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ORIGINAL ARTICLE **OPEN ACCESS**

Hypoglycemic and Protective Potentials of F16 Hexane Extract from the Air-dried Leaves of *Crescentia cujete* Linn. in Male *Mus musculus*

Gladys G. Paglinawan and Merell P. Billacura
Department of Chemistry, Mindanao State University-Main Campus, Marawi City
Lanao del Sur 9700, Philippines
Email: merelljohn@gmail.com

ABSTRACT

The hexane extract was concentrated in vacuo and further separated using gravity column chromatography. According to their color property, thirty fractions were obtained and pooled which labelled as F1-F30. However, only F17 and F16 has a sufficient volume needed to proceed for further tests. F16 and F17 were concentrated in vacuo and determined its toxicity using brine shrimp lethality test. The result shows that F16 and F17 have comparable LC_{50} of 7.025 x 10^{-13} and 0, respectively. F16 was administered to male Mus musculus via oral gavage. Twenty-four and thirty six hours after the four-day consecutive treatments, blood glucose level was determined using one touch glucometer. For hypoglycemic potential of the F16 in the Alloxan-induced male Mus musculus, the result shows no significant difference with the Alloxan group. However, for the protective potential, the results show a significantly lowering of the blood-glucose level of the male Mus musculus from a range of Alloxan, 120-106 mg/dL, to the treatment with samples, 93.3-61.6 mg/dL.

Keywords: *Miracle fruit leaves, Alloxan, Metformin, oral gavage*

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INTRODUCTION

The International Diabetes Federation in 2014 reported that IDF's Global Diabetes Scorecard of the Philippines has estimated to be 3.2 million cases in 2013. This has a prevalence rate of 5.9% and there are 1.7 million people of undiagnosed diabetes in adults between 20-79 years of age [1]. With this, Philippines can be considered as emerging diabetes hotspots in the world. Intensive lifestyle interventions are, however, resource intensive and difficult to sustain. Even the traditional insulin replacement therapy can be painful and time consuming for patients suffering with diabetes mellitus [2]. Microcomputer-controlled closed loop insulin delivery systems has been developed where continuous glucose monitors (CGMs) are used in conjunction with insulin pumps which automatically calculate and inject appropriate doses of insulin [3]. Even with technologies which improve patient's glucose control, there are still considerable drawbacks. Insulin pumps and CGMs which are expensive tools, and implanted sensors and cannulas increase the patient's risk of infection, inflammation and scarring. These also require frequent maintenance and replacement due to the foreign body response, and cost to patients [4].

Medicinal plants are frequently considered to be less toxic and free from side effects than the synthetic drugs. The World Health Organization has also recommended that this should be encouraged, especially in countries where conventional treatment of diabetes seems insufficient [5]. Medicinal plants are highly preferred than that of the synthetic one as of today especially at rural areas because synthetic drugs are expensive and sometimes has side effects. According to folklores, *Crescentia cujete* Linn. commonly known as Miracle Tree, has many potentials to cure many kind of diseases and illnesses. It is currently used as herbal medicine to treat diabetes mellitus in many rural areas, especially in Davao Region in Philippines. There were studies conducted that aqueous and alcoholic extracts of stem bark and leaves of *Crescentia cujete* has found to have antimicrobial potential against MDR isolates DKU-156 and JAL-1236 [6]. It also found that the crude ethanolic extract of the leaves and stem bark of *Crescentia cujete* has an

anti-inflammatory and antibacterial potentials [7]. Its fruit ethanolic extract has an antivenom potential [8]. It is also considered to have anti-helminthic, antimutagenic and hypoglycemic potentials [9-12].

To further discover the hypoglycemic potential of this tree, this study was conducted to assess the effectiveness of the *Crescentia cujete Linn.* leaves hexane extract as a possible source of hypoglycemic component. Specifically, to assess the hypoglycemic and protective potentials of the Fraction 16 (F16) hexane extract from the air-dried leaves of *Crescentia cujete* Linn. into the Alloxan-induced *Mus musculus.* The results of this study can be a baseline in the development of a drug to potentially cure many diabetic patients with a minimum cost and would be beneficial to the people who still rely on the traditional medicine.

