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Alternative to Conventional Diabetic Management: The Antihyperglycaemic potential of an Ethyl Acetate Fraction Extract of *Holarrhena floribunda*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BBN, BNG and JAS were involved with research concept, data collection, data analysis, interpretation of results and writing of manuscript. Authors KD, IAK, PA and IAG contributed to the research concept, interpretation of results and review of the manuscript. Authors DOO, AN, APY were also involved in the research concept, data analysis and review of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Antihyperglycaemic effects of an ethyl acetate fraction of the leaf extract of *Holarrhena floribunda* on normal and streptozotocin-induced diabetic rats.
Study Design: Healthy adult albino Wistar rats (weighing 100-150 g) were used for this study. The rats were divided into 5 groups (5 animals in each group).

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Place and Duration of Study: Laboratory of Physiology, Pharmacology and Phytotherapy Côte d'Ivoire; School of Pharmacy, Ghana and 8 months.
Methodology: A hundred grams (100 g) of the powdered leaves was macerated in 96% ethanol and water. The antihyperglycaemic effect of the extract was studied in normoglycaemic and Streptozotocin (STZ)-induced diabetic rats. The effect of the extract on the levels of cholesterol, triglycerides, total proteins and creatinine in STZ-induced diabetic rats was also investigated.
Results: The ethyl acetate fraction of the leaf extract of *Holarrhena floribunda* showed antihyperglycaemic effect after both short term and prolonged treatment of the diabetic rats.
The results indicate that the extract could moderate blood lipid abnormalities, which would be helpful to the prevention of diabetic complications through improving dyslipidemia.
Conclusion: The present study indicates that treatment with ethyl acetate fraction of *Holarrhena floribunda* ethanolic leaf extract has favourable effects on blood glucose levels, serum lipids and body weight.

Keywords: Diabetes mellitus; ethyl acetate extract; Holarrhena floribunda; antihyperglycaemic; biochemical profile; ethnopharmacology; medicinal plants.

ABBREVIATIONS

Ethanolic extract of Holarrhena floribunda leaves (HFE); Ethyl acetate fraction of Holarrhena floribunda leaves extract (EAcf); streptozotocin (STZ); glibenclamide (GLB); diabetes mellitus (DM); oral glucose tolerance test (OGTT); total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and triglycerides (TG).

1. INTRODUCTION

Diabetes mellitus (DM) is a global chronic metabolic disorder recognized as one of the leading causes of morbidity and mortality in the world [1]. The number of people affected with diabetes worldwide has increased dramatically over recent years. About 2.5 to 7% of the world's population has been diagnosed with diabetes mellitus and the International Diabetes Federation (IDF) predicts further increases in the future [2]. In Africa, diabetes is currently considered to be a public health problem [3,4]. At least, 78% of Africans with diabetes remain undiagnosed and they are unaware they are living with diabetes [2].

Diabetes Mellitus is characterized by hyperglycaemia and alterations in carbohydrate, fat and protein metabolism, associated with absolute or relative deficiencies in insulin secretion and/or insulin action [5]. It is often accompanied by symptoms of polyuria, polydipsia, polyphagia and weight loss [6]. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels [7].

Globally, the treatment of diabetes involves vast resources including medicines, diets, and physical training. In Africa, the use of plant medicines to treat diabetes mellitus is a very common practice. This practice is considered as safe and less expensive [8,9]. Therefore, there is a need for investigation of new therapeutic strategies which might be cheaper, safe, and convenient for treatment of diabetes [10].

