

Full Length Research Paper

Effect of solvent extracts of *Colatropis gigantea* leaf on oxidative parameters in diabetic rabbits

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The effect of *Colatropis gigantea* leaf extract was studied on alloxan-induced hyperglycaemic rabbits to evaluate the possible hypoglycaemic and antioxidant properties of *Colatropis gigantea* (*C. gigantean*) in diabetes mellitus. Significant decrease ($p < 0.05$) in the thiobarbituric acid reactive substance (TBARS) was observed in diabetic rabbits administered with the acetone fraction of *Colatropis gigantea* (*C. gigantean*) leaf when compared to diabetic control group. Consistent significant increases ($p < 0.05$) were noticed in the activities of erythrocyte antioxidant enzymes; catalase, superoxide dismutase, and glutathione peroxidase in diabetic rabbits treated with acetone fraction of *C. gigantea* leaf compared with diabetic control group, although higher activities of these enzymes were observed in diabetic rabbits treated with glibenclamide (reference drug) when compared to the group administered *C. gigantea* extract. It was also observed that *C. gigantea* extract had significant increase in vitamin C and protein concentrations when compared with untreated group. These results suggest that acetone extract of *C. gigantea* leaves possess hypoglycaemic and antioxidant properties.

Key words: *Colatropis gigantean*, TBARS, antioxidant enzymes; vitamin C, malondialdehyde (MDA), diabetic rabbits.

INTRODUCTION

Diabetes mellitus is the most important disease involving the endocrine pancreas. Its major manifestations include disordered metabolism and inappropriate hyperglycaemia. Currently there are over 150 million diabetics worldwide and this number is likely to increase to 300 million or more by the year 2025 due to increase in sedentary lifestyle, consumption of energy rich diet, and obesity (Yajnik, 2001). While management of diabetes mellitus includes diet, exercise, oral hypoglycaemic agents, and insulin. These treatments do not effectively prevent the complications of diabetes like nephropathy,

neuropathy, cataract, and hypertension (Palumbo, 2001). It is well known that in diabetes, oxidative stress has been found to be mainly due to an increased production of oxygen free radicals and a sharp reduction of antioxidant defences: catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) (Oberley, 1988). In addition, there is a relationship between diabetes and impairment of lipid metabolism; high density lipoprotein (HDL) protects low density lipoprotein (LDL) oxidation, and this protection is impaired in diabetic cases (Sharpe et al., 1998). The antioxidant

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activity of high density lipoprotein (HDL) depends on its associated antioxidant enzyme paraoxonase (PON) (Boemi et al., 2001).

Diabetes mellitus is a widespread disease with great social impact. It is a syndrome, initially characterized by a loss of glucose homeostasis resulting from defects in insulin secretion and insulin action, both resulting in impaired metabolism of glucose and other energy-yielding fuels such as lipids and protein (Scheen, 1997). Oxidant free radicals play a relevant role in the etiology and pathogenesis of a variety of diseases such as diabetes mellitus, cancer, hypertension, and cardio vascular diseases and are considered to be the principle causative agents of aging (Jeon et al., 2002). Diabetes mellitus and its sequels, neuropathy and angiopathy, are conditions in which free radicals are involved both in human and in the experimental model. The increase in oxygen free radicals in diabetes could be primarily due to increase in blood glucose levels, which upon autoxidation generate free radicals and secondarily due to effect of diabetogenic agents (alloxan) (Szkudelski et al., 2001).

In diabetes, hypoinsulinemia increases the activity of the enzyme, fatty acyl coenzymes (coenzyme A oxidase), which initiate β -oxidation of fatty acids resulting in lipid peroxidation (Baynes, 2000). Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity, and changing the activity of membrane-bound enzymes (Baynes, 2000). Its products (lipid radicals and lipid peroxide) are harmful to the cells in the body and are associated with atherosclerosis and brain damage. In diabetic rabbits, increased lipid peroxidation was associated with hyperlipidemia. Liver, an insulin dependent tissue that plays a pivotal role in glucose and lipid homeostasis is severely affected during diabetes mellitus.

Despite the great strides that have been made in the understanding and management of diabetes, the disease and disease related complications are increasing unabated (Tiwari and Madhusudana, 2002). In spite of the presence of known anti-diabetic medicine in the pharmaceutical market, remedies from medicinal plants are used with success to treat this disease (Bhattaram et al., 2002). Many traditional plants are used throughout the world for the treatment of diabetes mellitus. Plant drugs and herbal formulation (Bhattacharya et al., 1997) are frequently considered to be less toxic and freer from side effects than synthetic one. Based on World Health Organization (WHO) recommendations for hypoglycemic agents of plants origin used in traditional medicine are important. The attributed anti-hyperglycemic effect of these plants is due to their ability to restore the function of pancreatic tissues by causing an increase in the insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in the insulin dependent process. Treatment with herbal formulations has an effect on protecting β -cells and smoothing out fluctuation in glucose levels (Elder, 2004; Jia et al., 2003). There is

limited biological knowledge on the specific modes of action in the treatment of diabetes but most of the plants have been found to contain substances like glucosides, alkaloids, terpenoids, flavonoids etc. that are frequently implicated as having anti-diabetic effect (Loew and Kaszkin, 2002).

