Glycosylated haemoglobin values of alloxan-induced diabetic rats treated with graded doses of Cussonia arborea extract

Patrick Emeka Aba and Isaac Uzoma Asuzu

Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, Enugu State, Nigeria

ABSTRACT

This study investigated glycosylated haemoglobin values and fasting blood glucose (FBG) levels of diabetic rats treated with methanol extract of Cussonia arborea. A total of 72 male wistar rats were assigned into 6 groups of 12 rats per group. Groups 1–5 rats were made diabetic by intraperitoneal injection of alloxan monohydrate at the dose of 160 mg/kg and treated with 62.5, 125, 250 mg/kg of the extracts, 2 mg/kg glibenclamide and 10 ml/kg distilled water (DW), respectively, while the non-diabetic group 6 rats received 10 ml/kg DW and served as normal control rats. The treatment was daily through the oral route for 84 days. The glycosylated haemoglobin values were measured on days 42, 56 and 84 post treatment while the FBG levels were determined on 2 weeks interval post treatment till the end of the experiment. The mean FBG level of rats treated with 125 mg/kg of the extract showed the most significant reduction (p < .05) in FBG from 315.33 ± 10.08 mg/dl to 93.00 ± 8.50 mg/dl and equally ameliorated haemoglobin glycosylation when compared to the negative control group. It was concluded that the methanol extract of Carborea, at the dose of 125 mg/kg mitigated glycosylation of haemoglobin.

Introduction

Diabetes mellitus is a complex disease characterized by inability to regulate blood glucose as a result of relative or absolute deficiency in insulin (produced from pancreas) resulting to hyperglycaemia often accompanied by glucosuria, polydipsia and polyuria (Celik et al. 2002). Chronic hyperglycaemia and enhanced oxidative stress play major roles in diabetes pathogenesis. The disease is progressive and is associated with a high risk of complications (Dewanjee et al. 2008). It is one of the most common endocrine diseases and has a prevalence rate varying from 1 to 50% (King and Rewers 1993).

Glycosylated or glycated haemoglobin is the result of simple chemical reaction between haemoglobin and sugars after synthesis of haemoglobin is complete (Bunn et al. 1976). The reaction proceeds in two stages. Firstly, glucose combines with the globin chains to form aldimine compound (Schiitius and stress-induced hyperglycaemia (Goldstein et al. 1984).

Early workers confirmed that glycosylation begins during erythropoeiesis and continues slowly throughout the life of haemoglobin in the circulation. Concentrations reached in the red cell of diabetic subjects are consistent with their known life span of about 120 days (Bunn et al. 1976). An international expert committee (IEC), after an extensive review of both established and emerging epidemiological evidence, recommended the use of the glycosylated haemoglobin (HbA1c) test to diagnose diabetes with a threshold of ≥6.5% and American Diabetes Association (ADA) affirms this. The diagnostic HbA1c cut point of 6.5% is associated with an infection point for retinopathy prevalence (IEC 2009).

Plants have been used since time immemorial in the management of diabetes mellitus; however their effects are usually exaggerated, unscientific or unstandardized. Cussonia arborea is a tropical plant belonging to the family of Araliacea with about 20 species (Tennant 2010). Folklorically, the plant is used in the treatment of malaria (De villers et al. 2010) fungi diseases, chronic diarrhoea (Kisangau et al. 2007) and diabetes (Amadou et al. 2008).

This study was therefore tailored to investigate the effects of treating alloxan-induced diabetic rats with graded doses of methanol root bark extract of C. arborea on glycosylated haemoglobin values.

