PJHRD Philippine Journal of Health Research and Development

Bioassay-guided Fractionation of the Anti-diabetic Constituents of Star Apple Leaves

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RESEARCH ARTICLE

Abstract

Background and Objectives: The Philippines is one of the world's emerging diabetes hotspots. In 2014, there were 3.2 million Filipinos diagnosed with diabetes. By 2030, incidence is projected to increase by up to 7.8 million. This study aimed to determine the potential α-glucosidase inhibiting activity of the fractions of the ethanolic extract obtained from *Chyrsophyllum cainito* L. leaves.
Methodology: The research employed thin layer chromatography (TLC) and flash column chromatography techniques for the fractionation, and α-glucosidase inhibition test for the assessment of the activity.
Results and Conclusion: TLC optimization resulted to 15:65:10:10 hexane, ethyl acetate, methanol, and acetic acid as the mobile phase, with silica gel GF254 as the stationary phase. The plates were viewed under white light, UV (254 & 366 nm), and vanillin-sulfuric acid spray. Three different pools of fractions based on TLC Rf values were collected from the flash column chromatography using a gradient mix of hexane, ethyl acetate, methanol, and acetic acid with a flow rate of 3.9 mL per minute. These fractions exhibited higher percent inhibition than Quercetin standard at 30, 70, and 150 µg/mL, with Fraction 2 having the highest activity, followed by Fraction 1, then Fraction 3. Phytochemical tests suggest the presence of Anthraquinone glycosides on the three pooled fractions.

Keywords: *diabetes, α-glucosidase inhibition, star apple leaves, bioassay-guided fractionation*

Introduction

Diabetes is a serious condition that places people at risk for greater morbidity and mortality. In the Philippines, it is on the top ten leading causes of mortality. In 2014, there were 3.2 million among the 100 million Filipinos diagnosed with diabetes, and it is projected to increase to 7.8 million by 2030 [1,2]. Drugs for the management of diabetes include the α-glucosidase inhibitors which are used to prevent the increase in postprandial hyperglycemia. They delay the digestion of carbohydrates, leading to delay in absorption. They are not only limited to the management of diabetes, but also to viral therapy [3]. However, common side effects include flatulence, occasional diarrhea and abdominal pain as these complex carbohydrates become fermented instead in the large intestine. These adverse reactions are among the common causes of decreased patient medication compliance. Medication compliance of patients having Type 2 diabetes was found to be correlated with their glucose control hemoglobin HbA1c [4]. Several anti-diabetic constituents isolated from natural products have an important role in diabetic management. The natural constituents, on the other hand, exhibit less adverse effects, and researches are geared to the isolation of these compounds that have been found useful for diabetes management [5]. The Sapotaceae family contains species exhibiting α-glucosidase inhibiting property. One of the members of this family is star apple *(Chrysophyllum cainito L.)*. Star apple leaves were used by the local healers of Aboudé-Mandéké in Africa as decoctions for the traditional treatment of diabetes. Moreover, the phytochemical screening of the star apple leaves yielded alkaloids, flavonoids, sterols and triterpenes. Among these constituents, the alkaloids, sterols and triterpenes were inferred to cause the hypoglycemic effect on the rabbits [6]. Recent studies also have shown that the *C. cainito* leaves have anti-helminthic, anti-oxidant, anti-inflammatory, and anti-hypersensitive activities [7, 8].

The study aimed to isolate and characterize the fractions responsible for the anti-diabetic activity of *C. cainito* leaves.

Specifically, the study was designed to 1)determine the optimum chromatographic conditions to isolate the phytochemical constituents responsible for the anti-diabetic activity of *C. cainito* leaves 2)determine the fractions which exhibit the highest α-glucosidase inhibiting property and 3)characterize the phytochemical constituents of the active fractions.

This study focused on the fractionation and characterization of the *C. cainito* leaves ethyl acetate fraction. The method used in obtaining the ethyl acetate fraction was modified Kupchan liquid-liquid partitioning. Further fractionation was done using flash column chromatography, resulting into pooled fractions. The study also included identification of anti-diabetic property of the pooled fractions through *in vitro* enzyme inhibition assay, specifically yeast α-glucosidase inhibition assay. Phytochemical screening was done in order to further analyze and characterize the fractions.

Methodology

Experimental Design

The research involved post-test only design to assess the α-glucosidase inhibition activity of *C. cainito* leaves fraction. The method done to determine its anti-diabetic property was α-glucosidase inhibition assay. Phytochemical screening was employed for further characterization of fractions with the highest α-glucosidase inhibition activity obtained from flash column chromatography.

Materials or Instruments

The sample used were the *C. cainito* leaves. The reagent used to get the crude ethanolic extract of the leaves was technical grade 95% ethanol. The reagents used to obtain the ethyl acetate fraction through Kupchan Partitioning were distilled water, technical grade 95% ethanol, reagent grade methanol, reagent grade n-hexane, reagent grade dichloromethane, and analytical grade ethyl acetate. The composition of the solvent system used for thin layer chromatography (TLC) was reagent grade n-hexane, analytical grade ethyl acetate, reagent grade methanol, and reagent grade acetic acid. Silica gel GF254-coated aluminum-back plates were used as stationary phase for TLC. CAMAG ® UV Cabinet dual wavelength 254/366 and vanillin-sulfuric acid spray reagent were used to view the plates. The composition of the eluents for flash column chromatography were reagent grade n-hexane, analytical grade ethyl acetate, reagent grade methanol, and reagent grade acetic acid. Cotton, quartzsand, and silica gel 60 0.040 nm-0.063 nm (230-400 mesh), were used to pack the column and Pyrex ® glass chromatography column 30x450mm with 125 mL reservoir and PTFE stop cock as the column. The reagents used for the α-glucosidase inhibition assay were distilled water, analytical grade sodium hydroxide ≥97% and reagent grade monobasic potassium phosphate to make pH 6.8 phosphate buffer, reagent grade dimethylsulfoxide (DMSO), and sodium carbonate, anhydrous 99.9%. 4-nitrophenyl-D-glucopyranoside (pNPG) ≥99% was used as substrate, α-glucosidase from *Saccharomyces cerevisiae* was used as enzyme, and quercetin ≥95% was used as control. Disposable 96-well microplates were used as assay plates and high performance CLARIOstar® monochromator multimode microplate reader was used to read the absorbance.