MATERIALS AND METHODS

Plant

The leaves of the plant *Colatropis gigantea* were obtained from Ogadimma research farm in Effiom, Ebonyi State, Nigeria. The plant was identified at Department of Botany, University of Nigeria, Nsukka with the authority of taxon; L.W.T Aiton. The plant was selected for evaluating its anti-diabetic activity based on its traditional/local use in the management of diabetes and other ailments.

Animals

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland. Healthy, adult male rabbits with an average body weight of 700 ± 50 g obtained from the animal house of Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria, were used for the study. The mice used for LD₅₀ determination were 4 to 6 weeks old with average weight of about 25.50 ± 0.50 g. The animals were housed at room temperature ($25 \pm 3^\circ\text{C}$) with a 12 h reverse light cycle and had free access to water and food (standard laboratory diet).

Experimental design

The study was made up of two phases.

Phase 1

Phase 1 was the screening phase at which the most potent fractionated extract amongst the fractions was noted and used in the second phase.

The animals were fasted for 24 h after which the blood sugar levels were determined. Diabetes was induced by slow intraperitoneal injection of 1% solution of alloxan (200 mg/kg body weight) in normal saline and administered within few minutes of preparation. Diabetic state was confirmed after three days using glucometer. The animals were administered with the extract twice daily intraperitoneally at a dose of 300 mg/kg body weight for five days. Screening was done by estimating the fraction that reduced the elevated blood glucose level close to normal using glucometer.

A total of twenty seven (27) rabbits were used and was divided into nine groups:

Group 1: Normal rabbits fed on normal rabbit chow and water *ad libitum*.

Group 2: Diabetic rabbits not treated and given water *ad libitum*.

Group 3: Diabetic rabbits treated with glibenclamide (standard) and water *ad libitum*.

Group 4: Diabetic rabbits treated with n-hexane fraction and water *ad libitum*.

Group 5: Diabetic rabbits treated with chloroform fraction and water *ad libitum*.

Group 6: Diabetic rabbits treated with ethylacetate fraction and water *ad libitum*.

libitum.

Group 8: Diabetic rabbits treated with acetone fraction and water *ad libitum*.

Group 9: Diabetic rabbits treated with methanol fraction and water *ad libitum*.

Phase 2

This phase of the study was made up of four (4) groups of three (3) rabbits each. Diabetic induction was done as described in the phase 1. Treatment was administered twice daily intraperitoneally at a dosage of 300 mg/kg body weight for five (5) days. Blood samples were collected through ear vein for biochemical analysis. Liver and pancreatic tissues were dissected out, washed in normal saline and kept in formal calcium removed for histopathological study. A total of twelve rabbits were used and divided into groups.

Group 1: Normal rabbit fed on normal rabbit chow and water *ad libitum*.

Group 2: Diabetic rabbits not treated and water *ad libitum*.

Group 3: Diabetic rabbits treated with Glibenclamide (standard) and water *ad libitum*.

Group 4: Diabetic rabbits treated with most active fraction (acetone fraction) and water *ad libitum*.

Plant treatment

Fresh leaves of *C. gigantea* were dried under room temperature, crushed and 500 g of the powder soaked in 200 ml of analytical grade of methanol for 48 h, then filtered and was evaporated with rotary evaporator at room temperature to obtain the crude extract.

Extraction procedure

The crude extract was subjected to fractionation using different organic solvents. It was first extracted using n-hexane. The n-hexane soluble fraction was obtained and concentrated using rotary evaporator at an optimum temperature of 25°C. The resulting residue (residue A) was dried and then fractionated using chloroform. The soluble chloroform fraction was concentrated using rotary evaporator and obtained chloroform extract while the resulting residue (residue B) was subsequently fractionated using ethyl acetate. The ethyl acetate soluble fraction was concentrated using rotary evaporator and an insoluble residue (residue C). The insoluble residue obtained was re-suspended in acetone, filtered and the filtrate concentrated. The residue obtained (residue D) was further fractionated using methanol. The methanol soluble fraction was concentrated and the methanol concentrate was obtained while the insoluble portion obtained was dried; this was soluble in water.

