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Hypoglycemic Activities of Chromatographic Fractions of *Antidesma bunius* Fruit Ethanolic Extract on Alloxan-Induced Hyperglycemic Balb/C Mice

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ABSTRACT

Treatment of synthetic anti-diabetic drugs are costly and has high possibilities of unwanted effects such as nausea and jitters. Thus, there is a need to develop a drug of cheaper cost without, if not lesser, side effects. In this study, the *in vivo* hypoglycemic effect of partially purified *Antidesma bunius* fruit ethanolic extract was tested. Ethanolic fruit extracts were partially purified using liquid chromatography, obtaining four fractions (F1, F2, F3 and F4). These were administered orally (500 mg extract/kg body weight) to alloxan-induced diabetic female Balb/C mice to determine their hypoglycemic effects. Fasting blood glucose (FBG) levels were measured 5 days after alloxan injection, and on the 3rd, 7th, 10th, and the 14th day after treatment administration. Fractions F2 and F3 exhibited the highest blood glucose lowering activity, compared to the crude ethanolic extract and positive control. Phytochemical screening of F2 and F3 revealed the presence of tannins and indoles. These results demonstrate the partially purified *A. bunius* extract as a potential herbal drug candidate in diabetes therapy.

INTRODUCTION

Diabetes is a syndrome characterized by a deranged carbohydrate metabolism that results from failure of pancreatic beta cells in maintaining adequate insulin secretion to prevent abnormally high blood glucose level called hyperglycemia (Rother, 2007). High blood glucose levels can damage nerves and blood vessels, resulting to neuropathies and vascular diseases. Diabetes can lessen the body's ability to fight infection and various illnesses (Schaberg and Norwood, 2002). Additionally, diabetes is also linked with other long term body malfunctions of the eyes, kidneys, nerves, heart and blood vessels, and health complications, including renal failure, sexual dysfunction, heart disease, stroke, and blindness (Kadhirvel *et al*, 2010). At 2011, the number of cases of all types of diabetes was estimated to be 366 million worldwide while in the Philippines, the estimate was 4.2 million (8.2% prevalence) (Whiting *et al*, 2011). Aside from proper diet and exercise, diabetes can be prevented, or at least alleviated, by synthetic drugs, including sulfonylureas, meglitinide, biguanides, thiazolidinediones, and alpha-glucosidase inhibitors. Treatment of these drugs, however, is costly and has high possibilities of side effects such as nausea, vomiting, diarrhea, dizziness, headache, jittery feeling, acidity, hypersensitivity, abdominal upset, weakness, respiratory infections, sinusitis, muscle pain, and indigestion (Singh *et al*, 2009). Thus, for the past decades, scientists have conducted studies with the aim of finding alternative medications for diabetes mellitus. Today, an estimate of 1200 plants has already been experimentally proven to treat diabetes (Kambouche *et al*, 2009; Kaur and Arora, 2015). In the Philippines, several plant species have been studied for their anti-diabetic properties. Some of these are *Momordica charantia* and *Andrographis paniculata* (Reyes *et al*, 2006), *Bougainvillea glabra* (Adebayo *et al*, 2009), *Allium sativum* (Jelodar *et al*, 2005), cinnamon (Khan *et al*, 2003), *Grifola frondosa* (Kubo *et al*, 1994), *Ganoderma applanatum* and *Collybia confuens* (Yang *et al*, 2007).

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Antidesma bunius (Family *Phyllanthaceae*) commonly known as *bignay* in the Philippines, is an abundant invasive shrub tree with wide spreading branches, alternate leaves, and round fruits borne in grape-like pendent clusters. Although the bark is considered poisonous, the people in Thailand use *A. bunius* as medicinal plant for gastric intestinal problems, dysentery, indigestion, and constipation. Compounds in fruits and beverages from *A. bunius* have been observed to possess nutritive values in human health, particularly in healing of coronary heart disease (Hertog *et al*, 1997), reduce platelet aggregation (Knekt *et al*, 1996), and provide anti-carcinogenic protection (Stoclet *et al*, 2004).

The present study aims to demonstrate the hypoglycemic effects of partially purified *A. bunius* fruit extracts in alloxan-induced diabetic Balb/C mice, by comparing fasting blood glucose (FBG) levels before and after treatment. Also, the fractions were screened for phytochemical content.

MATERIALS AND METHODS

Plants and Animals

Ripe *A. bunius* fruits were purchased from the Bureau of Plant Industry, Manila, Philippines and authenticated by the Botany Division, National Museum, Manila, Philippines.

Female BALB/c mice of 5 to 6 weeks old were obtained from National Institute of Health, University of the Philippines Manila, Manila, Philippines. The mice were caged individually and were given rabbit pellets and tap water in their diet. All procedures involving animal subjects were approved by the Institutional Animal Care and Use Committee, National Institutes of Health, University of the Philippines Manila.

Chemical Reagents

All chemicals and solvents used were analytical grade. Alloxan monohydrate was purchased from Belman Laboratories (Quezon City, Philippines). Phytochemical testing reagents were prepared using chemicals bought from Belman Laboratories (Quezon City, Philippines). Silica gel G60 and solvents were bought from Merck, Philippines. Blood glucose levels were determined using the One Touch Ultra glucometer™ (Johnson & Johnson Co., USA).

