PJHRD Philippine Journal of Health Research and Development

RESEARCH COMMUNICATION

# Inhibitory effects of *Piper umbellatum* Linn. leaf extracts on α-amylase and α-glucosidase activities

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**ABSTRACT**

**Background:** Alpha-amylase and alpha-glucosidase are essential enzymes in converting food into energy inside the body. However, the proper management of these enzymes is needed to maintain normal blood glucose levels in the body. Thus, inhibiting these carbohydrate-hydrolyzing enzymes is necessary for managing hyperglycemia and alleviating Type 2 Diabetes Mellitus complications. Studies have reported that the Piper species have inhibitory properties on these digestive enzymes. However, the locally cultivated *P. umbellatum* has not yet been studied.

**Objectives:** The study evaluated the inhibitory potentials of the locally grown *P. umbellatum* leaf extracts against α-amylase and α-gluiosidase.

**Methodology:** The *P. umbellatum* leaf extracts were screened for *in vitro* inhibitory α-amylase and α-glucosidase tests. The most active crude extract was subjected to semi-purification and fractions were subjected to inhibitory enzymatic tests. Qualitative and quantitative phytochemical analyses were conducted.

**Results:** Among all crude extracts, the KMB-HE exhibited the highest activities comparable to that of the acarbose standard ($p>0.05$). The KMB-HE showed inhibitions of 81.01%±1.66 and 89.51%±3.03 on α-amylase and α-glucosidase at 250 µg/mL, respectively. In addition, it contained the highest phenolic (195.00 ±3.60 GAE mg/gram) and flavonoid (4.55±0.06 QE mg/gram) contents that may be linked to the observed activities. The semi-purification of KMB-HE collected 24 fractions with PF10 demonstrating inhibitions of 68.07%±13.48 and 74.21%±2.22 in α-amylase and α-glucosidase, respectively. Also, the PF10 contained the highest phenolic (204.75±2.42 GAE mg/gram) and flavonoid (4.00±0.07 QE mg/gram) compounds among all the active fractions tested. The PF10 satisfied the recommended criteria for plant natural inhibitors: a moderate-to-low α-amylase and stronger α-glucosidase inhibitory activity.

**Conclusion:** The *P. umbellatum* exhibited good inhibitory activities on carbohydrate-hydrolyzing enzymes. These may lead to further analysis of the *P. umbellatum* for future development of a safer, locally available natural remedy in managing Type 2 Diabetes Mellitus.

**Keywords:** *Piper umbellatum, α-amylase, α-glucosidase, type 2 diabetes mellitus*

## Introduction

Metabolism of dietary carbohydrates provides energy utilized to maintain body processes. However, an imbalance in carbohydrate consumption and utilization may alter the overall metabolic function of the body. Diabetes is one of these chronic metabolic diseases, characterized by sustained hyperglycemia – high glucose levels in the blood. This disease has affected over 537 million individuals worldwide [1,2]. If not treated, this disease may lead to significant secondary complications, such as obesity, dyslipidemia, non-alcoholic fatty liver disease, hypertension, systemic inflammation, and ketoacidosis [3,4].

The carbohydrate-hydrolyzing endopeptidases, such as α-amylase and α-glucosidase, play a vital role in the regulation

of dietary carbohydrates metabolism. The alpha-amylases produced by the mouth and pancreas split starch polymers to produce limit dextrins and maltoses; while α-glucosidase found on the intestinal brush border catalyzes the cleavage of disaccharides to form glucose. These enzymes' overall action results in the complete hydrolysis of complex carbohydrates to form monomeric glucose units before absorption. Thus, the inhibition of their action may delay the uptake of dietary carbohydrates by enterocytes and suppress a sudden increase in blood glucose level, known as postprandial hyperglycemia [5-7].

Currently, the use of medicinal plants with inhibitory activities against α-amylase and α-glucosidase has been promoted World Health Organization in treating hyperglycemia and diabetes. Studies have shown that the use of plants is much cheaper and safer when compared to synthetic inhibitors [8-11].

Studies have shown that plants under the *Piper* species have phytochemical constituents that may inhibit α-amylase and α-glucosidase enzymes. *Piper* plants such as *P. guineense*, *P. betel, P. trioicium*, *P. longum*, and *P. angustifolium* were found to have α-amylase and α-glucosidase inhibitory activities [12-14]. In the Philippines, an underexplored *Piper* species, *Piper umbellatum* – also known as 'Kamamba' has been highlighted for use as food and ethnopharmacological purpose to treat stomach disorders, malaria, edema, and kidney problems [15]. In addition, some studies have shown that this plant also exhibits bioactivities such as antioxidant [16], antibacterial [17], anti-fungal [18], and anti-atherogenic [19] properties. Thus, this study investigated the potential inhibitory activity of *P. umbellatum* on α-amylase and α-glucosidase.

## Methodology

*Chemicals and Reagents*

Hexane, ethyl acetate, methanol, ethanol, α-amylase, starch solution, 3,5-dinitrosalicylic acid, sodium hydroxide, sodium potassium tartrate, dimethylsulfoxide (DMSO), α-glucosidase, and 4-nitrophenyl-D-glucopyranoside (ρ-NPG) were purchased from Merck. All other chemicals used were of analytical grade.

Plant Sample

The *Piper umbellatum* Linn. leaves were collected from Lucban, Quezon. A voucher (PU 11162017) was submitted and authenticated by Jose Vera Santos Memorial Herbarium, Institute of Biology, University of the Philippines Diliman.

*Plant Sample Processing and Extraction*

The *P. umbellatum* leaves were washed with distilled water and air-dried for seven days. The air-dried leaves were manually comminuted and weighed. One hundred grams of air-dried *P. umbellatum* leaves were subjected to serial extraction using solvents of increasing polarity: hexane, ethyl acetate, methanol, and ethanol. The dried plant leaves were soaked in 500 mL of hexane at room temperature for 24 hours. The sample was then filtered, and the residue was re-extracted thrice with the same solvent using the same proportion. The *P. umbellatum* residue was re-extracted with the next solvent and was redone until all solvent extracts were gathered.

On a separate extraction set-up, 100 g of the *P. umbellatum* was soaked in 500-mL 1:1 hexane: ethyl acetate solvent system. All filtered extracts were concentrated using a rotary evaporator at 40˚C.

