Co-Administration of *Azadirachta indica a*nd *Calotropis procera* Methanolic Flower Extracts Ameliorate Hypozincaemia and Hyperglycaemia in Alloxanized Rats

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Abstract

Polyherbal formulations generally enhance the therapeutic action and reduce the concentrations of single herbs, thereby reducing adverse side effects. *Azadirachta indica* and *Calotropis procera* are multipurpose plants with much pharmacological potentials. The flower extracts of these plants were medicinally used in treatment of different diseases including diabetes. A number of studies have reported an association between diabetes and alterations in the metabolism of several trace minerals. These elements might have specific role in the pathogenesis and progress of the disease. Exploring antidiabetic plant combinations could be an alternative and new approach for effective diabetes treatment. The present investigation was carried out to study the potential antihyperglycemic effect of the combined methanolic flower extracts of these plants in alloxan induced hyperglycemic rats. The rats were divided into five groups, diabetes was induced using alloxan (150mg/kg b.w) and the treatments were carried out for a period of fourteen days. Phytochemicals screening, Fasting blood glucose levels, extracts and serum levels of zinc (Zn) and chromium (Cr) of the experimental rats were evaluated. Oral administration of methanolic extract of each plant (250mg/kg b.w) and the combination (1:1) of the two plants (250mg/kg b.w) shows significant reduction in blood glucose level ($p<0.05$), compared with diabetic control. Serum Zn and Cr showed significance increase at different time interval. The flower content of Zn and Cr was high in *A. indica* (0.29± 0.001mg/g for Zn) and (0.26± 0.06mg/g for Cr) than in *C. procera* (0.25 ±0.0014mg/g for Zn) and (0.21± 0.0014mg/g for Cr). This study revealed that combination of both extracts in 1:1 ratio has better hypoglycemic effect than the single plant treatment. Presence of phytochemical compounds (especially Saponins, Tannins, Phenols and flavanoids) as well as minerals (Zn and Cr) that act through various mechanisms may be responsible for the improved antihyperglycemic properties of the extracts and thus the usefulness of these plants in polyherbal diabetic medications.

Keywords: Hypoglycemia, Zinc, Chromium, Alloxan monohydrate, Co-administration

Introduction

Medicinal plants have been identified and used throughout human history. Indeed, chemical compounds in plants mediate their effect on human body through processes identical to those already well understood for chemical compounds in conventional drugs, thus herbal medicines do not differ greatly from conventional drugs in terms of mode of action. [1]

Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic syndromes in which there are high blood sugar levels over a prolonged period. Symptoms of high blood sugar include frequent urination, increased thirst and increased hunger. If left untreated, diabetes can cause many complications. Acute complications include diabetes ketoacidosis and nonketotic hyperosmolar coma.[2] Serious long term complications include cardiovascular disease, stroke,

Date received-29/09/2016 Date accepted- 26/10/2016

chronic kidney failure, foot ulcer and damage to the eyes. *Calotropis procera* and *Azadirachta indica* have been traditionally used by the people of Northern Nigeria to treat various diseases including DM.

The *Calotropis procera* has many medicinal properties due to the presence of numerous secondary metabolites. The milky latex of the plant is rich in lupeol, calotropin, calotoxin, and uscharidin, the latex protein.[3] The major phytochemicals viz. alkaloids, carbohydrates, glycosides, phenolic compounds/tannins, proteins and amino acids, flavonoids, saponins, sterols, acid compounds, resins in flower, bud, root of Calotropis has been screened.[3] The elements such as Al, As, Cu, Ca, Cr, Cd, Fe, K, Mn, Na, Pb, and Zn have been found in this medicinal plant in variable range. [4] It has also been reported by Silvania [5] and Khairnar *et al.* [6] that the plant has antidiabetic activity. The mechanism of action could be probably due to polyphenolic compounds which increase insulin activity and prevent oxidative damage, and thus, responsible for the hypoglycemic activity. [7]