Data Collection and Analysis

Fifty (50) kilograms of fresh *C. cainito* leaves were collected from a single tree in a backyard in Marikina City in December 2015 in the morning. Right after collection, the fresh leaves were sorted and selected [9], then washed and air-dried for 27 days in drying beds after garbling. The air-dried leaves were further dried in an oven at 40°C for 2.5 hours until a moisture content of 2.13% was achieved. After this, the leaves were milled, and subsequently sieved using sieve no. 20. This was stored in clean plastic bags and kept in a moisture-free environment prior to maceration after 24 hours of storage.

For the preparation of the crude ethanolic extract, the modified method in [10] was adopted. The sieved leaves were exhaustively macerated in ethanol in a 1:4 ratio (w/v) for a total of 168 hours. All liquid extracts were evaporated *in vacuo* (40oC) at 0.08-0.09 MPa after filtration, then evaporated to dryness (60oC) in water bath prior to lyophilization.

Modified Kupchan liquid partitioning [11] was used for the fractionation of the extract. Ten (10) grams of the lyophilized ethanolic crude extract were suspended in 200 mL 9:1 methanol: water solution in a separatory funnel, followed by extraction with 600 mL *n*-hexane, then two subsequent extractions of 200 mL *n*-hexane. The organic hexane layer was set aside. The aqueous layer was extracted with three portions of 200 mL dichloromethane. The dichloromethane fraction was set aside. The aqueous layer was extracted with three portions of 200 mL ethyl acetate.

The aqueous layer was set aside. The ethyl acetate fraction was evaporated *in vacuo* (40oC) and evaporated to dryness (60oC) in water bath to completely remove the solvent and obtain the extracts. The resulting concentrate was subjected to further fractionation using flash column chromatography and TLC.

The chromatographic condition for the TLC was optimized prior to running the flash column chromatography. The concentrated crude ethyl acetate fraction was dissolved in methanol to make a 10 mg/mL solution. Five (5.0) microliters of the solution were applied in duplicates to silica gel GF254-coated aluminum-back plates. The solvent systems used to develop the TLC plates of the crude ethyl acetate fraction can be seen in Table 1. The plates were removed from the chamber and allowed to dry after developing. Immediately after drying, visualization of spots was carried out using different visualization methods: under white light, UV detection at wavelengths 254 nm and 366 nm, and by spraying with vanillin-sulfuric acid. The Rf values of the spots detected were then obtained. The solvent system with the most efficient separation of spots was noted and prepared for the flash column chromatography.

The method for the flash column chromatography was adopted from [12, 13]. First, the cotton plug was placed in the column, then topped with 2 cm layer of quartzsand, followed by packing of silica gel weighing 24.50 g, packed by slurry pacing method using 50:50 hexane: ethyl acetate. Positive air pressure was used to drain most of the eluent until 2 cm above the silica packing off the column. After the column was packed, a layer of quartzsand was added, about 2 cm thick on top of the silica. Using positive air pressure,

Table 1. *Solvent systems explored to elute the crude ethyl acetate fraction in TLC*

Solvent System	Volume Ratio
Hexane: Ethyl Acetate	35 : 65
	30 : 70
Hexane: Ethyl Acetate: Methanol	20 : 75 : 5
Hexane: Ethyl Acetate: Acetic Acid	45 : 65 : 1
	40 : 60 : 1
	35 : 65 : 1
Hexane: Ethyl Acetate: Methanol: Acetic Acid	25 : 75 : 5 : 5
	15 : 65 : 10 : 10
	15 : 60 : 10 : 15
	10 : 65 : 10 : 15

Table 2. *Order of solvent gradient used in the flash column chromatography with corresponding polarity indices*

Solvent Order (100mL)		Solvent Gradient	Volume Ratio	Polarity Index
First Flash Column Chromatography Run	Second Flash Column Chromatography Run			
1st	Not Performed *	Hexane: Ethyl acetate	50:50	2.250
2nd	1st	Hexane: Ethyl acetate	25:75	3.325
3rd	2nd	Ethyl acetate: Methanol:	100	4.400
4th	3rd	Ethyl acetate: Methanol: Acetic acid	80:10:10	4.650
5th	4th	Ethyl acetate: Methanol: Acetic acid	70:20:10	4.720

*Not performed due to absence of spots eluted from 1st flash column chromatography run

