belonging to phenolics and flavonoids can be construed as a material essential for discovering a novel and better inhibitory activity.

3.3. Determination of glucose uptake by yeast cells.

Figures 1 to 3 show the % increase in glucose uptake of Pito-pito extracts relative to the reference drugs metronidazole and metformin. The glucose uptake remains unaffected by the presence of acetone extract (DQ) in all substrate concentrations and regardless of extract dosage strengths. Likewise, the extract of ethanol (CQ) at 500 µg glucose substrate load remains unchanged, as shown in Figure 1. However, an increase in glucose uptake was observed in 1,000 and 2,500 µg substrate load, wherein a significant increase was observed in the latter, as shown in Figure 3. The potency relative to the reference drugs is greater at 100 and 200 µg dosage strengths.

On the other hand, the ethyl acetate extracts (EQ) exhibited a contrasting trend to CQ. A high increase in glucose uptake relative to the reference drugs was observed in the 500 µg substrate load and diminished as the glucose concentration increases. In figure 3, only the EQ extract with 500 µg dosage strength caused an increase in glucose uptake. The extracts of water (AQ) and methanol (BQ) caused a relatively weak effect in increasing the glucose uptake compared to the reference drugs. On the contrary, a very high increase in glucose uptake was derived from the extracts of dichloromethane (FQ) and hexane (GQ) in 500 and 1,000 µg substrate load. The potency remains significant in 2,500 µg substrate load; however, the reference drugs and the extracts obtained from polar solvent caused a greater increase in glucose uptake.
Figure 1. Percent increase in glucose uptake by yeast cells at 500 µg glucose load in the presence of the reference drug, metronidazole (MT), metformin (MF), and Pito-pito extracted from water (AQ), methanol (BQ), ethanol (CQ), acetone (DQ), ethyl acetate (EQ), dichloromethane (FQ) and hexane (GQ) solvents (in increasing drug/extract load).

Figure 2. Percent increase in glucose uptake by yeast cells at 1,000 µg glucose load in the presence of the reference drug, metronidazole (MT), metformin (MF), and Pito-pito extracted from water (AQ), methanol (BQ), ethanol (CQ), acetone (DQ), ethyl acetate (EQ), dichloromethane (FQ) and hexane (GQ) solvents (in increasing drug/extract load).

The effect of individual plant ingredients on the glucose uptake in yeast cells is shown in Figures 4 to 6. The glucose uptake of water extracts from L. speciosa (AT), P. guajava (AX), and M. indica (AY) were indifferent from the positive control at 500 µg substrate concentration. An increase in glucose uptake relative to positive control was observed at a higher concentration of substrate (i.e., at 1,000 and 2,500 µg of glucose). An increasing trend holds to other plant ingredients except for the water extracts of C. sativum (AW). Furthermore, an increasing potency relative to the reference drug was observed when the substrate concentration increased.

In Figure 4, the % increase in glucose uptake in both of the reference drugs is higher than all of the extracts at 500 µg glucose concentration.
Figure 3. Percent increase in glucose uptake by yeast cells at 2,500 µg glucose load in the presence of the reference drug, metronidazole (MT), metformin (MF), and Pito-pito extracted from water (AQ), methanol (BQ), ethanol (CQ), acetone (DQ), ethyl acetate (EQ), dichloromethane (FQ) and hexane (GQ) solvents (in increasing drug/extract load).

A reversing trend was observed as the glucose concentration increases, wherein the water extracts of *P. odorata* (AR) and *P. anisum* (AS) become significantly high, and all other remaining extracts become comparable to the reference drugs. In addition, there was no additive effect observed to the glucose uptake activity of Pito-pito water extract as a result of combining the 7 plant ingredients.

Figure 4. Percent increase in glucose uptake by yeast cells at 500 µg glucose load in the presence of the reference drug, metronidazole (MT), metformin (MF), Pito-pito water extract (AQ), and the individual plant ingredient water extract *P. odorata* (AR), *P. anisum* (AS), *L. speciosa* (AT), *C. sativum* (AW), *P. guajava* (AX), *M. indica* (AY) and *P. amaryllifolius* (AZ) (in increasing drug/extract load).