Materials and methods

Experimental animal

Male albino rats weighing between 100 and 105 g were obtained from the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka laboratory animal house. The rats were randomly assigned into 6 groups of 12 rats per group. Diabetes was induced in groups 1–5 rats while 5 rats were made diabetic by intraperitoneal injection of alloxan monohydrate at the dose of 160 mg/kg and treated with 62.5, 125, 250 mg/kg of the extracts, 2 mg/kg glibenclamide and 10 ml/kg distilled water (DW), respectively, while the non-diabetic group 6 rats received 10 ml/kg DW and served as normal control rats. The treatment was daily through the oral route for 84 days. The glycosylated haemoglobin values were measured on days 42, 56 and 84 post treatment while the FBG levels were determined on 2 weeks interval post treatment till the end of the experiment. The mean FBG level of rats treated with 125 mg/kg of the extract showed the most significant reduction (p < .05) in FBG from 315.33 ± 10.08 mg/dl to 93.00 ± 8.50 mg/dl and equally ameliorated haemoglobin glycosylation when compared to the negative control group. It was concluded that the methanol extract of Carborea, at the dose of 125 mg/kg mitigated glycosylation of haemoglobin.
the rats in group 6 served as normal control rats. The rats were acclimatized for two weeks. The environmental temperature where the animals were housed varied between 28 and 32°C. The animals were kept in stainless wire mesh cages and provided with good clean water ad libitum. They were fed with standard commercial feed (Guinea grower). The experimental protocol used in this study was approved by the ethics committee of the University of Nigeria, Nsukka and conforms with the guide to the care and use of animals in research and teaching of the University of Nigeria, Enugu state, Nigeria (ECUN: 2015/58/702).

Plant material

The root bark of the plant material (C. arborea) used in this study was collected from Orukpa Local Government Area of Benue state and identified by a plant taxonomist, at the International Centre for Ethnomedicine and Drug Development, Echara, Aku road, Nsukka, Enugu state, Nigeria.

Preparation of the plant extract

Cold maceration method of extraction was employed. The root bark of C. arborea was air dried at a very low intensity of sunlight to avoid denaturation of the active ingredient. It was pulverized and stored in an air tight container pending its usage. About 1 kg of the powdered root bark was soaked in 5 l of 80% methanol (Sigma Aldrich, UK) with intermittent shaking every 2 h for 48 h. The mixture was filtered using Whatmann No 1 filter paper. The filtrate was concentrated using rotary evaporator and the extract stored as C. arborea extract (CAE) at 4°C.

Induction of experimental diabetes mellitus

Diabetes was induced in rats using the method described by Venugopal et al. (1998). The rats were injected with alloxan monohydrate (Sigma Aldrich, UK) dissolved in distilled water at a dose of 160 mg/kg body weight intraperitoneally, after overnight fasting (18 h). Before the injection with alloxan monohydrate, the FBG levels of the rats were taken using Accu-Check glucometer (Oh et al., 2013; Togashi et al., 2016 and Weitgasser et al., 1999). This was done by tail snip of the rats and allowing blood to drop on the glucometer strip. The value was read off on the screen of the glucometer. After induction, the rats were kept in clean stainless cages and fed with commercial feed and were also given clean water for about 2 days before they came down with diabetes. On the 2nd day, diabetes was confirmed. The rats were fasted overnight before the assessment of their blood glucose status on the 2nd day. The FBG values above 7 mMol/L (126 mg/dl) were considered diabetic (WHO 1980).

Treatments

The rats were treated as follows:
- Group 1. Diabetic rats treated with 62.5 mg/kg C. arborea extract (CAE).
- Group 2. Diabetic rats treated with 125 mg/kg CAE.
- Group 3. Diabetic rats treated with 250 mg/kg CAE.
- Group 4. Diabetic rats treated with 2 mg/kg glibenclamide.
- Group 5. Diabetic rats treated with 10 ml/kg distilled water.
- Group 6. Undiabetic rats treated with 10 ml/kg distilled water.

The rats were treated orally daily for 84 days and the FBG levels determined on days 14, 28, 42, 56, 70 and 84 while the glycated haemoglobin values were measured on days 42, 56 and 84 post treatments.

Glycosylated haemoglobin determination

Glycosylated haemoglobin kit (Spectrum, Egypt) was used.

Assay principle

A haemolysed preparation of whole blood was mixed continuously for 5 min with a weakly binding cation-exchange resin. The labile fraction was eliminated during the hemolysate preparation and during the binding. During this mixing, non-glycosylated haemoglobin binds to the ion-exchange resin leaving glycosylated haemoglobin free in the supernatant. After the mixing period, a filter separator was used to remove the resin from the supernatant. The percent glycated haemoglobin was determined by measuring absorbances of the ratio of the absorbances of the glycosylated haemoglobin (GHb) and the total haemoglobin fraction (THb). The ratios of the absorbances of GHb and THb of the control and test were used to calculate the percent GHb of the sample.