Determination of yield of extract

$$\% \text{ Yield} = \frac{\text{Weight (g) of Extract Evaporated}}{\text{Weight (g) Ground Sample Pulp}} \times 100$$

Induction of diabetes

Phase 1

after 12 h of fasting were injected intraperitoneally (i.p.) with 200 mg/kg body weight of freshly prepared alloxan monohydrate in normal saline (Yanarday and Colak, 1998). The animals were fed with Bendel Feed and Flour Mill Limited Pelletized Guinea Growers mash. After three days, triplicate determination of blood sugar concentrations were carried out using glucometer. Blood sugar concentrations found to be 150 mg/dl and above were used for determination of hypoglycemic effects of *C. gigantea* leaves extract. The diabetic rabbits were divided into eight groups of two animals each. The extract dissolved in the normal saline and was injected intraperitoneally.

Group 1 represented the control group, while group 2 represented diabetic not treated as described in the experimental design. Group 3 received glibenclamide as standard hypoglycaemic agent. Groups 4 to 9 received different fraction of the extract at the dose of 300 mg/kg body weight twice daily for five days. After five days treatment, Groups 4 to 9 were screened down to one group using blood sugar level as a marker that determined the most active fraction.

Phase 2

Twelve rabbits having blood sugar concentration of 60 to 90 mg/dl after 12 h of fasting were injected intraperitoneally (i.p.) with 200 mg/kg body weight of freshly prepared alloxan monohydrate in normal saline. The animals were fed with Bendel Feed and Flour Mill Limited Pelletized Guinea Growers mash. After 3 days, blood sugar was determined using glucometer. Blood sugar levels were found to be 150 mg/dl and above. The diabetic rabbits were divided into 4 groups of 3 rabbits each. Group 1 represented normal control, group 2 represented diabetic untreated, group 3 represented diabetic treated with glibenclamide (reference drug) while group 4 received the acetone fraction (most active fraction). After 5 days treatment, blood samples were collected through the ear veins of the animals for oxidative parameter assay.

Determination of lipid peroxidation concentration

The concentration of lipid peroxidation product malondialdehyde (MDA) was determined by the method described by Wallin et al. (1993).

Principle

The principle for the estimation is based on the fact that thiobarbituric acid (TBARS) reacts with malondialdehyde (MDA) to give a red or pink colour, which absorbs maximally at 532 nm.

Assay of glutathione peroxidase (GPX) activities (randox commercial kit)

The assay of GPX activities was determined by the methods described by Paglia and Valentine (1967).

Principle

GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. This method is based on that of paglia and valentine. In the presence of Glutathione Reductase (GR) and Nicotinamide adenine dinucleotide phosphate (NADPH), the oxidized glutathione (GSSG) is immediately converted to the

Table 1. Effect of solvent fractions of *C. gigantean* leaf on blood glucose level

Treatment Groups	Mean Fasting blood sugar level (mg/dl)	Mean sugar level (mg/dl) after induction	Mean sugar level (mg/dl) after treatment	% Maximum reduction
Control	95.00±4.24	—	—	—
Diabetic not treated	84.00±2.83	249.00±1.41	236.00±2.83	5.20
Diabetic treated with standard drug	96.50±0.71	153.00±1.41	112.00±4.24	26.80
Diabetic treated with n-hexane fractions	87.00±1.41	152.00±5.66	150.00±2.83	1.30
Diabetic treated with chloroform fractions	88.50±0.71	204.50±2.12	203.50±0.71	0.75
Diabetic treated with ethylacetate fractions	74.50±4.95	174.00±5.66	166.00±1.41	4.60
Diabetic treated with butanol fractions	98.50±0.71	307.50±0.71	290.50±20.51	5.53
Diabetic treated with acetone fractions	95.50±3.54	153.00±7.07	127.50±0.71	16.99
Diabetic treated with methanol fractions	74.00±5.66	165.00±7.07	281.00±7.07	14.70

The values are mean ± standard deviation of the triplicate determination (n = 3).

Assay of catalase (CAT) activities

The assay of CAT activities was determined by the method described by Aebi (1984).

Principle

The ultraviolet absorption of hydrogen peroxide can be easily measured at 240nm. On the decomposition of hydrogen peroxide with CAT, the absorption decrease with time and from this decrease CAT activity can be measured.

Assay of superoxide dismutase (SOD) activities

The assay of SOD activities was determined by the method described by Fridovich (1989).

Principle

The ability of superoxide dismutase to inhibit the autoxidation of adrenaline was the basis of the SOD assay. Superoxide generated by the xanthine oxidase reaction is shown to cause the oxidation of adrenaline to adrenochrome. The yield of adrenochrome produced per superoxide introduced increases with pH (Valerino and McCormack, 1971) and also with increasing concentration of adrenaline. This led to the proposal that autoxidation of adrenaline proceeds by at least two distinct pathways: one of which is a free radical chain reaction involving superoxide radical and hence could be inhibited by SOD.

