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RESEARCH ARTICLE

Antidiabetic effects of *Cycas riuminiana* leaf extracts on alloxan-induced diabetic ICR mice (*Mus musculus L.*)

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ABSTRACT

Background: The Philippines is one of the countries with high prevalence of diabetes mellitus. Although there are several drugs marketed to control it, there are unwanted side effects. Knowing this, alternative options have been sought for to control the disease. **Aims and Objectives:** To evaluate the effects of aqueous extracts of *Cycas riuminiana* on the blood glucose and cholesterol levels of alloxan-induced diabetic mice. **Materials and Methods:** Aqueous crude leaf extract from *C. riuminiana* was administered to 6 and 30 ICR mice (*Mus musculus)* to determine whether there are toxic effects and effects on the blood glucose and cholesterol levels, respectively. The 30 mice were divided into six groups and were either treated with double distilled water, glimepiride, or the *C. riuminiana* aqueous extract. **Results:** After performing the acute oral toxicity, there were no deaths signifying that the extracts may be safe to administer in mice. After 28 days of treatment, statistical analyses indicated that the treatment groups had anti-hyperglycemic effects on the diabetic mice. The high-dose group had the lowest mean blood glucose values, which surpassed the hypoglycemic effects of the positive control group, which was treated with glimepiride. Furthermore, there was no difference between the control and the treatment groups. On the other hand, blood cholesterol values of the treatment and control groups were within the normal range after the 28 days study. **Conclusion:** The results of the study shows that *C. riuminiana* leaves have antidiabetic properties by lowering glucose and cholesterol levels at doses between 250 and 1000 mg/kg body weight.

KEY WORDS: Anticholesteremic; Blood Glucose; *Cycas riuminiana*; Hemtatology; Hypoglycemic Agents; *Mus musculus*

INTRODUCTION

Diabetes mellitus, characterized by hyperglycemia, is a metabolic disease, which causes defects in the production or utilization of the insulin produced by the pancreas.[1] The Philippines is one of the many countries dealing with diabetes mellitus with approximately 7.2% of people aged 20 and above affected by the disease.[2] The year 2000 saw diabetes being declared as a growing health problem in the West Pacific region including the Philippines. The prevalence for crude diabetes was 5.1% which meant a 54% increase from 3.3% in a similar population in Luzon in 1982.[3] The alarming rate of progression of diabetes cases worldwide called for a collective effort in dealing with the disease. At present, metformin (1,1-dimethylbiguanide hydrochloride), the only biguanide available in the market, is used to reduce hepatic glucose output while amplifying the glucose uptake of muscle tissues.[4] The mechanism of action of metformin is mediated by activating the liver kinase B1, which regulates adenosine monophosphate coactivator, a transducer of regulator protein 2, which ultimately results in the downregulation of gluconeogenic enzyme synthesis. Metformin, though effective when initially taken on its own, is often taken in combination with other antidiabetic drugs such as sulfonylureas, insulin treatments, thiazolidinediones, and gliflozins.[5] However,

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known side effects of metformin include lactic acidosis, weight loss, and renal dysfunction.[6]

The genus *Cycas,* which solely constitute the Cycadaceae family is comprised around 100 species that are widespread throughout China, Australia, Malaysia, India, Japan, Madagascar, and Polynesia.[7] There are 10 species of *Cycas* that are endemic to the Philippines, but among those, *Cycas riuminiana* is an endangered species, which can be found exclusively in the Luzon portion of the Philippines.[8] Due to *C. riuminiana* being classified as endangered and endemic to the Luzon part of the Philippines, research on this species has been limited. However, various studies on other *Cycas* species have shown medicinal properties among various species of the plant. A study conducted by Laishram et al.[9] which aimed to determine antidiabetic molecules found in *Cycas pectinata* griff., was able to identify two biflavonoids amentoflavone and 2,3-dihydromentoflavone that exhibited high inhibitory potency against alpha-glucosidase and alpha-amylase. The study was able to conclude that antidiabetic molecules found in *C. pectinata* were able to significantly reduce the blood glucose levels of streptozotocin-induced diabetic rats. In a similar study, Yokoyama et al.[10] were able to purify antimicrobial peptides from *Cycas revoluta* seeds with the use of a CM cellulofine column, ion-exchange high-performance liquid chromatography (HPLC), and reverse-phase HPLC. Of the three antimicrobial peptides designated, two were able to inhibit the growth of plant pathogenic fungi by up to 50% while the other antimicrobial peptide had little to no antimicrobial property at all. Kalpashree and Raveesha[11] screened the antibacterial activities of *Cycas circinalis* ovules against human pathogenic bacteria *Bacillus cereus, Staphylococcus aureus, Escherichia coli,* and plant bacteria *Xanthomonas axonopodis pv malvacearum*. Despite not having significant antibacterial properties against human pathogens, extracts from the plant ovule was able to exhibit significant antibacterial properties against plant pathogenic bacteria which makes the plant a suitable candidate for further work on antibacterial plants against plant pathogens.

