HYPOGLYCEMIC AND PROTECTIVE POTENTIALS OF THE EXTRACTS FROM THE AIR-DRIED LEAVES OF *Crescentia cujete* Linn.

Al-Habsi S. Uhon and Merell P. Billacura

Mindanao State University-Main Campus, Marawi City, Lanao del Sur, 9700, Philippines

*For correspondence; Tel. + (63) 9177642446, E-mail: merelljohn@gmail.com

ABSTRACT: *Air-dried leaves of Crescentia cujete* Linn. *were extracted in 95% ethanol, sequentially underwent solvent partitioning then the extracts were concentrated in vacuo. Phytochemical screening of the extract showed the presence of alkaloids, saponins, reducing sugar, tannins, polyphenolics, and volatile oils. Brine shrimp lethality test of the partitioned solvent extracts indicates that ethyl acetate extract is the most bioactive extract. Ethyl acetate extract was administered to the male Mus musculus via oral gavage. Twenty-four and thirty-six hours after the three-day consecutive treatments, blood-glucose level was determined using one touch glucometer. For the hypoglycemic potential of the ethyl acetate extract in the Alloxan-induced male Mus musculus, it was observed that it significantly reduced the blood-glucose level. It also showed that the ethyl acetate extract has a protective potential by significantly inhibiting the increase of the blood-glucose level through administration of ethyl acetate extract prior to administration of Alloxan in the male Mus musculus.*

Keywords: Blood-glucose lowering, Metformin, Alloxan-induced, Phytochemicals

1. INTRODUCTION

Diabetes mellitus is a chronic disease and a predominant public health concern that has grown steadily worldwide [1-2]. It is caused by hereditary or acquired deficiency in the production of insulin by pancreatic β-cells, and/or by ineffectiveness of insulin produced. Wild *et al* [3] reported that it affects more than 170 million people worldwide and is predicted to affect over 365 million people by the year 2030. However, diabetes can be controlled by diet, exercise, insulin replacement therapy and by the use of herbal hypoglycemic agents [4]. Type 1 diabetes is classified as insulin dependent and type 2 diabetes as non-insulin dependent and the most common form of diabetes. Insulin therapy affords glycemic control in type 1 diabetes, yet its shortcomings such as ineffectiveness on oral administration, short shelf life, the requirements of constant refrigeration, fatal hypoglycemia in event of excess dosage, reluctance of patients to take insulin injection and above all the resistance due to prolonged administration limits its usage [5]. Similarly, treatment of type 2 diabetes patients with sulfonylureas and biguanides is almost always associated with side effects [6-7].

Medicinal plants are frequently considered to be less toxic and free from side effects than the synthetic one. The World Health Organization has also recommended that this should be encouraged, especially in countries where conventional treatment of diabetes seems insufficient [8]. *Crescentia cujete* Linn. has used and claimed by folklores in the Philippines as source of treatment of many diseases including diabetes but this somehow has no scientific reports. However studies conducted by Billacura Group reported that *Crescentia cujete* Linn. extracts from the leaves and fruits to have an antihelminthic, hypoglycemic and antimutagenic potentials but they have not reported yet the potential of the extracts from the dried-leaves of *Crescentia cujete* Linn. as a source of hypoglycemic agent [9-11]. Hence, this opens a search for possible hypoglycemic agent in the leaves of *Crescentia cujete* Linn. which may offer a less side effects compared to what is readily available in the market. This study generally aim to determine the various possible bioactive component, lethality and assess the hypoglycemic and protective bioactive potentials of the most bioactive extract from the air-dried leaves of *Crescentia cujete* Linn.

EXPERIMENTAL DETAILS

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, then was 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.