The reference drugs, metformin, and metronidazole increased the glucose uptake in yeast cells. The percent increase in glucose uptake is substantial for metronidazole at the highest dose administered (500 µg) and highest glucose load (2,500 µg). On the other hand, the highest increase in glucose uptake observed in metformin was on a glucose load of 500 µg and drug strength of 500 µg. Metformin is considered the first line of treatment for diabetic patients. Its mechanism of action is still not fully understood, and thus far, it has been shown to reduce the production of hepatic glucose as its primary mode of drug action [34].
Figure 5. Percent increase in glucose uptake by yeast cells at 1,000 µg glucose load in the presence of the reference drug, metronidazole (MT), metformin (MF), Pito-pito water extract (AQ), and the individual plant ingredient water extract *P. odorata* (AR), *P. anisum* (AS), *L. speciosa* (AT), *C. sativum* (AW), *P. guajava* (AX), *M. indica* (AY) and *P. amaryllifolius* (AY) (in increasing drug/extract load).

Moreover, the drug is known to increase glucose uptake into cells [35,36]. It has been reported that metformin increases the glucose uptake in peripheral tissues by regulating Glucose Transporter (GLUT) trafficking [37,38]. Recently, it has been shown that metformin enhances the glucose uptake into cells by inhibiting the lipid phosphatase Src homology 2 domain-containing inositol-5-phosphatase 2 (SHIP2) activity [39]. The experiment on glucose uptake in yeast cells can provide insights into its potential outcome in an organism with vascular supply. Furthermore, several studies utilized yeast as a model to understand glucose transport across the cell membrane [14,40-42]. And studies on the use of metformin as a reference drug that involves glucose uptake experiments have been reported [43-45]. On the other hand, other researchers used metronidazole as a reference drug [46-48]. Metronidazole is an antibiotic and antiprotozoal drug. However, it has been shown to increase the uptake of nutrients, including glucose, in rat animal models [49].

In general, a high increase in glucose uptake was observed in the extracts obtained from non-polar solvents. However, the effect diminished at a high concentration of substrate. It is
possible that the extracts from non-polar solvents gradually caused toxicity in the yeast cell. FQ and GQ are lipophilic extracts, and a large amount of the extract could have slowly deposited in the yeast intracellularly over time. As a result, the diffusion of glucose in the experiment was halted, and the manifestation can be observed when the substrate concentration is high. Thus, the impediment of diffusion caused by gradual cell mortality is probable. This claim, however, needs further verification and is beyond the scope of the study.

It is remarkable that the extracts obtained from polar solvents (water, methanol, and ethanol), at 2500 μg glucose concentration, exhibited a greater increase in glucose uptake relative to the extracts obtained from non-polar solvents and references drugs, except for metronidazole at the highest dose strength (500 μg). This result suggests that the extracts may have better efficacy in enhancing glucose uptake, and potential benefits could be observed when glucose concentration is high. Moreover, the water extract (AQ) exhibited an obvious trend of dose dependency. The glucose uptake of plant ingredient water extracts revealed no additive enhancement of activity observed as a consequence of its combination in the formula. However, a substantial increase in glucose uptake was observed in AR and AS in 1,000 and 2,500 μg glucose concentrations. This indicated that these plant ingredients are more effective in enhancing glucose uptake when administered alone rather than as included in the Pito-pito formula. There has been no reported study on the effect of *P. odorata* and *P. anisum* extracts on glucose uptake. However, a recent study has shown that acacetin, one of the extracted bioactive materials from *P. odorata*, enhances glucose uptake through insulin-dependent GLUT-4 translocation in L6 myotubes [50].


