Acta Manilana 65 (2017), pp. 11–16
Printed in the Philippines
ISSN: 0065–1370

A glucomannan with α-glucosidase inhibitory activity isolated from the leaves of *Antidesma microcarpum* Elmer (Euphorbiaceae)

John P. Paulin[1]*, Mario A. Tan[1–3], Grecebio Jonathan D. Alejandro[1–3], & Mafel C. Ysrael[1,2]

[1]The Graduate School; [2]Research Center for the Natural and Applied Sciences; [3]College of Science, University of Santo Tomas, 1015 Manila, Philippines

The α-glucosidase inhibition is seen as a possible approach to controlling post-prandial hyperglycemia in diabetes mellitus. Some species of the genus *Antidesma* L. are reported to be of use in the treatment of diabetes. In search of indigenous and/or endemic *Antidesma* species with potential use as adjuvants of antidiabetic agents the extracts of *A. curranii*, *A. fusicarpum*, *A. microcarpum*, and *A. montanum* were screened for α-glucosidase inhibitory activity and to isolate the active constituent from the most active extract. The *Antidesma* extracts were assayed by an in vitro method using yeast α-glucosidase. The in vitro α-glucosidase inhibitory assay was also used for the bioassay-guided isolation of the active constituent. *A. microcarpum* extract exhibited the highest glucosidase inhibitory activity with an IC_{50} of 1.42±0.03 μg/mL. The bioassay-guided isolation of the active constituent in the extract of *A. microcarpum* afforded a glucomannan having the structure β-D-mannopyranosyl-(1→3)-α-D-mannopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-D-glucopyranose and with an IC_{50} α-glucosidase inhibitory activity of 0.22 ±0.04 μg/mL which is higher than the inhibitory activity of standard acarbose.

Keywords: *Antidesma microcarpum*, Euphorbiaceae, α-glucosidase inhibitory activity, glucomannan

Introduction

Diabetes mellitus is one of the leading causes of mortality in the Philippines [1]. By 2030, around 552 million individuals will have type 2 diabetes mellitus corresponding to 7.8% of the adult population [2]. Diabetic individuals have glucose intolerance, and intake of dietary carbohydrates causes postprandial hyperglycemia, in which blood glucose is elevated to abnormal levels [3]. Carbohydrates are converted into glucose through a hydrolytic cleavage catalyzed by membrane-bound α-glucosidase found in the epithelium of the small intestines [4]. Inhibition of α-glucosidase will slow down the hydrolysis of carbohydrates into glucose and control the build-up of blood glucose. Thus, α-glucosidase inhibition is seen as a possible approach in improving postprandial hyperglycemia in type 2 diabetes mellitus.

The commonly prescribed α-glucosidase inhibitors are acarbose and miglitol which are

**To whom correspondence should be addressed:* paulin.johnp@gmail.com

obtained from microbial sources. Meanwhile, plants that are indicated to lower blood glucose levels can also serve as potential sources of α-glucosidase inhibitors. There are 47 species of plants belonging to 29 families exhibiting α-glucosidase inhibitory activity [5] and the compounds responsible for this activity are terpenes, alkaloids, quinine, flavonoids, phenol, phenylpropanoid, and steride structures containing carboxylic acid, ester, alcohol, and allyl functional groups [6].

The genus *Antidesma* has about 170 species distributed in the world [7, 8]. Locals in Mauritius [9], Northeast India [10], and the Philippines [11] use *Antidesma* species as an antidiabetic agent. Inhibitory effect of different *Antidesma* species *A. bunius* [12, 13], *A. celebicum* [12, 14], *A. madagascariense* [15, 16], *A. montanum* [12], and *A. nuerocarpum* [12] on α-glucosidase have been reported.

In an effort to search for α-glucosidase inhibitors from either indigenous or endemic *Antidesma* species, extracts of *A. curranii*, *A. microcarpum*, *A. montanum*, and *A. fusicarpum* were screened for α-glucosidase inhibition. The active constituent from the most active *Antidesma* extract was then isolated and identified.

Experimental

General. For 1D and 2D NMR, Bruker AV-500 (500 MHz) spectrometer was used; gel filtration chromatography was performed using Sephadex LH-20 (30 g 25–100 μ bead size, column diameter = 1.0 in., height = 10.0 in.) and preparative thin layer chromatography (TLC) using a pre-coated with silica gel 60 RP-8 F_{254} plate (0.25 mm, Merck) and pre-coated silica-gel 60 F254 (0.25 mm, Merck) plates.

