

In vitro α -glucosidase inhibition and antioxidant activities of partially purified *Antidesma bunius* fruit and *Gynura nepalensis* leaf extracts

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ABSTRACT

Diabetes mellitus is a metabolic disorder characterized by increased blood glucose levels. Current treatments involve the use of sulfonylureas, α -glucosidase inhibitors, and other synthetic drugs. The study demonstrated the α -glucosidase inhibition and antioxidant properties of partially purified ethanolic extracts of *Antidesma bunius* fruits and *Gynura nepalensis* leaves, as possible herbal drug candidates. After ethanol extraction, the extracts were fractionated using normal phase liquid column chromatography, with elution solvents ethyl acetate, methanol and water. Fractionation resulted in five fractions for *A. bunius* (A1, A2, A3, A4 and A5) and seven fractions for *G. nepalensis* (G1, G2, G3, G4, G5, G6 and G7). Fraction G1 showed the highest α -glucosidase inhibition activity ($90.61 \pm 8.05\%$) and possibly acted via a mixed mode of inhibition. For the antioxidant activities, Fraction A1 exhibited highest radical scavenging activity via DPPH assay ($97.39 \pm 2.48\%$), Fraction G7 exhibited highest iron (II)-chelating activity ($95.85 \pm 1.46\%$) and Fraction G6 exhibited highest ferric-ion reducing activity via FRAP assay ($272.60 \mu\text{g/mL FeSO}_4$ equivalents). Phytochemical screening revealed that flavonoids and tannins were common among all fractions. The results demonstrated the potential of these plants as an antidiabetic herbal treatment. However, further studies needs to be done, specifically focusing on isolating the active component(s), structure and mechanism elucidation and toxicity assays.

INTRODUCTION

Type 2 diabetes mellitus is a metabolic disorder caused by chronic hyperglycemia due to insulin resistance and loss of β -cell function. Its complete mechanism has not yet been elucidated; it is believed to be caused by a variety of genetic and environmental factors (Wilcox, 2005). In 2011, the number of cases of all types of diabetes was estimated to be 366 million worldwide (8.30% of the world population) while in the Philippines, the estimate was 4.2 million (8.2% prevalence) and are expected to rise in the future (Whiting *et al.*, 2011). Poor management of diabetes may lead to complications which

include cardiovascular diseases, retinopathy, nephropathy, neuropathy, and peripheral gangrene-making this disease the eighth leading cause of mortality in the Philippines (Chang *et al.*, 2013). Current drugs used in the treatment of diabetes include sulfonylureas, biguanides, thiazolidinedione, and α -glucosidase inhibitors, sodium-glucose co-transporter inhibitors, glucagon-like peptide-1 analogues and dipeptidyl peptidase-IV inhibitors. These may be costly, inadequate, and have side effects (Chang *et al.*, 2013; Kimmel *et al.*, 2005; White, 2008; Abdul-Ghani and Defronzo, 2011; Garber, 2011). α -glucosidase inhibitors such as the drugs acarbose, miglitol, and voglibose can decrease hyperglycemia by decreasing the digestion and absorption of glucose in the intestines via inhibition of α -glucosidases (Mogensen, 2007). Antioxidants, such as vitamin E and flavonoids, have also proven beneficial to diabetics as they decrease the risk of complications (Bonfont-Rousselot, 2004).

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In various studies, it has been shown that antidiabetic drugs and antioxidants delayed or prevented the onset of type 2 diabetes mellitus in patients with impaired glucose tolerance (Montonen *et al.*, 2004; Kawamori, 2009; Phung *et al.*, 2011). More than 400 plants, such as *Momordica charantia* and *Gynura* spp. have been shown to have antidiabetic activities *in vitro* and *in vivo* (Singh *et al.*, 2011; Tan *et al.*, 2013; Algariri *et al.*, 2013; Deng *et al.*, 2011; Liu *et al.*, 2010; Li *et al.*, 2009). Metformin, a biguanide widely used in the treatment of type 2 diabetes, has been developed based on a biguanide in French lilac (Chang *et al.*, 2013). Major classes of phytochemicals which have been shown to have antidiabetic effects are alkaloids, flavonoids, glycosides/steroids/terpenoids, polysaccharides/ proteins, and miscellaneous compounds (Lamba *et al.*, 2000). Various plant extracts have already been shown to exhibit positive effects in diabetic assays, particularly α -glucosidase inhibitory and antioxidant activities (Lawag *et al.*, 2012; Misbah *et al.*, 2013; Wang *et al.*, 2013; Shihabudeen *et al.*, 2011; Apea-Bah *et al.*, 2009).

