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Anticoagulant Activity of Ethanolic Leaf Extract of *Pithecellobium Dulce* Benth. (Fabaceae) in Alloxan-Induced Diabetic Rats

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ABSTRACT

Diabetes is associated with the changes in thrombotic and fibrinolytic coagulation factor level/activity that increases the risk of thrombus formation. Patients with diabetes mellitus (DM) have a higher risk of developing blood clots in the arteries that may lead to a series of complications associated, specifically, with the cardiovascular system. Blood clots in the arteries of the heart accounts for 80% mortality rate of diabetic patients. In this study, the researchers evaluated the anticoagulant effect of *Pithecellobium dulce* in diabetes and its roles in the hypercoagulation. Leaf extracts of *P. dulce* were orally administered to alloxan-induced diabetic male sprague-dawley rats for seven consecutive days. The induction of diabetes was described through glucose testing which was measured prior to the start of drug administration. The rats were divided into six groups consisting of six rats each. Normal saline solution and warfarin 3 mg/kg BW were administered as the negative and the positive controls, respectively. Doses of 200 mg/kg, 400 mg/kg, and 600 mg/kg BW were administered once daily for seven days. Samples of blood were drawn after seven days of treatment and were centrifuged at 5000 rpm for 15 minutes. Anticoagulant activity was measured using prothrombin time (PT), activated partial thromboplastin time (aPPT), thrombin time (TT). In conclusion, *P. dulce* did not exhibit anticoagulant effect as demonstrated by statistical significance by shortened period for clot formation compared to the positive control.

Keywords
Pithecellobium dulce (Benth); anticoagulant; anti-hyperglycemic.

1. INTRODUCTION

Diabetes mellitus (DM) has been affecting many worldwide. By 2025, it was predicted that India would have the highest number of DM patients in the world [1]. DM patients are typically associated with many other chronic diseases such as cardiovascular [2], renal [3], neurological [4], and ocular [5].

One of the clinical manifestations of diabetes mellitus is hypercoagulation. Coagulation is a process that slows blood clot formation in the presence of an injury such as cuts and wounds. Proteins in the blood such as fibrin, work with small cell fragments called platelets to form the clot. After the bleeding has stopped and the healing process has occurred, it is the body's normal response to break down and remove the clots. Hypercoagulation or excessive blood clotting is the occurrence of rapid uncontrolled formation of clot or prolonged dissolution of the mass. These blood clots can form in or travel out the arteries or the veins of the brain, heart, kidney, lungs, and limbs that can cause heart attack and stroke. According to the Department of Health [6], the prevalence of fatalities in the Philippines that is caused

by thrombosis belonged to ages 70 years old and above, contributing to 18,883 deaths or 39.2%. Diabetic patients have increased thrombotic tendency due to platelet hyper-reactivity and increased activation of prothrombotic coagulation factors coupled with decreased fibrinolysis. Premature artherosclerosis and more extensive vascular disease predisposes subject with diabetes to plaque and thrombus formation [7].

Recent development for modern medicines helps to identify constituents and develop novel therapies from traditional medicines to combat and lessen the effects of diabetes [8]. Several studies have been shown that medicinal and herbal plant has been used for the cure of diabetes mellitus.According to Rouzbehan*et al.*,[9] plants with high α-glucosidase inhibitors, has antidiabetic potentials. The combination of *Andrographis paniculata* and *Centellaasiatica* herb fraction has proven to increase glucose uptake and insulin sensitivity [10].Samoo et al. [11] reported that 1% consumption of barley, black plum and Chinese tree aqueous extract hadreduced glucose level by 43.92 %, 39.05 %, and 32.47% respectively. Results obtained from the study of *Gossypium herbaceum* Land its aqueous and ethanol extracts have promising anti-diabetic and hypolipidemic effects [12]. Generally, medicinal and herbal plants are widely preferred since it would be more affordable and have less side effects compared to the available synthetic drugs, and are more effective in treatment of diabetes mellitus [13].

P. dulce Benth. (Fabaceae) is a small to medium sized, evergreen, spiny tree that grows up to 18 m in height [8].

P. dulce, traditionally has been widely used to treat dysentery, intestinal disorders and ulcers. In addition, it has an anti-inflammatory property and the potential to combat protein malnutrition [14]. A study by Mule et al. [15] reported a significant reduction in blood glucose level among diabetic rats that were given *P. dulce* leaves extract. Reduction of elevated blood glucose level is an indicator of antidiabetic activity of *P. dulce* leaves. At present, research on their anticoagulant activity has not been yet reported. Since diabetes and hypercoagulation are linked, anticoagulant activity added to medications that are able to treat diabetes would be advantageous in the management of this condition. Thus, this research aimed to investigate the anticoagulant activity of ethanolic leaf extract of *Pithecellobium Dulce* Benth. (Fabaceae) in alloxan-induced diabetic rats.

