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Apigenin: A methanol fraction component of *Newbouldia laevis* leaf, as a potential antidiabetic agent

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ABSTRACT

The leaves of *Newbouldia laevis* is traditionally used to treat diabetes mellitus in southeast Nigeria. The apigenin isolated from the methanol fraction of dichloromethane/methanol (1:1) extract of the leaves was evaluated for antidiabetic and antihyperglycemic activity in alloxan-induced diabetic rats and on normal rats. Treatment of alloxan-diabetic rats with the compound (apigenin) significantly ($p < 0.05$) reduced blood glucose, and increased the liver and muscle glycogen content. The adrenaline-induced elevation of blood glucose of normal rats was significantly ($p < 0.05$) reduced by the isolated compound. These results suggest that apigenin may be the anti-diabetic principle in the leaves of *Newbouldia laevis*..

Keywords: Antidiabetic, *Newbouldia laevis*, Apigenin, Glycogen, Adrenaline.

INTRODUCTION

Diabetes mellitus is a metabolic endocrine disorder and has been recognized as one of the largest global health emergencies of the 21st century.[1] It is one of the major health problems confronting Africans. Management of diabetes is still met with clinical failures. Arrays of chemical agents are available to control and manage diabetes, but total recovery from diabetes has been elusive. The conventional synthetic anti-diabetic drugs available to manage the disease are costly and not readily affordable to the majority of the patients Consequently, a good number of patients now resort to medicinal plants as alternative sources of diabetic therapy, and a good number of these plants have been reported to demonstrated potent anti-diabetic effect [2-7] *Newbouldia laevis* (P. Beaux, Bignoniaceae) has a versatile application in traditional medicine and is used in more than 25 medical conditions throughout tropical Africa.[8-13]

Recently, the antihyperglycemic activity of the leaf extract and active fractions of the plant was reported.[14] In the present study, we have investigated the antidiabetic effects of a compound isolated from the active methanol fraction of *N. laevis* leaf extract.

MATERIALS AND METHOD

Collection of plant material

Mature fresh leaves of *N. laevis* were collected in the month of October from Igbo-Ukwu, Aguata Local Government Area of Anambra State, South-Eastern Nigeria. The whole plant was identified by Mr J.M.C. Ekekwe, a plant tanxonomist of the Department of Botany, University of Nigeria Nsukka. The leaves were washed to remove contaminants, air-dried under shade at room temperature until dry and pulverized into a coarse powder using locally fabricated hammer mill. The powdered plant material was kept in airtight containers until required.

Preparation of extract

The powdered leaf material (5 kg) was extracted by cold maceration in dichloromethane / methanol (1:1) with intermittent shaking and repeated filtration every 24 h for 48 h. The filtrate was concentrated *in vacuo* at 40°C to dryness with a rotary evaporator and labeled DME extract of *N. laevis*. The DME extract was stored in a refrigerator until required.

Experimental animals

Adult rats (150-250 g) and mice (15-30 g) of both sexes bred in the laboratory animal facility of the Department of Pharmacology and Toxicology, University of Nigeria Nsukka were used for the study. The animals were kept in steel cages and fed with standard livestock feeds and allowed free access to water. The animals were allowed 14 days acclimatization period on transfer to the research area before starting the experiments**.** All animal experiments were in compliance with National Institute of Health Guide for the care and use of Laboratory Animals (Pub No. 85-23 revised 1985).[15]

Acute toxicity (LD_{50}) test

The oral and intraperitoneal median lethal doses (LD_{50}) of DME in mice were determined using the method described by Lorke (1983).[16] The study was carried out in two phases. In the first phase, 9 mice were randomly divided into 3 groups (n=3) and treated orally with the extract at 10, 100 and 1000 mg/kg dose levels. The animals were observed for 24 hours and the number that died in each group was recorded. Based on this, the doses for the second phase were selected

In the second phase, 4 groups of mice (n=1) were treated with the extract at 1000, 1600, 2900 and 5000 mg/kg dose levels respectively. The LD_{50} was calculated as the geometric mean of the minimum lethal dose and the maximum non-lethal dose. The same procedure was carried out for the intra-peritoneal route using a separate group of mice.