Meanwhile, aqueous extracts were obtained through decoction. One hundred grams of air-dried leaves were boiled for 10 minutes. The solution was then filtered and subjected to lyophilization using a Martin Crist Freeze dryer BETA 2-8 LSC. All plant crude extracts were properly labeled and kept in tightly-sealed bottles at 4°C until use. The percent yield was calculated for all crude extracts.

*Qualitative Phytochemical Screening [20,21]*

The secondary metabolites such as proteins, alkaloids, glycosides, steroids and phytosterols, terpenes and terpenoids, polyphenols, flavonoids, and tannins were determined in each plant sample using preliminary and confirmatory tests. The presence of proteins was detected using Ninhydrin and Biuret test; alkaloids using Mayer's, Wagner's, Hager's, and Dragendorff's tests; glycosides using Modified Borntranger's and Keller Killiani; steroids using Liebermann-Burchard test; terpenes and terpenoids using Salkowski's test; flavonoids using alkaline reagent and Shinoda's tests; polyphenols using ferric chloride test; and tannins using ferric chloride and gelatin tests.

*Quantitative Phytochemical Tests*

*Total phenolic content [22]*

The total phenolic contents of the *P. umbellatum* leaf extracts were determined using the Folin–Ciocalteu method with modifications. A volume of 15.4µL of test

compounds and gallic acid standard (0-500 µg/mL) was mixed with 61.5µL of Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and was neutralized with 123µL of 7.5% sodium carbonate. The mixtures were allowed to stand at room temperature for 30 minutes. Absorbances were measured at 765 nm. The amount of phenolics was expressed as mg gallic acid equivalence per gram of the dried extract (mg/g) using a gallic acid standard curve. All determinations were performed in triplicates.

*Total Flavonoid Content [22]*

The total flavonoid content was measured using an aluminum chloride colorimetric assay. A volume of 100 µL of extracts or quercetin standard solutions (0-500 µg/mL) was mixed with 2% (w/v) $AlCl_3$ (100µL) in 96-well plates. After 15 minutes of incubation at room temperature, the absorbances were read at 435nm. The number of flavonoids was expressed as mg quercetin equivalence per gram of the dried extract (mg/g) using the quercetin standard curve. All determinations were performed in triplicates.

*Enzyme Inhibition Assays*

*α-Amylase enzyme inhibition assay [23]*

The extracts collected from the *P. umbellatum* were tested for their inhibitory activity against pancreatic α-amylase using 3, 5-dinitrosalicylic acid (DNS) method with slight modifications. In a 96-well plate, 100 µL of (250, 500, 1000 µg/mL) plant extracts (reconstituted on 1% DMSO), 200 µL porcine pancreatic amylase was added to the reaction mixture and incubated at 37 ºC for 20 minutes. Then, the reaction mixture was added with 100 µL (1%) starch solution and incubated at 37 ºC for 10 minutes. The reaction was stopped by adding 200 µL DNSA and kept in a boiling water bath for 5 minutes. The reaction mixture was diluted with 2.2 mL of water. Absorbance was read at 540 nm using a spectrophotometer.

Blank tubes were prepared for each concentration by replacing the enzyme solution with 200 µL buffer. The experiments were repeated 5 to 10 times per setup using the same protocol. The absorbance of the resulting mixture was read at 540 nm. The results were expressed as percentage inhibition using the formula:

% Inhibition = ([Abs control- Abs samples]/ Abs control) x 100

*α-Glucosidase enzyme inhibition assay [23]*

The α-glucosidase inhibitory activity of the extracts was determined. To 100 µL (250, 500, and 1000 µg/ml) of plant extracts (reconstituted on 1% DMSO), 200 µL α -glucosidase (Bacillus stearothermophilus) was added. The mixture was then incubated at 37°C for 20 minutes. The reaction mixture added 100 µL of 3mM ρ-NPG and then incubated at 37°C for 10 min. The reaction was terminated using 2 mL of 0.1 M $Na_2CO_3$. The α-glucosidase activity was determined spectrophotometrically at 405 nm. The acarbose was used as the positive control. The experiments were repeated 5 to 10 per setup using the same protocol. The alpha-glucosidase inhibitory activity was calculated as percentage inhibition, as mentioned for α-amylase enzyme inhibition.

*Semi-purification of Most Active Extract*

The most active crude extract exhibiting the highest enzyme inhibitory activity was partially purified using silica gel column chromatography. A column packed with silica gel was used to semi-purify the said extract. Varying proportions of hexane and ethyl acetate solvent systems with a volume of 100 mL per gradient were used as mobile phases in the set-up. Fractions were collected at a volume of 20 mL.

A thin-layer chromatography was conducted to single out or pool fraction/s with similar distinctions (bands and Rf value). A ratio and proportion ranging from 9:5:0.01-9:3:0.01 of the solvents hexane, ethyl acetate, and acetic acid was used in the fractions as a solvent system. Fractions were then used in the *in vitro* enzyme inhibitory assays to determine which fractions have α-enzymatic inhibitory activity.

*Statistical Analysis*

All experiments under *in vitro* studies were performed in duplicates or triplicates. The means, standard errors, and standard deviations were calculated from replicates within the experiments. The statistical analysis was done by one-way analysis of variance (ANOVA) with LSD post hoc test for multiple comparisons. The statistical significance was accepted at $p<0.05$.

## Results

In this study, the bioactivity of the *P. umbellatum* crude extracts was tested against carbohydrate-digesting enzymes. The screening of the extracts' phytochemical constituents, semi-purifying the most active extract, and identifying its fraction with the highest enzyme inhibitory activity were also conducted.

Extract names were coded for ease of recognition. Samples were labeled as KMB with corresponding letters for solvents used in the experiment.

*Plant Sample Extraction*

A high extraction yield was observed in polar solvents than in their non-polar counterparts (Table 1). The crude leaf extracts of the *P. umbellatum* showed extraction yields in the following descending order: KMB-Aq < KMB-EtOH < KMB-MeOH < KMB-EtOAc < KMB-H.