Several researches have reported that *A. indica* significantly lowered blood glucose in alloxan and streptozotocin induced diabetic rats.[8] Major chemical constituents of *A. indica* are Terpenes and Limonoids. The major active components in the Limonoids are a zadirachtin, 3- deacetyl-3-cinnamoylazadirachtin, I-tigloyl-3-acetyl-II-methoxyazadirachtin, 22, 23-dihydro-23β-methoxyazadirachtin, nimbanal, 3-tigloylazadirachtol, 3-acetyl-salannoV nimbidioV margocin, margocinin, margocilin and others. *A. indica* has also been reported to have anti-hyperglycaemic and hypocholestromic effects in rabbits. [9,10,11] Quercetin, rutin, and nimbidin contained in *A. indica* are reported to be active components that contribute to its hypoglycemic effect. [12] Azadratchtin and nimbin was also reported to have antidiabetic activities due to blocking the epinephrine action and thus glycogenolytic.[13]

Zinc (Zn) is an essential micronutrient which has an important role in the functioning of hundreds of enzymes [14], in insulin metabolism and acts as an efficient antioxidant.[15] Zn is considered important mainly because it plays a major role in the stabilization of insulin hexamers and the pancreatic storage of the hormone[16] and it is an efficient antioxidant[17], while oxidative stress is considered to be a main component in initiation and progression of insulin resistance and diabetes.[18] However abnormal zinc metabolism has been suggested to play a role in the pathogenesis of diabetes and its complications.

Chromium deficiency is relatively common in patients with type 2 diabetes. The significance of Chromium as a trace nutrient is well documented and its function in the control of glucose and lipid metabolism has been claimed.[19] Studies have shown that chromium can facilitate or potentiate the action of insulin.[20]

A current model postulates that trivalent chromium might be the cofactor of a low-molecular-weight chromium-binding substance (LMWCr) or chromodulin.[21] Chromodulin is thought to enhance the cascade of signalling events induced by the binding of insulin to extracellular α-subunit of the insulin receptor (IR). Upon insulin binding, the tyrosine kinase domain of the intracellular β- subunit of the IR becomes activated and causes the phosphorylation of tyrosine residues in the β-subunit itself. Subsequently, IR activation triggers a series of rapid phosphorylation reactions that activate many downstream effectors, eventually resulting in an increase in glucose uptake and storage.[21] Chromodulin binds to the IR and up-regulate insulin signalling molecules, ultimately increasing the translocation of glucose transporters (GLUT-4) from cytosolic vesicles to the cell membrane.[22]

In this modern era, herbal formulations have gained greater importance than ever before, mainly due to their efficacy and availability, [23] as well as less side effects as compared to the synthetic drugs. [24] These advantages have led the people move toward herbal preparations, for disease treatment and prevention, as they are claimed to display synergistic, potentiative, and agonistic/antagonistic actions and

the mixture of species in them shows better therapeutic effect than either species on its own.[25] *C. procera* and *A. indica* have been widely investigated and reported for its potential as antidiabetic agents, with different mechanism of actions but the hypoglycemic effect of the combination of both plants has not been reported. Polyherbal formulations generally enhance the therapeutic action and reduce the concentrations of single herbs, thereby reducing adverse side effects. As the knowledge of Combination therapy is becoming wider, combination of different plants with different mechanism of actions is justified. This study aimed to determine the antidiabetic potential of the methanolic flower extracts of *Calotropis procera* and *Azadirachta indica* combination in alloxan induced diabetic rats.

Materials and Methods

Plants Sample Collection and Identification

Flowers of *Calotropis procera* and *Azadirachta indica* were collected from Bayero University, Kano, Gwale L.G.A of Kano State in the month of June, 2015. It was identified and authenticated at Herbarium unit, Botany Department, Bayero University, with reference voucher no.: BUKAKHAN0132 (*C. procera*) and BUKAKHAN0312 (*A. indica*). The flowers were removed from the stalk and dried under shade at room temperature before grinding using mortar and pestle and stored in plastic containers.

Experimental Animals Acquisition and Care

A total of twenty five (25) albino rats of either sex were kept in the animal room, department of Biological Sciences, Bayero University, Kano. The animals used in this study had an average weight of 110g and were kept in a well-ventilated cage in the animal house. The animals were exposed to alternate 12hrs of darkness and light each, and were fed with standard rodents feed and water throughout the course of the experiment. All aspects of animal care complied with the ethical guidelines and technical requirements approved by the institutional animals ethics committee.

