ORIGINAL ARTICLE

Determination of Toxicity and Hypoglycemic Effect in Alloxan-induced Diabetic Mice of *Manihot esculenta* Crantz (Fam. Euphorbiaceae) Aqueous Crude Leaf Extract and its Fractions

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ABSTRACT

Objective. *Manihot esculenta* (Crantz) leaves have been used for the management of diabetes based on cited ethnopharmacological studies. However, scientific evidence to support its efficacy is lacking. The aim of the study was to assess the cytotoxicity of the aqueous leaf extract and fractions of *Manihot esculenta* and its potential hypoglycemic effect on male Swiss albino mice.

Methods. The cytotoxicity assay was necessary to screen which extract and fractions will be used for the *in vivo* hypoglycemic study.

Phytochemical screening identified the composition of the aqueous crude extract and its fractions. The phytochemical results showed the presence of alkaloids, anthraquinone glycosides, carbohydrates, phenolic glycosides, saponins, and flavonoids.

Cytotoxicity was screened using CytoTox 96® (Promega), a NonRadioactive Cytotoxicity Assay on liver cancer (HepG2) and normal kidney (HK-2) cell lines. Five samples were tagged as highly cytotoxic and were flagged for further assays. These samples were DCM fraction (100 ppm), n-Hexane fraction (1 and 10 ppm), Ethyl acetate fraction (1 ppm), and Aqueous fraction (100 ppm).

Results. The hypoglycemic activity was examined in alloxan-induced diabetic mice using *in vivo* hypoglycemic study. The aqueous crude extract at dose levels of 200 mg/kg and 300 mg/kg body weight showed significant reduction in blood glucose levels compared to the diabetic control but not exceeding the results in the metformin treatment group. A *p-value* of 0.05 set a priori was used to consider whether the intervention had a statistically significant difference compared to the diabetic control.

Conclusion. The effectiveness of aqueous crude extract in reduction of blood glucose in mice may be attributed to the synergistic effects of phytochemicals present, especially the alkaloids which were retained in the extract but were undetectable in the fractionated samples. The results of this study also support the findings of existing ethnopharmacological studies on *M. esculenta* leaf extract as a hypoglycemic agent.

Keywords: Alloxan-induced mice, HepG2, HK-2, NonRadioactive Cytotoxicity Assay, synergistic effects, alkaloids, hypoglycemic agent, ethnopharmacology, blood glucose reduction

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INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders that share a common attribute of hyperglycemia or elevation of blood glucose.[1] Hyperglycemia can be associated with or without impairment of insulin action to cells, or problems in the pancreatic insulin secretion. DM can be classified into four categories: type 1, type 2, gestational, and other diabetes

mellitus, with type 2 being the most common type.[2] The World Health Organization (WHO)[3] has reported the rise in the number of people with diabetes from 108 million in 1980 to 422 million in 2014. In 2017, diabetes in the Philippines had a prevalence of 3,721,900 adult cases based on the study conducted by the International Diabetes Federation (IDF).[4]

Due to the problems associated with current prescribed medications (side effects, availability, and cost), most Filipinos opt to use other alternatives such as herbal preparations; one of these is the plant *Manihot esculenta*.

Manihot esculenta (Crantz) from the family Euphorbiaceae, locally known as *kamoteng kahoy* or cassava, is one of the most important staple crops worldwide. However, several studies put more emphasis on its toxicity that can be attributed to linamarin which produces the toxic compound hydrogen cyanide (HCN), thus overshadowing its medicinal values. Cassava has been known to contain alkaloids, cyanogenic glycosides, flavonoid glycosides, and other phytochemicals. It is known that plant alkaloids are medicinally valuable for the management of diseases such as diabetes. Furthermore, dietary flavonoid has been reviewed to have antidiabetic potential improving glycolysis, mitochondrial functions, and insulin sensitivity, as well as causing reductions in gluconeogenesis and oxidative stress.[5] The use of natural substances as therapeutic alternatives became more widespread driven by the undeniable belief that natural substances are readily available and may have fewer side effects than pharmaceuticals. Traditional healers in South West Nigeria and in Laguna, Philippines use decoction of *M. esculenta* leaves for managing diabetes mellitus.[6,7] Other Philippine plant species such as *Momordica charantia* L. showed more significant increase in serum insulin level compared with glibenclamide and control hybrid group. In addition, *Asystasia gangetica*, *Chamaecostus cuspidatus*, and *Lagerstroemia speciosa* were also proven effective for the management of type 2 diabetes. The extract of *Manihot esculenta* leaves is being used as a hypoglycemic agent based on ethnopharmacological studies. However, few researches have studied its antidiabetic property. The study provided an *in vivo* evidence for the potential hypoglycemic effect of *Manihot esculenta* leaves and *in vitro* evidence for its toxicity.

MATERIALS AND METHODS

Materials

Plant

Manihot esculenta leaves were collected in Barangay Malapad na Parang, Lobo, Batangas. The plant specimens were authenticated and verified by the Bureau of Plant Industry in Malate, Manila with the certificate number PLT-ID-CRPSD-035-19 on January 10, 2019.

Animal

Fifty-six (56) healthy, 8-12-week-old male Swiss albino mice weighing 23-27g were used in the study. Mice were obtained from the Research Institute for Tropical Medicine (RITM) and housed in the Analytical/Animal Laboratory of the Department of Pharmacology and Toxicology, UP College of Medicine. The study was reviewed and approved by the Institutional Animal Care and Use Committee with the approval number 2018-029. The mice were acclimatized under a 12-hour light/dark cycle for 7 days before the study. They were fed standard feed (Breeder's Choice®) composed of 21% crude protein, 10% crude fat, 5% crude fiber, 10% moisture, and fortified with vitamin C, vitamin E, vitamin A, vitamin B12, vitamin D3, and riboflavin. Water was provided *ad libitum*.