Studies have been ongoing on traditional medicines with a view to discover other hypoglycaemic agents from plants [11]. *Holarrhena floribunda* (Apocynaceae) is one such plant that has been investigated for anti-diabetic properties. *H. floribunda* is a tropical tree that can grow to a height of 17 meter and a girth of one meter. It is found in deciduous forests and savanna woodlands [12]. It is widely distributed in Côte d'Ivoire, Ghana, Burkina Faso, Mali and some other parts of West Africa. Traditionally, the roots of *H. floribunda* are useful in the management of constipation, colic discomforts and sterility. The root and bark are used to manage diarrhoea and dysentery while the stem bark is used to treat various ailments such as abdominal pains, nausea, indigestion, and diarrhoea. The leaves of *H. floribunda* have been employed to treat malaria and dysentery [13]. The leaves and the bark are used for treating diarrhoea while the leaves are exclusively used for amenorrhea and management of diabetes [13,14]. However, in general, there are scanty experimental studies on the pharmacological properties of the plant.

This paper, therefore reports work done on the hypoglycaemic effects of an ethyl acetate fraction of the leaf extract of *Holarrhena floribunda* on normal and streptozotocin-induced diabetic rats.

2. MATERIALS AND METHODS

2.1 Plant Collection

The leaves of *Holarrhena floribunda* (G. Don) Dur. et Shinz (Apocynaceae) were collected in Sikensi (south region of Côte d'Ivoire) in August 2010. The plant was identified and authenticated by Professor Aké-Assi. A voucher specimen (n°13240) of the plant was deposited in the herbarium of the National Floristic Centre of the University of Cocody, Abidjan.

2.2 Preparation of Extract and Fractions

Leaves of *H. floribunda* were cleaned, washed with distilled water, sliced into small pieces, air dried at ambient temperature for two weeks and grounded into powder. A hundred grams (100 g) of the powdered leaves were macerated in an 80:20 (volume /volume) mixture of ethanol (96%) and water for 48 hours (with occasional stirring) at room temperature. This operation was repeated for another 48 hours. The extract (HFE) was filtered twice through cotton wool, then through Whatman filter paper (N°1). The filtrate was concentrated using a rotavapor apparatus (Buchi R110/NKE6540/2, Switzerland) at a temperature of 45°C to yield 12.86 g of semi-solid mass of HFE. A portion of the dried HFE (8.5g) was suspended in water and the clear supernatant was partitioned between hexane and water (1:1) to obtain an aqueous fraction (AF) and hexane fraction (HF). The AF was further partitioned using dichloromethane, ethyl acetate and n-butanol to obtain dichloromethane, ethyl acetate, n-butanol fractions. Each fraction was concentrated at 45°C, using the rotavapor and then freeze-dried to yield 0.64 g of HFE, 0.72 g of dicloromethane fraction, 1.8 g of ethyl acetate fraction, 1.08 g of butanolic fraction and 2.5 g of aqueous fraction. The ethyl acetate extract of *Holarrhena floribunda* leaves (EAcf) was selected for the present study based on preliminary studies where it showed the highest hypoglycaemic activity.

2.3 Animals

Healthy adult albino Wistar rats (weighing 100-150 g) of both sexes were provided by UFR Biosciences (University of Cocody-Abidjan, Côte d'Ivoire) and housed in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (Ivograin®, Abidjan, Côte d'Ivoire) and were given water ad libitum. They were allowed to acclimatize to standard laboratory conditions (temperature 24-28°C, relative humidity 60-70%, and 12 hour light-dark cycle) for one week before the commencement of the experiments. The animals were deprived of food for at least 18 hours prior to experiments but allowed free access to drinking water. The usage and handling of animals were performed in accordance with the European Council legislation 87/609/EEC for the protection of experimental animals [15]. The protocols for the study were approved by the Departmental Ethics Committee.

2.4 Chemicals

Streptozotocin (Sigma St Louis, Mo, USA), Glibenclamide (Daonil®), Commercial diagnostic kits: It was used as a single intraperitoneal injection of 55 mg/kg body weight of streptozotocin dissolved in citrate buffer pH 4.5. After 72 hours of induction of diabetes by streptozotocin, the diabetic rats (glucose level > 250 mg/dl) were separated and used for the study [16].