Preparation of Extracts

The powdered plant material (120g) of each plant (*Calotropis procera* and *Azadirachta indica* flower) was extracted each with 500ml of methanol for 3days (72hrs) at room temperature with constant shaking using incubator shaker. The extract was filtered and the filtrate was concentrated at 40^0C under reduced pressure in a rotary evaporator. The concentrate was then dried in desiccators to get the crude extract.[26]

Induction of Diabetes Using Alloxan

All animals were allowed to acclimatize for two weeks after which they were fasted for 12hrs and normal blood glucose level was determined. The animals were fasted for 12hrs the next day and a single dose of 150mg/kg of alloxan monohydrate freshly dissolved in normal saline was injected intraperitoneally.[27]

Phytochemicals Analysis

Determination of saponins, tannins, glycosides and alkaloids by the Method of Obasi *et al*. [28]; flavonoids, carbohydrate, phenol were determined by the method of Audu *et al*. [29]

Experimental Design

Grouping of Animals

Experimental rats were divided into five groups of five animals each and treated with the plants extract via oral administration of 250 mg/kg [9] for 14 days as follows.

Group I: Negative control received food and water only for 14 days.
Group II: Positive control were administered with 150mg/kg average body weight alloxan and were given food and water only for 14 days.
Group III: Administered with alloxan and 250mg/kg of methanolic flower extract of *A. indica* for 14 days.
Group IV: Administered with alloxan and 250mg/kg of methanolic flower extract of *C. procera* for 14 days.
Group V: Administered with alloxan and combination of 250mg/kg of methanolic extract of *C. procera* and *A. indica* at 1:1 ratio.

Fasting blood glucose levels were measured on the 48th hour after administration of alloxan which marked the zero day of the experiment. Only rats

with fasting blood glucose greater than 250mg/dl were considered to be hyperglycemic and were grouped in the experiment. Treatment (as stated under grouping above) commenced on day one of the experiment.

Determination of Serum Glucose

At 0 (zero) and after every 3 days (0^{th}, 3^{rd}, 7^{th}, 11^{th} and 14^{th} day of the experiment), rats in all 5 groups were fasted for 12hrs and fasting blood glucose was determined using *Accu-Chek* mobile blood glucose meter kit- Roche diagnostics. On the day of sacrifice i.e. the 14th day, a maximal amount of blood was collected in lithium heparin tubes. The collected blood sample were immediately centrifuged at 3500rpm for 10min, the serum separated was collected in fresh plain tubes and was then digested for analysis of serum zinc and serum chromium.

Methods

Digestion of Extract and Serum for Elemental Analysis

Each extract (5g) was put in a crucible and heated in an electronic furnace to become ash. 10ml of HNO_3 and $10cm^3$ of HCl was then added and heated in an electro-thermal heater until evolution of white fumes marking the end of the digestion process. The digest is then filtered through Whatmann No 1 filter paper and diluted to 50ml mark with distilled water. The heavy metals were determined using the atomic absorption spectrophotometer.[30] Digestion of serum for elemental analysis was carried out according to the method of Cui *et al*. [30]

Results and Discussion

Result

Phytochemical screening of methanol extracts of *C. procera* and *A. indica* presented in table 1 shows the presence of alkaloids, flavonoids, glycosides, saponins, phenols, and carbohydrate in the plants extract while tannin is only present in *C. procera.*

Table 1: Phytochemical Composition of Methanolic Extract of *Calotropis procera* and *Azadirachta indica* flowers

Phytochemicals	*Calotropis procera*	*Azadirachta indica*
Alkaloids	+	++
Flavonoids	+	+
Tannins	+	-
Saponins	+	+
Glycosides	+	+
Phenols	+	+
Carbohydrates	+	+