Plant materials and extraction. The leaves of *A. curranii* Merr. were obtained from Hermosa, Bataan while *A. fusicarpum* Elmer were collected from Pilar, Bataan. Samples of *A. microcarpum* Elmer and *A. montanum* Blume leaves were acquired from Lambunao, Iloilo. Collection was done between October 2014 and January 2015. The samples were authenticated by the UST Herbarium. Voucher specimens of *A. curranii* (USTH 012454), *A. fusicarpum* (USTH 012855), *A. microcarpum* (USTH 012455), and *A. montanum* (USTH 012456) were deposited at the Herbarium, Research Center for the Natural and Applied Sciences (RCNAS), University of Santo Tomas.

All air-dried leaves were extracted exhaustively with methanol based on the ratio of 1 kg dried leaves to 10 L solvent. After 3 days, the extracts were collected and subjected to rotary evaporation.

α-Glucosidase inhibitory assay. The α-glucosidase inhibition assay was performed according to a slightly modified procedure of Feng *et al.* [17]. In a 96-well plate, 20 μL of 0.8 U/mL yeast α-glucosidase was mixed with 120 μL of a test substance or acarbose in 0.5% DMSO. The solution was incubated at 37°C for 15 min then 20 μL of 5.0 mM *p*-nitrophenyl-α-D-glucopyranoside was added. The plate was returned to the incubator and the reaction was allowed to proceed at 37°C for 15 min. After incubation, 80 μL of 0.2 M sodium carbonate was added to stop the reaction. The absorbance of the contents of the plate were read at 405 nm using Hitachi SH-1000 microplate reader. Percent inhibition was calculated using the formula below.

$$Percent\ inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ and A_{sample} are the absorbance of the control and sample respectively.

To determine the IC_{50} of the extracts for α-glucosidase inhibition, a dose-response curve of five concentrations was prepared and each concentration was measured. The mean percent inhibition and standard deviation were reported. The IC_{50} values were calculated using four-

parameter logistic equation through GraphPad Prism 6.07. The mean IC_{50} of the extracts on α-glucosidase inhibition were compared to that of acarbose.

Isolation of the active glucomannan. The air-dried leaves of *A. microcarpum* (262.4 g) were soaked in methanol (2.6 L) for 36 h. The methanol extract was concentrated in vacuo to a green syrupy consistency. The concentrated extract (20.54 g) was suspended in water and successively partitioned with hexane (500 mL), ethyl acetate (300 mL), and *n*-butanol (300 mL) to obtain hexane (2.60 g), ethyl acetate (6.20 g), *n*-butanol (4.68 g) and aqueous (7.00 g) soluble-fractions after removal of solvents in vacuo. Each major fraction was tested for α-glucosidase inhibitory activity.

The most active *n*-butanol soluble-fraction (1.02 g) was subjected to gel filtration chromatography on LH-20 Sephadex column eluting with methanol (1 L) to give 20 pooled sub-fractions based on similar TLC profiles. One of two active sub-fractions labelled BF7 appearing as a single component was purified by preparative TLC silica gel 60 RP-8 F_{254} (Merck) plate developed in methanol:water (1:1, v/v) to obtain the active glucomannan. The structure of the active glucomannan was determined by 1D (^{1}H-NMR and ^{13}C-NMR) and 2D (HMQC and HMBC) NMR spectral analysis. The isolate was dissolved in deuterated methanol (CD_3OD) and its ^{1}H and ^{13}C NMR were recorded on a Bruker AV-500 (500 MHz) spectrometer using trimethylsilane as internal standard.

Results and Discussion

α-Glucosidase inhibitory activity. The α-glucosidase inhibitory effect of the four *Antidesma* species varied with the concentration of the methanol extracts (Fig. 1). Based on this concentration-response curve, the IC_{50} value which indicated the extract concentration needed to inhibit 50% of enzyme activity, was determined through interpolation. Table 1 shows the IC_{50} values of the four *Antidesma* extracts and acarbose on α-glucosidase. Among the *Antidesma* extracts, *A. microcarpum* was the most active with an IC_{50} at 1.42±0.03 µg/mL. The results prompted the bioassay-guided isolation of the constituent with α-glucosidase inhibitory activity from *A. microcarpum*.