Leaves of *A. bunius*, locally known as *bignay*, have been shown to possess α -glucosidase inhibitory activity (Lawag *et al.*, 2012). *G. nepalensis*, locally known as *ashitaba*, has been used traditionally to control hyperglycemia in diabetic patients but no experiment has been done to study its antihyperglycemic or antioxidant properties.

The study aims to determine α -glucosidase inhibitory and antioxidant activities of partially purified ethanolic extracts of *A. bunius* fruits and *G. nepalensis* leaves using *in vitro* assays, as well as determine the phytochemical constituents of the most active fractions.

MATERIAL AND METHODS

Plant samples

Ripe *A. bunius* fruits and *G. nepalensis* leaves were obtained from local plant cultivators (Luzon, Philippines). These were authenticated by the Botany Division, National Museum, Philippines.

Extraction of *A. bunius* fruits and *G. nepalensis* leaves

Two kilograms (2 kg) *A. bunius* fruits and 100 g dried *G. nepalensis* leaves were powdered using an electric blender, then each were extracted twice with 2 L 80% ethanol by maceration for 48 hours. These were filtered, and the filtrates were dried by rotary evaporation and lyophilization.

Partial Purification

To determine the appropriate solvent system for separation, crude extracts were subjected to thin layer chromatography. Solvents used were methanol, acetonitrile, ethyl acetate, chloroform, and hexane. Using the best solvent system, the extracts were separated via gradient elution in a silica gel column. For both extracts, the solvents were as follows: ethyl acetate, ethyl acetate-methanol (1:4, 2:3, 3:2, 4:1), methanol,

methanol-water (1:4, 1:1), and water. Fractions were collected and subjected to TLC with ethyl acetate-methanol (5:1) as the solvent system. Fractions with similar retardation factors were pooled together. Pooled fractions of each extract and the crude extracts were subjected to α -glucosidase inhibitory and antioxidant assays.

α -Glucosidase Inhibition Assay

Extracts were prepared at 1.0 mg/mL. In 250 μ L phosphate buffer (pH 6.8), 100 μ L of extract or fraction was incubated at room temperature for 5 minutes with 100 μ L 2.0 mM *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG) as substrate for the enzyme. Then 50 μ L of 1.0 unit/mL α -glucosidase from *Saccharomyces cerevisiae* was added and the solution was incubated at 37 °C for 7 minutes. The reaction was terminated by adding 250 μ L of 0.1 M sodium carbonate. Acarbose was used as the positive control.

Change in absorbance was measured at 405 nm after termination of the reaction. Percentage inhibition of each fraction was computed as:

$$\% \text{ inhibition} = \frac{C - (S_7 - S_0)}{C} * 100$$

where C refers to the blank control, S refers to the extracts, subscript 7 refers to absorbance after 7 minutes, and subscript 0 refers to initial absorbance.

The mode of inhibition of the fraction with the highest α -glucosidase inhibitory activity was determined using the same procedure described but varying the concentration of *p*-NPG (0.8, 1.0, 1.3, 1.5, 1.8 mM). Lineweaver-Burk plots were generated by plotting the reciprocal of the initial reaction rate with respect to the reciprocal of substrate concentration. The initial reaction rate was computed as the rate of formation of *p*-nitrophenol. The procedure was done first without any inhibitor and then in the presence of 250 μ g/mL of the fraction. Maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) for both conditions were computed from the Lineweaver-Burk plots.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Free Radical Scavenging Assay

Fractions were prepared to a concentration of 10 mg/mL and 375 μ L of this stock solution was added with 375 μ L 0.2 mM DPPH in ethanol. Mixture were shaken and incubated in the dark for 30 min. Absorbance was measured at 517 nm with ascorbic acid as the positive control. Percentage radical scavenging activity of each fraction was computed as:

$$\% \text{ radical scavenging activity} = \frac{C - (S_{30} - S_0)}{C} * 100$$

where C refers to blank control, S refers to the extracts, subscript 30 refers to absorbance after 30 minutes, and subscript 0 refers to initial absorbance.

Iron (II) Chelation Assay

The iron (II) chelation assay was done by adding 200 μ L of extract (10 mg/mL) and 20 μ L FeCl₂ (2 mM) in 740 μ L solvent. After which, 40 μ L 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine or

ferrozine (5 mM) was added and the solution was incubated at room temperature for 10 minutes. Absorbance was measured at 562 nm with quercetin as the positive control. Percentage iron (II) chelating activity of each fraction was computed as:

$$\% \text{ iron chelating activity} = \frac{C - (S_{10} - S_0)}{C} * 100$$

where C refers to blank control, S refers to the extracts, subscript 10 refers to absorbance after 10 minutes, and subscript 0 refers to initial absorbance.