Key: += Present = Absent

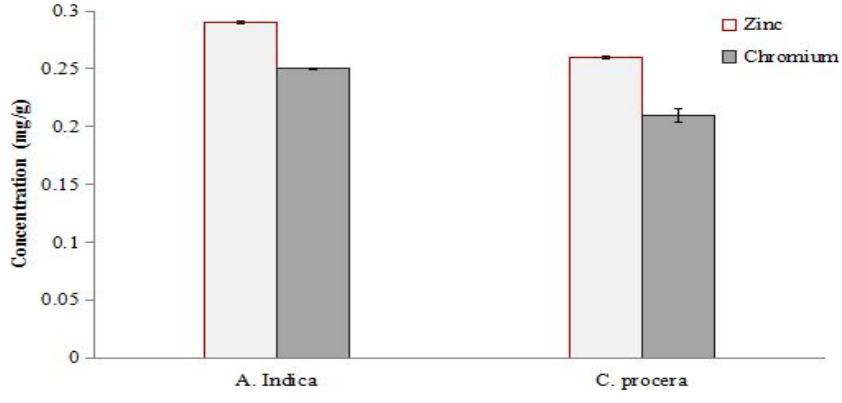

Figure 1: Content of Zinc and Chromium in the Methanolic Flower Extracts of *C. procera* and *A. indica* Results are presented as means ± SD; n=3.

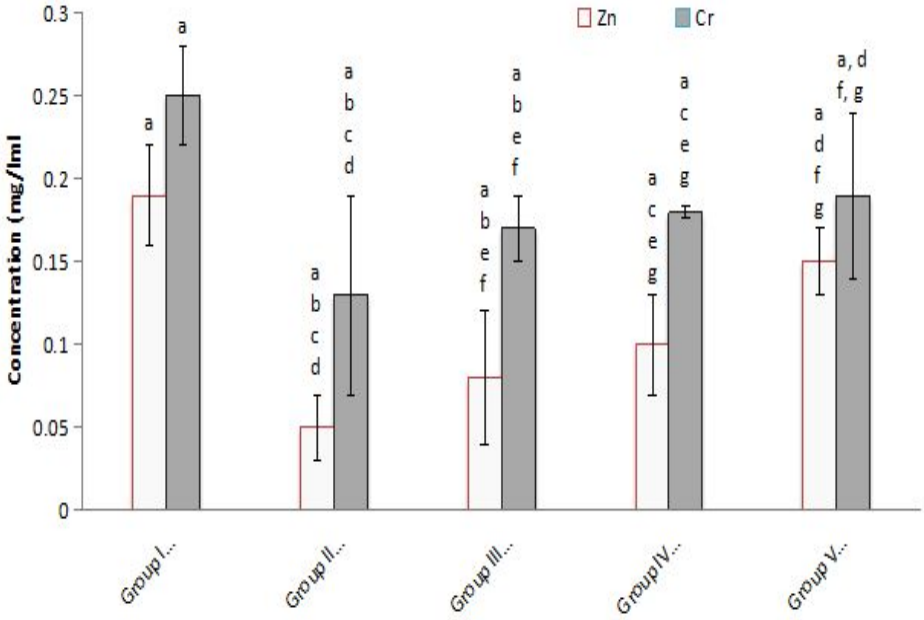

Figure 2: Serum concentration of Zn and Cr in Alloxan-induced hyperglycemic rats after 14 days treatment; Results are presented as means ± SD; n=3; Values bearing the same superscripts indicate significant different when compared with each other (P<0.005).