In this study, glibenclamide was used as positive control in a dose of 10 mg/kg body weight [16]. A single oral administration of anhydrous glucose (4g/kg body weight) was used to induce hyperglycaemia (Oral Glucose Tolerance test) in normal rats [17,18]. Commercial diagnostic kits were used to analyze total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), total protein, creatinine, urea and uric acid activities:

2.5 Phytochemical Analysis of Ethyl Acetate Leaf Fraction

The ethyl acetate leaf fraction of *Holarrhena floribunda* was subjected to phytochemical screening using standard procedures [19,20].

2.6 Acute Toxicity

Acute toxicity studies were conducted to determine the LD50 value of *H. floribunda* leaves (EAcf) according to the method described by Miller and Tainter [21]. Thirty five rats were divided in seven groups of five animals. The ethyl acetate extract of *Holarrhena floribunda* leaves (EAcf), was administrated orally at doses of 500, 1000, 1500, 2000, 2500 and 3000 mg/kg body

weight to the animal groups (one dose per group). The control group received the vehicle (10 ml/kg) and all the animals were observed continuously for 2 h [22] for (i) behavioural profile (alertness, restlessness, irritability, and fearfulness), (ii) neurological profile (spontaneous activities, reactivity, touch response, pain response and gait, postural abnormalities) and (iii) autonomic profile (defecation and urination). After a period of 24 h, 48 h and 14 days the animals were observed for lethality or death.

2.7 Study on Normoglycemic Animals

Wistar rats (weighing 100-150 g) of both sexes were divided into 5 groups (5 animals in each group). Group 1 served as the vehicle control group (Veh), and received dose of 10 ml/kg of distilled water. Group 2 comprised normal Wistar rats treated with a single dose of glibenclamide (10 mg/kg) as a standard hypoglycaemic agent. Groups 3, 4 and 5 were also normal Wistar rats treated with single doses of EAcf (250, 500 and 1000 mg/kg per os, dissolved in vehicle). The hypoglycaemic effect of the plant extract was assessed by measuring blood glucose level at 0 h (before drug administration), and at 1, 2, 4 and 8 h (after drug administration).

2.8 Study on Glucose-loaded Animals [Oral Glucose Tolerance Test (OGTT)

An oral glucose tolerance test (OGTT) was performed on rats by feeding them with glucose (4 g/kg) per os. Normal rats were deprived of food 12 h before and during the experiment but were allowed free access to water. The rats were divided into 5 groups (5 animals in each group). Control group received the vehicle. Three groups received the EAcf at the doses of 250, 500 and 1000 mg/kg per os, respectively. One group received 10 mg/kg of glibenclamide. EAcf, glibenclamide and vehicle were orally administered 1 hour before anhydrous glucose administration (4 g/kg). Blood glucose level was determined before drug and glucose administration (−60 and 0 min, respectively) and subsequently at 30, 60, 90 and 120 min after.

2.9 Induction of Experimental Diabetes

Wistar rats fasted overnight were made diabetic by i.p injection of freshly dissolved STZ, 55 mg/kg, in citrate buffer (0.01 M, pH 4.5) [23]. Diabetes was confirmed in the STZ-treated rats by measuring the fasting blood glucose concentration 72 hours post STZ injection. Rats with blood glucose level above 250 mg/dl were considered to be diabetic and were used in the experiment [16]. All animals had free access to food and water after the STZ injection.

The dose of EAcf that produced a maximum reduction in blood glucose levels in normal and glucose-loaded animals in normal and diabetic rats was used in this section of the study. The rats were divided into 4 groups (6 animals in each group). Groups 1 constituted the vehicle control diabetic group (and received distilled water 10 ml/kg, p.o); Group 2 was diabetic rats treated with EAcf 1000 mg/kg, po. Group 3 diabetic rats treated with glibenclamide (10 mg/kg) as a standard hypoglycaemic agent. A separate group of normal, non-diabetic rats of six rats were used as normal control to all the STZ-induced diabetic rats. This group received 10ml/kg distilled water. The hypoglycaemic effect of the EAcf was assessed in the diabetic rats after a single oral administration by measuring blood glucose levels at 0 h (before drug administration), 1, 2, 4 and 8 h (after drug administration).