Reagents

Alloxan monohydrate was purchased from Chemline Scientific Corporation. Glucometer (Glucoleader® Value) and glucometer strips (Value blood glucose test strips) for measurement of random blood sugar (RBS) were provided by the researchers. The analytical grade solvents used in fractionation, i.e., n-hexane, dichloromethane, and ethyl acetate, were purchased from Belman Laboratories. All reagents used in phytochemical screening were provided by the Pharmaceutical Chemistry Department of the UP College of Pharmacy.

Methods

Preparation of Aqueous Crude Extract and Lyophilization Process

The preparation of powdered crude extract was based on Bokanga's method.[8] Five hundred grams of fresh leaves were crushed for 15 minutes, followed by boiling in distilled water for another 15 minutes. Heat sped up the evaporation of hydrogen cyanide and cyanohydrin, rendering the solution free of cyanide or cyanohydrin. The boiled extract was filtered, and the filtrate was then subjected to lyophilization process for the removal of water which resulted in powder form.

Fractionation of Extract

The method was a modified procedure from Mendoza et al..[9] The aqueous crude extract was fractionated through Modified Kupchan Liquid Partitioning. Initially, five (5) grams of the powdered crude extract was dissolved in 250 mL 9:1 methanol:water solution and was transferred to a separatory funnel. The solution was then extracted with three portions of 100 mL n-hexane. The organic hexane layer was set aside. The aqueous layer was extracted with three portions of 100 mL dichloromethane. The dichloromethane fraction was set aside. The aqueous layer was then extracted with three portions of 100 mL ethyl acetate. The aqueous layer was set aside. All resulting fractions were concentrated in vacuo (40°C) and evaporated to dryness (60°C) in water bath to remove the solvent. The aqueous crude extract and all its fractions were then subjected to phytochemical screening and cytotoxicity assay (Figure 1).

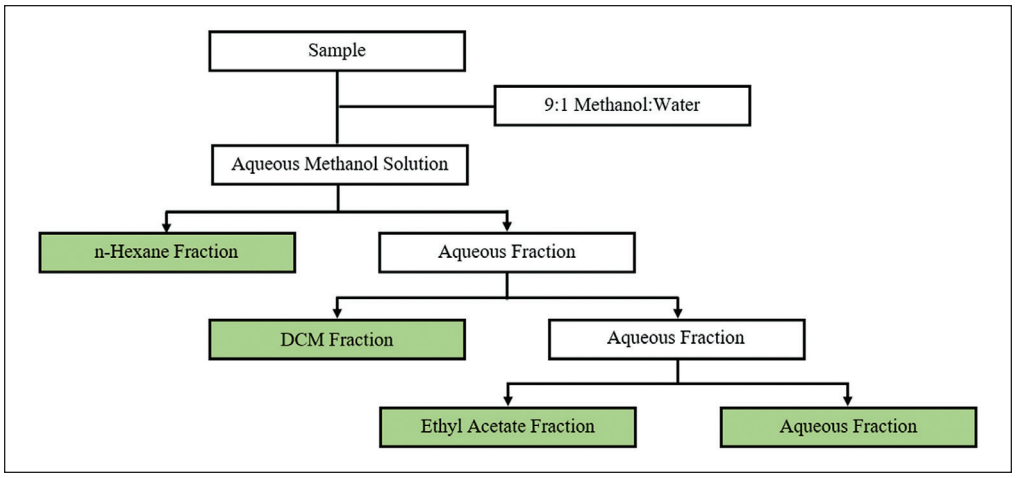

Figure 1. Schematic diagram of the modified Kupchan liquid partitioning method.

Phytochemical Screening of Aqueous Leaf Extract and its Fractions

M. esculenta aqueous crude extract and its fractions were screened qualitatively for alkaloids, glycosides (cardiac, anthraquinone, and cyanogenic), carbohydrates, flavonoids, saponins, tannins, and phenolic compounds using phytochemical tests adopted from the phytochemical procedures from the Pharmaceutical Chemistry 128 (Medicinal Chemistry I) Laboratory Manual.[10]

Cytotoxicity Assay

Sample Preparation

A total of fifteen (15) samples, 5 mg of aqueous crude extract and its fractions, were transmitted to the National Institute of Molecular Biology and Biotechnology laboratory in the University of the Philippines Diliman for analysis. The samples were stored at 4°C and were kept away from light.

Prior to the assay, the lyophilized samples were dissolved in DMSO to achieve a stock concentration of 20,000 ppm. Serial dilution was then performed to obtain 100, 10, and 1 ppm concentrations.

Assay Protocol

Liver cancer (HepG2) and normal kidney (HK-2) cell lines were cultured using the appropriate media and grown to 80% confluency. They were then seeded with a seeding density of 5,000 cells per well into 96-well plates for subsequent assay.

A commercially available kit, CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was used. It is a colorimetric assay that quantitatively measures extracellular lactate dehydrogenase (LDH) through the conversion of a violet tetrazolium salt into a red formazan product.

A negative control was used in which the wells do not contain any cells. This determines culture medium background. The average absorbance values obtained were subtracted from each experimental well. These were then regarded as the corrected values which were used for computation of the percent cytotoxicity.

The absorbance values were measured using a 96-well plate reader and read at wavelength 490 nm. The percent cytotoxicity of the samples was calculated based on the measured absorbance values, using the formula:

$$\text{Percent cytotoxicity} = 100 \times \frac{\text{Experimental LDH release } (OD_{490})}{\text{Maximum LDH release } (OD_{490})}$$

OD_{490} – optical density at 490nm

In vivo Antidiabetic Assay[11]

Induction of Diabetes by Alloxan

The dose of alloxan used to induced diabetes was based on the optimization study conducted by Bukhari et al..[12] Fifty-six (56) healthy 8-12-week-old male Swiss albino mice weighing 23-27 g were used in the study. The animals were kept in standard cages at room temperature and 12-hour light/dark cycle in the Analytical/Animal Laboratory at the Department of Pharmacology and Toxicology, UP College of Medicine. The animals were given standard feed, and water *ad libitum*. Induction of diabetes was done by intraperitoneal injection of 200 mg/dL of alloxan monohydrate, freshly prepared in normal saline and administered to overnight fasted mice. Mice with blood glucose levels greater than 200 mg/dL after 72 hours were considered diabetic and selected for the study. Induction procedure was also conducted in the Analytical/Animal Laboratory.