Haemolysate preparation

Into a test tube was dispensed 0.5 ml of lysing reagent and labelled as test and control. Thereafter, 0.1 ml of the reconstituted control or well-mixed sample was added into the appropriately labelled tubes and mixed until complete lysis was evident.

Glycosylated haemoglobin separation

The cap from the ion exchange resin was removed and labelled as control and test and 0.1 ml of hemolysate from hemolysate preparation above was added into appropriately labelled ion exchange resin tubes. Thereafter, a resin separator was inserted into each tube so that the rubber sleeve was approximately 1 cm above the liquid level of the resin suspension and the tube was vortexed continuously for 5 min. The resin was allowed to settle and the resin separator was pushed until the resin was firmly packed and the supernatant decanted directly into a cuvette and absorbance measured at 415 nm against distilled water.

Total haemoglobin fraction

Into test tubes labelled as ‘test’ and ‘control’ were dispensed 5 ml of distilled water and 0.02 ml of hemolysate from hemolysate preparation above and added to appropriately labelled
tubes. The mixture was mixed well and the absorbance read against distilled water.

**Calculations**

\[
\text{Ratio of control (R_c)} = \frac{\text{Absorbance control GHb}}{\text{Absorbance control THb}}
\]

\[
\text{Ratio of test (R_t)} = \frac{\text{Absorbance test GHb}}{\text{Absorbance test THb}}
\]

\[
\text{GHb} (\%) = \frac{\text{Ratio of Test (R_t)}}{\text{Ratio of control (R_c)}} \times 10
\]

10 is the value of the control.

**Statistical analysis**

Statistical package for social sciences (SPSS) version 20 was used for the analysis. One way Analysis of Variance (ANOVA) was used to compare the Means of the FBG levels and their difference separated using Duncans Multiple Range test while Data on glycosylated haemoglobin and FBG were correlated using Pearson parametric correlation. P-values <.05 were considered significant.

**Results and discussion**

The FBG of all the rats in groups 1–5 were significantly (p < .05) higher than that of the group 6 rats (normal control) post induction. On day 14 post treatment, the FBG levels of groups 2 and 4 rats were significantly (p < .05) lower than those of the group 5 rats but statistically comparable (p > .05) to that of the group 6 rats (normal control). These two groups (Groups 2 and 4) consistently compared very well (p > .05) with the normal control rats till the 84 d duration of the study (Table 1). The single intraperitoneal injection of alloxan monohydrate at the dose of 160 mg/kg body weight resulted in a significant (p < .05) elevation of the fasting blood glucose (FBG) levels when compared to the nondiabetic rat (Table 1).

Alloxan monohydrate and its reduced product, dialuric acid establish a redox cycle with the formation of superoxide radicals (Szukudelski 2001). These radicals undergo dismutation to hydrogen peroxide with a simultaneous massive increase in cytosolic calcium ion concentration which causes rapid destruction of pancreatic beta cells of the islets of Langerhans resulting in a decrease in endogenous insulin secretion and attendant elevation of the blood glucose levels (Akuodor et al. 2014). Rats with elevated glucose levels of ≥126 mg/dl (7 mMol/L) were considered diabetic (WHO 1980). The results indicated that treatment of the diabetic rats with *C. arborea* extract (especially at the dose of 125 mg/kg body weight) significantly (p < .05) reduced the elevated glucose levels (Table 1). The reductions achieved by the extract at this dose were comparable to that achieved by 2 mg/kg of glibenclamide, a known anti-diabetic drug. The result is in agreement with our earlier study (Aba et al. 2014) with methanol stem bark extract of *C. arborea* which demonstrated antihyperglycaemia. Other researchers (Khazraji et al. 1993; Lim et al. 2009; Liu et al. 2005) have also demonstrated hypoglycaemic potentials of root barks of different plants. Our earlier study indicated that the active principle responsible for the hypoglycaemic effect was a triterpenoid (Aba and Asuzu, 2016). Other researchers such as Min-jia et al., 2008; Santos et al., 2012 and Castellano et al., 2013 had earlier reported various degrees of hypoglycaemic activities of triterpenes isolated from different plant materials.