With the growing need for alternatives to conventional medicine, the need to use medicinal herbs to treat various maladies has been increasing. In the Philippines, rural areas such as Batan and Guimaras are using a wide array of medicinal plants that can be found in their respective vicinities.[12,13] There are many people who opt for herbal medicine as they consider herbal medicine to be natural and inexpensive alternatives to conventional medicine that are more commonly used in urban areas. Despite having potential medicinal properties, herbal medicine can still trigger adverse side effects which call for further research on the possible side effects of herbal medicines.

With various studies on other species of *Cycas* plants having been found to be medically beneficial, *C. riuminiana* may also contain the same phytochemicals that can serve medicinal purposes. Establishing the effectivity of *C. riuminiana* as a possible medicinal plant could provide an affordable alternative to the different prescription anti-hyperglycemic drugs available, pave the way for other medical studies to screen the other possible medical properties the plant may have, and hopefully bring about further collective efforts to further the preservation of this plant.

MATERIALS AND METHODS

Procurement of Plant Sample

C. riuminiana were obtained within the vicinity of Pampanga State Agricultural University, Pampanga, Philippines with coordinates 15°21'81.87" North 120°69'36.26" East. 7 kg of leaves from various plants of the said species were collected. Representative samples were then deposited at the Herbarium at the De La Salle University with acquisition number DLSUH3512 and verified by Dr. Ezperanza Maribel G. Agoo.

Preparation of Plant Extracts

The samples were washed with tap water and air-dried for 3 days then pulverized with the use of a blender. About 500 g dry weight of the samples were soaked in 2.5 l of water for 3 days. The solution was then filtered with the use of a Muslin cloth, and the resulting filtrate was filtered once more with the use of a Whatman No. 1 filter paper and a glass funnel. The collected filtrate was collected, freeze-dried, and lyophilized to obtain powdered extracts. The extract was weighed, stored in a waterproof and airtight amber bottle at 4°C. The extract was then reconstituted using distilled water for administration through oral gavage to the animals.

Phytochemical Analysis

The collected *C. riuminiana* leaf extract was subjected to phytochemical screening in the Institute of Pharmaceutical Sciences, National Institutes of Health, University of the Philippines, Manila. The presence of carbohydrates, flavonoids, tannins, glycosides, alkaloids, steroids and terpenoids, saponins, and resins was tested.

Procurement of Animals

Thirty six 8-week-old male ICR mice weighing at least 20 g were obtained from the Food and Drug Administration, Alabang, Muntinlupa City, Philippines. Six animals will be used for the acute oral toxicity test while the 30 animals will be used for the antidiabetic tests. The sample size was determined based on the methods of Charan and Kantharia.

The mice were placed in individual standard cages and were allowed to eat commercial rodent food placed on feeders and distilled drinking water placed on water bottles.

The cages were washed twice a week and were lined with autoclaved paddy husk. Before the experiment, all mice were acclimatized for 7 days. They were placed in the De La Salle University Animal house with a maintaining temperature of 23°C and 55% humidity at a 12 h light: 12 h dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of De La Salle University (reference #2015-003) following the standard guidelines for animal care as recommended by the Philippine Association of Laboratory Animal Science and the Department of Agriculture Bureau of Animal Industry. The experiment from extraction to the administration of samples and extrapolation of results was performed from September 2015 to April 2016.