Phytochemical screening

In the determination of the possible presence of the various bioactive components in the crude ethanolic extract of *Crescentia cujete* Linn., the method of Saidu and Garba [12] was modified and employed as follows:

Test for alkaloids

Three milliliter of 2M HCl was added to 1 mL of the sample extract. It was placed in a boiling water bath for 5 minutes and cooled. After cooling, 0.30 g of NaCl was added and filtered. It was washed with 2M HCl to bring the volume to 5 mL. About 2 to 3 drops of Dragendorff's reagent was added. The relative amount of turbidity was observed as follows: (+) indicated as slight turbidity, (++) as define turbidity, and (+++) indicated heavy precipitation.

Test for flavonoids

One milliliter of 0.1M NaOH was added to 0.1 mL of the plant extract. The appearance of yellow color would indicate the presence of flavonoids.

Test for saponins

One milliliter of the sample extracts was added in a test tube followed by addition of 5 mL of distilled water. The mixture was stoppered and shaken vigorously for 30 seconds then allowed to stand for 10 minutes. The presence of persistent honeycomb froth greater than 1.5 cm above the surface of the liquid was taken as an indication for the presence of saponins.

Test for steroids

In the test tube containing 2 mL chloroform, 1 mL of the extract was added. Concentrated H_2SO_4 was also added through the wall of the test tube to form layers. The indication of the presence of steroid ring would be the reddish brown color at the interface of the solution.

Test for reducing sugar

One milliliter of the extract was dissolved in 3 mL of distilled water followed by adding drops of Fehling's A and B solution. The resulting solution was mixed and placed in a boiling water bath for 1-5 minutes. Allowed to cool for several minutes. The appearance of red-brown precipitate indicates the presence of reducing compound.

Test for tannins and polyphenolic compounds

To 1 mL of sample extract, 15 mL of hot distilled water was added. The solution was mixed, cooled, and decanted. After addition of 3 drops of 10% NaCl solution the mixture it was filtered and divided into three separate test tubes. Test tube A (serve as control), test tube B and test tube C. To test tube B, gelatin test was done to discern the presence of tannins by adding 3 drops of gelatin salt reagent. While to test tube C, 3 drops of 1% $FeCl_3$ reagent was added. Formation of dark blue color was taken as an indication for the presence of hydrolysable tannins while brownish green color for the presence of condensed tannins.

Test for volatile oils

The extract was dissolved in 90% ethanol and drops of 1% $FeCl_3$ were added. Formation of green color indicates the presence of volatile oils.

Solvent partitioning

The concentrated crude ethanolic extract was partitioned using a polar and non-polar solvent. 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 was distinct and observable. The hexane and aqueous layers were then separated and collected. The aqueous layer was then subjected with 50 mL of hexane for several times until the organic layer becomes clear. All of the hexane extracts were collected.

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* and were refrigerated for storage. Only the hexane and ethyl acetate extracts were used in the study inasmuch as the aqueous extract was not properly stored.

Brine shrimp assay

Approximately 0.05 g sample was weighed and dissolve with 5 mL 95% methanol, this solution served as solution A. About 0.5 mL was pippeted 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 pippeted into a test tube and from the solution A, 0.05 mL and 0.5 mL were pipetted into a separate test tubes 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 3-inched 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

Results from the BSLT shows that ethyl acetate has a higher percentage mortality compared to the hexane extract. Ethyl acetate extract was further studied and used test for hypoglycemic potential using the methods of Shetti *et al* [14] and Billacura and Alansado [9] with minor modification as follows:

Test organism

Twenty seven *Mus musculus* were purchased from Manresa, Xavier University, Cagayan de Oro City, Misamis Oriental, Philippines. They were placed in a cage and acclimatized for a week with a 24-hour dark-light cycle at a normal weather condition in Mindanao State University, Marawi City, Lanao del Sur, Philippines. They were given unlimited access to water and fed 3-times a day.

Before introducing the solutions, the *Mus musculus* were fasted overnight with an unlimited access to water. The body weight of the *Mus musculus* were determined using a top loading balance.

Hypoglycemic activity determination

Blood analysis was done after four days of treatment. Blood glucose level of the *Mus musculus* were determined after 24- and 36-hours of the subsequent treatment period. Blood sampling was collected from the tail of the test organism then its blood glucose level was determined using one touch glucometer.