*Inhibitory Activity of Crude Extracts on Carbohydrate-hydrolyzing Enzymes*

The KMB crude extracts showed varying results in α-amylase and α-glucosidase enzymes. The non-polar extracts, KMB-H (70.85%±3.51 and 77.33%±5.59) and KMB-EtOAc (71.57%±5.44 and 79.03%±5.74), exhibited the most effective α-amylase inhibition at 250 and 500 µg/mL, respectively (Figure 1). In addition, these extracts were indicated to have no significant difference ($p>0.05$) with the inhibition of the positive control, acarbose (66.13%±3.46 and 66.44%±4.36) at the said

**Table 1.** *Percent Yield of P. umbellatum Leaf Extracts*

| Sample Number | Extracts | Sensory observations (Viscosity, color, texture) | Percentage Yield (%) |
|---|---|---|---|
| 1 | KMB-H (Hexane) | Viscous, dark green in color, oily in texture | 6.81 |
| 2 | KMB-EtOAc (Ethyl Acetate) | Viscous, dark green in color, oily in texture | 6.87 |
| 3 | KMB-MeOH (Methanol) | Dry and sticky in texture, dark green in coor | 10.09 |
| 4 | KMB-EtOH (Methanol) | Dry and sticky in texture, yellow to green in color | 11.50 |
| 5 | KMB-Aq (Water) | Powdery, brown | 40.47 |
| 6 | KMB-HE | Viscous, dark green in color, oily in texture | 18.00 |

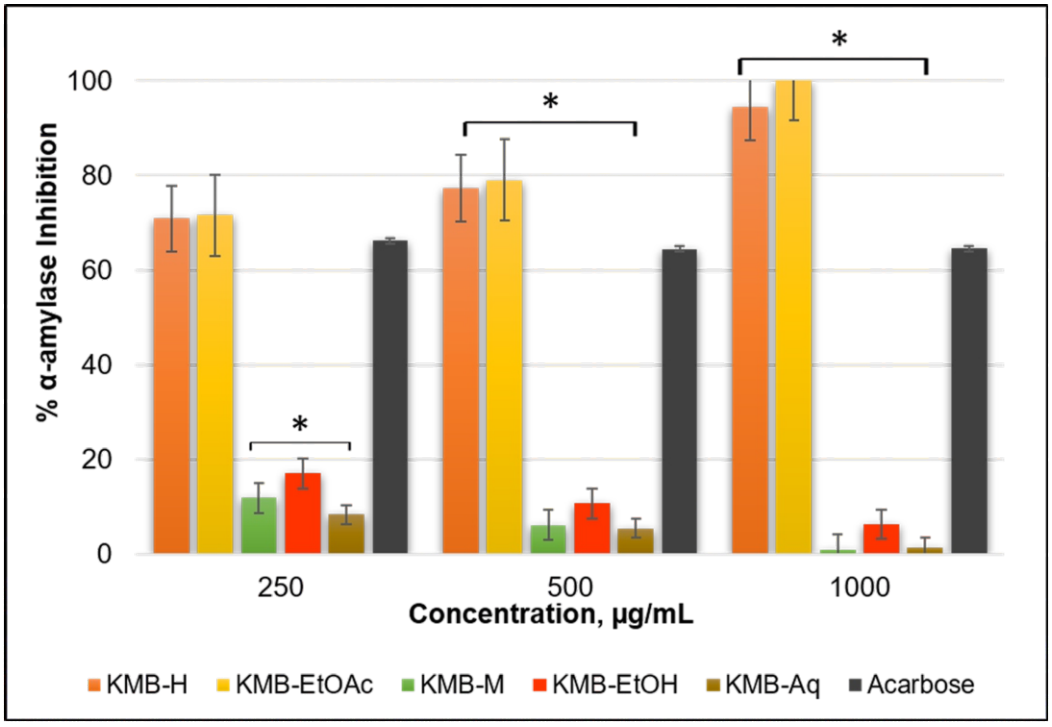


**Figure 1.** *Alpha-amylase inhibition of P. umbellatum crude leaf extracts; Values are mean ±SEM (n=3); * denotes p<0.05, comparison of treatment vs. Acarbose*

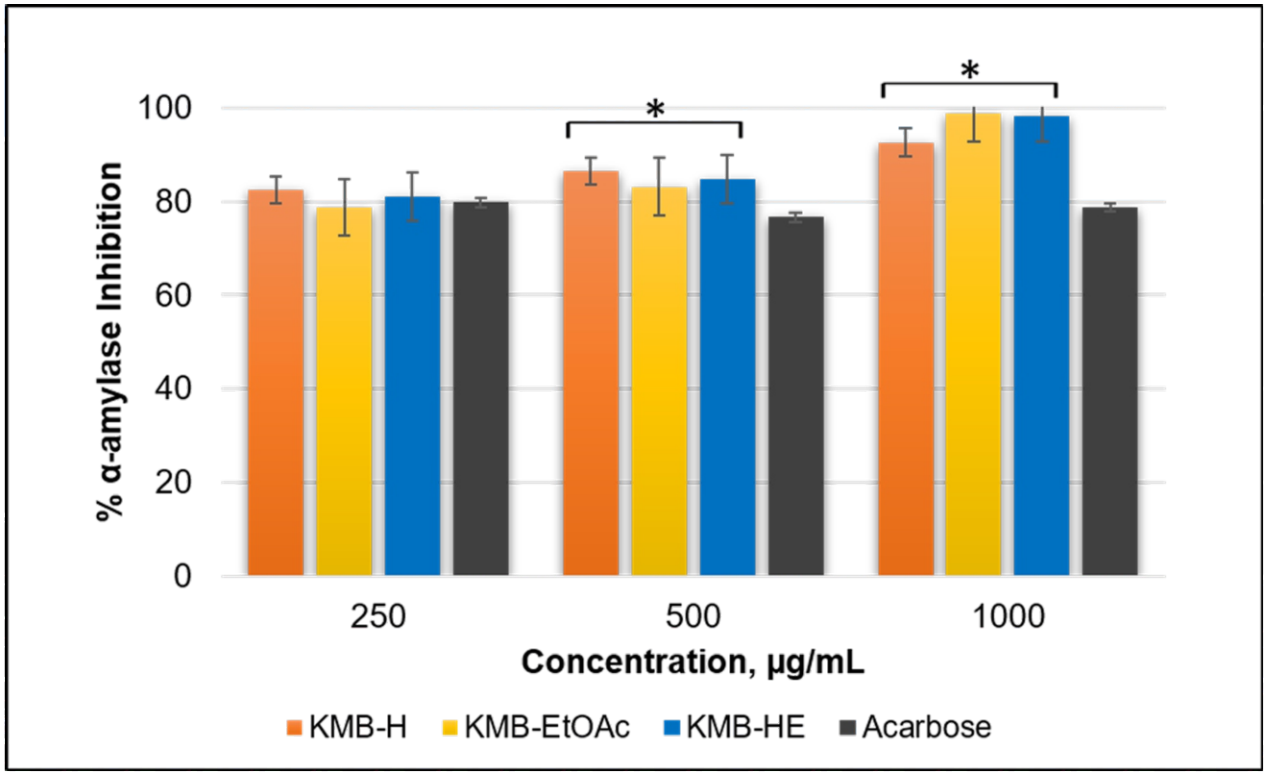


**Figure 2.** *Alpha-amylase inhibition of P. umbellatum crude leaf extracts of KMB-H, KMB-EtOAc, and KMB-HE; Values are mean ±SEM (n=3); * denotes p<0.05, comparison of treatment vs. Acarbose*