Determination of vitamin C concentration

The concentration of vitamin C was determined by the method described by Goodhart and Shils (1973).

Principle

This method involves the oxidation and conversion of ascorbic acid to diketogluconic acid in strong acid solution. A diphenylhydrazine is formed by the reaction sulphuric acid with 2, 4-dinitrophenylhydrazine. Cupric ions act as the oxidizing agent, followed by hydrazone formation. The hydrazone dissolves in strong sulphuric acid solution to produce a light red colouration,

whose intensity gives a measure of the concentration of ascorbic acid. The addition of thiourea as a reducing agent adds specificity by avoiding interference from non-ascorbate chromogens.

Statistical analysis

Data were expressed as mean ± standard error of mean and analysed statistically using one-way Analysis of Variance (ANOVA) and Student's t-test. A probability value of $p < 0.05$ was determined to be statistically significant. The means were separated using Duncan Multiple Test. The Statistical Products and Service Solutions (SPSS) version 20 was used for this analysis.

RESULTS

Percentage yield of the extract

The extraction of 500 g of the chipped dry leaves of *Colatropis gigantean* in methanol gave 30 g (6%) yield of the starting material before fractionation.

Effect of the fractionated extracts of *C. gigantea* leaves on blood glucose level in diabetic rabbits

The data in Table 1 shows the respective effects of 300 mg/kg body weight of different fractionated extract on mean fasting blood glucose level of diabetic rabbits. The effects on the blood glucose levels was the basis of determining the most potent fraction of *C. gigantean* leaves. Acetone fraction significantly ($p < 0.05$) lowered blood glucose levels of diabetic rabbits when compared to other solvent fractions.

Effect of acetone fraction of *C. gigantean* leaf extract on plasma MDA concentration

A significantly ($p < 0.05$) elevated level of MDA (Figure 1) concentration was observed in diabetic untreated rabbits when compared to group treated with acetone fraction.

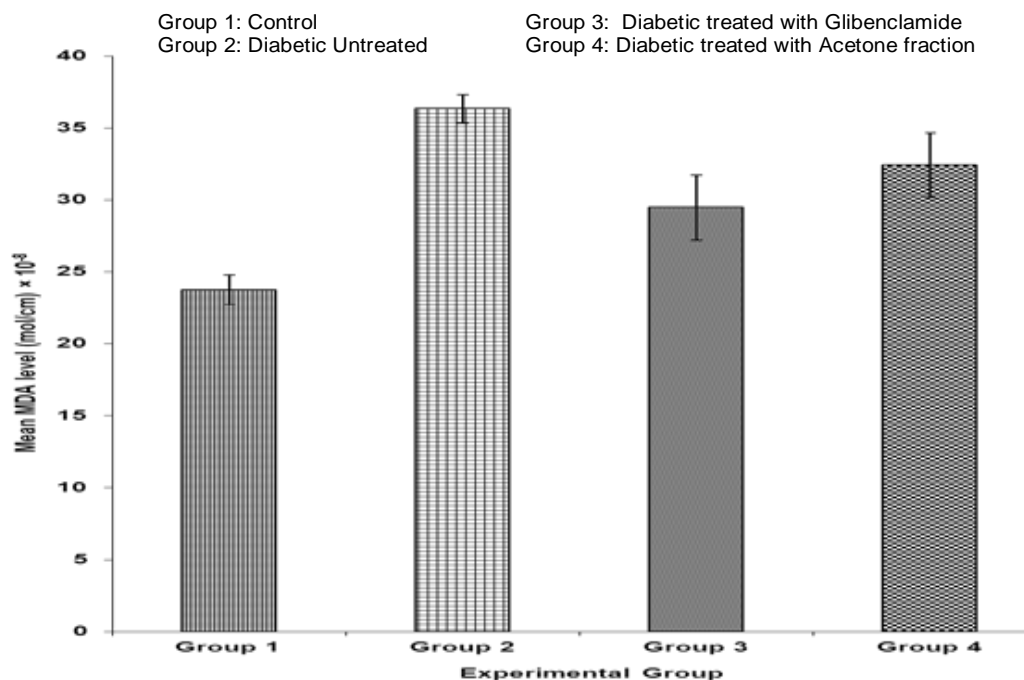


Figure 1. Effect of acetone extract of *C. gigantea* leaf on MDA concentration in rabbits.

treated with acetone fraction.

Effect of acetone fraction of *C. gigantea* leaf extract on CAT activity

As shown in Figure 2, there were significant differences ($p < 0.05$) in the CAT activities in normal and untreated diabetic groups when compared to diabetic group treated with acetone fraction of *C. gigantea* leaf extract. However the activity of CAT in diabetic rabbits treated with acetone fraction was lower than the diabetic control rabbits and diabetic rabbits treated with glibenclamide (reference drug).

