

## **Partial Purification of the Mammalian Alpha-glucosidase Inhibitor from *Melothria* sp. (Fam. Cucurbitaceae) Leaves and Stem Extract: *In Vitro***

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**Abstract:** Diabetes Mellitus Type 2 (DM2) is a chronic disease characterized by insufficient insulin levels, pancreatic beta cells function loss and insulin resistance in peripheral tissue. Voglibose and acarbose are the clinically used alpha-glucosidase inhibitor; however, adverse effects such as nausea, diarrhea, and bloating hinder the utilization of these agents. Hence, there is a need to explore for an alternative drug that has fewer side effects, better activity, and more affordable than the current commercial drugs. In this study, *Melothria* sp. was purified using normal phase column chromatography. Seven fractions (fraction A-G) were separated and tested for mammalian alpha-glucosidase assay in vitro and phytochemical screening was conducted to determine the metabolites present in each fraction. Fraction F showed a promising activity against the enzyme and comparable to other natural products and commercial alpha-glucosidase agent, acarbose. Phenolic compounds such as tannins and flavonoids based on the phytochemical screening were detected present in the sample which can be responsible in the inhibition activity of the semi-purified extracts.

**Keywords:** *Diabetes Mellitus; Mammalian alpha-glucosidase; Piri-pipino; Cucurbitaceae; Melothria* sp.

## 1. INTRODUCTION

Diabetes Mellitus Type 2 (DM2) is a chronic disease characterized by insufficient insulin levels, pancreatic beta cells function loss, and insulin resistance in peripheral tissue (American Diabetes Federation, 2009). The persistent high glucose level in the body leads to the development of comorbidities such as retinopathy, renal failure, neuropathy and limb amputation which increases the economic and emotional burden of the diabetic patient and their family (Deshpande, Harris-Hayes, & Schootman, 2008) Published literature from International Diabetes Federation 2018 (90% of the global population is suffering from DM2) and Food and Nutrition Research Institute-Philippines in (0.6% increase in diabetes prevalence in comparison with the same study conducted in 2008) strongly suggest that the disease is escalating at an alarming rate. In the Philippines, the prevalence of DM2 is dramatically increasing despite the availability of affordable antihyperglycemic medications and non-pharmacological program due to rapid urbanization, sedentary lifestyle, high-calorie diet, cigarette consumptions, aging population, and shift of Filipino culture to Western culture (Tan, 2015).

One of the key strategies in the management of postprandial glucose level is the inhibition of alpha-glucosidase, a membrane-bound enzyme at located in the brush border of the intestine (Subramanian, Asmawi, & Sadikun, 2008). Voglibose and acarbose are the clinically used alpha-glucosidase inhibitor; however, adverse effects such as nausea, diarrhea, and bloating hinder the utilization of these agents (Munasaroh, Tamat, & Dewi, 2018). Hence, there is a need to explore for an alternative drug that has fewer side effects, better activity, and more affordable than the current commercial drugs. A polyphenolic compound from plant extract will be investigated due application to its potential source of novel compound and promising.

The diverse bioactivity of polyphenolic compound such as tannins can be considered as alternative drug or supplements for Filipino patient suffering from DM2 by inhibition of alpha-glucosidase and alpha-amylase, increase intestinal Na<sup>+</sup> dependent glucose transporter (SGLT1 and 2) and improve the glucose utilization via insulin-signaling pathways mediators such as activation of Mitogen-Activated protein Kinase (p38 MAPK), phosphoinositide 3-kinase (p13K) and GLUT-4 translocation (Bahoran, Mirmiran, & Azizi, 2013). However, some reports limit the potential application of the compound due to non-selective protein binding and anti-nutritional activity (Kamantigue, Solidum, Quiming, Nicolas, & Pidlaon, 2017). Therefore, the aim of this investigation was to compare the mammalian alpha-glucosidase inhibition activity of condensed rich and tannin removed fractions obtained from separation techniques of *Melothria sp.* leaves and stem extract.