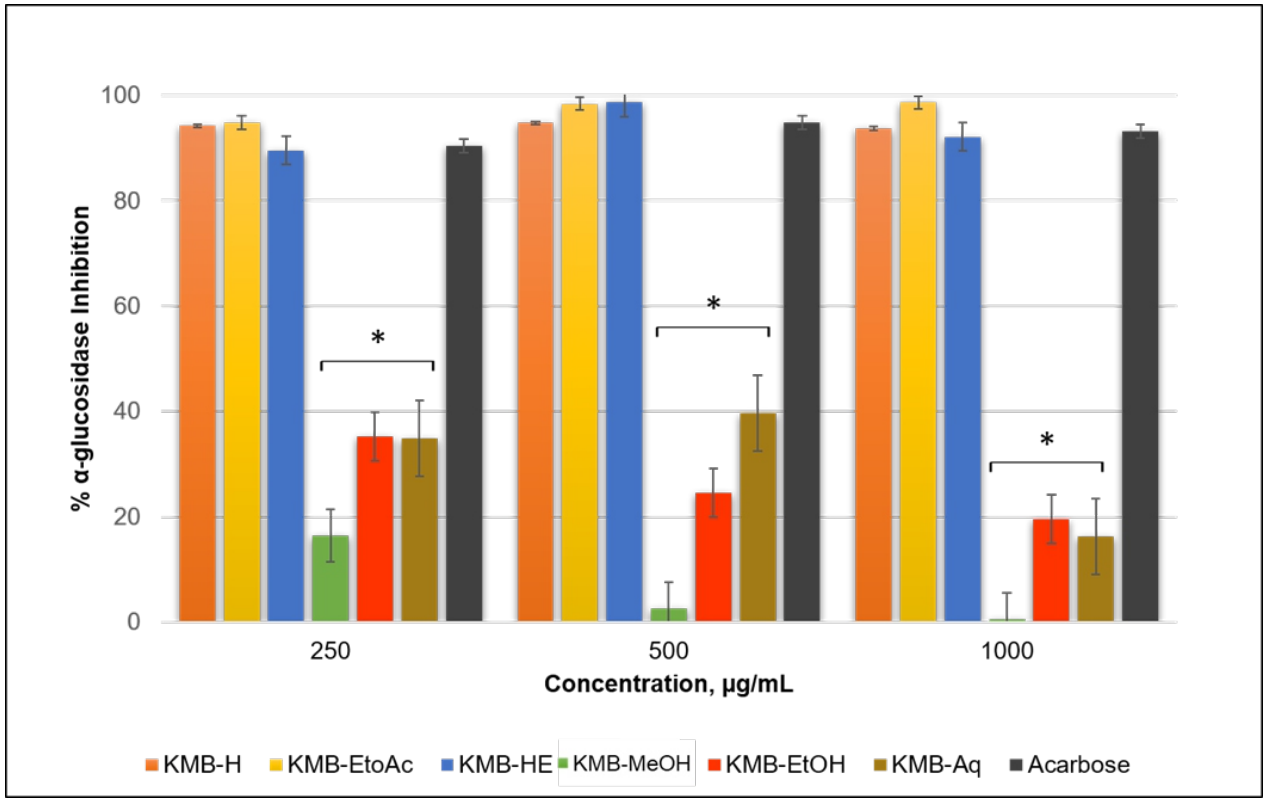


**Figure 3.** *Alpha-glucosidase Inhibitory Activity of P. umbellatum Crude leaf Extracts KMB-H, KMB-EtOAc, KMB-HE, KMB-MeOH, KMB-EtOH, and KMB-Aq; Values are Mean ±SEM (n=3); *denotes p<0.05, comparison of treatment vs. Acarbose*

concentrations. A greater activity than acarbose (64.52%±3.97) was even observed in both KMB-H (94.33%±0.40) and KMB-EtOAc (100%±0.00) at the highest concentration. On the other hand, the polar extracts showed <20% across all concentrations with a significantly lower inhibition on the said enzymes.

Since both non-polar extracts, KMB-H and KMB-EtOAc, decrease enzyme amylase's action significantly, the formulation and extraction of the KMB-HE crude extracts were performed to ensure that most of the bioactive compounds present in both extracts were captured into one sample. The KMB-HE was extracted using the proportion of 1:1 hexane-ethyl acetate solvents. The KMB-HE extract demonstrated comparable activities with KMB-H and KMB EtOAc. At 250 µg/mL, the KMB-H and KMB-EtOAc impeded α-amylase activity by about 82.42%±1.34and 78.72%±1.40, respectively. Similarly, the KMB-HE showed 81.01%±1.66 at the same concentration (Figure 2). The post-hoc test revealed that the KMB-H, KMB-EtOAc, and KMB-HE showed no significant difference with the inhibition of acarbose at 250 µg/mL ($p>0.05$). Interestingly, higher inhibitions than acarbose (78.75%±1.25) were demonstrated by the KMB-H (92.54%±5.33), KMB-EtOAc (98.67% ±1.01), and KMB-HE (98.06%±1.30) whereas high α-glucosidase inhibitory effects were detected in the KMB-H, KMB-EtOAc and KMB-HE extract with activities of 93.69%±1.65, 98.60%±0.94, and 92.10%±1.05, respectively (Figure 3). A significantly lower inhibition was observed from the KMB-H, KMB-EtOAc, KMB-HE, and acarbose against α-glucosidase across concentrations ($p>0.05$). With comparable or better activity than acarbose against carbohydrate-hydrolyzing enzymes, the KMB-HE was subjected to partial purification.

*Inhibitory Activities of Semi-purified KMB-HE Fractions*

From the KMB-HE extract, 66 fractions were collected. Of these, 24 fractions (10 single, 14 pooled) were gathered and subjected to *in vitro* enzyme inhibition assays.