Effect of acetone fraction of *C. gigantea* leaf extract on superoxide dismutase activity

The activities of superoxide dismutase (Figure 3) were significantly higher ($p < 0.05$) in diabetic treated with acetone fraction of *C. gigantea* leaf extract when compared to diabetic untreated group. Significant increase ($p < 0.05$) in the activities of SOD was observed in the diabetic group treated with glibenclamide compared to the diabetic group treated with *C. gigantea* leaf extract.

Effect of acetone fraction of *C. gigantea* leaf extract on glutathione peroxidase activities

GPX activity as shown in Figure 4 was significantly ($p < 0.05$) higher in diabetic rabbits treated with acetone fraction of *C. gigantea* when compared to diabetic untreated group. There was also significant ($p < 0.05$) increase of GPX activity in diabetic rabbits treated with glibenclamide when compared to group treated with acetone fraction of *Colatropis gigantea* leaf extract.

Effect of *C. gigantea* leaf extract on vitamin C concentration

A significant ($p < 0.05$) increase of vitamin C concentration, as shown in Figure 5, was observed in diabetic rabbits treated with *C. gigantea* leaf extract when compared to diabetic untreated rabbits. However, the group treated with glibenclamide (standard drug) elicited remarkable increase in vitamin C concentrations when compared to untreated group.

DISCUSSION

Diabetes mellitus is probably the fastest growing metabolic disease in the world and as the knowledge of the multifactorial/heterogenous nature of the disease increases so does the need for more challenging and appropriate therapies. Traditional plant remedies have been used for centuries in the treatment of diabetes (Akhtar and Ali, 1984), but only a few have been scientifically evaluated.

Alloxan induces hyperglycaemia by selective cytotoxic

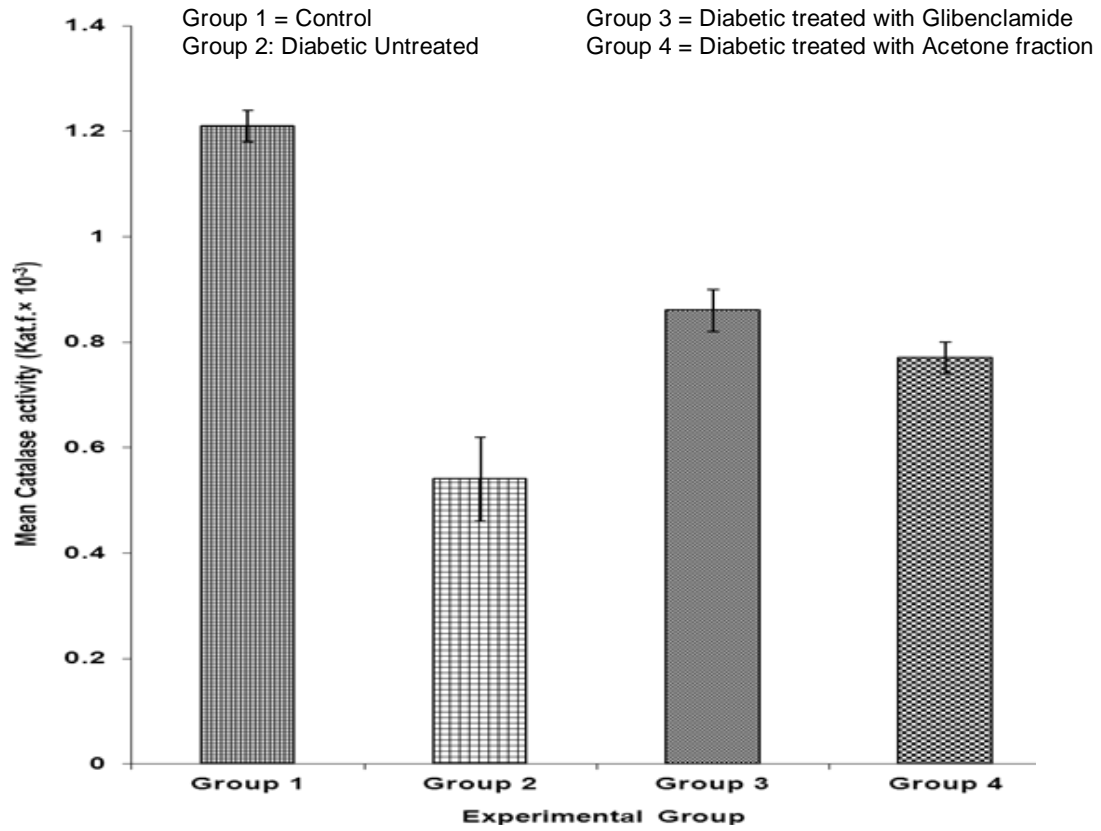


Figure 2. Effect of acetone extract of *C. gigantea* leaf on CAT activities in rabbits.