Experimental design

Twenty seven albino mice was divided into 5 groups with three male albino mice in each group and treated as follows:

Table 1. Experimental set-up for the hypoglycemic and protective potential of *Crescentia cujete* Linn.

Group number	Treatment	Test	Days
1	Distilled water	Hypoglycemia/ Protective	3 days
2	Distilled water + Alloxan	Hypoglycemia/ Protective	1 day distilled water + 2 days Alloxan
3	Alloxan + 10000 ppm EAE	Hypoglycemia	1 day Alloxan + 2 days EAE
4	Alloxan + 5000 ppm EAE	Hypoglycemia	1 day Alloxan + 2 days EAE
5	Alloxan + 2500 ppm EAE	Hypoglycemia	1 day Alloxan + 2 days EAE
6	Alloxan + Metformin	Hypoglycemia	1 day Alloxan + 2 days Metformin
7	10000 ppm EAE + Alloxan	Protective	2 days EAE + 1 day Alloxan
8	5000 ppm EAE + Alloxan	Protective	2 days EAE + 1 day Alloxan
9	2500 ppm EAE + Alloxan	Protective	2 days EAE + 1 day Alloxan

Legend: EAE- ethyl acetate extract

All the solutions induced depends on the body weight of the *Mus musculus*. For Alloxan solution, 20000 ppm was used. The concentration of ethyl acetate extract were: 10000, 5000, and 2500 ppm, and 2500 ppm for Metformin.

2. RESULTS AND DISCUSSION

Phytochemical screening

To identify the possible various bioactive components of the crude ethanolic extract from the air-dried leaves of *Crescentia cujete* Linn., phytochemical analysis as described by Saidu and Garba [12] was followed.

The results show the presence of alkaloids, saponins, reducing sugars, tannins polyphenolics, and volatile oils. Flavonoids and steroids are not detected.

Table 2. Phytochemical constituents present in the crude ethanolic extract of the air-dried leaves of *Crescentia cujete* Linn.

Phytochemical constituents	Result	Indication
Alkaloids	Slight turbidity	(+)
Flavonoids	No yellow coloration	(-)
Saponins	Persistent honeycomb froth was observed	(+++)
Steroids	No reddish brown color in the interface	(-)
Tannins and Polyphenolic	Formation of dark blue observed	(+++)
Reducing sugars	Heavy red precipitate was seen	(+++)
Volatile oils	Green coloration was seen	(+++)

Legend: +++ (copiously present), ++ (moderately present), + (trace), - (absent)

Brine shrimp lethality test

Brine shrimp lethality test is an assay to determine the cytotoxicity of the plant sample. Table 3 shows the results of the test of hexane and ethyl acetate extract.

Table 3. Toxicity results of *Crescentia cujete* Linn. against brine shrimp nauplii after 24-hours of exposure

Extracts	Dose (ppm)	After 24 hours	
		Average % Mortality	LC_{50}
Hexane	1,000	100	0.048254
	100	100	
	10	88	
Ethyl Acetate	1,000	100	0.00536
	100	100	
	10	92	
DMSO		30	
Seawater		20	

Brine shrimp lethality test were the first parameter used to determine the most bioactive extract. Table 3 shows that the ethyl acetate extract has a higher percentage mortality as compared to hexane extract. Hence, ethyl acetate was used as the most bioactive extract and considered to undergo various analyses.