Experimental design

The alloxan-induced diabetic mice were randomly grouped into nine groups of six each (n=6). Group 1 served as the normal control (non-diabetic mice) receiving only 0.1 ml normal saline. Group 2 served as the diabetic control, diabetic mice receiving only 0.1 ml normal saline. Group 3

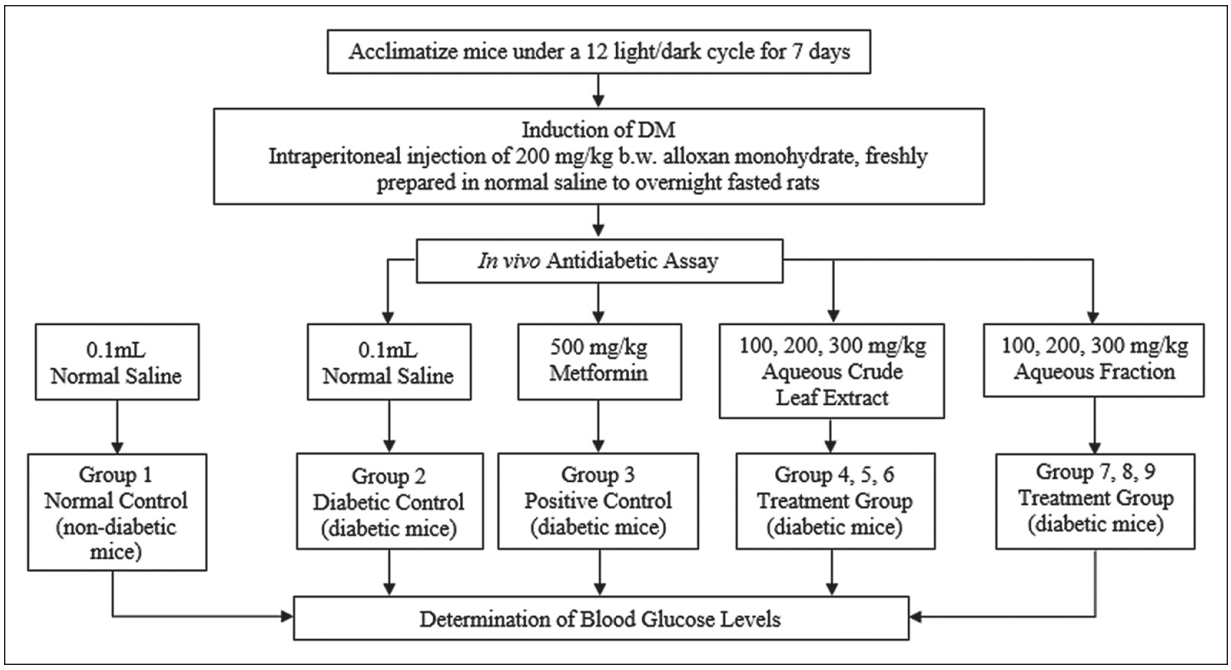

Figure 2. Schematic diagram for *in vivo* antidiabetic assay.

as the reference control, where diabetic mice received 500 mg/kg of metformin. Groups 4, 5 and 6 received 100, 200, and 300 mg/kg of the aqueous crude leaf extract, respectively while groups 7, 8, and 9 received 100, 200, and 300 mg/kg of the aqueous fraction. The doses used were based on other *in vivo* antidiabetic studies such as the study conducted by Akuodor et al. and Stephen et al.[11,13] The aqueous crude extract and aqueous fractions were administered once daily by oral gavage (Figure 2).

Determination of blood glucose levels

All blood samples were collected through the saphenous vein. This method of blood withdrawal was chosen as it is often used when a series of small samples are required. Anesthesia is no longer required with this method. Prior to blood withdrawal, the dorsal hind leg of the mice was shaved to have a better view of the lateral saphenous vein and to prevent infection. The exposed skin was disinfected with 70% isopropyl alcohol prior to puncture using a lancet and a drop of blood was applied to the glucose strip to determine the blood glucose level. The first drop of blood was discarded because it may be contaminated with tissue fluid or debris (slough-off skin). The puncture site was applied with cotton ball until bleeding stops.

The blood glucose level was measured using a calibrated GlucoLeader glucose kit at 0 hour (before extract/fraction/metformin administration) and at 4h on days 1, 7, and 14.

Statistical analysis

All data were expressed as Mean ± Standard Error of Mean (SEM). The significance of blood glucose reduction produced by the aqueous crude extracts and fractions compared to the control was determined by two-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons using Prism ver. 6.0, Graphpad software. A p value of < 0.05 was considered statistically significant.

RESULTS

Phytochemical Screening

Qualitative screening and identification of phytochemicals present in the aqueous crude extract and its fractions were performed. Results showed the presence of alkaloids, anthraquinone glycosides, carbohydrates, phenolic glycosides, saponins, and flavonoids. The phytochemical tests performed and the corresponding results from each fraction are shown in Table 1.

Cytotoxicity Assay

Percent cytotoxicity of the samples were computed based on absorbance values measured at 490 nm. Compounds greater than 10% cytotoxicity were tagged as highly cytotoxic; compounds within the range of 1-10% cytotoxicity were tagged as moderately cytotoxic and; compounds with positive percent cytotoxicity less than 1% were tagged as mildly cytotoxic. Compounds with negative percent cytotoxicity values were tagged as non-cytotoxic (Table 2).