Solvent-guided fractionation of DME and bioactivity-guided studies

The DME (500 g) was subjected to solvent-guided fractionation in a silica gel (60-200 mesh size) column and successively eluted with n-hexane, ethylacetate and methanol in order of increasing polarity to yield n-hexane fraction (HF), ethylacetate fraction (EF) and methanol fraction (MF). The fractions were concentrated using rotary evaporator (40-50^{o}C) under reduced pressure. The DME and the fractions (HF, EF, and MF) were subjected to biological activity studies. The antihyperglycemic effect 1n alloxan-induced diabetic rats were used as the activity guide. Methanol fraction (MF) was significantly ($p<0.05$) the most effective and it was therefore subjected to further fractionation. The MF (150 g) was fractionated by column chromatographic methods using gradient elution with n-hexane/ethylacetate (7:3) to obtain twenty five (25) sub-fractions of MF. Thin layer chromatography (TLC) was employed to pull the column chromatographic fractions together on the basis of the Rf values of the similar spots to obtain four sub-fractions: F_1, F_2, F_3 and F_4. The sub-fractions were subjected to antihyperglycemic activity studies on alloxan diabetic rats and F_4 was significantly ($p<0.05$) the most effective antihyperglycemic fraction. The sub fractionF_4 (50 g) was further separated in a silica gel column and eluted with n-hexane-ethylacetate (7:3) to yield 40 fractions ($F_{4(1)}$ – $F_{4(40)}$). Thin layer chromatography (TLC) was employed to pull the column chromatographic fractions together on the basis of the Rf values of the similar spots. Sub-fractions $F_{4(31)}$ - $F_{4(35)}$ which were the most effective fraction yielded F_5. Further purification ofF_5 (20 g) by repeated column chromatography on sephadex LH20 using methanol as eluent yielded a compound labeled CC2.

Determination of molecular structure of isolated compound CC2

Molecular structure was studied using nuclear magnetic resonance (NMR) spectroscopy. The NMR measurements were obtained at the Institute of Organic Chemistry, Heinrich-Heine- University. The ^{1}H NMR and ^{13}C NMR were recorded at 300^{o} K on ARX 300MHz NMR spectrometers (Bruker, Germany).All 1D and 2D spectra were obtained using the standard Bruker software (United Kingdom).Sample CC2 was dissolved in a deuterated methanol (CD_3OD), the choice of which was dependent on the solubility of the sample. Tetramethylsilane (TMS) was used as internal standard reference signal. The observed chemical shifts (δ) were recorded in ppm and the coupling constants (*J*) were recorded in Hz.

Hypoglycemic activity of the isolated compound CC2

Induction of diabetes

Diabetes was induced in adult albino rats of both sexes by a single intravenous injection of freshly prepared alloxan monohydrate (65 mg/kg) in 0.9% saline.[17,18] Glucose solution (50%) was used to prevent the initial hypoglycemia caused by alloxan monohydrate.[17,19] Blood samples were collected after 3 days from overnight fasted animals through the tail vein and blood glucose level was estimated using commercially available Accu-check Active® (Roche Diagnostics) glucometer. Rats with blood glucose levels above 250 mg/dl [19] were considered diabetic and selected for the study.

Five groups of diabetic rats (n=6) were used. Groups 1-3 were given graded doses of CC2 (25, 50 and 100 mg/kg body weight) respectively orally, group 4 received glibenclamide (5 mg/kg), while group 5 received 5 ml/kg 3% Tween 80 as the diabetic control. Blood samples were withdrawn through the tail vein at 0 (pre-treatment) and at 3, 6, 9, 12 and 24 h post treatment. The blood glucose level was estimated using commercially available glucometer (Accu-check Active® Roche Diagnostics). The percentage reductions in blood glucose levels were calculated relative to the pre-treatment values.[20,21]

Effects of CC2 on adrenaline - induced hyperglycemic

Normal adult rats of both sexes were randomly selected and divided into 4 groups of 6 rats per group. After blood withdrawal for FBS estimation, the rats were treated as follows:

Group 1 3% Tween 80; 5 ml/kg.

Group 2 CC2; 25 mg/kg.

Group 3 CC2; 50 mg/kg

.Group 4 glibenclamide 5 mg/kg.