MATERIALS AND METHODS

Preparation of crude ethanolic extract

Fresh leaves of *Crescentia cujete* Linn. were collected from Bayugan City, Agusan del Sur, Philippines. The *Crescentia cujete* Linn. leaves were washed, air dried for four weeks and cut to small pieces. Exactly 312.8 g of the cut sample was soaked in 95% ethanol for 72 hours, filtered and evaporated to dryness using a rotary evaporator at 40°C. The crude extract was remove and then transferred into a pre-labelled vial and was refrigerated for storage.

Solvent partitioning

The concentrated crude ethanolic extract was partitioned using polar and non-polar solvents. The crude extract was initially dissolved in a 10 mL distilled water and 5 mL 95% methanol. Then, the partially dissolved extract was transferred to the separatory funnel, then another 35 mL of distilled water was added to make a total of 45 mL of distilled water and 5 mL of 95% methanol and this was thoroughly mixed. Fifty milliliter of hexane was poured into the same separatory funnel. The separatory funnel was stoppered and shaken for several times, then the knob was opened after several agitation to release the pressure and allowed to stand until the separation between aqueous layer and organic layer is distinct and observable. The hexane and aqueous layers were collected and separated. The aqueous layer was subjected with 50 mL of hexane for several times until the organic layer becomes clear. All of the hexane extracts were collected and pooled. Then, the aqueous layer was further extracted using 50 mL ethyl acetate. The same procedures previously mentioned were followed in the extraction of the ethyl acetate extracts. The separately collected hexane, ethyl acetate and aqueous extracts were concentrated *in vacuo*. Only the hexane and ethyl acetate extracts were used in the study inasmuch as the aqueous extract was not properly stored. Only the hexane extract was used in further in this study because the other research students used the other extracts for several tests.

Gravity column chromatography

Approximately 40 grams of silica gel was heated for an hour to remove moisture content. After this, a slurry was prepared with hexane as the solvent then was packed. Sample was loaded to the column and elution process began. Approximately 10 mL was dispensed in every vial.

When the solvent's volume decreases, another volume of solvent with 5% increase in polarity was added in the reservoir of the column. The solvent system of this chromatography is hexane and ethyl acetate with 5% increment up to 100% ethyl acetate. Then, another solvent system was employed which was the ethyl acetate and methanol with 10% increment until it reaches 60% methanol. Fractions were obtained and pooled based on color. Fractions were gathered and dried *in vacuo* and underwent a brine shrimp lethality test.

Brine shrimp assay

Approximately 0.05 g sample was weighed and dissolved with 5 mL methanol, this solution served as solution A. About 0.5 mL was pipetted from solution A into a test tube and diluted with 10 mL methanol, this solution served as solution B. From solution B, 0.1 mL was pipetted into a test tube and from the solution A, 0.05 mL and 0.5 mL were pipetted into a separate test tube and allowed to dry at room temperature. The air-dried solutions were diluted to 5 mL with artificial seawater containing nauplii to make a final concentration of 10, 100 and 1000 ppm, respectively. Five replicates were prepared per concentrations. A shallow rectangular dish was filled with the prepared artificial seawater. Plastic divider with several holes was placed in the dish to divide it into unequal compartments. The brown brine eggs were sprinkled into the large compartment and the compartment was covered to keep away from light, leaving the smaller compartment open and illuminated with a light bulb. After 48 hours, the hatched nauplii were pipetted out ready for assay. With a Pasteur pipette, ten nauplii were transferred to each test tube containing air-dried solutions of hexane and ethyl acetate extracts of different concentrations. Artificial seawater was added to each test tube to make a total volume of 5 mL. Ten nauplii were also transferred to the control test tube, which has 5 mL artificial seawater and to another test tube, which has

5 drops of DMSO. A drop of yeast was added to each tube as a food. The test tubes were kept illuminated. The number of survivors was counted after 6-hours and 24-hours [13].

Hypoglycemic activity

Based on the results from the BSLT, Fraction 17 (F17) has a comparable percentage mortality with Fraction16 (F16). Test for hypoglycemic potential in F16 was studied further inasmuch as F17 has insufficient amount of volume.