All the samples were tested in duplicates in two (2) independent trials. Samples with a highly cytotoxic profile were given an extended margin of 5% only if the other trial exhibited moderately cytotoxic, mildly cytotoxic, or non-cytotoxic profile.

Hepatotoxicity Assay

The percent cytotoxicity of *M. esculenta* aqueous crude extract and fractions at different concentrations on HepG2 cells for two trials are shown in Table 3. Only DCM fraction 100 ppm was flagged for further assays since the fraction expressed a percent cytotoxicity greater than 10%.

Nephrotoxicity Assay

The percent cytotoxicity of *M. esculenta* aqueous crude extract and fractions at different concentrations on HK-2 cells are shown in Table 4. Hexane (1 and 10 ppm), DCM (100 ppm), Ethyl acetate (1 ppm) and Aqueous (100 ppm) fractions were flagged and were considered not safe for

Table 1. Phytochemical Screening Results of *Manihot esculenta* Aqueous Crude Extract and Fractions

Test	Crude	n-Hexane	DCM	Ethyl Acetate	Aqueous
Alkaloids					
Valser's Test	+	-	-	-	-
Mayer's Test	+	-	-	-	-
Wagner's Test	+	-	-	-	-
Dragendorff's Test	+	-	-	-	-
Anthraquinone Glycosides					
Modified Borntrager's Test	+	+	+	+	+
Shouteten Test	+	+	+	+	+
Carbohydrates					
Molisch's Test	+	+	-	-	+
Fehling's Test	-	+	-	-	+
Seliwanoff's Test	-	+	-	-	-
Tollen's phloroglucinol Test	-	-	-	-	+
Phenolic Glycosides					
Ferric Chloride Test	+	-	-	-	+
Saponins					
Froth Test	+	-	-	-	-
Tannins					
Gelatin Test	-	-	-	-	-
Cyanogenic Glycosides					
Sodium Picrate Test	-	-	-	-	-
Flavonoids					
Shinoda Test	+	-	-	-	+
Sulfuric Acid Test	+	-	-	-	+

Legend: (+): present; (-): absent

Table 2. Criteria for Classification of Cytotoxicity

Classification	Cytotoxicity
Non-cytotoxic	< 0%
Mildly cytotoxic	< 1%
Moderately cytotoxic	1 - 10%
Highly cytotoxic	> 10%

Table 3. Hepatotoxicity of *M. esculenta* on Liver Cancer (HepG2 or Hepatoblastoma G2) cells

Sample	Concentration (ppm)	Cytotoxicity (%)		Cytotoxicity
		Trial 1	Trial 2	
Aqueous Crude Extract	1	-22.317	4.196	Safe
	10	-16.574	4.495	Safe
	100	-23.831	2.066	Safe
n-Hexane Fraction	1	-20.759	3.865	Safe
	10	-21.460	5.084	Safe
	100	-12.044	11.572	Safe
DCM Fraction	1	-23.475	4.963	Safe
	10	-20.381	5.471	Safe
	100	-8.471	17.536	Flagged
Ethyl Acetate Fraction	1	-20.670	3.793	Safe
	10	-22.028	3.599	Safe
	100	-17.798	9.498	Safe
Aqueous Fraction	1	-23.764	1.517	Safe
	10	-23.197	2.816	Safe
	100	-19.691	2.485	Safe

further assays since they exhibited high cytotoxicity on kidney cell lines.

The probability to pursue similar assays that have closer physiological conditions depends on the concurrence of both hepatic and nephric cytotoxic classifications of the samples (Table 5).

In summary, all of the samples were recommended for further testing, except DCM fraction 100 ppm, n-Hexane fraction 1 and 10 ppm, Ethyl acetate fraction 1 ppm, and Aqueous fraction 100 ppm, because of the relatively high % cytotoxicity of these compounds.

In vivo studies

The effect of *M. esculenta* aqueous crude extract and aqueous fraction on blood glucose level at 0hr and 4hr on day 1, day 7, and day 14 are shown in Table 6.

Mean blood glucose (mg/dL) per group were plotted against time (Figure 3) to see changes in the blood glucose level over time after the 14-day treatment and to compare the changes in treatment groups to the diabetic control and to the hypoglycemic effect of metformin.

The blood glucose means per time point were also totaled and averaged for each group to compare levels of reduction (Figure 4). Furthermore, error bars were reported in Figure 4 as standard error means (SEM). The error bars represent the variability of values, indirectly implying the range of blood glucose reduction for one treatment compared to another.

Results of Dunnett's Test for multiple comparisons are summarized in Table 7. Based on the results, the normal control, metformin group, and the 200 mg/kg and 300 mg/kg crude extract groups were the only groups that exhibited a significant change in blood glucose as compared to the diabetic control.

The summary of the properties of the treatment groups (Crude Extract 200 mg/kg and Crude Extract 300 mg/kg) of *M. esculenta* that have significant difference in blood glucose reduction compared to the diabetic control are presented in Table 8. The summary includes significance of blood

Table 4. Nephrotoxicity of *M. esculenta* on Normal Kidney (HK-2 or Human Kidney-2) cells

Sample	Concentration (ppm)	Cytotoxicity (%)		Cytotoxicity
		Trial 1	Trial 2	
Aqueous crude extract	1	-52.806	-52.806	Safe
	10	10.995	9.005	Safe
	100	-0.663	1.937	Safe
n-Hexane Fraction	1	50.026	3.651	Flagged
	10	28.214	6.545	Flagged
	100	0.408	12.055	Safe
DCM Fraction	1	6.760	3.328	Safe
	10	5.179	5.154	Safe
	100	19.056	17.320	Flagged
Ethyl Acetate Fraction	1	103.597	0.623	Flagged
	10	-1.760	4.920	Safe
	100	0.918	8.437	Safe
Aqueous Fraction	1	-13.112	10.062	Safe
	10	-51.378	3.829	Safe
	100	33.878	2.037	Flagged