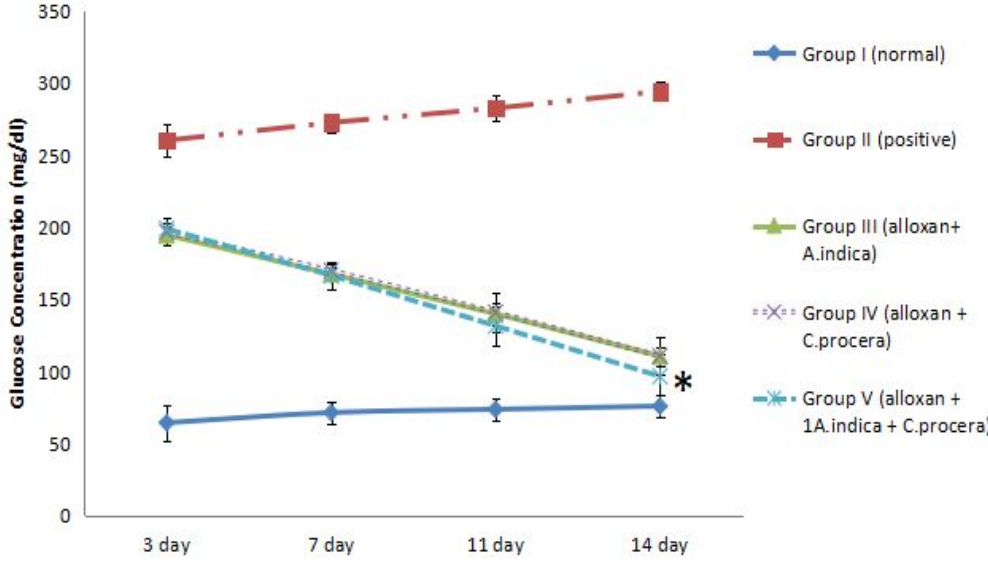

Figure 3: Serum Fasting Blood Sugar Levels of Alloxan-induced Diabetic Rats Administered with (250mg/kg) *A. indica* and *C. procera* Flower extracts; Results are presented as means ± SD; n=3; * indicate significant different when compared with group II (P<0.005).

Discussion

Preliminary phytochemical investigation of methanol extracts of *A. indica* and *C. procera* flowers have revealed the presence of alkaloids, flavonoids, saponins, glycosides, phenols, carbohydrates and tannins (in *C. procera*) while tannin is absent in *A. indica*. This finding was in conformity with the work of Suresh *et al*.[3] in *C. procera* flowers. The phytochemicals have the ability to neutralize the free radicals or reactive oxygen species or oxidants generated by alloxan responsible for the onset of the diabetes in rats. Phytochemical screening of these plants showed the presence of flavonoids and other polyphenolics which may be responsible for their antioxidant effect. Plant polyphenols were reported, also to inhibit alpha-amylase and sucrase activity and thus decreasing postprandial glycemia. [31,32] Phenolic compounds have an electron donor capability and are readily oxidized to form phenolate ion or quinone, which is an electron acceptor [33], thus, they have the ability to block or enhance specific enzymes responsible for digestion of carbohydrates. [34, 33, 35, 36]

Hyperglycemia results in overproduction of oxygen free radicals, which contributes to the progression of diabetes. The development of complications during diabetes is also associated with oxidative stress. Alloxan act as a generator of reactive oxygen –species which are thought to be the major cause of B-cells dysfunction in diabetic animals induced by alloxan.

Tannins have antioxidative effect, oxidative stress is one of the important factors in tissue injury in diabetes mellitus and hence potent antioxidants may protect beta cells and increase insulin secretion. [36] Hagerman *et al*.[34] reported that due to their binding ability with protein and carbohydrates, tannins can inhibit digestive enzymes and reduce the bioavailability of different proteins. Saponin present in some medicinal plants has been described to demonstrate glucagon decreasing effect which may enhance glucose utilization and lower blood glucose. It was equally reported that saponins stimulates insulin release from pancreas. [35] It also delays the transfer of glucose from stomach to the small intestine and subsequently glucose absorption.

The content of zinc and chromium in the flower extracts of *A. indica* was found to be 0.29 ± 0.0007mg/g and 0.25 ± 0.001mg/g respectively while for *C. procera*, Zinc (Zn) and Chromium (Cr) were found to be 0.26 ± 0.001mg/g and 0.21 ± 0.006mg/g respectively which shows that both zinc and chromium were high in *A. indica* than in *C. procera* (Fig.1)

Also, from figure 2, the serum Zn and Cr concentration in group 1 was found to be statistically significant ($p<0.05$) when compared with all the groups. The methanol flower extracts of *A. indica* and *C. procera* showed relatively high levels of zinc and chromium. Diabetes has been shown to be associated with abnormalities in the metabolism of Cr, Zn, and Mg. Impairment of Cr and Zn status has been reported as aggravating factors in the progression of diabetes. Present study reports that there is a significant decrease in serum concentrations when compared to control.