Ferric Reduction/Antioxidant Power (FRAP) Assay

The FRAP Assay protocols used were given by (Benzie and Strain, 1999). Absorbance was measured at 595 nm. A calibration curve of absorbance versus ferrous sulfate equivalents was prepared and tannic acid was used as the positive control for the assay.

Statistical Analysis

All measurements were done in triplicate and mean activities and standard deviations were computed. One-Way ANOVA with Tukey post-hoc test was performed to determine the most active fractions.

Phytochemical Screening

The protocol for phytochemical screening used was the guidelines given by Aguinaldo *et al.* (2005). Fractions of each extract were spotted on thin-layer silica gel plates and plates were developed in a chamber with chloroform-methanol (5:1). The chromatograms were air-dried and visualized using the reagents stated in the guidelines. Tests for the presence of flavonoids, steroids, phenols, tannins, alkaloids, cardenolides, coumarins, anthraquinones, indoles and sugars were conducted.

RESULT AND DISCUSSION

After gradient elution with ethyl acetate, methanol, and water, a total of 180 15-mL fractions were collected from each extract. From the 180 fractions, the *A. bunius* crude extract (ABCrude) was pooled into five fractions (AB1, AB2, AB3, AB 4, and AB5) while the *G. nepalensis* crude extract (GNCrude) was pooled into seven fractions (GN1, GN2, GN3, GN4, GN5, GN6, and GN7). These fractions, along with the *A. bunius* and *G. nepalensis* crude extracts were subjected to the different assays.

For the α -glucosidase inhibition assay, a change in absorbance at 405 nm is attributed to the release of *p*-nitrophenol after hydrolysis of *p*-NPG by the enzyme. Thus, a decrease in absorbance is correlated to an increase in inhibition activity. **Figure 1** shows the α -glucosidase inhibition activity of the crude extracts and fractions at 1 mg/mL. All *G. nepalensis* fractions showed inhibitory activity while only fractions AB 4 and AB 5 from *A. bunius* inhibited the enzyme. Moreover, fractions GN 1, GN 4, GN 5 and GNCrude had comparable activity compared to the positive control Acarbose. α -Glucosidase inhibition is important in diabetic

patients because these slow down the hydrolysis of polysaccharides and the absorption of free sugars in the intestine. Ultimately, this results in less drastic rise in blood glucose levels and decreased insulin sensitivity (Mogensen, 2007).

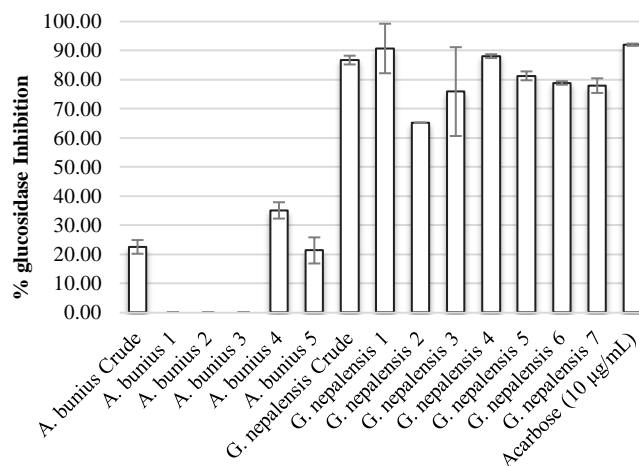


Fig. 1: α -glucosidase inhibitory activity of 1 mg/mL crude extracts and fractions of the *A. bunius* and *G. nepalensis* extracts and fractions, with Acarbose as positive control. No inhibition was detected from the *A. bunius* 1, 2 and 3 fractions.

Fraction GN 1 showed the highest activity among all fractions and its possible mode of inhibition was determined. **Figure 2** shows the Lineweaver-Burk plots of α -glucosidase in the absence and presence of GN 1.