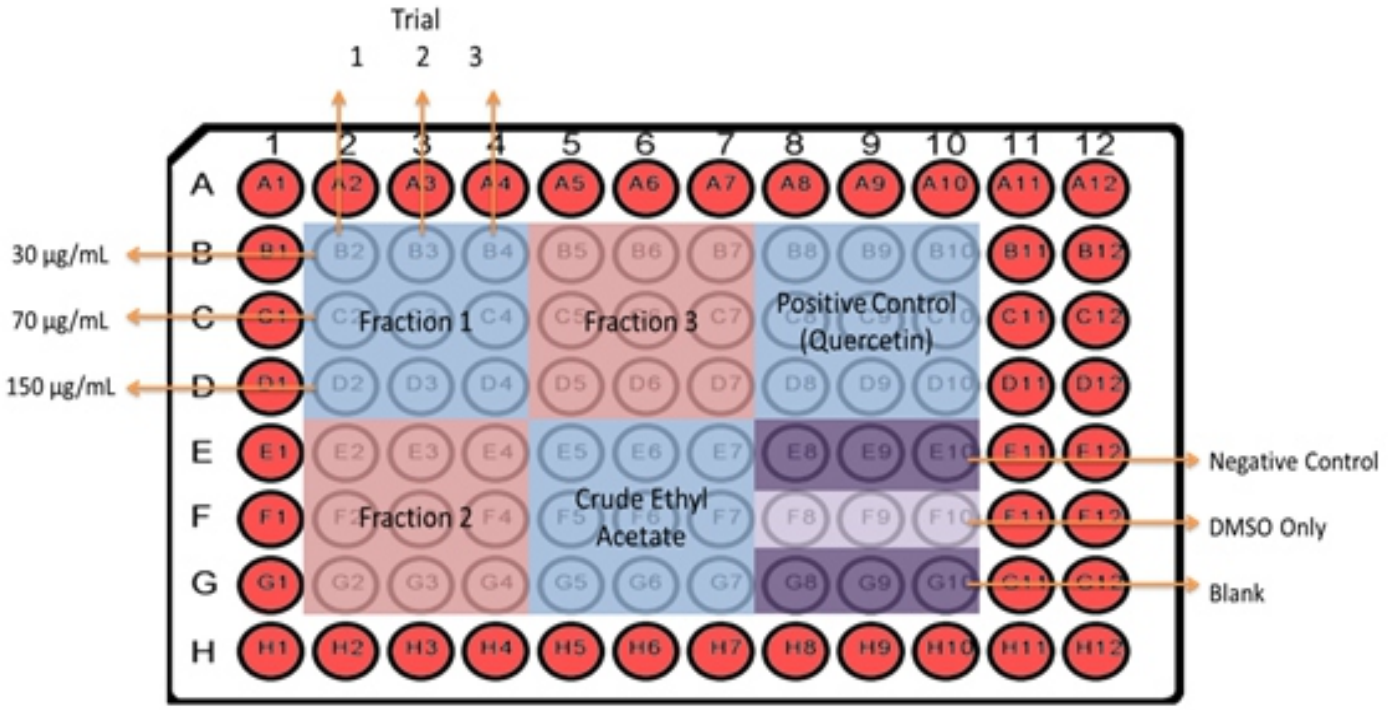

Figure 1. *Ninety-six well plate design*

the solvent was pushed again until the level was even with the height of the quartzsand. The overall solvent run was 410 mm. A 0.2300 g portion of the crude ethyl acetate fraction was dissolved in a minimal volume of ethyl acetate. The solution was allowed to adsorb to 2.00 g of silica gel, then the ethyl acetate was evaporated to dryness. The silica gel with the adsorbed sample was then applied on top of the quartzsand. Gradient elution was done, thereafter, with the application of positive pressure (2.90 psi), thus, having a flow rate of 3.9 mL per minute. The order of solvent gradient applied can be seen in Table 2. Two (2) runs of flash column chromatography were employed to obtain more samples. Test tubes were used to collect every 5 mL of the fraction eluted. Each fraction was subjected to TLC with the chromatographic condition previously optimized.

Ten (10.0) microliters of each 5-mL fractions were applied in silica gel GF254-coated aluminum-back plates and ran under the prepared optimized TLC condition. After reaching a solvent front of 8 cm, the plates were removed from the chamber and allowed to dry. Spots were detected using the previously mentioned visualization methods. Similar fractions based on TLC spots were pooled, evaporated to dryness at 40°C, then subjected to α-glucosidase inhibition screening.

The *in vitro* screening for α-glucosidase inhibition was adopted from [14]. Ten (10) milligrams each of the concentrated pooled fractions from flash column chromatography, and the concentrated crude ethyl acetate fraction were dissolved in 2.00 mL of DMSO, then filled to volume of 10.00 mL using 100 mM phosphate buffer (pH 6.8), to obtain stock solutions having a concentration of 1 mg/mL each. From the stock solutions, test solutions were prepared by adjusting the concentrations to 30, 70, and 150 µg/mL of the phosphate buffer. Quercetin standard was used as positive control, which was also adjusted to the previously mentioned various concentrations dissolved in the phosphate buffer. Fifty (50.00) microliters of each test solution were prepared in triplicates, and positioned in the 96-well plate as shown in Figure 1. Wells in triplicates were also allotted for negative control initially containing 50.00 microliters of the phosphate buffer, for DMSO test solution initially containing 50.00 microliters of DMSO, and for blank test solution initially containing 100.00 microliters of the phosphate buffer. To all the occupied wells, 50.00 microliters of the phosphate buffer, and 50.00 microliters of 2.5 mM p-nitrophenyl-D-glucopyranoside (pNPG) in the phosphate buffer were added to the mixture in the following order. These wells were followed by the addition of 50.00 microliters of the phosphate buffer containing 0.2 U/mL yeast α-glucosidase, except for the blank test solution wells. The plate was incubated at 37°C for 15 minutes, followed by the immediate addition of 150.00 microliters of 0.3 M sodium carbonate solution to stop the reaction. The plate was read using a microplate reader at 405 nm,

factoring in the wells allotted for negative control, DMSO, and blank test solutions. The percent inhibition was calculated and plotted against the concentration. The Inhibitory Concentration 50% (IC50) of each test fraction was derived using linear regression. The IC50 of the test extracts was compared to Quercetin standard.

Finally, phytochemical tests were conducted to characterize the crude ethyl acetate fraction, and pooled fractions that exhibited the highest α-glucosidase inhibitory activities. The following tests were used to confirm presence of Anthraquinone Glycosides (1) General Test (2) Schouteten Reaction and (3) Modified Bornträger. For Tannins and Phenolic Glycosides, the following tests were done (1) Ferric Chloride Test and (2) Gelatin Test.