Two hours after drug treatment, the animals were injected with adrenaline HCl (Martindale®) (0.8mg/kg ip).[22] Blood glucose levels were estimated at 30, 60, 90, 120, 180 and 240 min [23] and the percentage increase in blood glucose level calculated relative to 0 min.

Effect of CC2 on liver and muscle glycogen content of diabetic rats

Three groups of diabetic rats (n-6) were randomly selected and treated as follows: Group 1 (CC2 50 mg/kg/day), Group 2: (glibenclamide 5 mg/kg/day), Group 3: (3% Tween 80, 5 ml/kg/day) as diabetic control. A group of: normal non-diabetic rats (Group 4) treated with 3% Tween 80 (5 ml/kg/day) served as the normal control.

All treatments were by the oral route once daily for a period of seven days. Fasting blood sugar was estimated on day 0 (pre-treatment), day 3 and day 7. On day 7, the animals were sacrificed 2 hours after treatment and the liver and gastrocnemius muscles were excised for the estimation of glycogen content.[24] Glycogen was recovered from the tissue (liver/muscle) by repeated homogenization with 5 % trichloroacetic acid (TCA). Glycogen was precipitated from the TCA filtrate of the tissue by 95% ethanol and determined by the method of Caroll *et al.*, (1956).[25]

Statistical analysis

The results were analysed using SPSS Version 20.0 software (USA) and presented as mean ± SEM. The means were compared with the control groups by using one way analysis of variance (ANOVA) followed by LSD post hoc test. Differences between means were considered significant at $p < 0.05$.

RESULTS

Acute toxicity test

The results of the acute toxicity tests showed that the oral LD_{50} of MDE was greater than 5000 mg/kg, while the intraperitoneal LD_{50} was 3,807.9 mg/kg.

Structure of CC2

The structural elucidation of the isolated compound CC2 was established based on spectroscopic studies notably the 2D NMR spectra (Figures 1; 2; 3; Table 1). The compound was identified as apigenin (Figure 3).

Table 1: HNMR data of isolated compound (CC2)

Position	δ_H Compound (CC2)	δ_H (Apigenin Reported) Ersoz et al., 2002
1	-	
2	-	
3	6.60 s	6.58 s
4	-	
5	-	
6	6.21 d (*J= 2.1*)	6.71 d (*J= 2.1*)
7	-	
8	6.46 d (*J= 2.1*)	6.83 d (*J= 2.1*)
9	-	
10	-	
1'	-	
2'	7.86 d (*J= 8.9*)	7.83 d (*J= 8.8*)
3'	6.93 d (*J= 8.9*)	6.92 d (*J= 8.8*)
4'	-	-
5'	6.93 d (*J= 8.9*)	6.92 d (*J= 8.8*)

Hypoglycemic effect of CC2

Administration of the isolated compound (CC2) evoked a significant ($p < 0.05$) dose-related reduction in mean blood glucose levels (Figure 4). The hypoglycemic effect of CC2 (25 mg/kg) was comparable to that of glibenclamide.

Effect of CC2 on adrenaline induced hyperglycemia

The isolated compound (CC2) at the doses of 25 mg and 50 mg significantly ($p < 0.05$) reduced the hyperglycemic response to adrenaline on non-glycemic rats. The blood glucose increase at 30 min peak was 65.73 % and 52.88 % respectively when compared to the untreated control (97.86 %). The effect was comparable to that of glibenclamide (63.90 %) (Figure 5).

Effect of CC2 on liver and muscle glycogen of diabetic rats

Daily administration of CC2 (50 mg/kg) for 7 days significantly ($p < 0.05$) lowered the mean fasting blood glucose level. The reduction was comparable to that of glibenclamide (Figure 6).

The liver and muscle glycogen contents in diabetic control were significantly ($p < 0.05$) lowered by 61.39 % and 66.37 % respectively when compared with normal control (Figure 7).Treatment with CC2 (50 mg/kg) for 7 days caused a significant increase (162.80 % and 226.89 % respectively) in the liver and muscle glycogen contents when compared with the diabetic control ($p < 0.05$). This effect was significantly ($p < 0.05$) more than that of glibenclamide (131.52 % and 138.44 %) respectively.