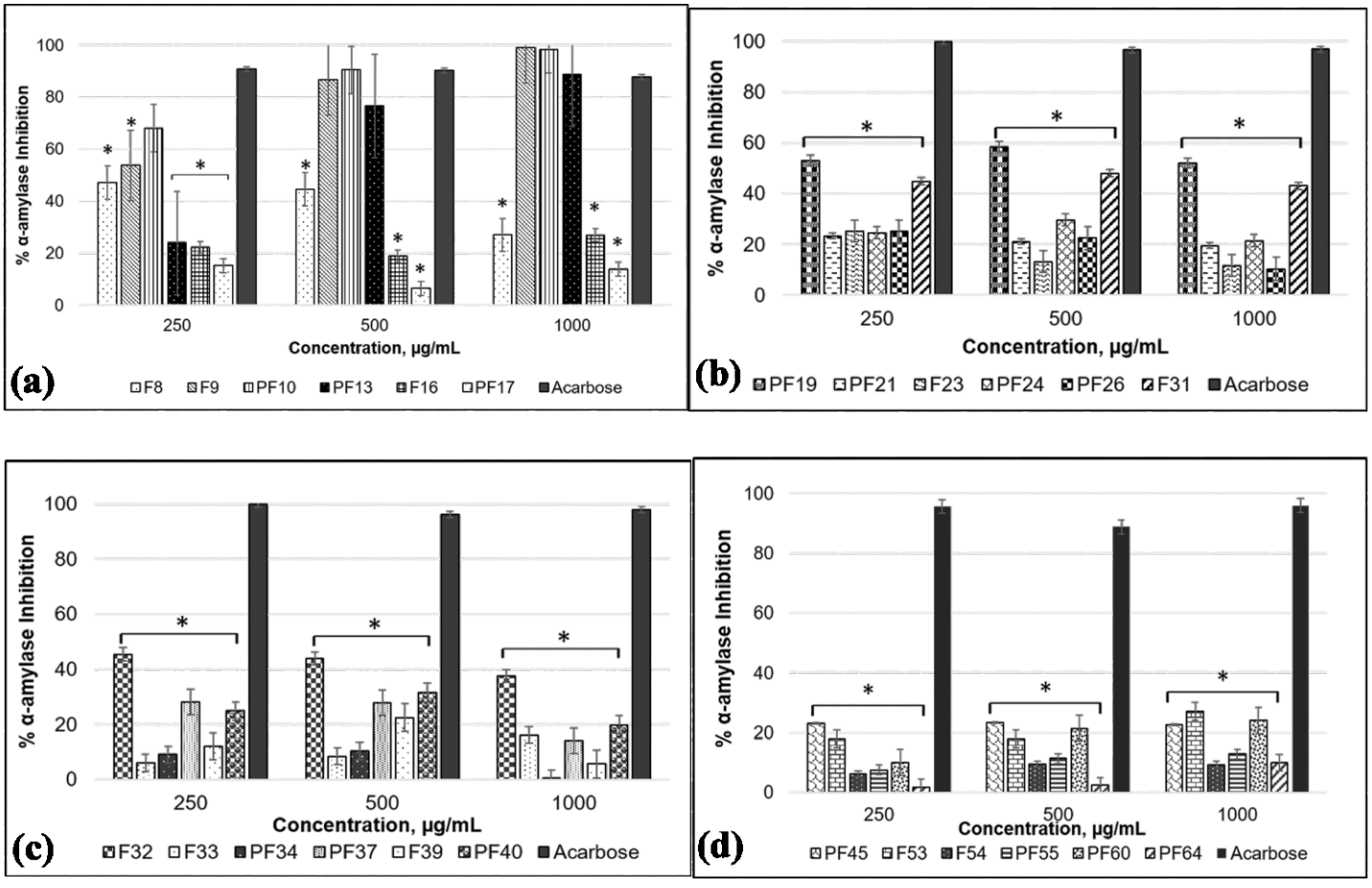


**Figure 4.** *Percent inhibitions of KMB-HE fractions on α-amylase activity (a) fractions 8,9,16 and pooled fractions 10,13,and 17, (b) single fractions 23 and 31 and pooled fractions 19,21,24, and 26, (c) single fractions 32,33 ,39 and pooled fractions 34, 34 and 40, (d) single fractions 53, 54, and pooled fractions 45, 55, 60 and 64; Values are mean ±SEM (n=3); * denotes p<0.05, comparison of treatment vs. Acarbose*

*α-Amylase inhibitory activity*

Among the fractions tested, pooled fraction 10 (PF10) showed remarkable inhibition of α-amylase activity (Figure 4). The PF10 demonstrated the most effective enzyme inhibition of 68.07%±13.48 compared with acarbose (90.75%±4.09) at 250 µg/mL ($p>0.05$). This fraction showed no significant difference with acarbose at 500 and 1000 µg/mL. Moderate inhibitions (>40-70%) were also observed in some fractions; namely, fraction 9 (F9; 53.70%±13.25), pooled fraction 19 (PF19; 53.18%±10.29), fraction 8 (F8;47.19%±2.35), pooled fraction 31 (PF31; 44.88%±6.39), and fraction 32 (F32; 45.48%±2.71) at the same concentration. On the other hand, most fractions displayed low (<30%) to no inhibition of α-amylase activity.

*α-Glucosidase inhibitory activity*

Higher inhibition percentages were demonstrated by all fractions in the α-glucosidase assay than in the α-amylase test. Among the 24 fractions (Figure 5), PF10 and PF26 exhibited the highest enzyme effects, significantly impeding α-glucosidase activity by 74.21%±2.22 and 87.19%±1.54 at 250 µg/mL, respectively. The inhibition of the said fractions was compared to the inhibitory activity of the acarbose standard, 89.69%±6.91. The posthoc test revealed that these fractions had no significant difference across all concentrations ($p>0.05$). Some of the fractions tested showed ~45-55% inhibition at 250 µg/mL; namely, PF24 (57.41%±4.15), followed by PF19 (53.25%±5.92), F9 (51.56%±4.84), PF34