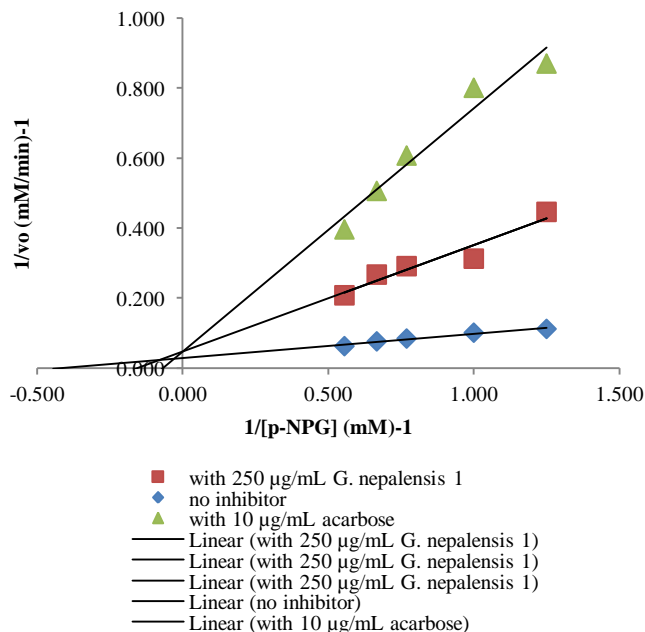


Fig. 2: Lineweaver-Burk plots of α -glucosidase with 10 mg/mL acarbose, 250 µg/mL G1, and without any inhibitor.

The increase in y-intercept is interpreted as a decrease in V_{max} of the enzyme caused by *G. nepalensis* 1 (**Table 1**). GN1 seems to exhibit a mixed mode of inhibition of α -

glucosidase which is not unexpected because GN1 has not been totally purified.

Table 1: V_{max} and K_m values of α -glucosidase with and without *G. nepalensis*.

Condition	K_m (mg/mL)	V_{max} (mg/mL•s)
without inhibitor	2.431	35.03
with <i>G. nepalensis</i> 1	6.551	21.47
with Acarbose	14.947	21.28

For the antioxidant activities, three assays were performed: DPPH assay, iron (II)-chelating assay and FRAP assay. **Figure 3** shows the radical scavenging activity of crude extracts and fractions via DPPH assay. All *A. bunius* fractions showed high radical scavenging activity with AB 1 having the highest. Compared to the positive control Ascorbic acid, all *A. bunius* extract and fractions, as well as GN 1, 2 and 4, had comparable activity.

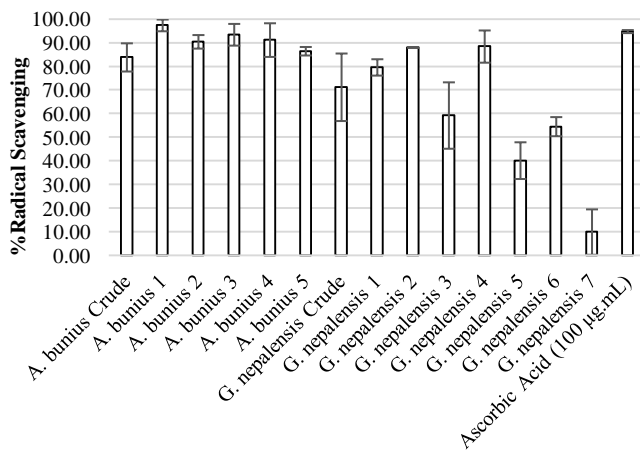


Fig. 3: Free radical scavenging activity of 10 mg/mL crude extract and fractions of the *A. bunius* and *G. nepalensis* determined via DPPH assay using Ascorbic acid positive control.

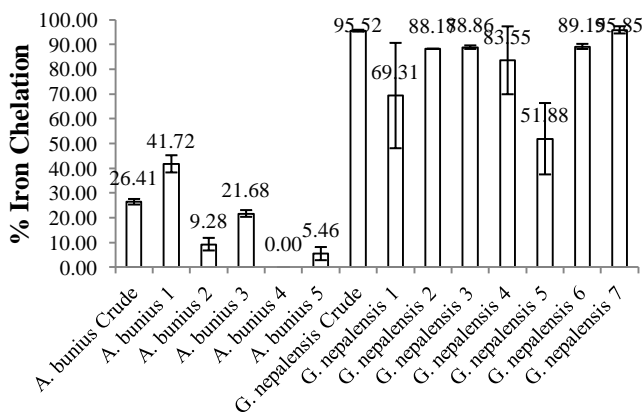


Fig. 4: Iron (II) chelating activity of 10 mg/mL crude extracts and fractions of the *A. bunius* and *G. nepalensis*.