2.10 Estimation of Blood Glucose Level

Blood samples were obtained by nicking the tails of rats with a sharp razor [24] and glucose concentrations were determined using a one-touch glucometer (Accu-Chek Go®, Roche Diagnostics, Mannheim, Germany).

2.11 Collection and Processing of Blood for Analyses of Biochemical Parameters

At weekly intervals until the end of the experimental period (28 days or four weeks), the rats were anesthetized with diethyl ether following a 12 h overnight fast. The blood samples were collected from retro orbital sinus of the eye with a Pasteur pipette and transferred into tubes. Sera obtained from the blood samples by centrifugation (3000× g for 10 min) were stored at 4°C and analysed for biochemical parameters. Total cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine, measured at 500 nm, was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase [25]. The triglycerides were determined after enzymatic hydrolysis by lipases. The quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol reactions under peroxidase activity was

measured at 500 nm [25]. The total proteins were determined based on the reaction, in an alkaline medium, of cupric ions with protein peptide bonds, resulting in the formation of a coloured complex that indicates proteins concentration at 540 nm [26]. Urea was measured based on the reaction of salicylate and hypochlorite with the ammonium ions to form a green complex (2.2 dicarboxylindophenol) which can be read at 600 nm [27]. Uric acid was quantified uricase/peroxidase reaction by colorimetric enzymatic [28]. The high-density lipoprotein cholesterol (HDL) was quantified phosphotungstic and magnesium chlorure reaction by enzymatic method [29].

2.12 Data Analysis

Results were expressed as mean±SEM. Data were analysed for statistical significance with one and two-way ANOVA followed by the Fisher Snedecor test or a Bonferroni's post hoc test or a Dunnett's Multiple Comparison Test. GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered statistically significant in all analysis. The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

3. RESULTS

3.1 Phytochemical Analysis of Ethyl Acetate Leaf Fraction

Phytochemical screening of the ethyl acetate fraction of the *H. floribunda* ethanolic leaf extract revealed the presence of flavonoids, alkaloids, terpens, tannins and polyphenols.

3.2 Acute toxicity Effects

The acute toxicity studies revealed that the ethyl acetate leaf fraction was non-toxic below the dose of 3000 mg/kg body weight. There were no signs of toxicity after 3 – 5 hours of extract administration. No reduction in general locomotor activity, less sensitivity to touch, a decline in food intake and prostration occurred after 5 h of extract administration.

3.3 Effects of Eacf on Fasted Normoglycaemic Animals

The effect of EAcf on fasting blood glucose levels of normal rats is presented in Fig. 1. A significant decrease in blood glucose levels was observed in the animals from the first and second hour to the fourth hour following the administration of EAcf at doses of 500 and 1000 mg/kg (P< 0.05-0.0001), respectively. The maximum reduction in blood glucose was noted 4 h after the administration of the extract, 26.94% and 30.69% for 500 and 1000 mg/kg dose levels, respectively. Neither distilled water nor EAcf (250 mg/kg) showed hypoglycaemic effect on normal rats ($P > 0.05$).

Treatment of normal rats with glibenclamide (GLB) produced a significant hypoglycaemic effect from the first to the fifth hour, reaching a 38.44% maximum fall ($P < 0.01$) in the blood glucose, compared to the normal control group.