Results

Optimum chromatographic conditions were determined to fractionate the phytochemical constituents responsible for the anti-diabetic activity of *C. cainito* leaves crude ethyl acetate fraction. Among the solvent systems used for developing the TLC plates, the 15:65:10:10 volume ratio of hexane: ethyl acetate: methanol: acetic acid produced the most number and separation of spots. The TLC profile of the crude ethyl acetate fraction under different visualization methods can be seen in Table 3. To further fractionate the crude ethyl acetate fraction, it was subjected to flash column chromatography. The 5-mL fractions collected after each flash column chromatography run were pooled based on their TLC chromatograms as seen in Figure 2. The TLC profile of the fractions from the flash

Table 3. *TLC profile of the crude ethyl acetate fraction under different detection methods*

Rf Value	White Light	UV 254 nm	UV 366 nm	Vanillin H2SO4
0.1969	No spot	Light grey spot	No spot	Light reddish brown spot
0.4188	No spot	Grey spot	No spot	Light reddish brown spot
0.5844	No spot	Light grey spot	No spot	Light reddish brown spot
0.7063	No spot	Light grey spot	No spot	No spot

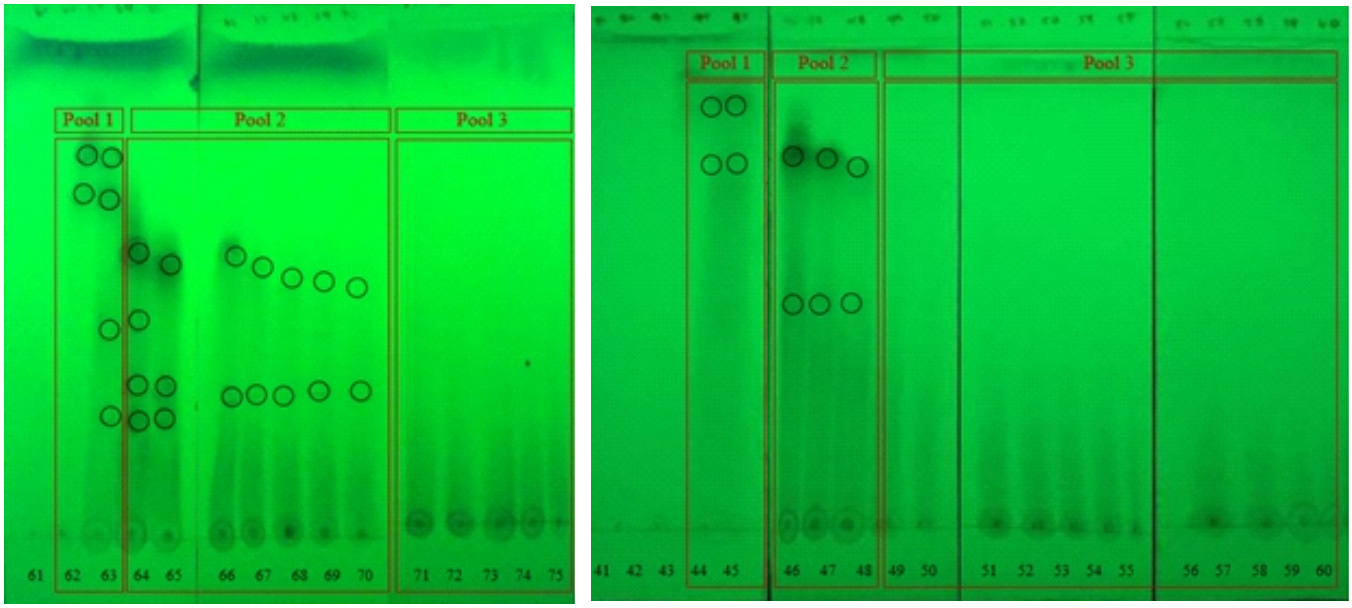

Figure 2. *TLC chromatograms of the fractions from the first (left) and second (right) runs of flash column chromatography ran in 15:65:10:10 hexane: ethyl acetate: methanol: acetic acid visualized using UV 254.*
**Red boxes indicate the pooled fractions*