Table 5. Summary of Hepatotoxicity and Nephrotoxicity of *M. esculenta* and Recommendation to Proceed to further Assays (i.e., orthogonal assay)

Sample	Concentration (ppm)	Hepato-toxicity	Nephro-toxicity	Recommen-dation
Aqueous Crude Extract	1	Safe	Safe	Proceed
	10	Safe	Safe	Proceed
	100	Safe	Safe	Proceed
n-Hexane Fraction	1	Safe	Flagged	Discontinue
	10	Safe	Flagged	Discontinue
	100	Safe	Safe	Proceed
DCM Fraction	1	Safe	Safe	Proceed
	10	Safe	Safe	Proceed
	100	Flagged	Flagged	Discontinue
Ethyl Acetate Fraction	1	Safe	Flagged	Discontinue
	10	Safe	Safe	Proceed
	100	Safe	Safe	Proceed
Aqueous Fraction	1	Safe	Safe	Proceed
	10	Safe	Safe	Proceed
	100	Safe	Flagged	Discontinue

Table 6. Effects of Orally Administered *M. esculenta* Aqueous Crude Extract and Fraction on Blood Glucose Levels in Alloxan-induced Diabetic Mice after Acute Treatment

Treatment	Time			
	0 hr	4 hr	7th day	14th day
Normal Control	114.50±5.92	116.83±5.88	116.17±11.34	115.17±2.92
Diabetic Control	521.17±27.82	541.50±11.87	531.33±28.44	519.83±22.43
Metformin (500 mg/kg)	523.00±7.20	375.50±22.30	262.83±15.69	134.33±7.65
Crude Extract (100 mg/kg)	536.83±9.43	487.67±11.216	464.50±10.77	390.67±21.61
Crude Extract (200 mg/kg)	467.17±23.33	440.67±10.13	378.67±7.84	349.17±17.76
Crude Extract (300 mg/kg)	519.33±14.13	419.00±10.47	347.83±23.92	278.17±20.16
Aqueous Fraction (100 mg/kg)	532.83±11.08	526.67±11.83	518.17±16.76	501.83±11.25
Aqueous Fraction (200 mg/kg)	451.67±11.93	452.00±10.11	398.17±14.09	446.17±7.44
Aqueous Fraction (300 mg/kg)	478.33±20.44	488.17±18.54	468.17±18.27	475.67±16.53

Values here are reported as Means ± SEM; n = 6.

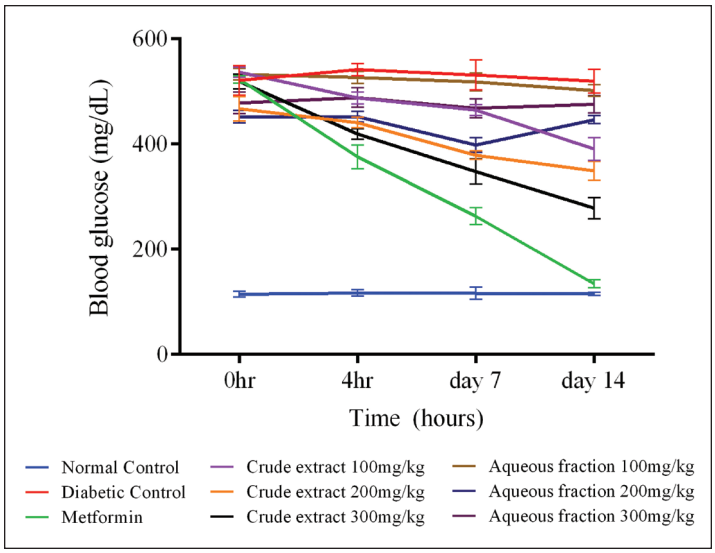

Figure 3. Blood glucose level of alloxan-induced diabetic mice after treatment grouped according to time; n=6 per group.

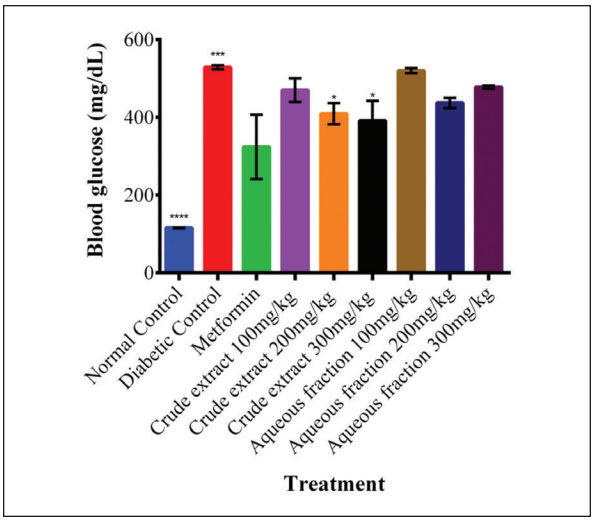

Figure 4. Comparison of the means of blood glucose level of alloxan-induced diabetic mice per group for the two-week study; n=6 per group.