2. MATERIALS AND METHODS

2.1 Reagents and Materials

The diabetogenic agent – alloxan was purchased at Belman Laboratories, Quezon City, Metro Manila. While the commercially available warfarin (Coumadin) was purchased as the reference drug.

2.2 Preparation of the Plant Extract and Standard Drug

Taxonomically identified leaves of *P.dulce* were collected from Barangay Aya, San Jose, Batangas. The collected parts were shade dried for seven (7) days and its size were reduced to a coarse powder using grinder and passed through sieve no. 80. It was extracted through soxhlet apparatus with 95% ethanol until the solution in the thimble becomes clear. The extract was concentrated to dryness under reduced pressure at 40°C with the use of a rotator vacuum evaporator. The researchers computed the percentage yield and the organoleptic characteristics were recorded [16].

The percentage yield of the ethanolic extract of *P. dulce* is calculated using Equation 1.

$$percentage\ yield = \frac{weight\ of\ the\ extract}{weight\ of\ the\ dried\ plant} \times 100 \qquad \text{(Eq. 1)}$$

2.3 Animals

Healthy male sprague-dawley rats aged 7 to 8 weeks and weighing 150-300 grams were purchased at Lipa City, Batangas and was certified free from evidence of dangerous communicable animal diseases by Dr. Neil Norman A. Cruz. The animals were acclimatized at LPU-Batangas animal house for 1 week prior to experimental use. The animals were provided with standard pellet diet and distilled water *ad*

libitum. Standard laboratory environment were provided, by implementing a 12-hour light and dark cycle and an environmental temperature of 25°C or below [15, 17]

2.4 Induction of Experimental Diabetes Mellitus

Alloxan monohydrate was used to induce diabetes in overnight fasted rats. Prior to induction, blood glucose level checking was performed to get the baseline glucose level. An intraperitoneal route of administration was used at a dose of 150 mg/kg BW. Due to the fatal hypoglycemia that is caused by massive pancreatic insulin release, the rats were given 10% (w/v) glucose solution after 1 hour of induction for the following 24 hours to prevent drug-induced hypoglycemia. After 48 hours, animals that presented blood glucose levels of $\geq$200 mg/dl were considered diabetic and were included in the study. Food and water were given *ad libitum* following alloxan injection [17-19].

2.5 Measurement of Blood Glucose Levels

The use of a glucometer (Value®) was utilized in measuring blood glucose levels. Rats were fasted for 8 hours prior to testing. Administration of normal saline (control); the reference drug warfarin 3 mg/kg BW, and three different doses of the extract at 200 mg/kg, 400 mg/kg and 600 mg/kg BW of the leaves of *P. dulce* were administered orally by gavage [15-17].

2.6 Experimental Design

The experimental rats were grouped in six, comprising of six rats in each group as follows:

Group I: Normal control group given NSS only PO. (NC)

Group II: Diabetic control group given NSS only PO. (DC)

Group III: Diabetic group, treated with reference drug Warfarin, 3 mg/kg BW.(DW)

Group IV: Diabetic group, treated with extract of *P.dulce* at a dose of 200 mg/kg BW. (D200)

Group V: Diabetic group, treated with extract of *P.dulce* at a dose of 400 mg/kg BW. (D400)

Group VI: Diabetic group, treated with extract of *P.dulce* at a dose of 600 mg/kg BW. (D600)

Blood glucose levels and body weight were monitored before the induction of diabetogenic agent, Day 0, Day 4 and Day 7. On the Day 7, blood collection was done via tail veinwith a volume of 1.8 mL in 8-hour fasted rats. The withdrawn blood was then placed in tubes containing sodium citrate. Samples were subjected to centrifugation for 15 minutes at 5000rpm at 4°C to obtain the plasma. The plasma obtained was then used for clotting time assays [16, 17, 21].

2.7 Anticoagulant Activity

Clotting time assays were used to determine the anticoagulant effect of *P. dulce* extract on rat plasma. The anticoagulant activity was evaluated by measuring the PT, aPTT and TT using a coagulometer (Thrombotimer, BehnkElektronik, Germany).