Hypoglycemic activity

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

Table 4. Hypoglycemic activity of ethyl acetate extract (EAE) from the air-dried leaves of *Crescentia cujete* Linn. by oral gavage in *Mus musculus* after 24- and 30-hours

Treatment	Blood glucose level (mg/dL)	
	After 24 hours	After 30 hours
A	70^{b}	72^{b}
B	145^{a}	115^{a}
C	69^{b}	71^{b}
D	77^{b}	76^{b}
E	91^{b}	76^{b}
F	80^{b}	72^{b}

Legend: treatment A-control (distilled water), treatment B-Alloxan treatment, negative control (200 mg/kg body wt.),treatment C-Alloxan + 10000 ppm EAE, treatment D-Alloxan + 5000 ppm EAE, treatment E-Alloxan + 2500 ppm EAE, treatment F-Alloxan + Metformin, positive control. Means having same letter mean insignificantly different at $\alpha = 0.05$ DMRT

Twenty-four hours after the last treatment, it shows that treatment B has the highest blood-glucose level relative to treatment A (control-distilled water). It also shows that the blood-glucose level of the Alloxan-induced *Mus musculus* with EAE in treatments C, D and E are lower compared to treatment B. Also, it is clear that treatment F (alloxan + Metformin) has a lower blood-glucose level compared to treatment B.

Metformin 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 [15].

Upon doing the statistical analysis, it shows that treatment B is significantly different from all other treatments (A, C, D, E

and F). Hence, the blood-glucose level of the Alloxan-induced *Mus musculus* was reduced by the different concentrations of EAE. It also shows that treatment A, C, D, E and F are not significantly different from each other.

As for the observation after 30 hours from the last treatment, results from the statistical analysis showed similar pattern to that of the observed treatments after 24 hours, the blood glucose level for treatments A, C, D, E and F are not significantly different from each other. While that for treatment B showed to be significantly different from all other treatments.

Protective potential of EAE against Alloxan in *Mus musculus*

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

Twenty-four hours after the last treatment of Alloxan, it shows that treatment B has the highest blood-glucose level. However, *Mus musculus* treated with a double dose of EAE has a lower blood-glucose level 24-hours after the treatment of Alloxan. Statistical results revealed that treatment B is significantly different from other treatment (A, G, H and I).

After 30 hours, another blood sampling was done, the observed results was the same, only the treatment B has a highest glucose level compared to treatment A, G, H and I, however, there is a discrepancy in treatment H after an evaluation using T-test as there is an increase in the blood glucose level which cannot be explain by the researcher.

Table 5. Protective potential of ethyl acetate extract (EAE) from the air-dried leaves of *Crescentia cujete* Linn. by oral gavage in *Mus musculus* after 24-and 30-hours.

Treatment	Average blood glucose level (mg/dL)	
	After 24 hours	After 30 hours
A	70^{b}	72^{b}
B	145^{a}	115^{a}
G	58^{b}	39^{b}
H	63^{b}	78^{b}
I	92^{b}	90^{b}

Legend: treatment A- control (distilled water), treatment B-Alloxan treatment (200mg/kg body wt.), treatment G- 10000 ppm + Alloxan, treatment H- 5000 ppm + Alloxan, treatment I-2500 ppm + Alloxan. Means having the same letters are insignificantly different at α=0.05 DMRT

Based on the results of phytochemical screening, it reveals the presence of alkaloids, saponins, reducing sugars, volatile oils, and, tannins and polyphenolics. Jayaraman *et al* [16] reported that saponins, flavonoids and glycosides can have an antihyperglycemic action through stimulation of β-cells of islets of Langerhans to release more insulin.

3. CONCLUSIONS

Preliminary phytochemical screening of the air-dried leaves of the crude ethanolic extract of the *Crescentia cujete* Linn. showed the presence of alkaloids, saponins, reducing sugar, tannins, polyphenolic compounds and volatile oils. The air-dried leaves of *Crescentia cujete* Linn. can normalize the blood glucose level of the Alloxan-induced *Mus musculus* and can prevent the increase of the blood glucose level in a pre-orally treated *Mus musculus* with ethyl acetate extract. The effect of the 10000 ppm and 5000 ppm on the air-dried leaves of *Crescentia cujete* Linn. is comparable to the effect of the commercially available hypoglycemic agent, Metformin.

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*For correspondence; Tel. + (63) 9177642446, E-mail: merelljohn@gmail.com