DISCUSSION

The structural elucidation of the isolated compound (CC2) was established using spectroscopic studies notably the 2D NMR spectra and H^1NMR. The compound was identified as 4', 5, 7 trihydroxy flavone (apigenin). Eyong *et al.* (2006) [26] reported the isolation of apigenin from the root bark of *N. laevis.* This study is probably the first report of isolation of apigenin from the leaves of *N. laevis.*

The effect of CC2 on adrenaline induced hyperglycemia revealed a significant dose- related reduction of the hyperglycemic response induced by adrenaline. Adrenaline produces hyperglycemia by inhibiting insulin release, stimulating glucogenolysis in muscle, stimulating glucagon secretion and stimulating adrenocorticothrophin hormone (ACTH).[27] It is also reported that adrenaline produces hyperglycemia by increasing glucose uptake from both the large and small intestine.[28] The compound (CC2) probably prevented the rise in blood sugar by inhibiting adrenaline induced stimulation of α_2 receptors present in the pancreatic β-cells thus helping insulin release.[29,30]

Estimation of glycogen level in animal studies may be considered as the best marker for assessing antihyperglycemic activity of any drug in experimental diabetes.[31] Conversion of glycogen in the liver cells is dependent on the extracellular glucose concentration and on the availability of insulin which stimulates glycogen synthesis over a wide range of glucose concentrations.[32] Glycogen content in liver and skeletal muscle was evaluated in this study to explore the possible mechanism of action of the isolated compound (CC2). In this study, a reduction in the liver and muscle glycogen was observed in alloxan-induced diabetic rats. This observation is consistent with earlier reports.[32, 33] Reduced liver and muscle glycogen content in diabetic control rats has been attributed to reduced activity of glycogen synthase and increased activity of glycogen phosphorylase [34]. Reversal of the activities of these two key enzymes was reported in diabetic rats treated with hyperin- (a flavonoid compound) leading to the accumulation of liver glycogen.[32] Activities of hepatic glucose-6 phosphatase and fructose – 1, 6 – biphosphatase were also reported to be increased significantly in diabetic rats.[32] These two enzymes are the regulatory enzymes in gluconeogenetic pathway. Although these glucose metabolizing enzymes were not measured in this study, the antihyperglycemic effect and increased liver glycogen content in treated diabetic rats may be taken as a direct evidence of the influence of the compound (CC2) on these enzymes.