## 2. METHODOLOGY

### 2.1 Research design

An experimental method post-test was utilized in the study to determine the mammalian alpha-glucosidase inhibition activity of *Melothria sp.* semi-purified extract or locally known as *Piri-pipino* in Calabanga, Camarines Sur, Philippines.

### 2.2 Collection of plant sample

*Melothria sp.* leaves with stem were tested as sample mixtures and was harvested from Siba-o, Naga using single cluster random sampling. The processed voucher specimen was submitted to the Institute of Biology, University of the Philippines Diliman for authentication purpose. The plant materials were washed with running tapped water to remove adhering dust particles and other contaminants and air-dried for 2 weeks at room temperature until crisp. The dried plant materials were grabbed immediately to avoid cross contamination from other plant parts, reduced into fine powder using an osterizer and the moisture content ( $\leq 10\%$ ) was maintained by storing the samples in airtight resealable plastic bag with desiccant.

### 2.3 Extraction of selected botanical materials

The powdered plant materials were exhaustively macerated thrice with absolute ethanol for 3 successive days with constant shaking using an improvised mechanical shaker for optimum secondary metabolite extraction. The resulting mixtures was filtered using Whatmann filter paper no.40, filtrate was combined and concentrated to about of its original volume using a rotary evaporator. Crude extract was stored at  $-4^{\circ}\text{C}$  for further processing.

Mammalian alpha-glucosidase assay and phytochemical screening was described from the previous publication of Kamantigue et al., 2017.

### 2.4 Column chromatography of the crude extract

The reported work from Sheng et al. (2014 with modifications was used for initial purification of crude extract. Two grams (2 g) of crude extract was mixed with two grams of silica slurry and allowed to dry under the fume hood. A silica slurry was prepared by dissolving 100 grams of normal phase silica in an organic solvent. The silica mixture was packed in the column and tapped continuously to remove the bubbles during pouring. After packing, 250 ml of optimized solvent systems consisting of (80:20 Chloroform: Ethyl acetate, 100 Ethyl acetate, 50:50 Methanol: Chloroform, 100 Methanol, 50:50 methanol: water and deionized water for purging) were loaded to the solvent reservoir to separate the phytoconstituents.

Ten milliliters of the eluate were collected. Each fraction was spotted in normal phase TLC plates with aluminum backing, developed with the optimized solvent system and fractions with the same  $R_f$  value were pooled together. Six semi-purified fractions, labeled Fraction A-F were obtained.

The concentrated samples were tested for enzymes inhibition ( $\alpha$ -glucosidase) and fraction F; the sample with the highest inhibition was further purified.

### 2.5 Biostatistical Analysis

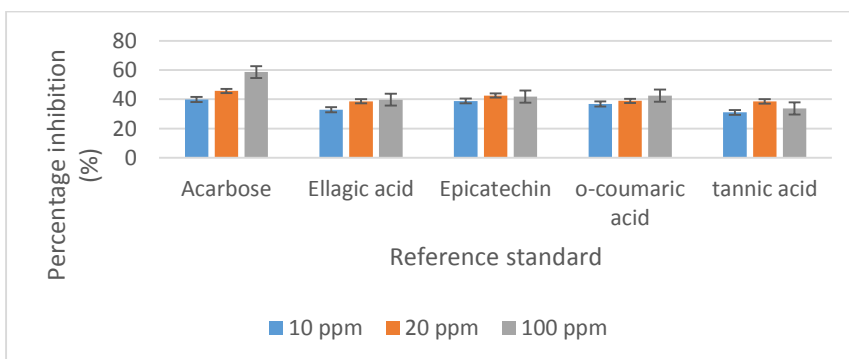
All measurements were conducted in triplicate and express the percentage inhibition as mean, and the standard deviation was calculated. Kruskal Wallis test followed by the Mann-Whitney "U" test ( $\alpha=0.05$ ). Inhibitory concentration 50 ( $IC_{50}$ ) was computed using 5-parametric logistic fit if the sample reached at least 50% inhibition at 100 ppm.