The survival rate of RAW 264.7 cells in MTT and % NO inhibition is presented in Table 2. The inhibition activity of the extracts derived from various solvents decreases from polar to solvents with intermediate polarity and then increases as polarity decreases (polar > intermediate polarity < non-polar). The highest inhibition activity was obtained from the hexane extract (91 ± 2.05%), followed by the water extract (88.83 ± 1.4%). The lowest inhibition activity was observed in acetone extract (66.49 ± 4.35%). The highest and lowest survival rate was observed from the extracts of non-polar solvents. The lowest was obtained from dichloromethane extract (0.91 ± 1.84%), while the highest was hexane extract (91.89 ± 0.85%). The water extract exhibited a 73.09 ± 1.67% survival rate. Based on the results, the hexane extract is potentially the most promising extract, followed by the water extract.

<table>
<thead>
<tr>
<th>Extract</th>
<th>MTT Survival Rate (%)</th>
<th>NO Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQ</td>
<td>73.09±1.67</td>
<td>88.83±1.4</td>
</tr>
<tr>
<td>BQ</td>
<td>72.86±0.3</td>
<td>87.38±1.44</td>
</tr>
<tr>
<td>CQ</td>
<td>71.57±1.69</td>
<td>74.11±5.56</td>
</tr>
<tr>
<td>DQ</td>
<td>2.66±5.1</td>
<td>66.49±4.35</td>
</tr>
<tr>
<td>EQ</td>
<td>59.64±0.38</td>
<td>73.21±4.38</td>
</tr>
<tr>
<td>FQ</td>
<td>0.91±1.84</td>
<td>72.75±4.69</td>
</tr>
<tr>
<td>GQ</td>
<td>91.89±0.85</td>
<td>91.1±2.05</td>
</tr>
</tbody>
</table>

The extracts of Pito-pito caused significant inhibition of NO. The highest of which is the hexane extract (GQ), followed by water (AQ) and methanol (BQ) extracts, respectively. The MTT survival rate in GQ extract is also substantially high, followed by AQ and BQ, respectively. The MTT survival rate in FQ extract is substantially low. Based on the result, the
extracts from hexane, water, and methanol demonstrated a promising nitric oxide inhibition activity.

4. Conclusions

The highest α-amylase inhibitory activity was observed in alcoholic extracts of Pito-pito. The water extract was inactive at a 40 mg/mL concentration and below. Likewise, the water extracts from the plant ingredients revealed that 4 out of 7 ingredients were inactive, and the remaining 3 are several folds less potent than acarbose. The α-glucosidase inhibitory activity of Pito-pito extracted from all of the 7 seven solvents were several folds potent than acarbose. The highest activity was observed in water extract with an IC$_{50}$ value of 28.70 ± 0.08 µg/mL, which is 160-fold more potent than acarbose in terms of IC$_{50}$. Among the plant ingredients, L. speciosa (IC$_{50}$ = 10.57 ± 0.28 µg/mL), P. guajava (IC$_{50}$ = 11.40 ± 0.28 µg/mL) and M. indica (IC$_{50}$ = 23.55 ± 0.72 µg/mL) were the most active. The water extract of P. amaryllifolius demonstrated activity with an IC$_{50}$ value of 179.47 ± 2.25 µg/mL, while the extracts of P. odorata, P. anisum, and C. sativum were inactive. Except for the ethyl acetate extract, Pito-pito extracts enhanced the glucose uptake in yeast cells. The extracts demonstrated a degree of dose-dependence activity, except for dichloromethane extract and hexane extract, in which the latter exhibited an unclear trend towards dosage strength. All the plant ingredient's water extracts caused an increase in glucose uptake. The activity was exceptionally high for P. odorata and P. anisum water extracts in relation to the other water extracts. A dose-dependent trend was observed in the water extracts of L. speciosa, C. sativum, and P. amaryllifolius. The plant ingredients caused no apparent additive potency when combined as the Pito-pito formula. The hexane, water, and methanol extracts demonstrated promising nitric oxide inhibition activity.

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Conflicts of Interest

The authors declare no conflict of interest.

References