2.8 Data Analysis

The results were presented as means ± Standard Error of Mean. The data were analyzed using independent student t-test to compare the baseline data. Paired t-test was applied to test the changes within groups for statistical significance. Two-tailed probability value of $p<0.05$ was considered significance.

3. RESULTS AND DISCUSSION

3.1 Percentage yield obtained from *P. dulce*

Extraction of 1,084.62 grams of ground *P. dulce* leaves produced an approximate of 137.32 grams of crude extract with a percentage yield of 12.66% as shown in Figure 1. The crude extract was dark-green in color, has a pungent odor and a sticky consistency.

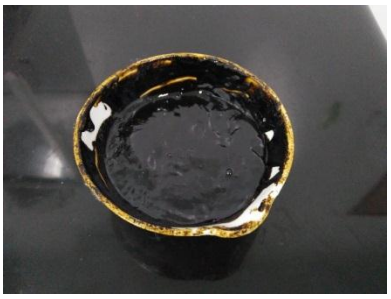

Figure 1. *Pithecellobium dulce* crude extract

3.2 Anticoagulant Activity of *P. Dulce* in Normal and Alloxan-Induced Diabetic Rats

The anticoagulant effect of *P. dulce* leaves was evaluated using PT, aPTT, and TT as parameters. Clotting time was compared against warfarin.

In diabetic rats, the leaf extracts showed significant differences in clotting time when compared to the positive control group. As the statistics in Table 1 implicated, the leaf extract of all doses manifested a faster clotting time having PT, aPTT,TT significant p-values 0.035, 0.000, 0.000 for the 4th day and 0.000, 0.000 and 0.000 for the 7th day for all the mentioned tests, respectively. These values were compared to the baseline which was measured prior the experimentation proper. According to Daud et al. [17], the extract may take time to be activated and reacted with thrombin since in the first week of their study, an increase in clot formation was observed.

Table 1. p-values for the *P. dulce* leaves extract for PT, aPTT and TT tests for day 4 and day 7.

Days	*P. dulce* leaves extract	p-values		
		PT	aPTT	TT
4	Diabetic + *P. dulce* extract 200 mg/kg	0.035	0.000	0.000
	Diabetic + *P. dulce* 400 mg/kg	0.035	0.000	0.000
	Diabetic + *P. dulce* 600 mg/kg	0.035	0.000	0.000
7	Diabetic + *P. dulce* extract 200 mg/kg	0.000	0.000	0.000
	Diabetic + *P. dulce* 400 mg/kg	0.000	0.000	0.000
	Diabetic + *P. dulce* 600 mg/kg	0.000	0.000	0.000

Comparison between the clotting time of normal and diabetic rats showed shorter clotting time for the latter as per described in the pathophysiology of diabetes in relationship to coagulation (Table 2).

With these results, it can be deduced that 200mg/kg, 400mg/kg, and 600mg/kg BW ethanolic extracts may not contain anticoagulant constituents compared to the positive control. The leaf extract of *P. dulce* did not manifest anticoagulant effect since the formation of clot was shortened over time.

Table 2. Comparison between coagulation parameters in normal and diabetic rats.

Group	PT	aPTT	TT
After 4 days			
Normal + NSS	12.67 ± 7.39	22.19 ± 7.97	16.05 ± 3.05
Diabetic + NSS	14.99 ± 6.46	44.23 ± 7.98	15.33 ± 3.13
Diabetic + Warfarin 3 mg/kg	137.78 ± 6.50*	115.56 ± 8.20*	63.93 ± 3.14*
Diabetic + *P. dulce* extract 200 mg/kg	21.55 ± 6.48	53.68 ± 8.47	24.38 ± 3.16
Diabetic + *P. dulce* 400 mg/kg	17.83 ± 6.45	40.15 ± 8.26	16.40 ± 3.10
Diabetic + *P. dulce* 600 mg/kg	17.01 ± 6.49	36.86 ± 8.25	15.77 ± 4.25
After 7 days			
Normal + NSS	13.07 ± 1.46	37.75 ± 1.41*	17.61 ± 1.36
Diabetic + NSS	12.62 ± 1.28	6.92 ± 1.41*	13.38 ± 1.40
Diabetic + Warfarin 3 mg/kg	110.25 ± 1.29*	107.85 ± 1.46*	17.48 ± 1.40
Diabetic + *P. dulce* extract 200 mg/kg	14.23 ± 1.28	9.86 ± 1.50*	64.89 ± 1.40*
Diabetic + *P. dulce* 400 mg/kg	11.68 ± 1.28	9.28 ± 1.46*	21.46 ± 1.38
Diabetic + *P. dulce* 600 mg/kg	10.18 ± 1.28	6.82 ± 1.46*	18.37 ± 1.89