Table 7. Multiple Comparison Analysis of the Different Groups to Diabetic Control Using Dunnett's Test

Dunnett's Multiple Comparisons Test	Mean Diff.	95% CI of Diff.	Significant?	Summary
Diabetic Control vs Normal control	412.8	295.2 to 530.4	Yes	****
Diabetic Control vs Metformin (500 mg/kg)	204.5	86.93 to 322.2	Yes	***
Diabetic Control vs Crude Extract (100 mg/kg)	58.54	-59.07 to 176.2	No	ns
Diabetic Control vs Crude Extract (200 mg/kg)	119.5	1.931 to 237.2	Yes	*
Diabetic Control vs Crude Extract (300 mg/kg)	137.4	19.76 to 255.0	Yes	*
Diabetic Control vs Aqueous Fraction (100 mg/kg)	8.583	-109.0 to 126.2	No	ns
Diabetic Control vs Aqueous Fraction (200 mg/kg)	91.46	-26.15 to 209.1	No	ns
Diabetic Control vs Aqueous Fraction (300 mg/kg)	50.87	-66.74 to 168.5	No	ns

*Values are statistically significant at *p≤0.05, **p≤0.01, ***p<0.001, ****p<0.0001, and not significant=ns at p>0.05, vs. diabetic control (ANOVA)*

Table 8. Summary of *M. esculenta* Aqueous Crude Extract and Aqueous Fraction with Corresponding Blood Glucose Reduction Significance, Toxicity Classification, and Phytochemical Constituents Present

Sample	Daily Dose	Blood Glucose Reduction Significance	Hepatotoxicity	Nephrotoxicity	Phytochemical Constituents
Aqueous Crude Extract	100 mg/kg	No	Safe	Safe	Alkaloids, Anthraquinone Glycosides, Carbohydrates, Phenolic glycosides, Saponins, Flavonoids
	200 mg/kg	Yes	Safe	Safe	
	300 mg/kg	Yes	Safe	Safe	
Aqueous Fraction	100 mg/kg	No	Safe	Safe	Anthraquinone Glycosides Carbohydrates, Phenolic glycosides, Flavonoids
	200 mg/kg	No	Safe	Safe	
	300 mg/kg	No	Safe	Flagged	

glucose reduction, hepatotoxicity, nephrotoxicity, and the phytochemicals present.

DISCUSSION

Fractionation and Determination of Phytochemical Constituents

The subsequent fractionation of the aqueous crude extract used the following solvents in the following order: n-hexane, dichloromethane, and ethyl acetate.

The order of solvent use was based on the increasing polarity indexes of these solvents. According to Harris, they are 0.1, 3.1 and 4.4, respectively.[14]

Phytochemical studies of leaves such as in the reports of Ogbuji and David-Chukwu have varying results of present constituents depending on the variety of cassava.[15,16]

Among the samples, the crude extract had the greatest number of constituents. It tested positive for all four alkaloid tests, Valser's, Mayer's, Wagner's, and Dragendorff's tests, indicating presence of alkaloidal compounds. After

fractionation, no fraction yielded positive for the alkaloids. It appears that the alkaloids were separated to the added initial 9:1 methanol-water extract even after consequent addition of the solvents.

For the testing of anthraquinone glycosides, the modified Borntrager's test and Shouteten test were used. The crude extract and all fractions tested positive. Presence of anthraquinones according to the molecular docking study of Jung, Ali, and Choi indicated potential antidiabetic property via the inhibition of protein tyrosine phosphatase 1B (PTP1B) and α-glucosidase enzymes.[17]

Results of the carbohydrates vary among the crude extract and fractions. Absence of starch in the crude extract was also confirmed using the Iodine Test.

Crude extract and aqueous fraction were the only ones positive for ferric chloride test indicating presence of phenolic glycosides. According to Rawat et al., antihyperglycemic activity for phenolic glycosides among eight of their tested compounds showed significant lowering of blood glucose level on diabetic rats induced with low dose streptozotocin.[18]

Saponins were only observed in the crude extract. A review done by Elekofehinti reported saponin's ability to regulate plasma glucose level, possibly due to its antioxidant property.[19]

Tannins and cyanogenic glycosides were negative for the crude extract and all fractions. Negative results of the cyanogenic glycosides through the sodium picrate test, proves qualitatively that no apparent cyanide and its organic forms are present in the sample after the preparation method as described by Bokanga.[8]

Flavonoids were observed in the crude and aqueous extracts only through the Shinoda and Sulfuric Acid test. Flavonoids are secondary metabolites that have a wide range of bioactivity for diabetes treatment. The effects include increase in insulin secretion, suppression activities on α-amylase and -glucosidase, restoration of high glucose-attenuated intracellular cAMP and ATP production, and many more.[20]

Cytotoxicity Assay

CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) is a colorimetric assay that is used as an alternative to radioactive cytotoxicity assays. The assay measures quantitatively the extracellular lactate dehydrogenase (LDH) through the conversion of a violet tetrazolium salt (INT/iodonitrotetrazolium) into a red formazan product. Lactate dehydrogenase is a cytosolic enzyme released during cell lysis. Cells undergo necrosis and lysis when exposed to cytotoxic compounds. The released LDH reacts with the tetrazolium salt supplied by the assay in the cell culture medium. The change in color of the medium (i.e., from violet to red), as measured by an increase in absorbance values, is proportional to the number of the lysed cells.

Percent cytotoxicity of the samples were computed based on absorbance values measured at 490 nm, following a logarithmic model of *in vitro* cytotoxicity versus compound concentration. The value of 10% cytotoxicity was arbitrarily chosen as the threshold for flagging compounds for testing discretion. This level of cytotoxicity implies that subsequent increases of extract concentration to increase bioactivity may potentially lead to cytotoxicity of 50%. Samples tagged as moderately cytotoxic, mildly cytotoxic, and non-cytotoxic may be safely pursued for further fine-tuned downstream experiments and assay while samples that are tagged as highly cytotoxic are flagged against such pursuit.

The only sample exhibiting a highly cytotoxic effect on liver cells (HepG2 cells) was the 100 ppm DCM fraction. The toxicity of the DCM fraction may be attributed to the presence of anthraquinone glycosides that tested positive in the preliminary phytochemical screening. Anthraquinones are a group of secondary metabolites produced by plants that possess a wide array of bioactivity, such as anti-inflammatory, anticancer, antimicrobial, diuretic, cathartic, vasorelaxing, and phytoestrogen activities. Despite its beneficial effects, the close similarity in structure between anthraquinones and the toxic analogue, anthracene may potentially produce damage to cells. Anthraquinone glycosides and its derivatives have been known to cause toxicity in various plants and in some biological models.[21] The concentration of the DCM fraction may also contribute to its toxicity. Meanwhile, all other fractions were considered safe for fine-tuned experiments based on the hepatotoxicity assay.