3.4 Effect of EAcf on Oral Glucose Tolerance Test (OGTT)]

The effect of EAcf on glucose tolerance is presented in Fig. 2. A dose-dependent effect was observed. In glucose-fed animals treated with distilled water, there was a significant increase in blood glucose levels after 30 to 90 minutes following administration of glucose ($P < 0.001$). The maximum increase in blood glucose was observed 30 minutes after administration of glucose. The EAcf (500 and 1000 mg/kg) and glibenclamide (10 mg/kg) significantly prevented a rise of the blood glucose level ($P < 0.05$-0.001) after 30 and 120 minutes compared to the control group. Following the administration of the extract at doses 500 and 1000 mg/kg, there was a percentage reduction of glucose levels of of 17 and 31.60% respectively compared to the normal. In addition, the EAcf at a dose of 250 mg/kg reduced the blood glucose ($P < 0.05$) at 30 and 60 min after glucose administration. Treatment of the rats with glibenclamide (GLB) produced a significantly reduction ($P < 0.01$-0.001) in blood glucose at 30 to 120 min after glucose administration. GLB produced a maximum reduction of 37.33% in blood glucose at 90 min after administration of glucose.

3.5 Acute Effects of Eacf on Blood Glucose of STZ-diabetic Rats

The effect of single dose administration of EAcf on fasting blood glucose levels of normal and STZ-induced diabetic rats is presented in Fig. 3. Following a 48 hours post streptozotocin injection, all diabetic rats exhibited hyperglycaemia, which ranged between 330 and 400 mg/dl compared to normal control non-diabetic rat level of 95-96 mg/dl.

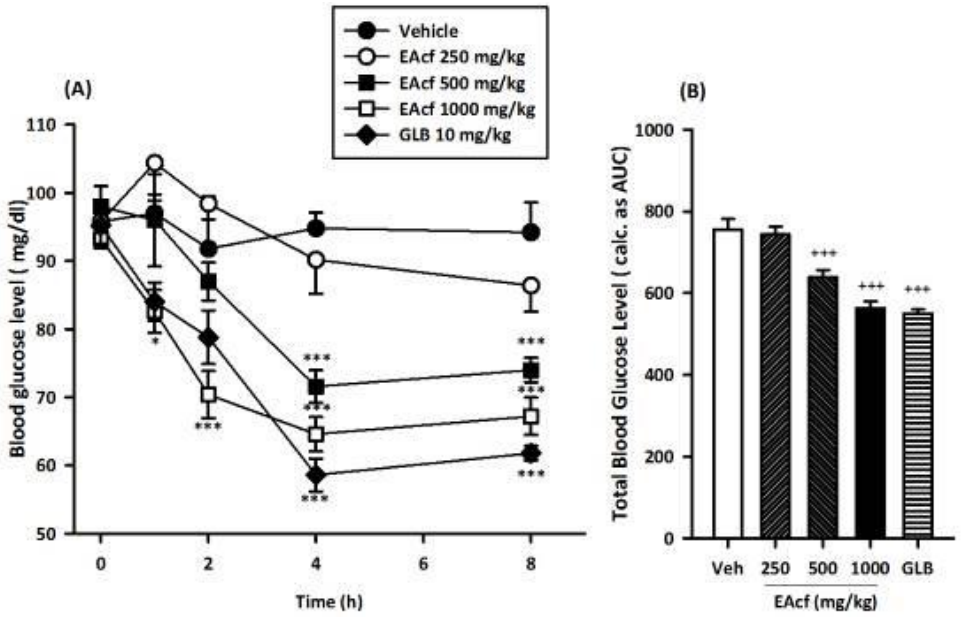

Fig. 1. Dose-response effects of EAcf (250-100mg/kg, *p.o.*) and glibenclamide, GLB (10 mg/kg) (A and B) on blood glucose concentration in normal rats. Chart A shows the time course effects over the eight (8) hour-period while chart B shows the total blood glucose level from AUC's over the eight hour- period

Data are Means±SEM (n = 6). $^{*}P < 0.05$, $^{***}P < 0.001$ compared to vehicle treated group (two-way ANOVA followed by a Bonferroni's post hoc test), $^{+++}P < 0.001$ compared to vehicle treated group (one-way ANOVA followed by a Dunnett's Multiple Comparison Test)