Extraction and Partial Purification

Approximately 1 kg fruits were extracted twice with 2 L 80% ethanol by maceration for 48 hours. These were then filtered, and the solvent removed by rotary evaporation and lyophilization. Purification was done via normal phase liquid chromatography. Silica gel G60 was used as the stationary phase, whereas a solvent gradient of increasing polarity (100% hexane; 1:1 hexane-ethyl acetate; 100% ethyl acetate; 80:20 ethyl acetate-methanol; 50:50 ethyl acetate-methanol; 20:80 ethyl-acetate methanol; 100% methanol) were used as the mobile phases. The slurry used to pack the column was composed of silica gel and hexane. The packed column was 1.1 cm in diameter and 35 cm in height. A total of 50 eluates was collected and pooled into 4 fractions. The fractions were the dried completely by rotary evaporation.

Hypoglycemic activity assay using *in vivo* mouse model

A 2% solution of alloxan monohydrate, prepared by dissolving 1g of the compound in 50mL of 0.9% NaCl solution. The alloxan solution was injected intraperitoneally (200 mg/kg) into 21 Balb/C mice. The animals were given the normal diet after this. Five days after the alloxan injection, the fasting blood glucose (FBG) levels of the animals were determined. The test subjects were divided into groups as shown in **Table 1**.

Table 1: Treatment distribution of the Balb/C mice.

Group	Treatment*
Normal	Untreated group: Normal mice treated with distilled water
Metformin	Positive control: Hyperglycemic mice treated with Metformin
D. H_2O	Negative control: Hyperglycemic mice treated with distilled water
Crude	Hyperglycemic mice treated with crude extract
F1	Hyperglycemic mice treated with fraction 1
F2	Hyperglycemic mice treated with fraction 2
F3	Hyperglycemic mice treated with fraction 3
F4	Hyperglycemic mice treated with fraction 4

*Each group consists of three 5-6 week old female Balb/C mice (n=3).
**Each group was treated with a total concentration of 500 mg treatment/kg body weight.

Administration of the extracts was performed after the animals became diabetic. Before the actual treatment, the animals were fasted for 4-5 hours. The extract and fractions were dissolved in normal saline solution at a concentration of 10 mg/mL. The treatments (total administered concentration of 500 mg extract or fraction/kg body weight) were administered via oral gavage.

FBG levels were determined 5 days after alloxan injection, and on the 3^{rd}, 7^{th}, 10^{th}, and the 14^{th} day after administration of treatments. The weight of each mouse was also determined on the same days.

Data Analysis

The data obtained from FBG level determination were analyzed using the statistical software Stata version 10.

Phytochemical Screening

The fractions were tested for alkaloids, anthraquinones, tannins, indoles, and flavonoids using standard spray tests (Aguinaldo *et al*, 2005).

RESULTS AND DISCUSSION

After gradient elution (100% hexane; 1:1 hexane-ethyl acetate; 100% ethyl acetate; 80:20 ethyl acetate-methanol; 50:50 ethyl acetate-methanol; 20:80 ethyl-acetate methanol; 100% methanol) with hexane, methanol and ethyl acetate, the crude extract yielded four fractions: F1, F2, F3 and F4. These fractions and the crude extract were tested for hypoglycemic activity and phytochemical screening.

Table 2: FBG levels of alloxan-induced diabetic mice before and after alloxan injection, and after administration of *A. bunius* extracts and metformin.

Group	FBG level (mg/dl)*				
	Day 0	**Day 3**	**Day 7**	**Day 10**	**Day 14**
Normal	98.33±11.37	102.33±18.34	99.33±18.58	93.67±14.15	95.33±7.57
Metformin	505.33±29.19	581±32.91	458.33±185.33	185.33±3.51	122.67±7.02
D. H_2O	419.67±144.72	455±140.54	501.33±86.02	567±22.52	594±4.58
Crude	540.4± 48.52	453.8±78.55	355.8 ± 49.76	305.2±36.78	214.6±3.54
F1	361.67±24.99	500.67±84.56	212.67±78.01	190.33±40.87	136.67±14.29
F2	402.67±159.09	598.33±2.89	177.33±119.72	42.33±15.63	22±3.46
F3	412.67±159.09	575±43.30	283±176.58	80.67±96.50	33.67±20.31
F4	455.33±123.14	548±55.24	406±61.53	363.33±53.59	343.33±44.05

*n = 3 replicates for each treatment group. Data presented in average Fasting blood glucose (FBG) level (mg/dL) ± SD
**Each group was treated with a total concentration of 500 mg treatment/kg body weight.