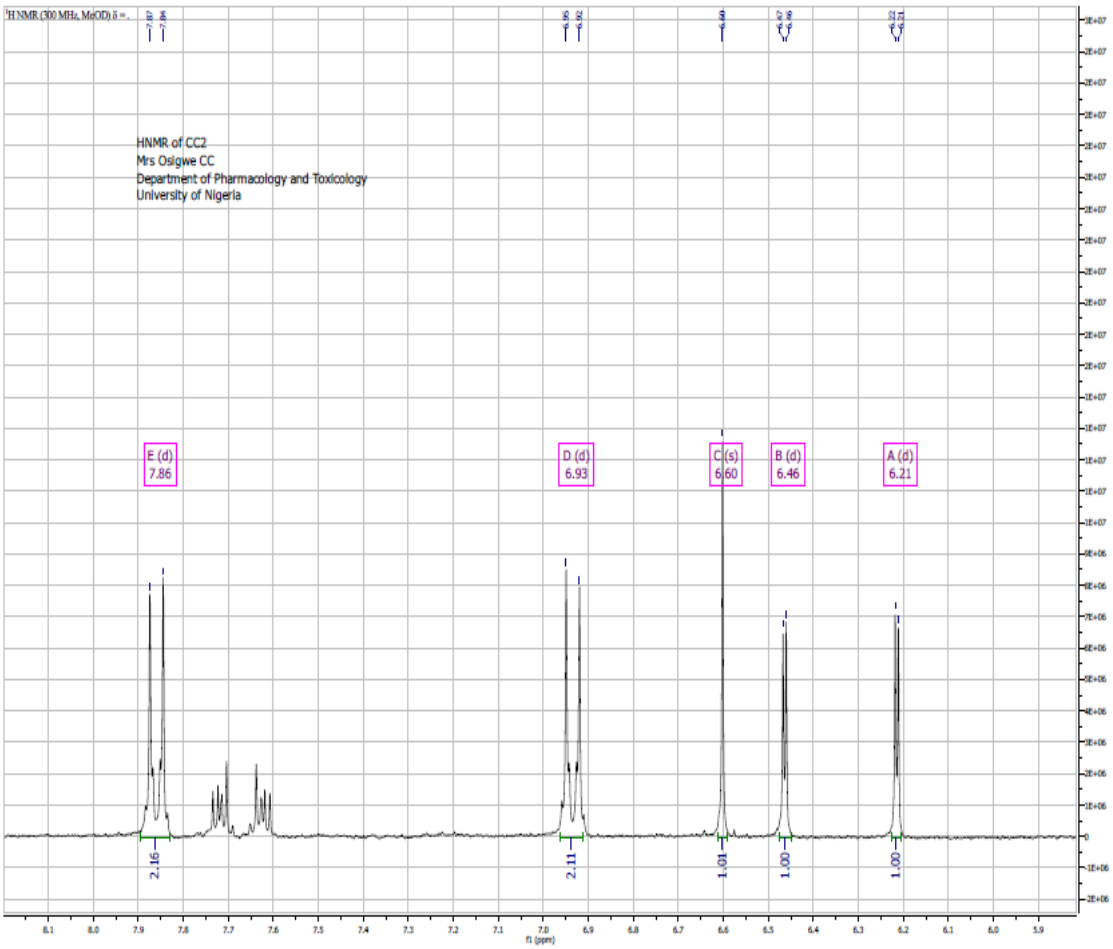

Figure 1: HNMR of isolated compound (CC2)

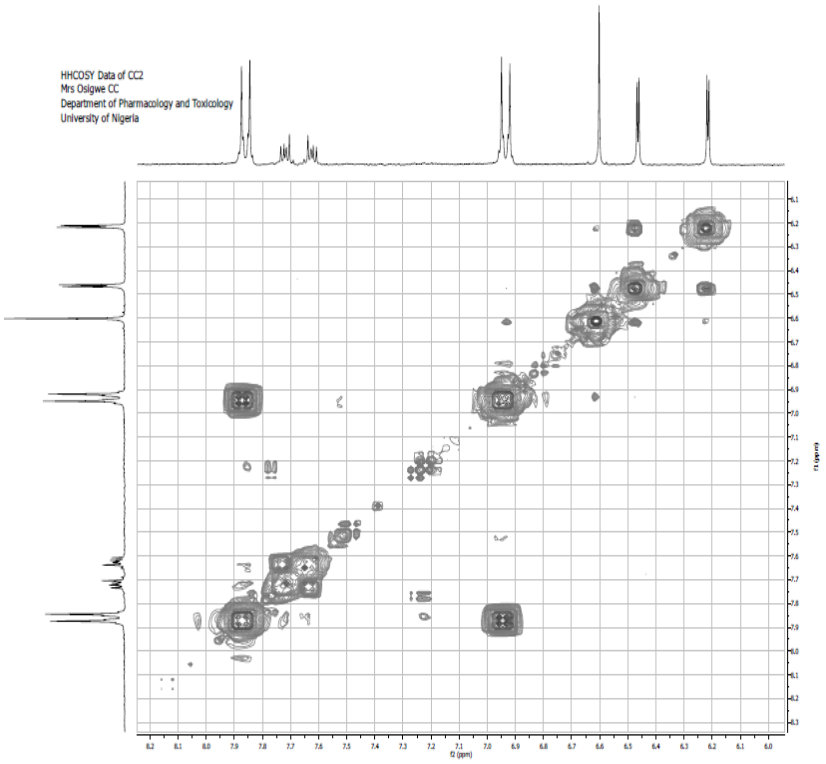

Figure 2: HHCOSY data of isolated compound (CC2)

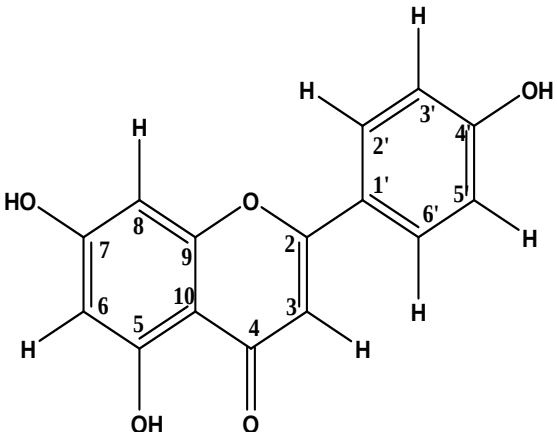

Figure 3: Structure of apigenin (4', 5, 7 trihydroxy flavone).