Experimental Groupings

Five groups with five male albino mice in each group of mice were divided and treated as follows:

Table 1: Experimental groupings

Group number	Treatment	Test	Days
1	Distilled water	Hypoglycemia/ Protective	4 days
2	Distilled water + Alloxan	Hypoglycemia/ Protective	2days Distilled water + 2 days Alloxan
3	Alloxan +5000 ppm F16	Hypoglycemia	2 days Alloxan + 2 days F16
4	Alloxan +2500 ppm F16	Hypoglycemia	2 days Alloxan + 2 days F16
5	Alloxan +1250 ppm F16	Hypoglycemia	2 days Alloxan + 2 days F16
6	Alloxan + Metformin	Hypoglycemia	2 day Alloxan + 2 days Metformin
7	5000 ppm F16 + Alloxan	Protective	2 days F16 + 2 days Alloxan
8	2500 ppm F16 + Alloxan	Protective	2 days F16 + 2 days Alloxan
9	1250 ppm F16+ Alloxan	Protective	2 days F16 + 2 days Alloxan

All the solutions induced depend on the body weight of the *Mus musculus*. Twenty thousand ppm was prepared for Alloxan. The concentration of F16 fractions were 5000, 2500, and 1250 ppm while for Metformin was 2500 ppm.

Hypoglycemic activity determination

Four days after the treatment, blood analysis was conducted. Blood glucose level of the *Mus musculus* were determined after 24- and 36-hours of the subsequent treatment period. Blood glucose level was determined using one touch glucometer. The data obtained was analyzed using One-way ANOVA.

Before introducing the solutions through oral gavage, the *Mus musculus* were fasted overnight with an unlimited access to water. Its body weight was determined using top loading balance [12].

RESULT AND DISCUSSION

Brine shrimp lethality test

Brine shrimp lethality test is an assay to determine the cytotoxicity of the plant sample. Table 2 shows the results of the brine shrimp lethality test of F16 and F17 fractions.

Brine shrimp lethality test is the initial parameter used to determine the most bioactive extract. Preliminary counting of the number of survivor nauplii was done after 6 hours and calculated the percentage mortality. Results show that F17 has a comparable percentage mortality to F16. After 24 hours, the numbers of nauplii survivors were further counted. Percentage mortality was calculated from the data obtained. LC_{50} was calculated using probit analysis to identify the most bioactive fraction. From the data, F17 exhibited comparable percentage mortality after 6 hours and 24 hours. However, since F16 was comparable to F17, F16 undergo further analysis, also because F17 has insufficient volume.

Table 2: Toxicity results of Crescentia cujete Linn. against brine shrimp nauplii after 6- and 24-hour of exposure

Samples	Dose (ppm)	% Mortality after 6 hours	LC_{50}	% Mortality after 24 hours	LC_{50}
F16	10	12	344.4	98	4.63×10^{-22}
	100	24		100	
	1000	90		100	
F17	10	38	213.10	100	0
	100	46		100	
	1000	90		100	
DMSO		10		10	
Saline		10		20	

Hypoglycemic activity

Diabetes is a disorder of carbohydrates, fat and protein metabolism attributed to the diminished production of insulin mounting resistance to its action. Oral hydroglycemic drugs are widely used for controlling hyperglycemia whereas Alloxan causes a massive reduction in insulin release by the destruction of ß-cells of the Islets of Langerhans and thus induces hyperglycemia.

In hypoglycemic acitivity, F16 was used instead of F17 because of the insufficient amount of F17 to proceed with this test. The highest concentration considered was 5000 ppm because of the minute amount left for the F16 to proceed with this test.

Based on the results, 24 hours after the last treatment, it shows that treatment B Alloxan has the highest blood-glucose level relative to control treatment A. However, it also shows that the blood-glucose level of the treatments D, E and F are lower compared to treatment B.