Acute Oral Toxicity Test

Acute oral toxicity tests were administered in 6 ICR mice. A dose of 5,000 mg/kg, which is the maximum that can be given to any animal subject, was administered orally to the subjects. The following responses were observed in the mice: Awareness, grooming, corneal reflex, pupillary reflex, gripping strength, skin color, urination, lactation, touch response, pain response, convulsion, tremors, writhing reflex, salivation, lacrimation, and hyperactivity. If death was observed, it was recorded from the time of administration. After testing the substance, food and water were not given for 2 h. The number of survivors was noted within 24 h then daily for 2 weeks. If deaths were observed, a lower dosage was used until no mortality will be monitored for 2 weeks.

Induction of Diabetes

A group of male ICR mice was abstained overnight for at least 8 h. Hyperglycemia was induced in each mice by administering 150 mg/kg alloxan monohydrate intraperitoneally in normal saline. The sham control group was given with normal saline intraperitoneally. After a week of inducing hyperglycemia, blood glucose was measured using an EasyTouch® glucose cholesterol uric acid (GCU) meter (Bioptik Technology, Inc., Taiwan). Mice with established hyperglycemia, those with blood glucose over 300 mg/dL, were included in the study.

Experimental Design

Thirty ICR mice were randomly assigned to six groups (n = 5). The different treatments were administered through oral gavage for 28 days (Table 1).

Blood Glucose and Cholesterol Collection and Testing

Blood was collected using the tail tip nicking method. Blood glucose levels were determined using an EasyTouch® GCU meter (Bioptik Technology, Inc., Taiwan) at baseline, and days 0, 14, and 28 after induction of diabetes. Blood cholesterol levels were determined using an EasyTouch® GCU meter (Bioptik Technology, Inc., Taiwan) at day 28.

Euthanasia

Each mice were euthanized by cervical dislocation and death was confirmed by the absence of righting reflex, corneal reflex, pupillary reflex, and pain withdrawal reflex.

Data Analysis

Repeated measures analysis of variance (ANOVA) was used to analyze the differences within groups in blood glucose and cholesterol levels (mean ± SD). One-way ANOVA was also used to check the differences between groups. The means were compared using Tukey's test to determine significant differences among the treatment groups at $P < 0.05$. All statistical analysis was performed using STATA v.12.

RESULTS

Phytochemical Analysis

Phytochemical analysis was performed to determine the presence of different phytochemicals present in the leaves of *C. riuminiana*. After analysis, results show that carbohydrates, reducing sugars, tannins, alkaloids, and saponins were present in the extract (Table 2).

Acute Oral Toxicity Test

An acute oral toxicity test was performed to determine any adverse effects that can be acquired from the plant extract. After inducing a dosage of 5000 mg/kg of the aqueous crude leaf extract of *C. riuminiana*, it shows that the response of five mice was normal according to their awareness, grooming, corneal reflex, pupillary reflex, gripping strength, skin color, urination, lactation, touch response, pain response, convulsion, tremors, righting reflex, salivation, lacrimation, and hyperactivity. There were no significant deaths observed for 14 days. According to OECD,[14] a dosage of 5000 mg/kg is only allowed if the results of the test have a direct relevance to the health of the animal.

Table 1: Experimental design of the study

Group	Treatment
Group 1 Sham control	Distilled water
Group 2 Negative control	Distilled water
Group 3 Positive control	Glimepiride
Group 4 Low-dose	250 mg/kg
Group 5 Mid-dose	500 mg/kg
Group 6 High-dose	1000 mg/kg

Antihyperglycemic Test

Three different doses of *C. riuminiana* leaf extract (250 mg/kg, 500 mg/kg, and 1000 mg/kg), glimepiride as the positive control 500 mg/kg and distilled water as the negative control 500 mg/kg were administered to 30 ICR mice (*Mus musculus)*. A total of four blood collections were done to quantify blood glucose levels (mg/dL) spanning 28 days (Figure 1 and Table 3).