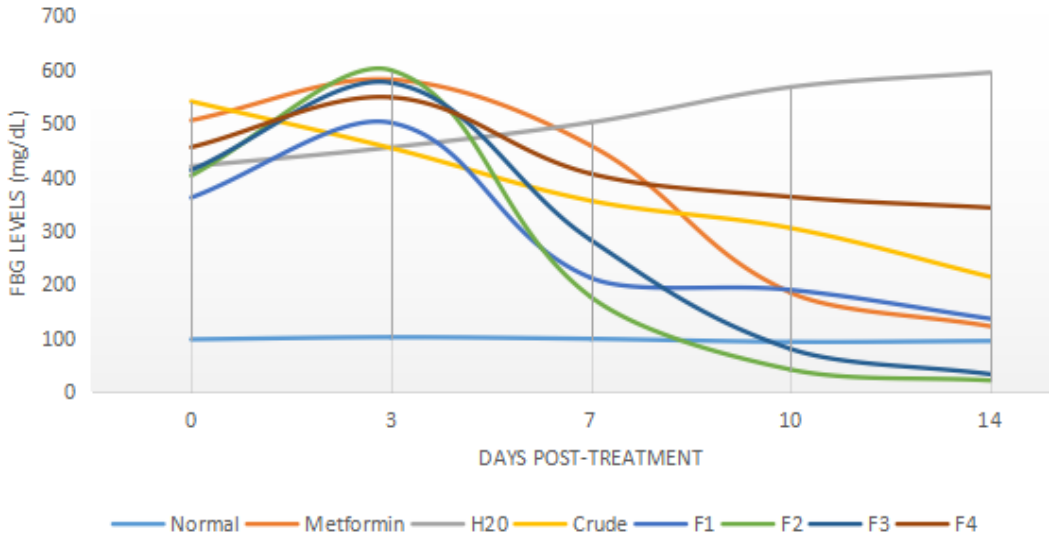

Fig 1: Trend in FBG levels of alloxan-induced diabetic mice in each treatment group after alloxan injection (0^{th}) and 3^{rd}, 7^{th}, 10^{th}, and 14^{th} day after administration of treatments. Each group was treated with a total concentration of 500 mg treatment/kg body weight.

Table 2 summarizes the FBG levels of the test groups throughout the duration of the experiment. Treatments with the fractions, crude extract as well as metformin significantly decreased the FBG levels of the animals during the 7^{th}, 10^{th}, and 14^{th} day following the administration. On the 3^{rd} day, however, only the crude extract gave a decrease in FBG level. The diabetic untreated mice, continued to display increases in their FBG levels during the same period of time.

Figures 1 shows the trend in FBG levels. FBG levels decrease in metformin, all pooled fractions and crude ethanolic extract-treated groups from 7^{th} day onwards. The FBG levels of mice treated with the negative control (distilled H_2O) were observed to increase continuously as shown by positive sign in the values while the normal untreated mice did not exhibit significant changes in their blood glucose levels and showed no specific trend.

Among the pooled fractions, F2 and F3 were found to cause the largest mean decrease in FBG of diabetic mice (pairwise comparison between groups show *p-value* <0.05). This indicates that pooled fractions F2 and F3 had the most significant effect in lowering the FBG levels of mice. Nevertheless, their activities are comparable to the positive control and crude fraction (*p-value* >0.05). On the other hand, pooled fractions F1 and F4 had significant differences compared to the negative control, but were statistically insignificant compared to the crude extract, metformin, F2 and F3.

With the induction of diabetes through the use of alloxan, a reduction of body weight of the test animals was observed in most studies (Reyes *et al*, 2006; Tanquilut *et al*, 2009). However, no significant changes in the mean body weights of the mice were observed after alloxan injection and extract administration.

The hypoglycemic activity of *A. bunius* have been studied before. Its α-glucosidase inhibitory activity were found to be moderately active (Lawag *et al*, 2012). Moreover, the ethanolic fruit extracts have been shown to be effective in lowering FBG levels in alloxanized ICR mice (Alvarado *et al*, 2015).

Tannins and indoles were found to be common in the crude extract and fractions, but anthraquinones were only found in the crude extract and fraction F1 (**Table 3**). Tannins isolated from plants have been observed for strong antioxidant activity (Nobre-Junior *et al*, 2008). Another study suggests that alkaloids, flavonoids, cardiac glycosides, and saponins are also anti-diabetic

agents while tannins act as alpha-glucosidase inhibitors reducing the absorption of carbohydrates in the gut (Adebayo *et al*, 2009).

Table 3: Phytochemical components of crude ethanolic extract, F1, F2, F3 and F4 according to the phytochemical screening performed. Legend: '++' = intense reaction; '+' = moderate reaction; '-' = no reaction.

Constituent Tested	Crude	F1	F2	F3	F4
Anthraquinones	+	+	-	-	-
Tannins	+	+	+	+	-
Indoles	+	+	++	+	-
Flavonoids	-	-	-	-	-
Alkaloids	-	-	-	-	-

CONCLUSION

The hypoglycemic effect of partially purified *A. bunius* fruit ethanolic extract on the alloxanized Balb/c mice was evaluated in this study. The data showed that fractions were able to lower the blood glucose levels of five-to-six-week old diabetic mice at 500 mg/kg body weight, compared to the positive control used. Analysis of the bioactive compounds in *A. bunius* fruits revealed that the most active fractions (F2 and F3) contained tannins and indoles. Isolation and structural elucidation of the active compounds are now being undertaken.

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