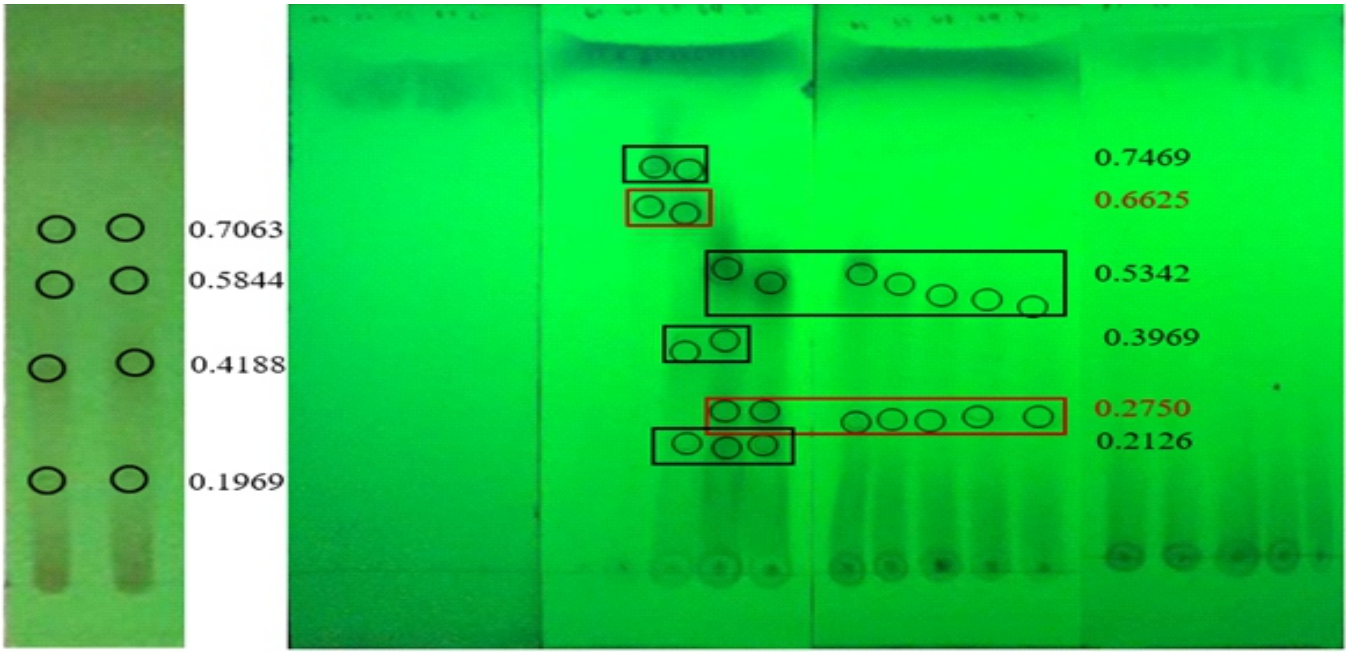

Figure 3. *TLC chromatogram of the crude ethyl acetate fraction (left), compared to the TLC chromatogram of the fractions from the flash column chromatography run (right) showing two new groups of spots (in red boxes) which were not initially seen on the TLC chromatogram of crude ethyl acetate fraction.*

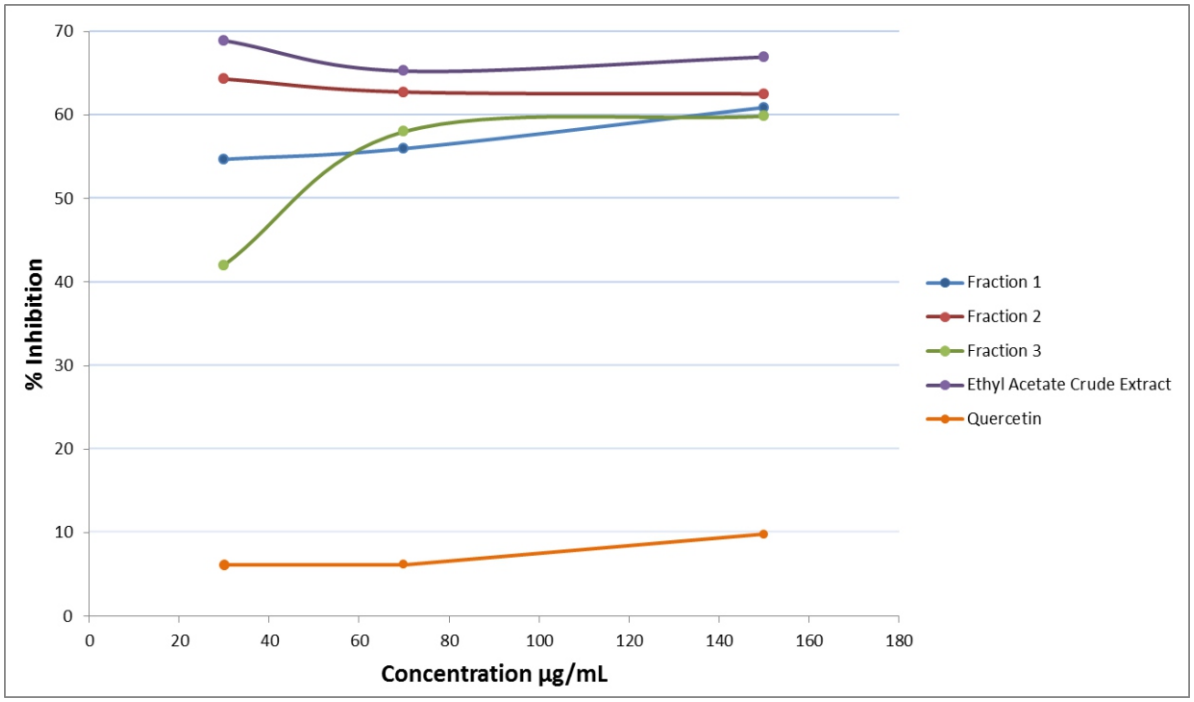

Figure 4. *Alpha-glucosidase inhibition activity profile of three pooled fractions from flash chromatography and crude ethyl acetate fraction (30, 70, 150 µg/mL) of C. cainito leaves, compared to Quercetin standard.*

column chromatography can be seen in Table 4. Upon visual inspection and comparison of Rf values, two new groups of spots were detected from the TLC chromatograms of flash column chromatography fractions, which were not initially detected in the TLC chromatogram of the crude ethyl acetate fraction.