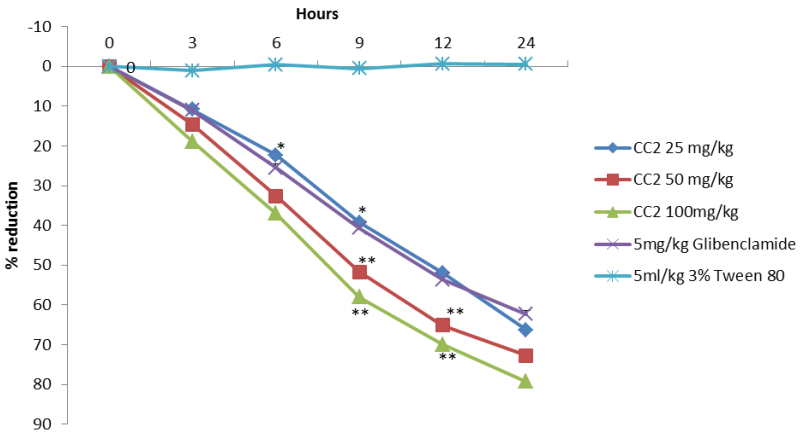

Figure 4: Effects of isolated compound (CC2) on blood glucose levels of diabetic rats. *$p<0.05$ compared to diabetic control. **$p<0.05$ compared to glibenclamide treated group,.n=6 .

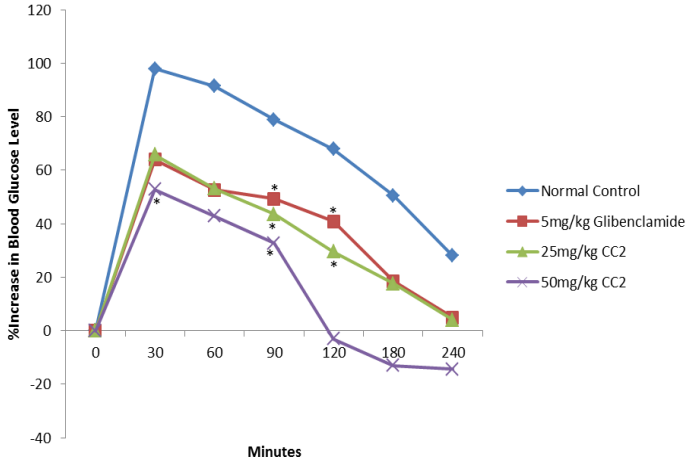

Figure 5: Effects of isolated compound (CC2) on adrenaline induced hyperglycemia in non-diabetic rats. *$p<0.05$ compared to untreated control

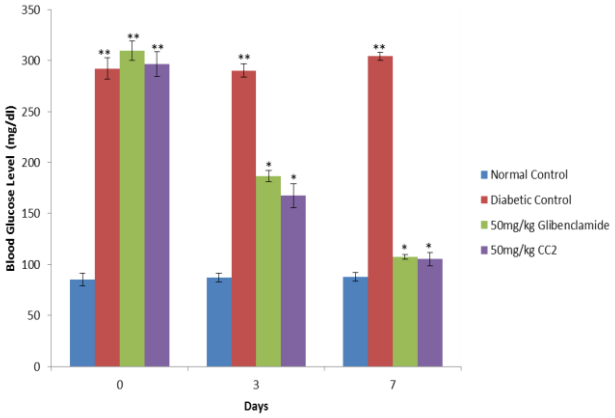

Figure 6: Effect of isolated compound (CC2, 50 mg/kg) on blood glucose levels of diabetic rats.Values are mean ± SEM (n=6) *$p<0.05$ compared to diabetic control; **p<0.05 compared to normal control