Table 3: Hypoglycemic activity of F16 from the hexane extract of the air-dried leaves of C*rescentia cujete* Linn. by oral gavage in *Mus musculus*

Treatment	Blood glucose level after 24 hours*	Blood glucose level after 24 hours*
A	72.3^{a}	62.3^{a}
B	120^{cd}	106.3^{cd}
C	117.3^{d}	123.7^{d}
D	99.3^{bc}	97.7^{bc}
E	72.6^{a}	66.3^{a}
F	88.3^{ab}	80^{ab}

Legend: treatment A-Control (distilled water), treatment B-Alloxan treatment (200 mg/kg body wt.), treatment C-Alloxan + 5000 ppm F16, treatment D-Alloxan + 2500 ppm F16, treatment E-Alloxan + 1250 ppm F16, treatment F-Alloxan + Metformin. Means having same letters have no significant difference at α = 0.05. *Average of three trials

Metformin is a commercially available drug used in treating diabetes. It activates AMP-activated protein kinase, a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats. In addition, it increases low-affinity and high-affinity receptors of insulin, and improves insulin resistance.

By comparing the effectiveness of the F16 in different treatments C, D and E to treatment F, it shows that at low concentration, treatment E, the blood glucose level of male *Mus musculus* is reduced and is comparable to treatment F, but at higher concentration, the blood glucose level of male *Mus musculus* increases.

With statistical analysis, treatments A, E and F have no significant difference. It also shows that treatments F and D have no significant difference, however, D, A, and E have significant differences. Also, D and B have no significant difference but F and B do have significant difference. Furthermore, B and C have no significant difference but D and C do have a significant difference. However, it is noted that treatments E and F can somehow normalize the glucose level of the Alloxan-induced mice since it has no significant difference with treatment A.

As for the observation after 36 hours from the last treatment, results from the statistical analysis showed similar pattern to that of the observed treatment after 24 hours. The blood glucose level for treatments B, C and D are significantly different to treatments A, E and F.

Protective potential of F16 against alloxan in *Mus musculus*

Protective potential activity was conducted by introducing the F16 for two consecutive days at 24-hours interval prior to the oral gavage of Alloxan in *Mus musculus.*

Table 4: Protective potential of F16 of the hexane extract from the air-dried leaves of *Crescentia cujete* Linn.by oral gavage in *Mus musculus* after 24-and 36-hours.

Treatment	Blood glucose level after 24 hours*	Blood glucose level after 24 hours*
A	72.3^{a}	62.3^{a}
B	120^{c}	106.3^{c}
G	61.6^{a}	63^{a}
H	65.3^{a}	62.6^{a}
I	93.3^{b}	73.3^{b}

Legend: treatment A-control (distilled water), treatment B-Alloxan treatment (200mg/kg body wt.), treatment G-5000 ppm F16 + Alloxan, treatment H- 2500 ppm F16 + Alloxan, treatment I- 1250 ppm F16 + Alloxan. Means having same letters have no significant difference at α = 0.05.

Table 4 shows the protective potential of F16. Twenty-four hours after the last treatment of Alloxan, results show that treatment B has the highest blood-glucose level followed by treatment I then by A and the by H and G. The possible factor of this is that, treatment I has a lower concentration of F16, so, it has a higher glucose level compared to that of treatment G and H. Thus, G and H have the protective potential since the range of normal glucose level is from 70-120 mg/dL and it even lowers down to 61.6 and 65.3 mg/dL, respectively.

After 36 hours, another blood sampling was done, the observed results was the same. Treatment B has the highest glucose level compared to treatment A, G, H and I. Based on the data gathered, F16 is more effective when introduced first to the organism prior to the introduction of Alloxan.

It is possible that introduction of F16 prior to Alloxan can protect the pancreas and can regenerate the damaged pancreatic cells by maintaining the level of blood insulin and blood glucose levels by elimination of insulin excretion for the undamaged pancreatic cells.

CONCLUSION

F16 of the hexane extract from the air-dried leaves of *Crescentia cujete* Linn. can potentially normalize the blood glucose level of the Alloxan-induced *Mus musculus*. In addition, the protective effect of F16 of hexane extract of *Crescentia cujete* Linn. leaves is effective in lowering the glucose concentration in the blood of the organism prior to its treatment of the organism.

CONFLICT OF INTEREST

No conflict of interest.

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