*Note : Values are expressed as means ± S.E.M. n=6. *p-value <0.05 vs. normal group.*

3.3 Effects of *P. Dulce* in Blood Glucose and Body Weight in Alloxan-Induced Diabetic Rats

The fasting blood glucose levels of the rats were measured prior to induction of alloxan. Table 3 presented the comparison between post-induction and during-treatment fasting blood glucose level. Based on the data in Table 3, with the consumption of *P.dulce*leaves extract, the rats appeared to show fluctuations in weight in the course of the study especially after day 4. The highest weight loss recorded was approximately 42.64g which was for consumption of P. *dulce* 200mg/kg. The decreased in body weight in diabetic rats clearly showed a loss or degradation of structural proteins due to diabetes. Generally, diabetic rats lost weight but over time, with the administration of the leaf extract, the rats were able to gain weight. According to Arumugam and Paneerselvam[22], the ability of the *P.dulce* to protect from maximum body weight loss appeared to be due to their ability to reduce hyperglycemia. Besides, statistics showed no significant difference as compared to the baseline.

In addition, there was also a significant reduction in the blood glucose level upon intake of *P.dulce* after the 7th day compared to the control groups. Similar findings were reported by Mule et al. [15] who mentioned that the reduced blood glucose level may be possibly due to increase in insulin release or due to increase in peripheral glucose uptake.

Table 3. Comparison between body weight and fasting blood glucose level after 4 and 7 days

Group	Body Weight (g)		Fasting blood glucose level	
	Before	**After**	**Before**	After
After 4 days				
Normal Group + NSS	144.33 ± 0.33	167.07 ± 10.17	121.33 ± 29.35	86.93 ± 102.84
Diabetic Group + NSS	178.67 ± 12.78	138.57 ± 10.26	352.67 ± 63.30	65.00 ± 62.87
Diabetic Group + Warfarin 3mg/kg	162.00± 4.58	163.28 ± 8.79	419.67 ± 17.32	309.11 ± 65.34
Diabetic Group + *P. dulce* 200mg/kg	176.67 ± 5.49	134.03 ± 9.96	463.33 ± 49.97	240.74 ± 70.82
Diabetic Group + *P. dulce* 400mg/kg	142.00± 8.14	140.21 ± 10.54	454.33 ± 77.31	309.38 ± 69.48
Diabetic Group + *P. dulce* 600 mg/kg	163.67 ± 11.84	158.51 ± 8.82	379.00± 62.93	223.83 ± 62.91
After 7 days				
Normal Group + NSS	144.33 ± 0.33	150.87 ± 8.67	121.33 ± 29.35	60.76 ± 33.43
Diabetic Group + NSS	178.67 ± 12.78	161.41 ± 8.74	352.67 ± 63.30	111.93 ± 20.44
Diabetic Group + Warfarin 3mg/kg	162.00± 4.58	164.93 ± 7.50	419.67 ± 17.32	314.44 ± 21.24*
Diabetic Group + *P. dulce* 200mg/kg	176.67 ± 5.49	146.93 ± 8.49	463.33 ± 49.97	161.52 ± 23.02
Diabetic Group + *P. dulce* 400mg/kg	142.00± 8.14	140.08 ± 8.99	454.33 ± 77.31	106.90 ± 22.58
Diabetic Group + *P. dulce* 600 mg/kg	163.67 ± 11.84	153.78 ± 7.52	379.00± 62.93	134.45 ± 20.45

*Note: Values are expressed as the means ± S.E.M. n=6. *p-value <0.05 vs. normal group*

4. CONCLUSION

P. dulce did not manifest anticoagulant effect in a 7-day experimental period as demonstrated by statistical significance by shortened period for the aPTT, PT, and TT compared to the positive control. Although the plant did not manifest anticoagulant activity, it exerted its antihyperglycemic effect with a statistical significance of 0.000 after 7 days of treatment.

5. RECOMMENDATIONS

Based on the study done, the researchers conducted the anticoagulant therapy in a seven-day period which showed no increase in clotting time of diabetic rats. The researchers recommended a longer

duration of anticoagulant therapy with *P. dulce* leave extracts which can contribute to the possible reduction of formation of blood clots. In the current study, the researchers made use of a once daily dosing for seven days. The future researchers are advised to explore the possible effects of the extract on a multiple daily dosing.

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