## 3. RESULTS AND DISCUSSION

Before the analysis of the fractions, different standards for  $\alpha$ -glucosidase inhibition were explored in the study to represent the different polyphenols that can be obtained from natural product and serve as a reference standard for enzyme inhibition aside to acarbose (pesudotetrasaccharide). Ellagic acid (non-flavonoid polyphenol), Epicatechin (monomeric flavanols), o-coumaric acid (phenolic acid) and tannic acid (tannin) (Tsao, 2010) were included as additional standards.

Table 1 summarizes the calculated percentage inhibition of reference standards at different concentrations. Kruskal-Wallis test revealed that at 10 ppm ( $p>0.047$ ) and 100 ppm ( $p>0.032$ ) concentrations have significant differences in inhibiting the mammalian enzymes. However, at 20 ppm there was no statistical difference between the group of standards ( $p<0.133$ ). The calculated  $IC_{50}$  of acarbose was 1.474 ppm. Additional statistical analysis using Mann-Whitney test of individual reference standards reveal that at all concentrations, there were no statistical differences between each standard ( $p>0.05$ ). The results indicate that the inhibitory activities of acarbose, epicatechin, tannic acid, ellagic acid, and o-coumaric acid with respect to mammalian  $\alpha$ -glucosidase were the same.

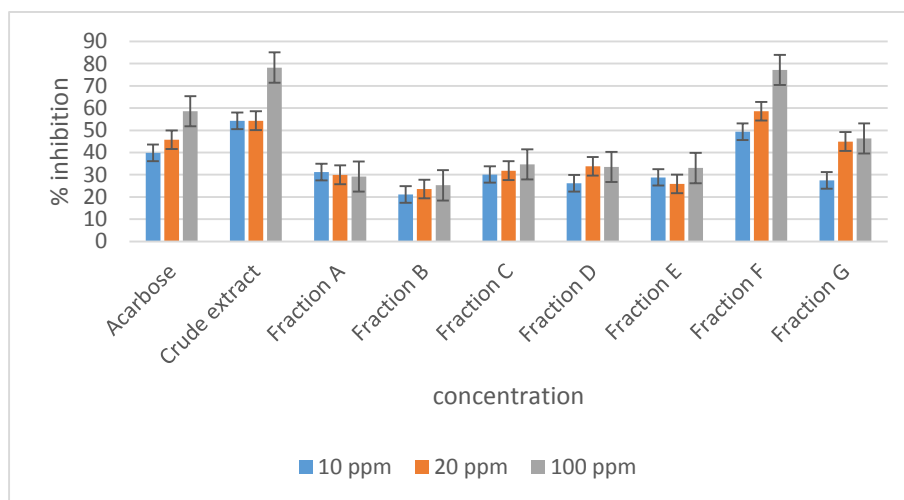
Table 1  
*Percentage Inhibition of Mammalian Alpha-glucosidase of Reference Standards*



The developed partial purification method utilized flash chromatography for rapid preparatory fractionation of crude extract due to the ease of use and high sample capacity. Seven fractions, labeled Fractions A-G, were obtained from this 1<sup>st</sup> purification step. These semi-purified samples were subjected to mammalian  $\alpha$ -glucosidase inhibitory assay, and the results are presented in Table 2. The fraction F was recovered from elution of 50:50 methanol: water had the highest inhibition at 100 ppm concentration followed by fraction G (recovered after the elution of white bands) and C (recovered from 100 % ethyl acetate). The assay results signify that after purifying the crude extract, the bioactivity remains the same. An  $IC_{50}$  of 1.898 ppm and 1.446 ppm were the calculated values for crude extract and fraction F respectively. Kruskal-Wallis test displayed that there was a statistical difference in inhibiting the mammalian glucosidase between all test groups and all concentrations ( $p < 0.05$ ). Mann-Whitney “u” test demonstrated that there was no difference between the inhibition ( $p > 0.05$ ).