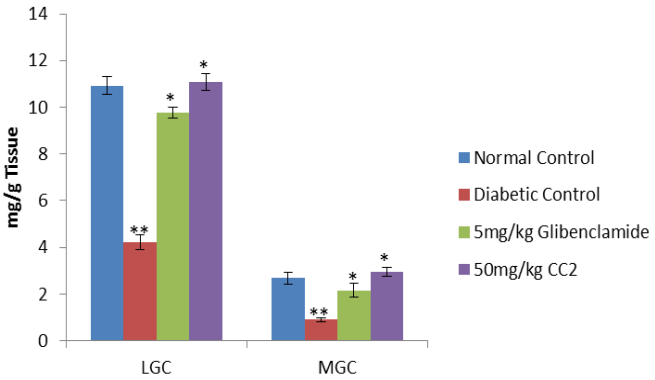

Figure 7: Effects of isolated compound (CC2) of on liver and muscle glycogen content of diabetic rats. Values are mean ± SEM (n=6) *$p<0.05$ compared to diabetic control; **$p<0.05$ compared to normal control. LGC = Liver glycogen content; MGC = Muscle glycogen content.

The increase in the glycogen level in the alloxan–induced treated diabetic rats may also be due to direct action on the pancreas to release insulin. Insulin activates the enzyme glycogen synthetase and glukokinase both of which are responsible for glycogenesis while inhibiting glycogen phosphorylase and glucose-6-phosphatase responsible for glycogenolysis.[35] The increased concentration of glycogen in skeletal muscle could also be attributed to increased phosphoinositol-3-kinase (PI-3K) activity and increased expression and translocation of GLUT-4 in skeletal muscle.[29]

Apigenin is a non-mutagenic flavone of a subclass of flavonoids with a very little toxicity.[36] Apigenin has been shown to possess remarkable anti-inflammatory, antiproliferative, free radical

scavenging, immunomodulatory, antioxidant, antitumor and cancer chemo-preventive properties.[37] Apigenin was reported to exhibit anxiolytic and sedative activities.[38] Antibacterial attribute of apigenin has also been reported.[38] Previous reports on apigenin in experimental diabetes are consistent with the result of this study. Apigenin was reported to enhance GLUT-4 translocation and downregulation of CD38 enzyme to improve diabetes [39], and to possess a potent antihyperglycemic activity and a strong inhibitory action on DPP-IV enzyme.[40] It has been reported that apigenin lowered pro-inflammatory cytokines and chemokines in plasma and improved hyperglycemia, hyperinsulinemia and insulin resistance) [41]. The improved glucose metabolism by apigenin was reported to be mediated through the inhibition of hepatic gluconeogenic enzyme activities [41]. A significant down regulation of gluconeogenic and lipogenic gene expression by apigenin in cells from human liver carcinomas has been reported) [42] which makes it a promising therapeutic option for lowering hepatic glucose production and reducing hepatic steatosis.[40] Apigenin has been reported to improve glucose tolerance.[43] Apigenin fucopyranoside has been reported to stimulate insulin secretion and glycogen synthesis.[44] Apigenin showed an antihyperglycemic effect and reduced glycemia of STZ-induced diabetic rats after 7 days of treatment.[45] It has been demonstrated that apigenin has a protective effect on pancreatic β-cell destruction in a model of STZ-induced diabetes and significantly increased insulin release.[46, 47] The result of this study is consistent with the earlier reports.

An elimination half-life of 91.8 h after a single oral administration in rats has been reported for apigenin.[48] In this study it was observed that the antihyperglycemic principle (apigenin) has a long and sustained duration of action (24 h) after a single oral dose in rats justifying the long elimination half-life earlier reported. In vitro studies have shown that apigenin undergoes transformation into luteolin (tetrahydroxyflavone).[48] The antidiabetic potential of luteolin has been reported.[42, 49] This transformation may also be true in vivo and may have contributed to the sustained antidiabetic effect of apigenin observed in this study.

CONCLUSION

The results of this study reveal that *Newbouldia laevis* leaf possesses antihperglycemic, antidiabetic effects, probably via enhanced insulin sensitivity, and insulin secretion. Apigenin was isolated for the first time from the leaves of *N. laevis* and it appears to be one of the antihyperglycaemic principles in *N. laevis*.

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