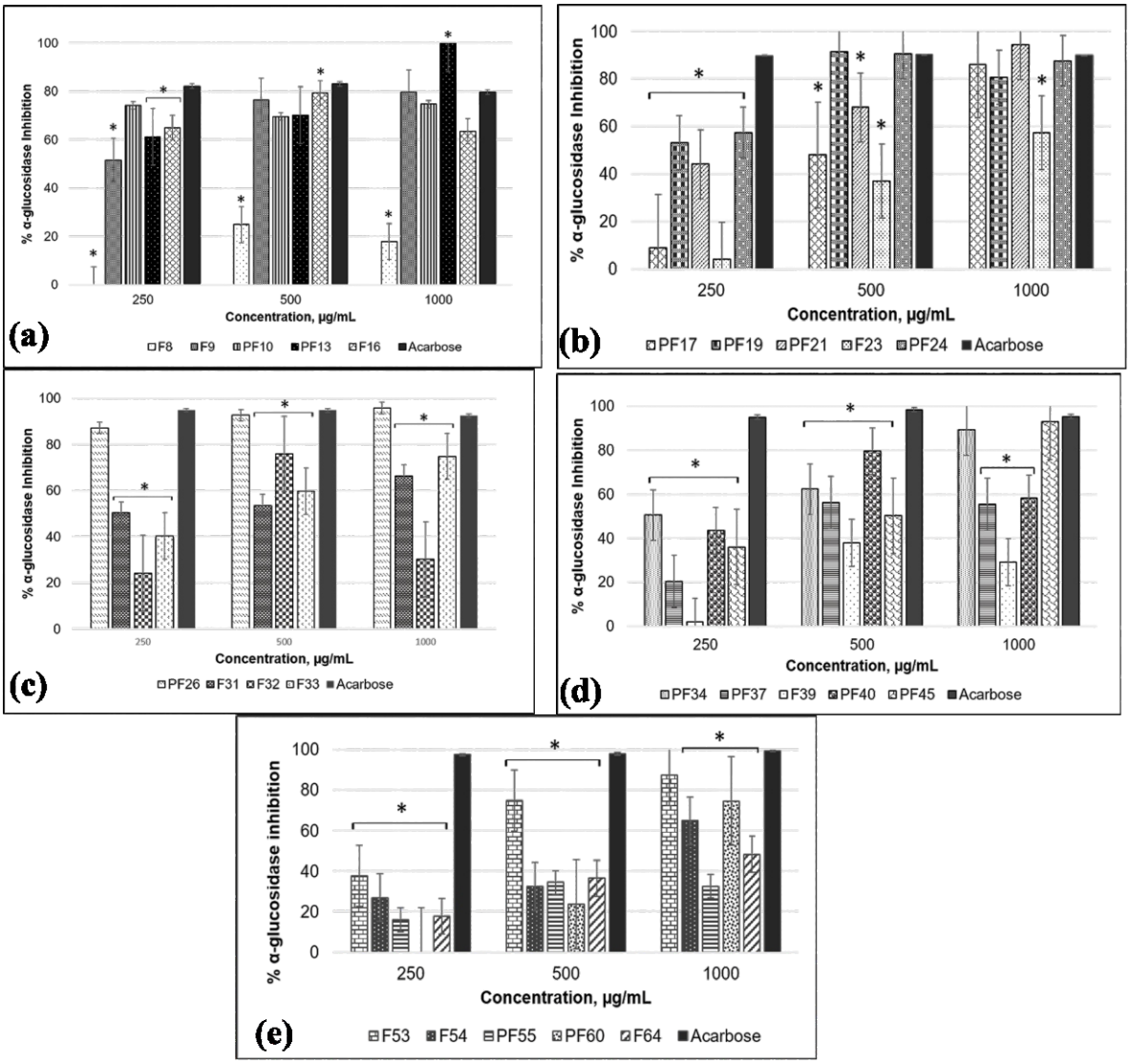


**Figure 5.** *Percent inhibitions of KMB-HE fractions on α-glucosidase activity (a) fractions 8,9,16 and pooled fractions 10,13 (b) single fractions 23 and 31 and pooled fractions 17, 19, 21, and 24, and 26, (c) single fractions 31, 32, 33, and pooled fractions 26 (d) single fractions 53, 54, 64 and pooled fractions 55 and 60 (e) single fractions 53, 55, 64, and pooled fractions 55 and 60 ; Values are mean ±SEM (n=3); * denotes $p<0.05$, comparison of treatment vs. Acarbose*

(50.44%±11.5), and PF31 (50.27%±2.53). Most of the fractions displayed no significant difference with the positive control at 1000 µg/mL ($p>0.05$).

*Phytochemical Analysis of Crude and Most Active Fractions*

*Phytochemicals in KMB crude extracts*

The qualitative phytochemical analyses showed that all KMB crude extracts contained steroids, flavonoids, cardiac glycosides, and phenols shown in Table 2. On the other hand, alkaloids, saponins, glycosides, amino acids, quinones, and anthraquinones were absent in all KMB crude extracts. The terpenoids were present in the KMB-H, KMB-EtOAc, KMB-HE, and KMB-Aq. The triterpenes were only found on the KMB-EtOH. Only the KMB-EtOAc, KMB-EtOH, and KMB-Aq have tannins. Betacyanins were only found in the KMB-MeOH and KMB-EtOH. The coumarins were only found in the KMB-EtOAc, KMB-EtOH, and KMB-HE.

The total phenolic content (TPC) and total flavonoid content (TFC) of the KMB crude extracts were determined using the Folin-Ciocalteau and aluminum chloride methods, respectively. The non-polar extracts, KMB-H, KMB-EtOAc, and KMB-HE had the highest phenolic and flavonoid content with KMB-HE containing the highest flavonoid and phenolic contents of 4.55±0.063 QE mg/g and 195.00 ±3.6 mg GAE/g, respectively. In contrast, the polar extracts KMB-MeOH, KMB-EtOH, and KMB-Aq had the least quantified phytochemical contents (Table 3).