Table 1. The α-glucosidase inhibitory activity of the four *Antidesma* species with *A. microcarpum* having the highest activity

Samples	IC_{50} values ($\times 10^3$ ng/mL) $n = 9$
A. curranii	10.03 ± 0.26
A. fusicarpum	8.53 ± 0.14
A. microcarpum	1.42 ± 0.03
A. montanum	5.02 ± 0.07
Acarbose	0.64 ± 0.03

*Results were reported as mean ± standard deviation ($n = 9$)

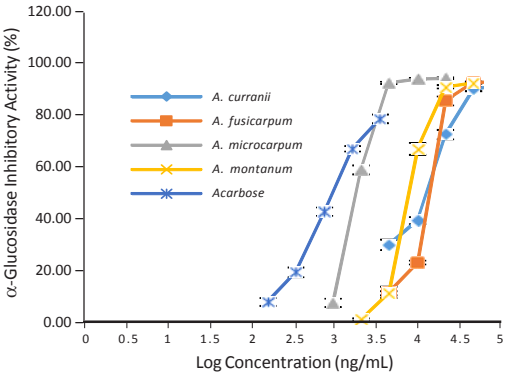

Figure 1. Concentration response curve of the *Antidesma* extracts and acarbose for α-glucosidase inhibitory activity. Values are expressed as mean ± standard deviation ($n = 9$).

The α-glucosidase inhibitory activity of the major fractions of the methanolic extract of *A. microcarpum* decreased with the concentration of the solutions of the fractions, as shown by the plots in Fig. 2. The IC_{50} values of the different fractions are presented in Table 2. The *n*-butanol fraction was found to be the most active α-glucosidase inhibitor with IC_{50} at 0.04±0.02 µg/mL.

Table 2. The α-glucosidase inhibitory activity of the major fractions of *A. microcarpum*

Fraction	IC_{50} values ($\times 10^3$ ng/mL) $n = 9$
Hexane	9.70 ± 0.27
Ethyl Acetate	9.78 ± 0.28
n-Butanol	0.04 ± 0.02
Aqueous	4.68 ± 0.11

*Results are reported as mean ± standard deviation ($n = 9$).

Table 3. ^{13}C and ^{1}H NMR Chemical Shifts of β-D-Mannopyranosyl-(1→3)-α-D-mannopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-D-glucopyranose

Monosaccharide	Chemical Shift ^{13}C NMR, 400 MHz	Chemical Shift ^{1}H NMR, 400 MHz
β-D-Mannopyranose		
1	98.3	4.55 ($J = 9.0$ Hz)
2	71.9	3.80
3	74.9	3.50
4	66.0	3.59
5	78.1	3.28
6	62.8	3.53, 3.55
α-D-Mannopyranose		
1	99.2	5.11 ($J = 4.5$ Hz)
2	73.0	4.02
3	71.3	3.69
4	64.6	3.59
5	76.3	4.04
6	62.9	3.53, 3.55
β-D-Glucopyranose		
1	103.2	4.48 ($J = 9.0$ Hz)
2	76.9	3.28
3	78.2	3.50
4	71.8	3.38
5	77.6	3.47
6	64.4	3.59
α-D Glucopyranose		
1	94.0	5.40 ($J = 4.5$ Hz)
2	71.9	3.55
3	83.4	3.66
4	69.5	3.85
5	73.9	4.04
6	64.2	3.59

Gel-filtration chromatography of the *n*-butanol fraction yielded two active sub-fractions, one of which is a single component (BF7) having an IC_{50} at 0.22±0.04 μg/mL which is higher than acarbose. The structure of purified BF7 is elucidated in this report. The other sub-fraction with higher activity than BF7 but with two

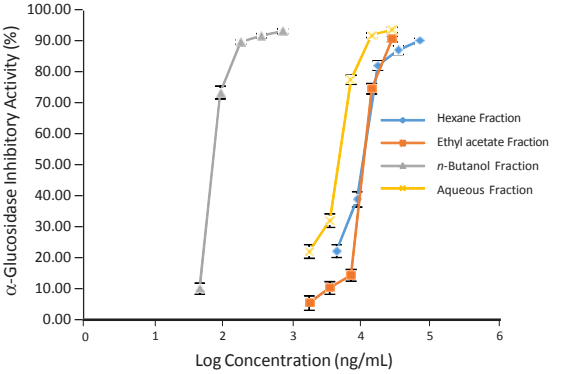

Figure 2. Concentration response curve by the *A. microcarpum* fractions for α-glucosidase inhibitory activity. Values are expressed as mean ± standard deviation ($n = 9$).

unidentified components acting together with BF7 to account for the high α-glucosidase inhibitory activity of the butanol fraction.