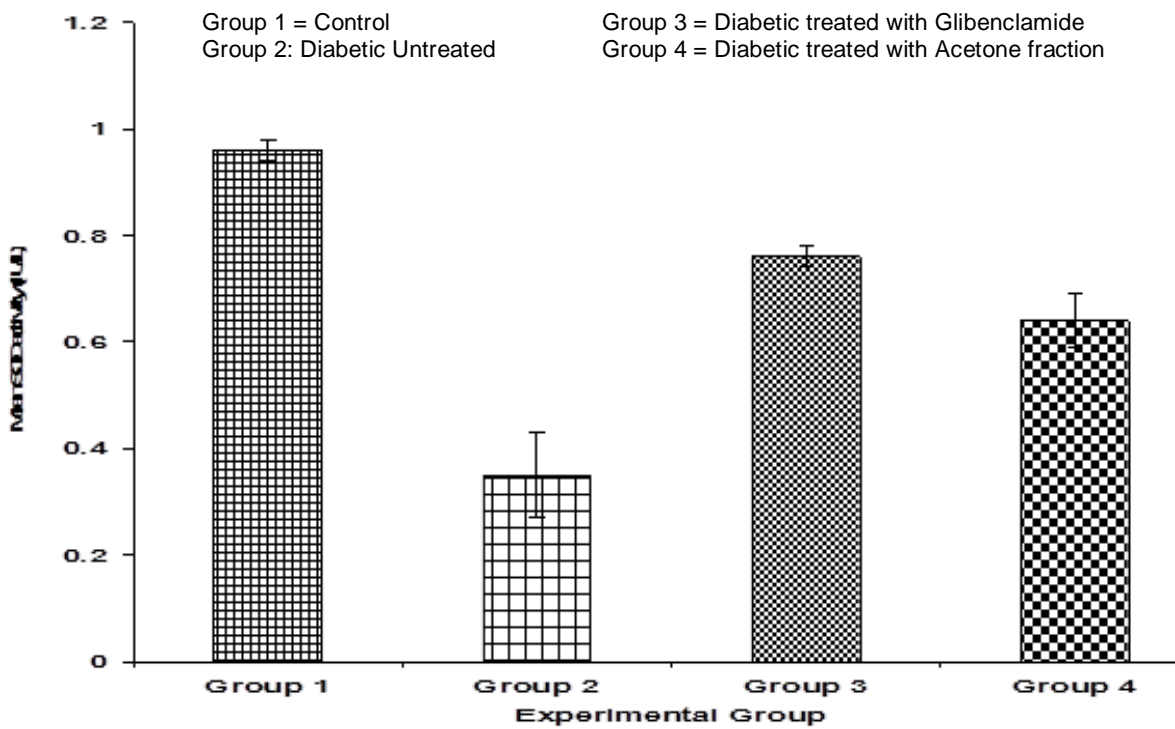


Figure 3. Effect of acetone extract of *C. gigantea* leaf on superoxide dismutase activities in rabbits.
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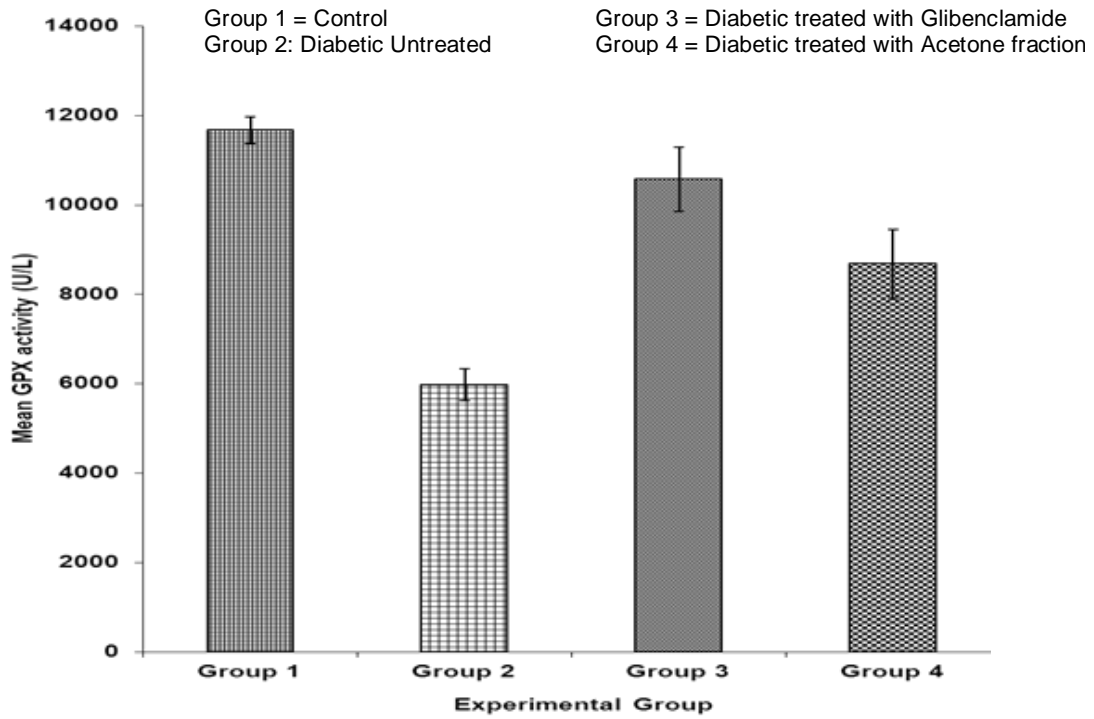
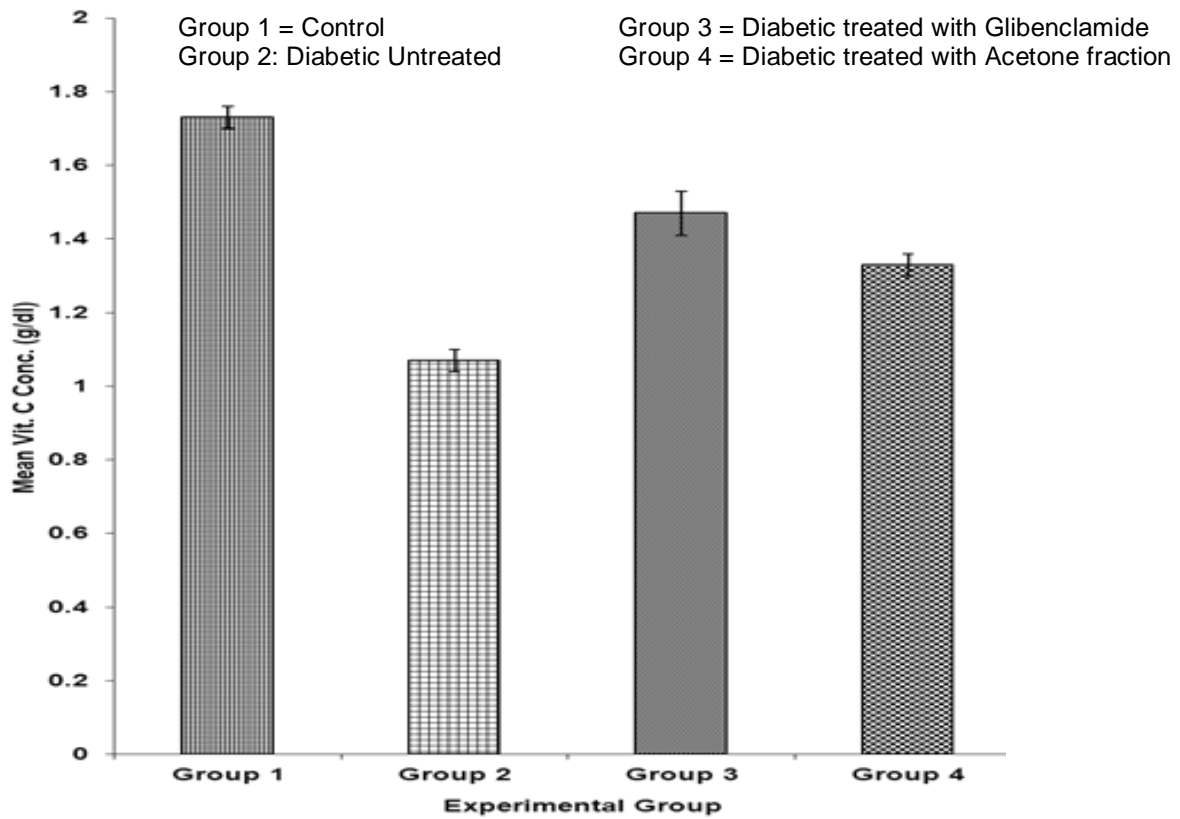


Figure 4. Effect of acetone extract of *C. gigantea* leaf on glutathione peroxidase activities in rabbits.



mg/kg of alloxan used in this study caused moderate diabetes (Grover et al., 2002). It has been reported that glibenclamide was not very effective after the occurrence of complete destruction of β -cells; hence more effective in moderate diabetic rabbits than in severe diabetes (Sharma et al., 1997). The acute hypoglycaemic effect of glibenclamide has been shown to be the stimulation of production of the residual β -cells of the pancreas in addition to enhancement of glucose utilization (Moller, 2001). This suggests that the extracts may have similar mechanism of action with glibenclamide and may in addition possess an insulinomimetic effect on peripheral tissues either by promoting glucose uptake and metabolism or inhibiting hepatic gluconeogenesis (Djomeni et al., 2006). This postulation correlates with that of Farjou et al. (1987) on the work with Artemisia extract.