**Table 2.** *Phytochemical Content of the P. umbellatum Leaf Extracts.*

| Test | Extracts | | | | | |
|---|---|---|---|---|---|---|
| | KMB-H | KMB-EtOAc | KMB-MeOH | KMB-EtOH | KMB-Aq | KMB-HE |
| Alkaloids | - | - | - | - | - | - |
| a. Steroids | + | + | + | + | + | + |
| b. Terpenoids | + | + | - | - | + | + |
| Flavonoids | + | + | + | + | + | + |
| Triterpenes | - | - | - | + | - | - |
| Tannins | - | + | - | + | + | - |
| Saponins | - | - | - | - | - | - |
| Phenols | + | + | + | + | + | + |
| a. Glycosidase | - | - | - | - | - | |
| b. Cardiac gycosidase | + | + | + | + | + | + |
| Amino acids | - | - | - | - | - | - |
| Quinones | - | - | - | - | - | - |
| Anthraquinones | - | - | - | - | - | - |
| Betacyanin | - | - | + | + | - | - |
| Coumarins | - | + | - | + | - | + |

(+) represents the presence of the photochemical; (-) means the absence of photochemical

**Table 3.** *Total Flavonoid and Phenolic Content of the P. umbellatum Leaf Extracts*

| KMB Leaf Extracts | TFC[a] Total flavonoid content QE mg/gram | TPC[b] Total phenolic content GAE mg/gram |
|---|---|---|
| KMB-H | 4.31±0.05 | 148.00±3.60 |
| KMB-EtOAc | 2.72±0.02 | 165.13±3.60 |
| KMB-HE | 4.55±0.06 | 195.00±3.60 |
| KMB-MeOH | 1.92±0.04 | 41.50±0.71 |
| KMB-EtOH | 2.15±0.02 | 57.88±1.29 |
| KMB-Aq | 1.81±0.03 | 29.33±1.47 |

a Total flavonoid content expressed as 1g of QE/mg of dry plant material.
b Total phenolic content expressed as 1g of GAE/mg of dry plant material.
Values represent mean ± SEM (n=3).

*Phytochemicals in KMB crude extracts*

The most active fractions, PF10 and PF26, were subjected to qualitative phytochemical tests. The PF10 contained flavonoids, phenolics, and tannin compounds while the PF26 showed no detectable colorimetric changes (Table 4). The quantitative analyses revealed that the PF10 contained high phenolic (204.75±2.42 GAE mg/gram) and flavonoid content (4.00±0.07 QE mg/gram) while the PF26 showed low phenolic (19.66±3.74 QE mg/gram) and flavonoid content (2.15±0.01 QE mg/gram) as shown in Table 5.

## Discussion

The *P. umbellatum*, also known as Kamamba, is commonly consumed as food with its leaves eaten raw, steamed, or boiled as a vegetable [15]. However, studies regarding its biological significance are not much known. Some studies on the traditional and medicinal uses of the *P. umbellatum* in different places have been reported. Decoction of dried leaves is utilized throughout Africa to relieve menstrual and stomach problems, dyspepsia, and constipation [15]. The aerial parts of the *P. umbellatum* have

**Table 4.** *Phytochemical Content of the P. umbellatum Leaf Extracts and Fractions*

| Test | Extracts | | | | |
|---|---|---|---|---|---|
| | KMB-H | KMB-EtOAc | KMB-HE | PF10 | PF26 |
| Alkaloids | - | - | - | - | - |
| a. Steroids | + | + | + | - | - |
| b. Terpenoids | + | + | + | - | - |
| Flavonoids | + | + | + | + | - |
| Triterpenes | - | - | - | - | - |
| Tannins | - | + | - | + | - |
| Saponins | - | - | - | - | - |
| Phenols | + | + | + | + | - |
| a. Glycosidase | - | - | - | - | - |
| b. Cardiac gycosidase | + | + | + | - | - |
| Amino acids | - | - | - | - | - |
| Quinones | - | - | - | - | - |
| Anthraquinones | - | - | - | - | - |
| Betacyanin | - | - | - | - | - |
| Coumarins | - | + | + | - | - |

(+) represents the presence of the photochemical; (-) means the absence of photochemical

**Table 5.** *Total Flavonoid and Phenolic Content of the P. umbellatum Leaf Extracts*

| KMB Leaf Extracts | TFC[a] Total flavonoid content QE mg/gram | TPC[b] Total phenolic content GAE mg/gram |
|---|---|---|
| KMB-H | 4.31±0.05 | 148.00±3.60 |
| KMB-EtOAc | 2.72±0.02 | 165.13±3.60 |
| KMB-HE | 4.55±0.06 | 195.00±3.60 |
| PF10 | 4.00±0.07 | 204.75±2.42 |
| PF26 | 2.51±0.01 | 19.66±3.74 |

a Total flavonoid content expressed as 1g of QE/mg of dry plant material.
b Total phenolic content expressed as 1g of GAE/mg of dry plant material.
Values represent mean ± SEM (n=3).

also been used as anti-parasitic and vermifuge. In the Philippines and some parts of Indochina, the *P. umbellatum* has also been shown as a treatment for conjunctivitis, edema, colic, and kidney problems [15]. Also, it has a wide spectrum of bioactivities including antioxidant, anti-inflammatory, antimalarial, cytotoxic, and anti-tumoral [10,15,24-27].

The *P. umbellatum* extracts showed no toxicity, were safe for consumption, and provided health-protective effects on animals tested for acute toxicity tests [28]. This is similar to the findings noted with the KMB-H and KMB-EtOAc (data not presented in the paper). Both non-polar extracts were shown to be safe for consumption even at a high dose in mice models from the acute toxicity test. Not only was it safe for consumption but it was also shown to have bioactive advantages when eaten. Consequently, this also led to its pharmacological advantages such as the inhibition of α-amylase and α-glucosidase activities.

The *P. umbellatum* contains a vast amount of phytochemical content that may have a connection to its inhibitory activity against carbohydrate-hydrolyzing enzymes. Among the phytochemical constituents, phenols are the greatest in number. These compounds are involved in the growth and reproduction of the plant and may possess numerous bioactive properties that provide health-protective effects [29]. Phenolic compounds are secondary metabolites containing benzene rings with one or more hydroxyl substituents that range from simple molecules to highly complex polymerized compounds [30]. Studies have also shown that phenolic compounds from plants have different anti-diabetic effects. Thus, it has been shown to modulate carbohydrate metabolism, attenuate hyperglycemia, and insulin resistance, stimulate insulin secretion, and inhibit α-amylase and α-glucosidase activity [31,32].

Under phenolic compounds are their derivatives, flavonoids, and tannins. Flavonoids and tannins are complex polyphenols with different medicinal advantages that may aid in managing diabetes. Flavonoids are the most numerous phenolics found throughout the plant kingdom responsible for the color and aroma of plants. Studies have shown that flavonoids have anti-diabetic effects that reduce blood sugar levels, protect the pancreas, and inhibit α-glucosidase [33]. Meanwhile, tannins are complex water-soluble phenolic structures that protect the plant from bacterial and fungal infections. Tannins enhance glucose uptake, reduce glucose levels, and improve insulin activity [34]. Thus, these compounds have the important property of inhibiting α-amylase and α-glucosidase which are key enzymes responsible for the digestion of dietary carbohydrates to glucose [30].