Structure elucidation of BF7. The isolate BF7 (4.4 mg) was subjected to different NMR experiments to determine its structure. Table 3 presents the spectral data from the ^{1}H NMR and ^{13}C NMR experiments. Initial assessment of the spectra suggested that the isolate is a carbohydrate. Analysis of the ^{1}H NMR spectrum showed that the isolate contained four anomeric protons at δ_H 4.48 (d, $J = 9.0$ Hz), δ_H 4.55 (d, $J = 9.0$ Hz), δ_H 5.11 (d, $J = 4.5$ Hz), and δ_H 5.40 (d, $J = 4.5$ Hz) suggesting the presence of four monosaccharide residues. The tetrasaccharide consisted of two α-anomers (5.11 and 5.40 ppm) and two β-anomers (4.48 and 4.55 ppm). Methine and methylene protons of the carbohydrate were observed between δ_H 3.11 and δ_H 4.13 ppm.

The ^{13}C NMR spectrum showed four signals between 94.0 and 103.2 ppm which were characteristic signals for anomeric carbons. This confirmed the presence of four monosaccharide residues. Among the four anomeric carbon signals, δ_C 94.0 corresponds to the reducing end of the tetrasaccharide. By comparing with known chemical shifts for ^{13}C, for the anomeric carbons

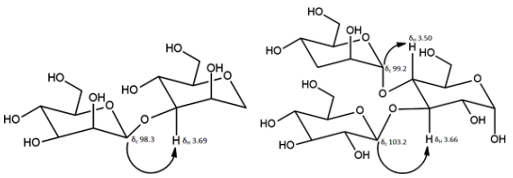

Figure 3. HMBC correlations at the glycosidic linkage

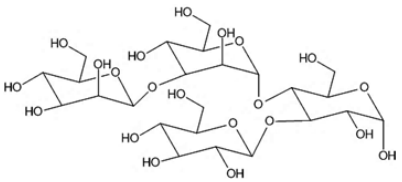

Figure 4. Structure of β-D-mannopyranosyl-(1→3)-α-D-mannopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-D-glucopyranose

of monosaccharides, δ_C 94.0 was characteristic for α-D-glucose. Signals of the other anomeric carbons were shifted downfield. This indicated that these carbons were involved in a glycosidic linkage.

Also, the ^{13}C NMR spectrum showed four signals between δ_C 62.8 and δ_C 64.4 ppm. These signals corresponded to four hydroxymethylene structures of aldopyranose. Based from known chemical shifts for C-6 of hexoses, the monosaccharide units were identified. The carbon signals δ_C 64.2 and δ_C 64.4 corresponded to two D-glucose units while signals δ_C 62.9 and δ_C 62.8 corresponded to two D-mannose units. The reducing end was glucose while the other residues had glycosylated C-1.

The HMBC spectrum provided information on how the four monosaccharide units were linked (Fig. 3). The C-1 of β-D-mannose (δ_C 98.3) was correlated to H-3 of α-D-mannose (δ_H 3.69). The C-1 of α-D-mannose (δ_C 99.2) was correlated to H-4 of α-D-glucose (δ_H 3.50). The C-1 of β-D-glucose (δ_C 103.2) was correlated to H-4 of α-D-glucose (δ_H 3.66).

Based on the spectral data, the active constituent from *A. microcarpum* was found to be β-D-mannopyranosyl-(1→3)-α-D-mannopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)]-α-D-glucopyranose (Fig. 4).

The structure of the isolated glucomannan may explain its α-glucosidase inhibitory activity. The α-glucosidase enzyme specifically breaks α-(1→4) linkages of oligosaccharides of glucose units. Thus the mannose units linked by β-(1→3) in the structure of the glucomannan are not susceptible to hydrolysis by α-glucosidase. The glucomannan structure may instead act to inhibit the activity of the α-glucosidase by competition at the active site of the enzyme. This is the first report on the isolation of a glucomannan with α-glucosidase inhibitory activity from *A. microcarpum*.

Conclusion

The α-glucosidase inhibitory activity of *A. microcarpum* is an evidence for its potential utility as an adjuvant to post prandial hyperglycemia in diabetes mellitus. The structure of the active component, a glucomannan may account for the possible mechanism of α-glucosidase inhibitory activity. The elucidation of the glucomannan may lead to alternative designs to the currently available α-glucosidase inhibitors which have reports of undue gastro intestinal side effects.

Acknowledgements

The researchers would like to thank Dr. Atsushi Nakayama of Tokushima University for the NMR experiments and the Research Center for the Natural and Applied Sciences for the use of the facilities.

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