The dried pooled fractions, as well as the crude ethyl acetate fraction and controls, were subjected to α-glucosidase inhibition assay after determining the optimum chromatographic conditions. The pooled fractions in the 96-well plate resulted to lighter yellow solutions, as compared to Quercetin standard. Based on the α-glucosidase inhibition

Table 4. *TLC profile of the fractions from the flash column chromatography under different detection methods*

Run	Fraction	Pooled Fraction	Rf Value	White light	UV 254 nm	UV 366 nm	Vanillin H2SO4
First run of Flash Chromatography (FCC)	1-61	N/A*	N/A*	No spot	No spot	No spot	No spot
	62-63	1	0.2126	No spot	Light grey spot	No spot	Light yellowish brown spot
			0.3969	No spot	Light grey spot	No spot	Light yellowish brown spot
			0.6625	No spot	Light grey spot	No spot	No spot
			0.7469	No spot	Light grey spot	No spot	No spot
	64-70	2	0.2126	No spot	Grey spot	No spot	Light yellowish brown spot
			0.2750	No spot	Light grey spot	No spot	Light yellowish brown spot
			0.3969	No spot	Light grey spot	No spot	Light yellowish brown spot
			0.5342	No spot	Grey spot	Dark violet spot	Yellowish brown spot
	71-89	3	N/A* (traces)	No spot	No spot	No spot	No spot
Second run of FCC	1-43	N/A*	N/A*	No spot	No spot	No spot	No spot
	44-45	1	0.8625	No spot	Light grey spot	Dark violet spot	No spot
			0.9187	No spot	Grey spot	Dark violet spot	No spot
	46-48	2	0.4250	No spot	Light grey spot	Dark violet spot	Yellowish brown spot
			0.7375	No spot	Dark grey spot	Dark violet spot	Yellowish brown spot
	49-65	3	N/A* (traces)	No spot	No spot	No spot	No spot

*Not applicable due to absence/traces of spots detected

activity profile of each fraction, all fractions exhibited higher percent inhibition than Quercetin standard at 30, 70, and 150 µg/mL as seen in Figure 4. Among the fractions, only Fraction 3 exhibited a concentration dependent activity like the Quercetin standard which is characterized by having positive slope and IC50 values computed via linear regression. The IC50 of Fraction 3, 58.02 µg/mL, was significantly higher than the IC50 of Quercetin standard, 1399.06 µg/mL. Fraction 1 also exhibited a positive slope, however, its IC50 value was negative. On the other hand, Fraction 2 and crude ethyl acetate fraction exhibited negative slopes despite having positive IC50 values.

Table 5. *Phytochemical screening of the fractions obtained from C. cainito leaves*

Glycosides	Test	Crude Ethyl Acetate Fraction	Flash Column Chromatography Fraction 1	Flash Column Chromatography Fraction 2	Flash Column Chromatography Fraction 3
Anthraquinone	General	(+) Clear, red orange solution	(+) Clear, light red orange solution	(-) Clear, very light yellow solution	(+) Clear, very light red orange solution
	Modified Bornträger	(-) Turbid top layer; opaque white bottom layer	(+) Turbid top layer; opaque yellow bottom layer	(-) Turbid top layer; opaque white bottom layer	(-) Turbid top layer; opaque white bottom layer
	Schouteten Reaction	(-) No fluorescence	(-) No fluorescence	(+) Bright purple fluorescence	(-) No fluorescence
Tannins and Phenolic	Ferric Chloride	(-) Absence of precipitate	(-) Absence of precipitate	(-) Absence of precipitate	(-) Absence of precipitate
	Gelatin	(+) Reddish brown curdy precipitate	(-) Absence of precipitate	(-) Absence of precipitate	(-) Absence of precipitate

To complete the characterization of the inhibitory activity, fractions were subjected to phytochemical screening. As seen in Table 5, the crude ethyl acetate fraction tested positive for Anthraquinone glycosides, and Tannins and Phenolic glycosides. On the other hand, all the flash column chromatography fractions tested positive for Anthraquinone glycosides only.

Discussion

The sieved leaves of *C. cainito* underwent exhaustive maceration using ethanol as the solvent. Ethanol was chosen due to its ability to dissolve both polar and non-polar molecules, and its high volatility which enable complete removal of the solvent from the extract. Soaking of fresh plant material with organic solvents like in cold maceration, is recommended since enzymes will be denatured, preserving the stability of the solute [15].

Following the exhaustive crude ethanolic extraction was the modified Kupchan partitioning. It was modified by substituting chloroform with DCM as one of the extraction solvents. This was done since DCM has almost the same polarity index, 0.42, as chloroform, 0.40 [16]. DCM is among the least toxic of the chlorinated methanes and has lesser toxicity than chloroform [17]. Another modification was the usage of 600.0 mL *n*-hexane instead of the usual 200.0 mL on the first partitioning. This was done since the initial resulting solution had a very dark color that when only 200.0 mL of *n*-hexane was used, the layers were not evident, thereby, disabling separation. The order of using n-hexane, DCM, then ethyl acetate increases in polarity. The crude ethyl acetate fraction was subjected to TLC and flash column chromatography.

In order to find the best TLC condition to efficiently separate the components of *C. cainito* leaves, 10.0 µg/mL of the crude ethyl acetate fraction was developed in different solvent systems. The first solvent system used was composed of 25:75 hexane: ethyl acetate with hexane as the non-polar solvent and ethyl acetate as partially polar component. Since the spots barely move from the origin, the polarity of the solvent system was increased by increasing the portion of ethyl acetate, and eventually by adding polar solvents methanol and acetic acid with polarity indexes of 5.1 and 6.2 respectively [18]. The portion of hexane, ethyl acetate, methanol, and acetic acid was varied until the ratio reached 15:65:10:10 which gave the most number of well-defined and well-separated spots. This volume ratio was considered the optimal chromatographic condition for TLC separation of *C. cainito* leaves components. Spots were detected using different

visualization methods. Compounds that absorb 254 and 366 nm include most compounds with aromatic rings and conjugated double bonds leaving the colored spots, while vanillin-sulfuric acid spray, known for its general detection method for steroids, higher alcohols, phenols, and essential oils (terpenoids, phenylpropanoids) [19], reacted with the components of C. cainito leaves giving reddish brown spots.