In the nephrotoxicity assay, five (5) samples were identified to be highly cytotoxic to kidney cell lines (HK-2 cells). Hexane (1 and 10 ppm), DCM (100 ppm), Ethyl acetate (1 ppm) and Aqueous (100 ppm) fractions were flagged for further fine-tuned assays. The nephrotoxicity of the said samples may also be attributed to anthraquinones present in the plant, particularly free anthraquinones such as physcion, chrysophanol, rhein, emodin, and glycoside derivatives.[21]

The decision to pursue (discontinue or proceed) similar assays that have closer physiological conditions is based on the concurrence of both hepatic and nephric cytotoxic classifications of the samples. Overall, there were 10 samples that were recommended for further testing and five samples that were flagged.

In vivo Antidiabetic Assay

In vivo Antidiabetic Assay was used in the study to assess the hypoglycemic effect of *Manihot esculenta.* Many animal models of diabetes can be used for *in vivo* antidiabetic assay and it is important that it reflects the natural history and pathogenesis of diabetes.[22] The researchers used the optimal diabetogenic dose of alloxan monohydrate (200 mg/kg b.w.) according to the optimization study conducted by Bukhari et al.[12] In the study, the optimized dose, 200 mg/kg body weight, was found to lower the mice mortality by 50%.

After induction of diabetes in mice, those that had blood glucose level greater than 200 mg/dL after 72 hours were considered as diabetic and selected for the study. The diabetic

mice also exhibited the common diabetic symptoms such as polyuria, polyphagia, and polydipsia. Mice that reverted to the normal (healthy state) blood glucose level were excluded from the study. The procedures in the *in vivo* antidiabetic assay used in the study was patterned from the procedures done by Akuodor et al., and Sikarwar and Patil with some modifications.[11,23]

The results shown in Table 6 show that severe hyperglycemia was successfully induced for the diabetic control. The diabetic control received only normal saline as treatment, thus their blood glucose level remained high and did not improve over the two-week study. The group receiving metformin had significant blood glucose reduction. Metformin is a biguanide that lowers both the basal and the postprandial plasma glucose (PPG).[24]

In Figure 3, metformin and crude extracts have shown a constantly decreasing blood glucose level as time passes while the aqueous fractions had varying trends in its measurements. To confirm such observations and conclude which groups are statistically different from the diabetic control, two-way ANOVA followed by Dunnett's test was done. The results are given in Table 6 which also shows the mean differences of each treatment group compared to the diabetic control. The diabetic control and normal (healthy) control clearly showed a significant difference as it presented the strongest significance with the p value of < 0.0001. Metformin 500 mg/kg, crude extracts 200 mg/kg and 300 mg/kg also showed significant differences but used a decreased threshold of significance. A p value of < 0.001 for metformin and a p value of < 0.05 for the crude extracts were set a priori.

The graphical representation of results from Table 6, are presented in Figure 4. The vertical bars represent the average of all the means of the time points for each treatment and it appears from the graph that metformin, compared to the other treatments, achieved the largest reduction of blood glucose compared with the diabetic control. Thus, metformin was shown to possess good control on the blood glucose of mice. Next to metformin with a significant blood glucose reduction was the crude extract 300 mg/kg followed by the crude extract 200 mg/kg.

In summary, Table 8 compares the results for the significance in the blood glucose reduction, toxicity studies, and the presence of phytochemical constituents of the plant extract. The two crude doses 200 mg/kg and 300 mg/kg b.w. had a significant hypoglycemic effect. The aqueous crude and aqueous dose extracts were also low on toxicity except aqueous fraction 300 mg/kg. Results of the toxicity assays showed that it was also not dose-dependent. In terms of safety, decoctions prepared as crude extract did not pose any harm since none were flagged for both cytotoxicity assays. The absence of alkaloids and saponins in the aqueous fraction compared to crude extract was noted. These phytochemicals may play an important role in blood glucose reduction and that the removal of these compounds may have resulted in the absence of hypoglycemic effect of the aqueous fraction. This is supported by other studies proving that plant alkaloids and saponins are medicinally valuable for the management of diabetes.[19,25]

CONCLUSION

The Non-Radioactive Cytotoxicity Assay showed that the concentrations of the aqueous crude extracts and aqueous fractions were safe and thus could proceed to further assays, except for the highest concentration (100 ppm) of aqueous fraction which was classified as highly cytotoxic. Since the assay used liver cancer cell lines, it is recommended to also use normal cell lines to assess its effect on the liver of a healthy individual. An increase in the number of trials is also recommended for both cytotoxic tests.

The hypoglycemic effect of *M. esculenta* were exhibited by the 200 mg/kg and 300 mg/kg b.w. aqueous crude extract. However, doses of the aqueous fraction did not exhibit a significant blood glucose reduction which may be attributed to the loss of alkaloids and saponins during fractionation. The hypoglycemic effect of the aqueous crude extract may also be due to the synergistic effect of the phytochemicals present.

Despite the significant reduction by the 200 mg/kg and 300 mg/kg crude leaf extracts, metformin manifested greater blood glucose reduction showing its superior action over the extracts. Furthermore, it is recommended to determine the cassava variants that would exhibit hypoglycemic effect.

The study gave *in vitro* evidence of the cytotoxic effect *M. esculenta* leaf extract providing evidence for the safety and effectiveness of the crude extract. It supports the findings of ethnopharmacological studies on *M. esculenta* leaf extract regarding its use for managing diabetes.