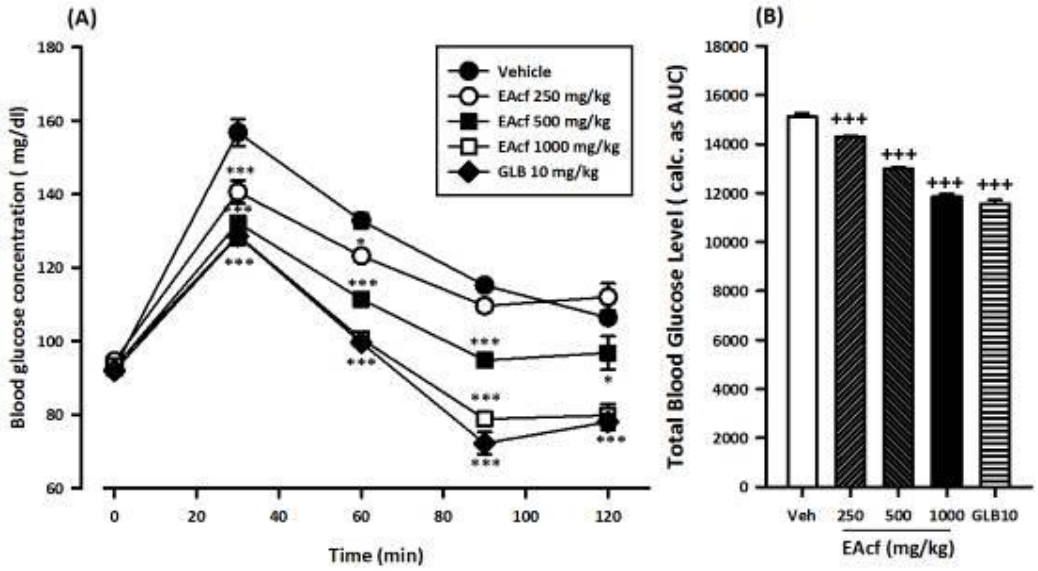

Fig. 2. Effect of oral administration of EAcf (250-1000 mg/kg) and Glibenclamide, GLB (10 mg/kg) (A and B) on blood glucose level of oral glucose loaded rats. Chart A shows the time course effects over the eight (8) hour-period (120 min) and B shows the total blood glucose level from AUC's over the eight hour- period

Data are Means±SEM (n = 6). $^{*}P < 0.05$, $^{***}P < 0.001$ compared to vehicle treated group (two-way ANOVA followed by a Bonferroni's post hoc test), $^{+++}P < 0.001$ compared to vehicle treated group (one-way ANOVA followed by a Dunnett's Multiple Comparison Test)