The glycosylated haemoglobin values of group 2 rats were significantly (p < .05) lower than that of the group 5 rats (negative control) but were statistically comparable (p > .05) to those of the normal control rats and to those treated with standard drug (Glibenclemide) 42 days post treatment. On days 56 and 84, all the treatment groups showed significantly (p < .05) lower values of glycosylated haemoglobin when compared with the negative control groups (Table 2).

The glycosylated haemoglobin (HbA1c) values of the diabetic untreated rats were significantly (p < .05) higher than that of the normal control rats. This could be attributed to the effect of alloxan which induced hyperglycaemia by ultimately leading to decreases in insulin secretion consequent upon pancreatic beta cell destruction (Szukudelski, 2001). Persistent or prolonged increase in blood glucose leads to non-enzymatic addition of glucose to the free amino groups at the N-terminal of beta chain of haemoglobin thereby forming glycosylated haemoglobin (Kilpatrick et al. 2009). However, all treatment groups recorded significantly (p < .05) lower HbA1c values at the end of the experiments (Table 2). Group 2 rats, treated with 125 mg/kg of the extract demonstrated the highest ameliorative effects with regards to glycosylation of haemoglobin. Glycosylated haemoglobin is a measurement that reflects both fasting and postprandial glucose concentrations over a 3-month period (Diana et al. 2010). Glycosylation of haemoglobin (non-enzymatic addition of glucose moiety to haemoglobin) starts six weeks following persistent hyperglycaemia (Nagisa et al., 2003; IEC 2009). It then lasts till the life span of red blood cell (which is about 3 months). Reductions in the values of HbA1c by the treated groups indicate the hypoglycaemic effect of the extracts. The

---

**Table 1.** Effects of administration of *C. arborea* root bark extract on Fasting blood glucose levels of alloxan-induced diabetic rats.

| Group | Preinduction | 0 h post treatment (pt) | 14 days pt | 28 days pt | 42 days pt | 56 days pt | 70 days pt | 84 days pt |
|-------|--------------|------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| 1     | 78.00 ± 1.15a | 310.00 ± 15.27b       | 118.33 ± 4.40b | 127.00 ± 3.21c | 113.66 ± 3.17b | 111.66 ± 6.00a | 115.66 ± 2.90b | 108.66 ± 4.37b |
| 2     | 79.33 ± 1.45a | 315.33 ± 10.08b       | 93.00 ± 8.50a | 94.66 ± 4.33b | 80.00 ± 3.78b | 79.00 ± 2.51a | 74.00 ± 5.67a | 82.33 ± 8.8a |
| 3     | 78.33 ± 1.76a | 318.33 ± 16.42b       | 117.66 ± 6.48b | 127.33 ± 1.76c | 118.00 ± 9.01b | 117.00 ± 5.68b | 76.33 ± 2.40a | 79.00 ± 1.20a |
| 4     | 77.66 ± 2.33a | 307.33 ± 7.33a        | 87.00 ± 1.52a | 87.66 ± 2.72c | 80.00 ± 5.77a | 73.66 ± 1.20a | 73.66 ± 2.02a | 73.33 ± 1.20a |
| 5     | 77.66 ± 5.23a | 311.33 ± 9.61b        | 288.66 ± 6.96a | 292.66 ± 3.92b | 303.66 ± 3.17b | 295.66 ± 2.84a | 292.33 ± 7.17b | 289.66 ± 4.91b |
| 6     | 76.33 ± 6.02a | 78.33 ± 4.05b         | 81.66 ± 4.09a | 77.66 ± 2.10a | 82.66 ± 1.45a | 76.66 ± 2.02a | 73.33 ± 1.20a | 73.00 ± 5.71a |

Note: Means with different superscript indicate significant difference at p < .05.
The correlation between the glycosylated haemoglobin and fasting glucose levels indicated that FBG and HbA1c significantly (p < .01) correlate with each other with R^2 value of 0.914 on day 84 post treatment. The implication of this strong positive correlation between fasting blood glucose and glycosylated haemoglobin is that, in the absence of glycosylated haemoglobin (which is a gold standard for diabetes monitoring), fasting blood glucose can be a reliable alternative. Measurement of HbA1c has advantage over fasting blood glucose since it does not depend on the erratic prandial fluctuations in the blood glucose (Musenge et al., 2016). However, since the average life span of RBC is about 3 months, HbA1c assay may encounter limitations when there are decreases in RBC counts following senescence and consequent lysis of RBCs (Roszylk et al., 2007). The finding of strong positive correlation between FBG and HbA1c suggests that both parameters can be used simultaneously to compensate for each other and increase accuracy.