Before administering alloxan monohydrate to the mice, the blood glucose and cholesterol levels of the mice were determined to be not more than the normal range which is 160 mg/dL[15] and 58 ± 0.04 mg/dL,[16] respectively. Accordingly, this indicates that before alloxan administration, the mice exhibited no hyperglycemic states.

Blood glucose levels were determined 4 days after inducing hyperglycemia. Blood glucose levels of mice across all treatment groups were compared to the baseline values, and all treatment groups were found to exhibit hyperglycemia with mean blood glucose values above 330 mg/dL. Comparing all treatment and control groups to the sham group where no alloxan was introduced, the treatment and control groups exhibited glucose values above the accepted normal value of 160 mg/dL indicating that the hyperglycemia exhibited by the mice was due to the effects of alloxan-monohydrate.

Table 2: Phytochemical analysis of *C. riuminiana* by the Institute of Pharmaceutical Sciences, National Institutes of Health, University of the Philippines Manila

Phytochemical	Indication
Carbohydrates	(+)
Reducing sugars	(+)
Flavonoids	(−)
Tannins	(+)
Glycosides	(-)
Alkaloids	(+)
Steroids and terpenoids	(−)
Saponins	(+)
Resins	(−)

C. riuminiana: Cycas riuminiana

On comparison of blood values collected at day 14 with the baseline values, the blood glucose levels of all treatment groups decreased except for the sham group, which exhibited an increase in blood glucose levels. The negative control and the high-dose group had the same mean blood glucose values.

At day 28, the high-dose group exhibited the lowest blood glucose values among all treatment groups. The high dose group was also the only treatment group to be significantly different from all the treatment groups. These results are suggestive that *C. riuminiana* extracts may be at par or better than glimepiride in terms of its hypoglycemic effects.

The blood glucose levels in all treatment groups declined throughout the study. The positive control served as a basis of comparison in determining the potential of *C. riuminiana* extracts to lower blood glucose levels while the negative control served as a basis that the decrease in blood glucose levels was either from the glimepiride or the *Cycas* extracts and that blood glucose tend to fluctuate since only water was only introduced to them. Based from Table 3, negative control had the highest blood glucose levels among the other groups after inducing alloxan monohydrate. At the end of the experiment, all treatment groups' and the positive control's blood glucose levels were lower than the values obtained after inducing alloxan before administering the treatments. This supports the claim that the decrease in blood glucose levels in all treatments may be due to the *C. riuminiana* leaf extract.

The cholesterol levels for the high- and mid-dose are significantly lower than the cholesterol level of the sham group. The cholesterol level of the low dose is not significantly different than the cholesterol level of the sham group. Cholesterol levels of treatment groups low, mid, and high dose groups do not have a significant difference with the cholesterol level of the positive control group. The normal cholesterol values for a male ICR mice are 98-186 mg/dL (Weibust).[16]

DISCUSSION

The decrease in blood glucose of mice after 28 days of treatment with *C. riuminiana* leaf extract may be attributed to the

Table 3: Blood glucose and cholesterol levels (mean±SD) of mice at different treatment levels

Group	Baseline (mg/dL)	Day 0 (mg/dL)	Day 14 (mg/dL)	Day 28 (mg/dL)	Cholesterol (mg/dL)
Sham	55.6±24.6	107.8±13.8	117±32.8^{b}	118.4±18.3^{a}	191.8±24.9^{b}
Positive control	119.8±53.8	445.2±112.3^{a}	309.4±158.9^{a,b}	196.8±227.9^{a}	146.2±42.2^{a,b}
Negative control	107±105.2	552±57.7^{a}	448.8±139.1^{a}	568.4±48.5^{b}	138.2±51.1^{a,b}
Low-dose	143.6±41.5	533.2±73.1^{a}	301.8±177.8^{a,b}	273.2±176.5^{a}	143.3±32.4^{a,b}
Mid-dose	154±63.5	531±122.^{7a}	313.2±92.2^{a,b}	328.6±163.1^{a,b}	122.4±35.2^{a}
High-dose	160.6±43.8	552±57.7^{a}	448.8±139.1^{a}	165.8±63.7^{a}	103±5.4^{a}