Table 2

*In Vitro Alpha-glucosidase Inhibition of Reference Standards, Crude Extract and Fractions from Column Chromatography*



Diverse types of metabolite were monitored in the column chromatography fractions using TLC-Bioautography (Table 3). Qualitative screening of fractions suggested the presence of polyphenols such as tannin and flavonoid in all fractions while the steroidal compound was detected in Fractions A to D only. Also, indole containing compound was noted in Fractions A to D. Coumarins which chemically known as 2H-1-benzopyran-2-one (consist of fused benzene and  $\alpha$ -pyrone rings) was detected in Fractions B and C. In Fraction A and C, the presence of anthrones was observed. On the other hand, low molecular weight lipophilic compound (Steroid) in Fractions A to E were present. A positive result in vanillin-sulfuric acid

verified the presence of phenolic and steroidal compounds and indicated the probable presence of higher alcohols and essential oils. Sugar was also noted in Fraction A to D and absence of cardenolides, alkaloids, anthraquinones, and peptides in all fractions.

Table 3  
*Phytochemical screening of Fractions A-G from flash chromatography*

Metabolites	Spray Reagent(s)	Fractions						
		A	B	C	D	E	F	G
Flavonoids	Antimony (III) Chloride	+	+	+	+	-	-	-
Tannins, flavonoids, phenolic	Potassium-ferricyanide-ferric chloride	+	+	+	+	+	+	+
Alkaloids	Dragendorff's	-	-	-	-	-	-	-
Cardenolides	3,5-Dinitrosalicylic acid:Kedde reagent	-	-	-	-	-	-	-
Coumarins, Anthraquinone, Anthrones, phenols	Borntrager's	+	+	+	-	-	-	-
Anthraquinones	Magnesium acetate	-	-	-	-	-	-	-
Indoles	Van Urk-Salkowski test	+	+	+	+	-	-	-
Higher alcohols, phenols, steroids, essential oils	Vanillin-sulfuric acid	+	+	+	+	+	-	-
Sugars	Alpha-naphthol-sulfuric acid	+	+	+	+	-	-	-
Peptides	Ninhydrin	-	-	-	-	-	-	-

(Legend: + detected – negative for the presence of the constituents)

Although the analysis of Fraction F resulted in 77.18% at 100 ppm inhibition of mammalian  $\alpha$ -glucosidase activity in vitro, the presence of abundant and common natural product such as condensed tannin was the only metabolites detected present in the fraction and additional purification may lead to a high false positive result due to non-specific enzyme precipitation of the compound (Beutler, 2009). Due to relative high sample yield of Fraction C (3<sup>rd</sup> highest inhibitor at 100 ppm 34.68% inhibition) in comparison to Fraction G (2<sup>nd</sup> highest inhibitor with 46.32% inhibition, recovered from 50:50 methanol: water and the sample yield was 2 mg only). Polyphenols such as tannin differ in their interaction to a specific protein due to their diversity in conformation, molecular size, and stereochemistry. Interaction between enzyme and tannins can be attributed from the multiple hydrogen bonds and hydrophobic associations and further supported by the

conformational congruency of the active pocket of the protein and tannin functional group (Barrett, Farhadi, & Smith, 2017).

Another study published by Nack, Pegg, and Ammarowicz (2010) discussed that the ingested tannins from the diet probably bind to salivary proteins, mucosa and epithelium gut. In vitro study of the human microbial intestinal flora shown that the microorganisms can degrade the non-absorbable tannins which limit the activity of the compound. The concentration of the tannins also plays a role in the bond formation between the tannin-protein complex. At the low protein concentration, protein can be precipitated by tannin by hydrophobic monolayer of compound on the enzyme surface. However, at high protein concentration, the combination of polyphenol on the protein surface and crosslinking of different protein molecules with polyphenols forms a hydrophobic surface on the protein (Nack, Ammarowicz, Zadernowski, & Shahidi, 2000).

#### 4. CONCLUSION

The study exhibited the potential application of the partially purified *Melothria sp.* leaves and stem extract as an alpha-glucosidase inhibitor as observed in the fraction *F* which was composed of phenolic compounds such as tannins and flavonoids based on the phytochemical screening. However, further study must be conducted to characterize the mechanism of inhibition of the metabolites through enzyme inhibition kinetics, structure elucidation of the isolated compound and DNA barcoding to determine the species of *Melothria sp.*

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