Following the TLC optimization is the flash column chromatography. Gradient elution was done to enhance the separation of the compounds in the crude sample matrix. The gradient solutions were composed of a variety of mixtures of hexane, ethyl acetate, methanol and glacial acetic acid having varying polarity indexes and strengths. The gradient started from the least polar, that was the hexane: ethyl acetate (25:75), which was followed by solvent mixtures with increasing polarity indexes, up to ethyl acetate: methanol: acetic acid (70:20:10) having the highest polarity index among the solvent systems. The same set of eluents were used on the first and second flash column chromatography run except that 50:50 hexane: ethyl acetate was omitted on the second flash column chromatography run. The eluent, 50:50 hexane: ethyl acetate was omitted since on the first flash column chromatography run, there were no components eluted by the said solvent which can be reflected on the TLC profile of fractions 1 to 20. The first solvent gradient used in the flash column chromatography has the least polarity index, hence the first fractions extracted were expected to contain the least polar components having high Rf values, while low Rf values for the polar substances.

Upon visual inspection and comparison of Rf values, two new groups of spots were seen in the flash column chromatography TLC chromatograms which were not initially present in crude ethyl acetate fraction TLC chromatogram. This indicated the improved separation power of the flash column chromatography which was done twice to provide enough samples for the α-glucosidase bioassay. Those fractions with similar chromatograms from the two runs were pooled resulting into three pooled fractions. The TLC profiles of the chromatograms were not exactly the same but followed the same trend where least polar compounds were contained in the first pooled fraction, mid-polar compounds in the second pooled fraction, and the most polar compounds in the third pooled fraction. Rf values of the spots decreased as the fractionation continued.

After the two flash column chromatography runs, the α-glucosidase inhibition assay was executed to determine the enzymatic inhibition activity of the fractions. The α-glucosidase is an enzyme secreted from the intestinal chorionic epithelium, responsible for the degradation of carbohydrates in the intestines. Thus, this enzyme became drug targets for anti-diabetic drugs and the *in vitro* test served as a determinant for compounds' enzyme inhibition capabilities, hence, their anti-diabetic properties [20]. The principle behind the assay involved the reaction of p-nitrophenyl-D-glucopyaranoside with the α-glucosidase to form α-D-glucopyranoside and p-nitrophenol, which is yellow-colored in solution. Compounds with α-glucosidase inhibitory activity stops the reaction, thereby, producing less coloration dependent on their α-glucosidase inhibitory power. The wells containing the crude ethyl acetate fraction together with all the pooled fractions produced less intense yellow coloration as compared to Quercetin standard, indicating relatively greater enzyme inhibition activity compared to standard. The color produced in the reaction was measured through its absorbance using a microplate reader at 405 nm.

The crude ethyl acetate fraction and the three pooled fractions from the flash column chromatography were tested for their enzyme inhibition activity compared against the Quercetin standard. This standard and its glycosides were identified in numerous studies to possess high inhibitory effects against the yeast α-glucosidase enzyme having an IC50 of 0.017 mmol/L, greater as compared to acarbose which has an IC50 of 0.091 mmol/L. Although acarbose has been frequently used as the standard for α-glucosidase bioassays, a study has shown that acarbose is a potent inhibitor of mammalian sucrose and maltase, and is inactive against the α-glucosidase from the yeast and bacteria [21]. In the study, the Quercetin standard exhibited a concentration dependent activity which was reflected by a positive slope and positive IC50 value of 1399.06 µg/mL. Based on the enzymatic activity profile, all fractions exhibited higher percent inhibition than Quercetin standard, however, only Fraction 3 exhibited a concentration dependent activity like the Quercetin standard.

The concentration dependency of Fraction 1, Fraction 2, and Crude ethyl acetate fraction cannot be established from the data generated because of the strong activity exhibited by the said fractions even at the lowest concentration used in the study. This was characterized by either negative slopes and/or negative IC50 values, therefore, smaller concentrations than 30 µg/mL may be explored for toxicity assays. The IC50 of Fraction 3, 58.02 µg/mL, was significantly higher compared to the IC50 of the Quercetin

standard, 1399.06 µg/mL. Crude ethyl acetate fraction exhibited the highest α-glucosidase inhibition activity, next to it is Fraction 2, followed by Fraction 1, then Fraction 3 with the least α-glucosidase inhibition activity relative to the other fractions. It can be said that Fraction 2 contributed mostly to the α-glucosidase inhibition activity of the Crude ethyl acetate fraction.

To complete the characterization, the pooled fractions and the crude ethyl acetate fraction of *C. cainito* leaves were tested for their phytochemical contents as seen in Table 5. All of the samples tested positive for Anthraquinone glycosides, however, only the crude ethyl acetate fraction tested positive for the gelatin test identifying the presence of Tannins and Phenolic glycosides. This may be due to the flash column chromatography extraction wherein tannins and phenols, being polar compounds, did not elute to the fractions since the column chromatogram gradient favored the elution of non-polar compounds, thereby, retaining the polar compounds to the stationary phase [22]. The pooled fractions which tested positive to the phytochemical screening for Anthraquinone glycosides, specifically Modified Borntrager's Test, were Fraction 1 and Fraction 3. Negative results on the rest of the fractions may indicate the presence of a very stable form of Anthraquinone glycosides or a reduced derivative of Anthraquinone–the Anthranols [23].

A test used to confirm the presence of Anthraquinone glycosides and their forms is the Shouteten reaction. Combined forms or dimer give a slight fluorescence while reduced forms give off intense fluorescence. Fraction 2 gave the most intense fluorescence, thus, it can be said that Fraction 2 was composed of reduced derivative of Anthraquinone in combined forms or dimer.