The combination of these plants may have increased the Zinc and Chromium content in the serum of the experimental animals compared with diabetic control. Zinc plays an important role in insulin metabolism and acts as an efficient antioxidant. [15] Therefore, serum levels of zinc are usually low in diabetic patients[37] as observed in diabetic control (Group II; Figure 2) when compared with test groups (Group III-V). The study showed that combined dose of *C. procera* and *A. indica* have elevated serum zinc levels which are higher than the individual plants and hence may justify the hypoglyceamic effect of the plants. Zinc affects the antigenic properties of insulin and the binding of insulin to hepatocytes membranes and a deficiency can lead to increased insulin resistance and hyperglycemia.[37] It is necessary for optimum insulin action as zinc is an integral constituent of insulin. It has also been reported that Zn has insulin-like effects that cause enhanced glucose uptake by inhibiting glycogen synthesis. Studies have shown that diabetes is accompanied by hypozincemia. Elevated glucose in turn produces

hyperzincuria. Low zinc leads to poor or slowed wound-healing common in diabetic patients; zinc is required for insulin storage and cellular binding, although high concentrations can lead to a reduction in insulin release. [38] The low gastrointestinal absorption and high urinary excretion of Zn in diabetic patients may explain hypozincemia seen in the DM group.

Chromium as part of a compound known as 'glucose tolerance factor' (GTF) is needed for appropriate metabolism, and insulin receptor glucose use. [39] Also Chromium decreases the level of blood glucose which functions in the control of glucose and lipid metabolism.[19] It has been observed that serum chromium levels significantly increases after the administration of plant extracts for the period of 14days and hence may decrease blood glucose levels as reported by Jeejeebttoy. [19] Studies have shown that chromium can facilitate or potentiate the action of insulin. [20] As such it may improve blood glucose levels in individuals with a tendency toward blood glucose fluctuations associated with diabetes (hyperglycemia).The biological activity of chromium (Cr) depends on its valency and the chemical form of the complex of which it is a part. In such cases Cr supplementation becomes absolutely essential.[40]

The blood glucose level of group I shows significantly ($p<0.05$) lower values when compared with all the groups; group II also shows significant increase when compared with group III, IV and V. Blood glucose level of group III animals after administration of 250mg/kg of *A. indica* flower extract decreases significantly ($p<0.05$) when compared to group IV (250mg/kg *C.procera*) group V (250mg/kg of *A. indica* and *C. procera)*. This might be due to the phytochemicals, Zinc and Chromium present in both plants and are known for hypoglycemic activities. *A. indica* and *C. procera* flowers have various biological and pharmacological activities including antioxidant. *A. indica* significantly lowered blood glucose in alloxan induced diabetic rats. *C.procera* also significantly lowered blood glucose level in alloxan induced diabetic rats.[5] The screening of the *C. procera* and *A. indica* flowers extracts revealed the presence of saponins and others with antioxidative potential such as phenols and flavonoids. These active compounds possess high antioxidant activity and are fingered in lowering blood glucose level and improving the morphology of islets of Langerhans and β-cells.[41] Oxidative stress plays a role in the pathogenesis of diabetes mellitus.[42] This causes the increase of insulin production and decreased blood glucose levels. These active compounds also have protection against oxidative damage and preserves pancreatic beta cell integrity.[43] This study shows that the hypoglycemic effect of the combination of *A. indica* and *C. procera* flower extracts is better than that of single treatments (Figure 3). The various active compounds (phytochemicals) and mineral elements (especially zinc and chromium) in the extract of both plant flowers may have collectively increased the hypoglycemic effect of the co-administered formulation as seen in the lowered FBS levels in the 7^{th}, 11^{th} and 14^{th} days of the study.

Conclusion

From the result of this study, the methanol flower extracts of *A. indica* and *C. procera* showed hypoglycemic effect on alloxan induced hyperglycemic experimental rats, individually as well as the combination which may be due to various phytochemicals and mineral elements (especially zinc and chromium) present in both plant extracts that have variety of biochemical and pharmacological functions. The combined effect of the plants is more potent in reducing blood glucose level. Because of the cost and toxicity of synthetic drugs used to treat diabetes, it is therefore essential to use bioactive plant extracts as an alternative; which are less toxic and less expensive and hence, the 1:1 combination of the flowers extracts of both plants may be recommended as anti-diabetic formulation and potentially be developed as antidiabetic agent as it possesses blood glucose lowering activities than the separate plant flowers extract.

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