On the other hand, **Figure 4** shows the iron (II) chelating activities of the crude extracts and fractions. Most of the *G. nepalensis* fractions showed high iron chelating activity with GN 7 having the highest activity among all fractions. AB 4 was the only

fraction from *A. bunius* with no iron chelating activity. Iron chelation is an important mode of antioxidant activity particularly because the ferrous ion is involved in the Fenton reaction, which produces the hydroxyl radical, a very strong reactive oxygen species (ROS). With ferrous ions chelated to certain compounds in the extracts, the Fenton reaction is prevented.

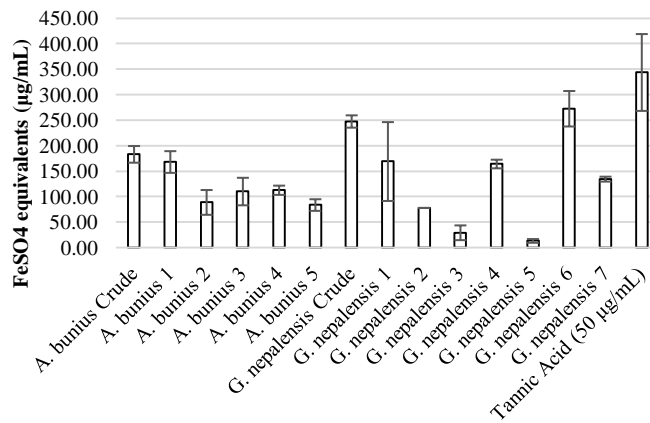


Fig. 5: Ferric reduction activity of 1 mg/mL crude extracts and fractions of the *A. bunius* and *G. nepalensis* expressed as $FeSO_4$ equivalents, with Tannic acid positive control.

Finally, **Figure 5** shows the ferric ion reducing power of the crude extracts and fractions in terms of the ferrous sulfate equivalents in the reacted solution. All fractions exhibited ferric reduction with GN 6 having the highest activity. Moreover, only GN Crude and GN 6 had comparable activity with the positive control Tannic acid. Any substance with a higher reducing potential than ferric ion will reduce this to ferrous ion. In the process, the compound is oxidized and prevents the oxidation of biological structures (Benzie and Strain, 1999). It is to be noted that ferrous ions are involved in the production of hydroxyl radicals. Thus, the iron chelating activity of the extracts comes hand in hand with ferric reduction.

Table 2: Results of phytochemical screening tests. Legend: ‘+’: Positive; ‘-’: Negative.

Fraction	Flavonoids	Tannins	Alkaloids	Cardenolides	Coumarins	Antraquinones	Indoles	Sugars
A. bunius Crude	+	+	-	-	-	-	-	+
A. bunius 1	+	+	-	-	-	-	-	-
A. bunius 2	+	+	-	-	-	-	-	-
A. bunius 3	+	+	-	-	-	-	-	+
A. bunius 4	+	+	-	-	-	-	-	+
A. bunius 5	+	+	-	-	-	-	-	+
G. nepalensis Crude	+	-	-	-	+	-	+	+
G. nepalensis 1	+	-	-	-	-	-	-	+
G. nepalensis 2	+	-	-	-	-	-	-	-
G. nepalensis 3	+	-	-	-	-	-	-	-
G. nepalensis 4	+	-	-	-	-	-	+	-
G. nepalensis 5	-	-	-	-	-	-	+	-
G. nepalensis 6	-	+	-	-	+	-	-	-
G. nepalensis 7	-	+	-	-	-	-	-	-

Phytochemical screening revealed the presence of tannins and flavonoids to be common among the fractions (**Table 2**). The phytochemical classes found in the fractions with highest activity can be responsible for such activity. GN1 and AB1 contained flavonoids and this might be responsible for the high α -glucosidase inhibitory activity in GN1 and high antioxidant activity in the other fractions as observed in other studies (Pereira *et al.*, 2011; Pietta, 2000). AB1 and GN7 have been found to contain tannins which have been shown to be efficient in free radical scavenging, iron chelation, and ferric reduction (Gulcin *et al.*, 2010). In addition to tannins, GN6 contains coumarins which have been shown to reduce ferric ions effectively (Mladěnka *et al.*, 2010). It appears that the role of phenolic compounds is important in the α -glucosidase inhibitory and antioxidant activity of plants.

CONCLUSION

This study demonstrates the antioxidant and α -glucosidase activities of partially purified *A. bunius* fruit and *G. nepalensis* leaf extracts *in vitro*. The group recommends testing the inhibition effects against other enzymes (*e.g.* α -amylase) or testing the extracts *in vivo* in hyperglycemic rats. Additionally, it should also be a goal to isolate the active components for structure elucidation and chemical synthesis, and use them as possible antidiabetic drug candidates.

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