Means±SD with the same superscript within the same column are not significantly different at $P<0.05$. SD: Standard deviation

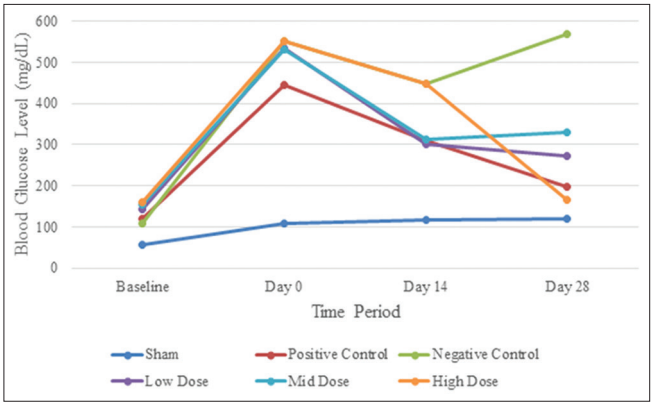

Figure 1: Blood glucose levels of each treatment and control groups across different time periods

collective work of the plant's different phytochemicals. After being subjected to phytochemical screening, *C. riuminiana tested* positive for the phytochemicals that are known to provide antihyperglycemic and antihypercholesterolemic properties, namely, saponins, tannins, and alkaloids.

The different phytochemicals found in the leaves of *C. riuminiana* have also been found in different *Cycas* species. A preliminary phytochemical screening of *C. circinalis* done by Babu et al.[17] found that alkaloids were present in the plant's leaves whereas Mourya et al.[18] found that *C. revoluta* leaves contained alkaloids, steroids, tannins, saponins, and sugars.

Alkaloids are nitrogen-containing secondary plant metabolites that are found in approximately 20% of vascular plant species and are frequently found in herbaceous dicot than in monocots and gymnosperms. Most alkaloids including pyrrolizidine alkaloids are toxic to some degree and can act as a defense mechanism in herbivore attack and microbial infection. The mode of action of alkaloids in animals is variable on a cellular level. Some alkaloids interfere with components of the nervous system especially the chemical transmitters. Others can affect protein synthesis, membrane transport, and miscellaneous enzyme activities.[19] Specifically, a study conducted in *Acanthus montanus* isolated alkaloids revealed that there is a 42.68% dose-dependent reduction in blood glucose levels of treated hyperglycemic rats compared with the control glibenclamide.[20] Mechanisms of action that has been attributed to alkaloid fractions may be due to its protein tyrosine phosphatase-1B inhibition activity in *Catharanthus roseus*[21] or its attenuating activity on glucose-6-phosphatase in *Capparis decidua*.[22] Berberine, a plant alkaloid, has been shown to significantly decrease total cholesterol, triglycerides, and low-density lipoprotein (LDL) – cholesterol, and an increase in high-density lipoprotein (HDL) -cholesterol levels. This effect is similar to that of statins currently used in the market without any major adverse effects.[23] Another study in rabbits on aerial part of *Anethum graveolens* L. presented the same results that alkaloids can prevent a rise in serum total cholesterol, triglycerides, LDL-cholesterol, very LDL (VLDL)-cholesterol, atherogenic index and significantly increase total antioxidant capacity and HDL-cholesterol.[24]