Conclusion
It was concluded that treatment of diabetic rats with methanol extract of *C. arborea* (especially at the dose of 125 mg/kg) for 84 days lowered the FBG levels of the rats and ameliorated haemoglobin glycosylation. The effects on both FBG and glycosylated haemoglobin were positively and strongly correlated.

Aknowledgements
We wish to heartily appreciate the effort of Dr Ignatius Maduka, Department of Human Biochemistry, Nnamdi Azikiwe University, Nnewi campus, Anambra state, Nigeria for helping in procurement of glycosylated haemoglobin kit used in this study.

Disclosure statement
No potential conflict of interest was reported by the authors.

References
Aba PE, Asuzu IU. 2016. ^1^H-Proton NMR spectra of antihyperglycemic triterpenoid isolated from *Cussonia arborea*. J Nat Prod. 9:5–13.
Aba PE, Asuzu IU. 2018. Mechanisms of actions of some bioactive antidiabetic principles from phytochemicals of medicinal plants: a review. Indian J Nat Prod Resour. 9(2):85–96.
Aba PE, Asuzu IU, Odo RI. 2014. Antihyperglycemic and antioxidant potential of *Cussonia arborea* in alloxan-induced diabetic rats. J Comp Clin Pathol. 23:451–458.
Akuodor GC, Udia PM, Bassey A, Chikala KC, Okozie OA. 2014. Antihyperglycemic and antihyperlipidemic properties of aqueous root extract of *Isocoma senegalensis* in alloxan-induced diabetic rats. J Acute Dis. 3(2):99–103.
Amadou MD, Anna SN, Diop MN, Guata YM, Diarall HR, Gaffary AN, Babacar F. 2008. Screening of plants for antidiabetic properties. Fund Clin Pharmacol. 22:211–216.
Anand P, Murali KY, Tandon V, Chandra R, Murthy PS. 2007. Preliminary studies on antihyperglycemic effect of aqueous extract of *Brassica nigra* (L.) Koch in streptozotocin induced diabetic rats. Indian J Exp Biol. 45:696–701.
Bunn HF, Hancy DN, Kamin S, Gabbay KH, Gallop PM. 1976. The biosynthesis of human hemoglobin A1c. Slow glycosylation of hemoglobin in vivo. J Clin Invest. 57:1652–1659.
Castellano JM, Guinda A, Delgado T, Mirela R, Cayuela JA. 2013. Biochemical basis of antidiabetic activity of oleandric acid and related triterpenes. Am Diabetes Assoc. 62(6):1791–1799.
Celik I, Yegin E, Odabasoglu F. 2002. Effect of experimental diabetes mellitus on plasma lactate dehydrogenase and glutamic oxaloacetic transaminase levels in rabbits. Turkish J Biol. 26:151–154.
De villiers BJ, Vuuren SF, Van zyl RL, Van wyk BE. 2010. Antimicrobial and antimalaria activity of *Cussonia arborea*. J Ethnopharmacol. 7:216–216.
Demanjek S, Bose SK, Sahu R, Mandal SC. 2008. Antidiabetic effect of matured fruits of *Diospyros peregrine* in alloxan induced diabetic rats. Inter J Green Pharm. 2:95–99.
Diana S, Kara N, Eleanor P, Jimmy C, Hertzcl C. 2010. The effect of oral anti-diabetic agents on A1c levels: systematic review and meta-analysis. Diab Care. 33(8):1859–1864.
Goldstein D, Wield-Mayer HM, England JD, Little RR, Parker KM. 1984. Recent advances in glycosylated hemoglobin measurements. CRC Crit Clin Lab Sci. 52:282–291.
International Expert Committee (IEC). 2009. International expert committee on the role of the HbA1c assay in the diagnosis of diabetes. Diab Care. 32:1327–1334.