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Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

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REFERENCES

1. Fauci A, Hauser S, Jameson J, Kasper D, Longo D, Loscalzo J. Harrison's Manual of Medicine. 19th ed. New York: McGraw-Hill Education; 2016. p. 904.
2. Katzung BG. Basic & Clinical Pharmacology. 14th ed. USA: McGraw-Hill Education; 2018. pp. 751-752,761.
3. World Health Organization. Diabetes [Internet]. 2018 [cited 2019 Feb 14]. Available from: http://www.who.int/news-room/fact-sheets/detail/diabetes.
4. International Diabetes Federation. The Philippines [Internet]. 2018 [cited 2019 Feb 14]. Available from: https://www.idf.org/our-network/regions-members/western-pacific/members/116-the-philippines.html.
5. Nwose EU, Onodu BC, Anyasodor AE, Sedowo MO, Okuzor JN, Culas RJ. Ethnopharmacological values of cassava and its potential for diabetes and dyslipidaemia management: knowledge survey and critical review of report. J Intercult Ethnopharmacol. 2017 Jun;6(3):260-6. doi: 10.5455/jice.20170606094119.
6. Abo KA, Fred-Jaiyesimi AA, Jaiyesimi AEA. Ethnobotanical studies of medicinal plants used in the management of diabetes mellitus in South Western Nigeria. J Ethnopharmacol. 2008 Jan;115(1):67-71. doi: 10.1016/j.jep.2007.09.005.
7. Fiscal RR. Ethnomedicinal plants used by traditional healers in Laguna, Philippines. Asia Pac J Multidiscip Res. 2017 Nov;5(4):132-7.
8. Bokanga M. Processing of cassava leaves for human consumption. Acta Hortic. 1994;375:203-8. doi: 10.17660/ActaHortic.1994.375.18
9. Mendoza CMT, Ramirez RF, So CM, Toralba JV. Bioassay-guided fractionation of the anti-diabetic constituents of star apple leaves. Philippine Journal of Health Research and Development. 2016;20(4):52-62.
10. Pharmaceutical Chemistry Department. Laboratory Manual in Pharmaceutical Chemistry 128 - Medicinal Chemistry I. Manila: College of Pharmacy, University of the Philippines Manila; 2016.
11. Akuodor GC, Udia PM, Bassey A, Chilaka KC, Okezie OA. Antihyperglycemic and antihyperlipidemic properties of aqueous root extract of Icacina senegalensis in alloxan induced diabetic rats. J Acute Dis. 2014;3(2):99–103. doi: 10.1016/s2221-6189(14)60025-1.
12. Bukhari SSI, Abbasi MH, Khan MKA. Dose optimization of alloxan for diabetes in albino mice. Biologia. 2018;61(2):301-5.
13. Stephen NM, Wycliffe AM, Alex MK, Joseph NJN, Eliud NNM. In vivo antidiabetic activity of aqueous and ethyl acetate leaf extract of Senna singuena (Delile) in alloxan induced diabetic mice. J Phytopharm. 2017;6(2):84-92.
14. Harris DC, Lucy CA. Quantitative chemical analysis, 9th ed. New York: Macmillan Higher Education; 2015. p. 677.
15. Ogbuji CA, David-Chukwu NP. Glycemic indices of different cassava food products. European Journal of Basic and Applied Sciences. 2016;3(3):1-7.
16. Ogbuji CA, David-Chukwu NP. Phytochemical, antinutrient and mineral compositions of leaf extracts of some cassava varieties. IOSR J Environ Sci Toxicol Food Technol. 2016;10(1):5-8. doi: 10.9790/2402-10110508.
17. Jung HA, Ali MY, Choi JS. Promising inhibitory effects of anthraquinones, naphthopyrone, and naphthalene glycosides, from Cassia obtusifolia on α-glucosidase and human protein tyrosine phosphatases 1B. Molecules. 2016 Dec;22(1):28. doi: 10.3390/molecules22010028.
18. Rawat P, Kumar M, Rahuja N, Srivastava DSL, Srivastava AK, Maurya R. Synthesis and antihyperglycemic activity of phenolic C-glycosides. Bioorg Med Chem Lett. 2011 Jan;21(1):228–33. doi:10.1016/j.bmcl.2010.11.031.
19. Elekofehinti OO. Saponins: anti-diabetic principles from medicinal plants – a review. Pathophysiology. 2015 Jun;22(2):95–103. doi: 10.1016/j.pathophys.2015.02.001.
20. Hasan MM, Ahmed QU, Mat Soad SZ, Tunna TS. Animal models and natural products to investigate in vivo and in vitro antidiabetic activity. Biomed Pharmacother. 2018 May;101:833–41. doi: 10.1016/j.biopha.2018.02.137.
21. Shukla V, Asthana S, Gupta P, Dwivedi PD, Tripathi A, Das M. Toxicity of naturally occurring anthraquinones. Advances in Molecular Toxicology. 2017;11:1–50. doi: 10.1016/b978-0-12-812522-9.00001-4.
22. Radenković M, Stojanović M, Prostran M. Experimental diabetes induced by alloxan and streptozotocin: The current state of the art. J Pharmacol Toxicol Methods. 2016 Mar-Apr;78:13–31. doi: 10.1016/j.vascn.2015.11.004.
23. Sikarwar MS, Patil MB. Antidiabetic activity of Pongamia pinnata leaf extracts in alloxan-induced diabetic rats. Int J Ayurveda Res. 2010 Oct;1(4):199-204. doi: 10.4103/0974-7788.76780.
24. Gong L, Goswami S, Giacomini KM, Altman RB, Klein TE. Metformin pathways: pharmacokinetics and pharmacodynamics. Pharmacogenet Genomics. 2012 Nov;22(11):820–7. doi: 10.1097/fpc.0b013e3283559b22.
25. Li WL, Zheng HC, Bukuru J, De Kimpe N. Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus. J Ethnopharmacol. 2004 May;92(1):1–21. doi: 10.1016/j.jep.2003.12.031.