Tannins are heterogeneous high molecular weight polyphenolic compounds with the capability of forming complexes with proteins, polysaccharides, alkaloids, nucleic acids, and minerals. They are commonly found in fruits, and health benefits of intaking tannins have been known to decrease the frequency of chronic diseases.[25] Tannins are usually divided into two groups, namely, the hydrolysable tannins, and the flavonoid derived condensed tannins. Hydrolysable tannins are also divided into two groups which are the ellagitannins and gallotannins which includes meta-depsides.[26] Tannic acid, a specific form of tannin has been shown to decrease body weight, glucose, insulin, glycogen and creatine levels, which shows that they possess antihyperglycemic effects in streptozotocin-induced diabetic rats.[27] Tannin extracts analyzed for its antihyperglycemic activity has been found to be potential inhibitors of alpha-amylase and alpha-glycoside which can attribute to its action.[28] Plasma total cholesterol, triacylglycerol, LDL cholesterol, and VLDL concentrations were found to be lower in treated rats when given with dietary grade seed tannins. These effects were attributed to a higher lipoprotein lipase and hepatic lipase activities found in treated animals.[29] The administration of tannins from *Ficus racemosa* has been showed to lower blood glucose, total cholesterol, triglycerides, LDL and also significantly restore the insulin and HDL in the serum.[30]

Saponins are secondary metabolites that are widely distributed in kingdom plantae. They consist of glycosylated steroids, triterpenoids, and steroidal alkaloid. Saponins are also considered part of the defense system of the plants and have been included in a group of molecules named phytoprotectants. They exhibit antimicrobial properties and provide plants protection against insect attacks.[25] Plants that have been found with saponins have been used to treat diabetes, hepatitis, high blood pressure, high cholesterol, and physical stress.[31] Saponins have been found to possess antimicrobial[25] and hypoglycemic[32] properties. A study on *Entada phaseoloides* (L.) saponins showed that it was able to reduce fasting blood glucose and serum insulin levels, which in turn reduce hyperglycemia.[32] The effects of *Scoparia dulcis* (L.) on alloxan-induced diabetic mice has also been attributed to the extract being rich in saponins.[33] The antidiabetic mechanism of saponins stems from the phytochemical being able to stimulate AMP-activated protein kinase (AMPK) and insulin receptor (IR)/IR substrate-1/P13K/Akt signaling pathways which consequently increases the activity of the enzymes hexokinase and pyruvate kinase to promote glucose breakdown. AMPK signals act by stimulating glucose synthesis in skeletal muscles, reducing hepatic glucose production, and β-oxidation of fatty acids in adipose tissues.[34] Saponins have also been used to treat hypertension,

hypercholesterolemia, and physical stress.[31,35] Particularly, saponins have been implicated in preventing the absorption of cholesterol, which may be the reason for its cholesterol-lowering activity.[36]

The study focused on the antihyperglycemic and antihypercholesterolemic effects of *C. riuminiana* aqueous extracts. It can then be inferred based on the results that presence of phytochemicals, particularly saponins, alkaloids and tannins may work synergistically or independently to lower blood glucose and cholesterol levels. Further, isolation and characterization of the specific compounds are warranted to determine the specific mechanisms of action.

CONCLUSION

The aqueous crude leaf extract of the *C. riuminiana* was obtained using the freeze-dry method. 36 male ICR mice weighing at least 20 g were used in the experiment. Six were subjected for acute oral toxicity test. 30 were separated into 6 groups, namely, sham, positive control, negative control, low dose, mid dose, and high dose. The sham group and the negative control group was administered with distilled water. The positive control group was administered with glimepiride. The low dose, mid dose, and high dose were administered with the aqueous crude extract of *C. riuminiana* with 250 mg/kg, 500 mg/kg, and 1000 mg/kg, respectively. Water, extract, and glimepiride were administered using oral gavage every day for 28 days. Blood glucose was measured on days 0, 14, and 28 while blood cholesterol was measured on day 28 only.

The study showed that no significant deaths occurred after 14 days of the acute oral toxicity test. Making the aqueous crude leaf extract of *C. riuminiana* non-toxic. All treatment groups: Low-dose, mid-dose, and high-dose treated with 250 mg/kg, 500 mg/kg, and 1000 mg/kg of aqueous crude leaf extract of *C. riumiana,* respectively, were successful in lowering the blood glucose level of the ICR mice. The high-dose group exhibited the lowest blood glucose values among all treatment groups; even lower than the positive control group which was treated with glimepiride. The cholesterol values of all the treatment and control groups after the 28 days study are within the normal range.

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