Khazraji SM, Shamaony LA, Twaij HA. 1993. Hypoglycaemic effect of artemisia herba alba: effect of different parts and influence of the solvent on hypoglycemic activity. J Ethnopharmacol. 40:163–166.

Kilpatrick ES, Bloomgarden ZT, Zimmet PZ. 2009. Is haemoglobin A1c a step for diagnosing diabetes? Br Med J. 339:4432.

King H, Rewers M. 1993. Global estimates from prevalence of diabetes mellitus and impaired glucose tolerance in adults. WHO ad HO diabetes reporting group. Diab Care. 16(1):157–177.

Kinsangau DP, Hosea KM, Herbert VM, Cosam CJ, Zakaria HM, Pax JM, Catherine BG, Lenta NB, Krishna PD, Nobert S. 2007. Plant species used in treating various HIV/AIDS-related conditions in Bukoba rural district. J Ethnobiol Ethnomed. 3:29.

Liu TP, Liu IM, Cheng JT. 2005. Improvement of insulin resistance by panax ginseng in fructose-rich chow-fed rats. Horm Metab. Res. 37:146–151.

Min-Jia T, Ji-ming Y, Nigel T, Cordula H, Chang-Qiang K, Chung-Ping T. 2008. Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. Chem Biol. 15(3):263–273.

Musenge EM, Manankov A, Michel C, Mudenda B. 2016. Relationship between glycated haemoglobin and fasting plasma glucose among diabetic out-patients at the University Teaching Hospital, Lusaka, Zambia. Tanz J Health Res. 18(3):1–9.

Nagisa Y, Kato K, Watanabe K, Murakoshi H, Odaka H, Yoshikawa K, Sugiyama Y. 2003. Changes in glycated haemoglobin levels in diabetic rats measured with automatic affinity HPLC. Clin Exp Pharmacol Physiol. 30(10):752–758.

Oh TJ, Shin YJ, Kang GH, Park KS, Cho YM. 2013. Effect of the combination of metformin and fenofibrate on glucose homeostasis in diabetic Goto-kakizaki rats. Exp Mol Med. 45:30–58.

Rang HP. 2006. The receptor concept: pharmacology’s big idea. British J Pharmacol. 147:9–16.

Roszyk L, Faye B, Sapin V, Somda F, Taueron I. 2007. Glycated haemoglobin (HbA1c): today and tomorrow. Annals Endocrinol. (Paris). 68:357–365.

Santos FA, Frota JT, Arruda BR, de Melo TS, da Silva AA, Brito GA, Chaves MH, Rao VS. 2012. Antihyperglycemic effects of α and β-amin, a triterpenoid mixture from protium, heptaphyllum in mice. Lipids Health Dis., 11:98.

Szkudelski T. 2001. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol. Res. 50(6):537–546.

Tennant JR. 2010. Cussonia arborea Hochst ex. A.Rich. Flora Trop East Afri. 1:621.

Togashi Y, Shirakawa J, Okuyama T, Yamazaki S, Kyohara M, Miyazawa A, Suzuki T, Hamada M, Terauchi Y. 2016. Evaluation of the appropriateness of using glucometer for measuring the blood glucose levels in mice; Sci. Rep. 6:6255.

Venogopal PM, Prince PSM, Pari L. 1998. Hypoglycemic activities of Syzigium cumini seeds effect on lipid peroxidation in alloxan diabetic rats. J Ethnopharmacol. 61:1–7.

Weitgasser R, Davalli AM, Weir CC. 1999. Measurement of glucose concentations in rats: differences between glucose meter and plasma laboratory results. Diabetologia. 42:256.

WHO. 1980. WHO expert committee on Diabetes Mellitus. Second Report. Technical Report Series 646, Geneva, Switzerland.

Yu J, Zhang Y, Sun S, Shen J, Qiu J, Yin X, Yin H, Jiang S. 2006. Inhibitory effects of astragaloside IV on diabetic peripheral neuropathy in rats. J Physiol Pharmacol. 84:579–587.