Relatively few studies focus on the potential of Anthraquinone glycosides for their pharmacologic use [24], however, in one study, four Anthraquinone compounds namely emodin, aloe-emodin, physcion, and rhein were found to exhibit strong α-glucosidase inhibition activity with IC50 values ranging from 4.12 µM to 5.68 µM [25]. It is possible that the said Anthraquinone glycosides, or other compounds with similar structure, were the ones responsible for the α-glucosidase inhibition activity of the pooled fractions.

Acknowledgment

We would like to extend our deepest gratitude to the people who have contributed in making this study possible, and to the University of the Philippines-National Institutes of Health (UP-NIH) for the research grant allotted for the study.

References

1. International Diabetes Federation. IDF treatment algorithm for people with type 2 diabetes. 2011.
2. World Health Organization. Country and regional data on diabetes. 2015.
3. Cannon KS, Hebert DN, Helenius A. Glycan-dependent and –independent association of vesicular stomatitis virus G protein with calnexin. Journal of Biological Chemistry 1996;271(24):14280–14284. doi: 10.1074/jbc.271.24.14280
4. Krapek K, King K, Warren SS, *et al.* Medication adherence and associate hemoglobin A¬1c in type 2 diabetes. Annals Pharmacotherapy 2004;38(9):1357-1362. doi: 10.1345/aph.1D612
5. Osadibe PO, Odon EU, Uzor PF. The search for new hypoglycemic agents from plants. African Journal of Pharmacy and Pharmacology 2014; 8(11):292-303. doi: 10.5897/AJPP2014.3933
6. N'guessan K, Amoikon KE, Tiébré MS, Kadja B, Zirihi GN. Effect of aqueous extract of *Chrysophyllum cainito* leaves on the glycaemia of diabetic rabbits. Afr J Pharm Pharmacol 2009;3:501–506.
7. Meira NA, Klein LC, Rocha LW, Quintal ZM, Monache FD, Cechinel Filho V, Quintão NLM. Anti-inflammatory and anti-hypersensitive effects of the crude extract, fractions and triterpenes obtained from *Chrysophyllum cainito* leaves in mice. Journal of Ethnopharmacology 2014;151(2):975–983. doi: 10.1016/j.jep.2013.12.014.
8. Shailajan S, Gurjar D. Pharmacognostic and phytochemical evaluation of *Chrysophyllum cainito* Linn. Leaves. Int J Pharm Sci 2014;26(1):106–111.
9. Florida Growables. Star Apple, Caimito – *Chyrsophyllum cainito*. 2013.
10. Muhit A, Tareq SM, Apu AS, Basak D, Islam MS. Isolation and identification of compounds from the leaf extract of *Dillena indica* Linn. Bangladesh Pharmaceutical Journal 2010;13(1):49-53. doi: 10.1071/APP9850032.
11. Franz JK, Shea KL. Laboratory manual: 5.301 chemistry laboratory techniques. 2013. Massachusetts: MIT.
12. Cannel RJP. Methods in biotechnology: Natural products isolation. 1998. New Jersey: Human Press Inc.
13. Li J, Song F, Xing J. Screening and structural characterization of α-glucosidase inhibitors from hawthorne leaf flavonoids extract by ultrafiltration LC-

DAD MSn and SORI-CID FTICR MS. Journal of American Society for Mass Spectrometry 2009;20(8):1946-1503. 0.1016/j.jasms.2009.04.003
14. Bart HJ. Extraction of natural products from plants – an introduction in industrial scale natural products extraction. 2011. Wiley-VCH Verlag GmbH & Co. KGaA. doi: 10.1002/9783527635122.ch1
15. Chaves AL, Vergara CE, Mayer JE. Dichloromethane as an economic alternative to chloroform in the extraction of DNA from plant tissues. Plant Molecular Biology Reporter 1995;13(1):18-25. doi: 10.1007/BF02668389
16. Williams PL, James RC, Roberts SM. Principles of toxicology: Environmental and industrial applications (3rd ed.). 2015. New Jersey: John Wiley & Sons.
17. Idaho State University (ISU). Polarity Index. 2016.
18. School of Chemical Sciences University of Illinois. CHEM 536 - Thin Layer Chromatography. 2016.
19. Yin Z, Zhang W, Feng F, Zhang Y, Kang W. α-Glucosidase inhibitors isolated from medicinal plants. Food Science and Human Wellness 2014;3(3-4):136–174. doi: 10.1016/j.fshw.2014.11.003
20. Li YQ, Zhou FC, Gao F, *et al.* Comparative evaluation of quercetin, isoquercetin and rutin as inhibitors of α-glucosidase. Journal of Agricultural and Food Chemistry 2009;57:11463-11468. doi: 10.1021/jf903083h.
21. Shinde J, Taldone T, Barletta M, Kunaparaju N, Hu B, Kumar S, Placido J, Zito SW. Alpha-glucosidase inhibitory activity of *Syzygium cumini* (Linn.) Skeels seed kernel in vitro and in Goto-Kakizaki (GK) rats. Carbohydrate Research 2008;343(7):1278–1281. doi: 10.1016/j.carres.2008.03.003.
22. Waksmundzka-Hajnos M, Sherma J, Kowalska T. Thin layer chromatography in phytochemistry. 2008; 99. Florida, CRC Press.
23. Evans WC. Trease and Evans Pharmacognosy (16th ed.). 2009. London: Saunders Elsevier.
24. Kouam SF, Khan SN, Krohn K, *et al.* α-Glucosidase inhibitory anthranols, kenganthranols A–C, from the stem bark of *Harungana madagascariensis*. Journal of Natural Products 2006;69(2):229-33. doi: 10.1021/np050407n.
25. Yang D, Zhao J, Liu S, *et al.* The screening of potential α-glucosidase inhibitors from the Polygonum multiflorum extract using ultrafiltration combined with liquid chromatography-tandem mass spectrometry. Analytical Methods 2014;6(10):3353-3359. doi: 10.1039/C4AY00064A