There are several reports describing elevations in specific oxidant stress markers in both experimental alloxan-induced diabetes and human diabetes mellitus, together with reduced total antioxidant defense and depletion in individual antioxidants (Laight et al., 2000). Such a pro-oxidant environment was demonstrated in our study by the increased lipid peroxidation products, accompanied by inhibition of the activity of antioxidative enzymes such as CAT, SOD and GPX in the diabetic untreated rabbits. Our results were in agreement with that reported by Piconi et al. (2003) who stated that "overproduction of reactive oxygen species (ROS) could lead to a decrease in cell/organism antioxidant defenses in diabetes". An increase in TBARS was presumably associated with an increase in ROS, confirming the fact that the singlet oxygen and peroxy radicals inhibited the activity of SOD. Fridovich (1989) mentioned that overproduction of superoxide radicals during oxidative stress could inhibit the activity of glutathione GPX, while the activity of CAT was inhibited by production of singlet oxygen, superoxide and peroxy radicals (Escobar et al., 1996).

A significantly elevated level of MDA was observed in diabetic control rabbits compared with the group treated with acetone fraction of *C. gigantea* leaf extract ($p < 0.05$). However, MDA levels in diabetic rabbits treated with glibenclamide (a reference drug) was lower than those treated with acetone fraction of *C. gigantea* leaf extract. This result confirmed that the untreated diabetic rabbits were subjected to uncontrolled oxidative stress as indicated by significantly abnormal lipid peroxidation (high MDA levels) present in the serum. There were significant differences in the catalase activities ($p < 0.05$) in normal and untreated diabetic group compared with diabetic group treated with acetone fraction of *C. gigantea* extract. However, lower activity was observed in diabetic rabbits treated with acetone fraction of *C. gigantea* extract when compared with diabetic rabbits treated with glibenclamide (a reference drug).

0.05) in diabetic treated with acetone extract of *C. gigantea* compared with diabetic untreated group. Differences in the activities were observed among the diabetic treated with glibenclamide and diabetic administered with acetone fraction of the plant extract. GPX activity was significantly higher ($p < 0.05$) in diabetic rabbits treated with acetone extract compared with diabetic control group. There was significant increase ($p < 0.05$) of GPX activity in diabetic rabbit treated with glibenclamide compared with diabetic rabbits treated with acetone fraction of *C. gigantea* leaf extract. The increased level of GPX that was recorded in the diabetic treated animals could equally be a compensatory mechanism by the animals to overcome the effect of oxidative stress as a result of repetitive bleeding. The present study suggests that exogenous administration of 300 mg/kg body weight of acetone fraction of *C. gigantea* leaf extract to hyperglycaemic rabbits caused decrease of lipid peroxidation as well as increase in the activity of antioxidant enzymes. This result indicates the important role of acetone extract of the plant in the reduction of oxidative stress. However, the mechanism by which *C. gigantea* leaf extracts could affect free radical and peroxide production, as well as antioxidant enzymes (CAT, SOD, GPX) activities *in vitro* is not clearly defined. *Colatropis gigantea* leaf extract may exhibit antioxidant activity capacity based on increasing the upregulation of gene expression in the antioxidant enzymes (CAT, SOD and GPX) (Jeon et al., 2002).

Consequently, the enhancement of the scavenging of reactive oxygen species accompanying with significantly lowering of lipid peroxidation suggests that administration of *C. gigantea* leaf extract increase the antioxidant potential. In addition like all the flavonoid compounds, presence of hydrogen donating substituent attached to aromatic rings may enable *C. gigantea* leaf extracts to scavenge free radicals. The result of the antioxidant vitamin C concentration showed a significant increase ($p < 0.05$) in diabetic rabbits treated with acetone fraction of *C. gigantea* leaf extract compared with diabetic untreated rabbits. It was observed that exogenous administration of *C. gigantea* leaf extract boost vitamin C concentration in extra cellular fluid which has a sparing effect on vitamin E as it regenerate vitamin E from the tocopheroxyl radical after neutralization of free radicals

Conclusion

The results of this study have shown that intraperitoneal administration of *C. gigantea* leaf extract revealed their action on antioxidant enzymes through reduction of oxidative stress intensity. The protective effect of *C. gigantea* leaf extract may be connected with the inhibition of autoxidation and, as a result, exhibited a potent

significant amelioration of oxidative stress in diabetic animals.

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Conflict of interest

Authors have none to declare.

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