Studies showed that phenolic-enriched extracts are associated with α-amylase and α-glucosidase inhibitory activities. The ellagic acid (phenolic acids) and catechin (flavonoids) isolated from the *R. idaeus* exhibited potent inhibition of α-glucosidase with $IC_{50}$ from 14.7 to 64.5 μg/mL but no inhibition occurred against the α-amylase activity [35]. The isolated compounds of *R. crenulate*, epicatechin-(4β,8)-epicatechingallate, epicatechin gallate, and 2-(4-hydroxyphenyl)ethyl 3,4,5-trihydroxybenzoate demonstrated significant α-glucosidase inhibitory activity with $IC_{50}$= 0.31 ± 0.01, 0.71 ± 0.01, and 4.77 ± 0.22 μM, respectively, when compared to quercetin ($IC_{50}$= 5.30 ± 0.11 μM) [36]. On the other hand, a curcuminoid isolated from *C. longa* showed strong inhibitory activity on α-glucosidase ($IC_{50}$= 23.0 μM) and has been proven to be a non-competitive α-glucosidase inhibitor [37]. Two coumarin derivatives isolated from the branch extract of the *L. pinceana* Hook showed strong α-glucosidase non-competitive inhibitory activity with Ki of 2.44 and 167.83 μg/mL. Genistein, an isoflavone, a well-known soybean constituent, demonstrated a reversible, slow binding, and non-competitive inhibitor of yeast α-glucosidase with a Ki value of 0.057 μmol/L and $IC_{50}$ value of 50 μmol/L [38].

Different *Piper* species with high phenolic content have also been shown to have α-amylase & α-glucosidase inhibitory activity. In a study, the *P. guineense* oil seeds showed inhibiting α-glucosidase ($EC_{50}$ = 68.29 ±4.48) more than α-amylase ($EC_{50}$ = 86.06 ±4.51). This reveals that the oil showed more potent inhibition of α-glucosidase activity than α-amylase activity [39]. On the other hand, methanolic and ethanolic extracts from the *P. betel* leaves showed high phenolic content and α-amylase inhibition activity [40]. The aqueous leaf extract of *P. angustifolium* showed high phenolic content but displayed moderate α-glucosidase inhibition [41]. The Nigerian variety of the *P.umbellatum* showed the presence of alkaloids, phenols, flavonoids, tannins, and steroids [42]. On the other hand, the Brazilian variety of plants displayed the presence of phenolic compounds such as rosmarinic acid, kaempferol, protocatechuic acid, and ferulic acid [43]. Similarly, the KMB-HE crude extract and active fractions from the plant showed the presence of phenolic compounds, flavonoids, and tannins. This can be a great interpretation where the plant grows in different geographical origins but still shows the same phytochemical contents.

From the findings presented above, it can be hypothesized that phenolic compounds have a similar structure as acarbose. The acarbose has a similar structure as oligosaccharides which have hydroxyl groups and a nitrogen group between the 1st and 2nd glucose molecules. On the other hand, the phenolic groups have phenyl rings and hydroxyl groups attached to them. The similarities in the presence of hydroxyl groups that may bind to the target enzyme may be a possible explanation for the inhibitory activities observed in KMB-HE and its fractions to α-amylase and α-glucosidase. The binding of the hydroxyl group in phenolics to the target enzyme may post a conformational change to the enzyme's native conformation, resulting in ineffective binding of the substrate. Thus, the presence of the hydroxyl groups in the compounds may greatly affect the inhibition potency of the compounds to α-amylase and α-glucosidase action [44]. However, further studies and experimentation are needed to confirm this hypothesis.

Previous reports have indicated that excessive or high inhibition of α-amylase could result in bacterial fermentation of undigested carbohydrates in the colon and may eventually result in abdominal distention, flatulence, and diarrhea [45]. Therefore, studies suggest that plant natural inhibitors should have moderate or lower inhibitory α-amylase activity and a stronger α-glucosidase inhibitory activity [24,25,35]. However, some studies also suggest no specific range of inhibition for α-amylase and α-glucosidase from plant extracts. In a study by Chipiti *et al.* (2015), the *A. antunesiana* extract showed 35% α-amylase inhibition and 70% α –glucosidase inhibition [46]. Another study displayed the *H. speciosa* extract with 57.32% α-amylase inhibition and 98.6% α –glucosidase inhibition. The same study showed that the *P. caimito* extract exhibited 53.43% α-amylase inhibition and 100% α –glucosidase inhibition [47]. In addition, the *T. occidentalis* extract displayed 50% α-amylase inhibition and 70% α –glucosidase inhibition [33]. However, it was reported that the effective inhibition of acarbose on both enzymes ranged from 50 to 82% in *in vitro* studies [48]. Thus, this was used as a criterion in choosing the most active fraction that showed inhibition of both enzymes in the specific range.

From the fractions in the study, the fraction that inhibited both enzymes close to the criterion was the PF10. This fraction inhibited α-amylase by 68.07%±13.48 and 74.21%±2.22 for α-glucosidase activity. The PF10 also showed no significant difference from acarbose in inhibiting both enzymes. Meanwhile, the rest of the fractions showed a lower inhibition of α-amylase ranging from 20 to 25% at 250 µg/mL, which was significantly different from the inhibition of acarbose. On the other hand, fractions showed comparable inhibitions on α-glucosidase activity from KMB-HE crude extract.

In conclusion, the study revealed that the *P. umbellatum*, which contains adequate amounts of secondary metabolites, such as phenolics and flavonoid components, exhibited good inhibitory effects on carbohydrate-hydrolyzing enzymes, α-amylase, and α –glucosidase. Through *in vitro* tests, the *P. umbellatum* may be a potential source of plant-based enzyme inhibitors that offer a prospective therapeutic approach for managing diabetes. However, it is very much recommended that *in vivo* studies may be performed to determine the inhibitory activity of the KMB-HE and active fractions on an animal model. In addition, the isolation and characterization of the compounds present in this extract and its fractions are deemed necessary to fully identify the specific molecule/s responsible for the observed bioactivity.

## Acknowledgement

The authors wish to acknowledge the National Institutes of Health (NIH) and Department of Science and Technology - Accelerated Science and Technology Human Resource Development Program (DOST-ASTHDRP) for the financial grants which were used in the study.

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