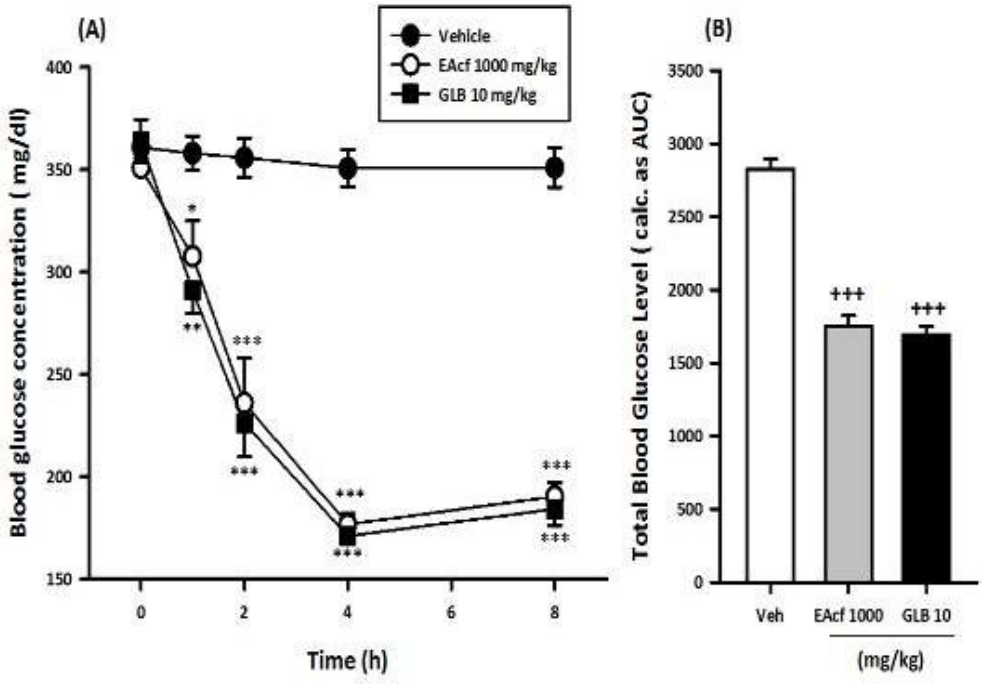

Fig. 3. The effect of EAcf (1000 mg/kg) and GLB (10 mg/kg) (A and B) on blood glucose kinetics of oral glucose tolerance test in STZ-induced diabetic rats. Panel A shows the time course effects over the eight (8) hour-period while panel B show the total blood glucose level from AUC's over the eight hour- period

Data are Means±SEM (n = 6). $^{***}P < 0.001$ compared to vehicle treated group (two-way ANOVA followed by a Bonferroni's post hoc test), $^{+++}P < 0.001$ compared to vehicle treated group (one-way ANOVA followed by a Dunnett's Multiple Comparison Test)

The extract (EAcf at 1000 mg/kg) produced a significant hypoglycaemic effect in STZ-induced diabetic rats ($P< 0.05$). In the STZ diabetic rats, a reduction of 50.58% was observed after 4 hours following administration of the plant extract. Treatment of diabetic rats with glibenclamide (GLB) also produced a significant hypoglycaemic effect ($P< 0.01$-0.001) from the 1st to the 8th hour. There was a maximum decrease 51.96% ($P < 0.001$) in the blood glucose after the 4th hour, as compared with the diabetic control group. Distilled water had no significant effect ($P>0.05$) on the blood glucose concentrations of the STZ-induced diabetic rats after acute treatment.

3.6 Long Term Effect of Leaf Extract of Daily Administration of *H. floribunda* on Blood Glucose Level in STZ-induced Diabetic Rats

Effects of the ethyl acetate fraction of the *H. floribunda* ethanolic leaf extract on fasting blood glucose levels of STZ-induced diabetic rats are shown in Fig. 4. After 7 days of daily administration of the extract, blood glucose levels of the diabetic rats decreased significantly ($P <0.001$). However, no significant differences were recorded ($P>0.05$) for blood glucose concentrations in STZ-induced diabetic control rats after 15-28days. At a dose of 1000 mg/kg EAcf produced significant lowering of blood glucose levels plant ($P <0.001$) at days 7, 14, 21 and 28 following treatment compared to control. Maximum reduction (43.08%) of glucose levels occurred on 14th day of treatment. The reference drug glibenclamide (10 mg/kg) caused a significantly ($P < 0.001$) decreased in blood glucose levels at 1, 7, 14, 21 and 28th days of treatment. There were no significant differences observed between the extract EAcf (1000 mg/kg) and glibenclamide (10 mg/kg).

3.7 Long Term Effect of Leaf Extract of Daily Administration of *H. floribunda* on Body Weight and Some Biochemical Parameters

Changes in the body weight in different groups are shown in (Table 1). Significant weight loss was observed in diabetic vehicle control group

than in the normal vehicle control. The groups of rats treated with EAcf (1000 mg/kg) and GLB (10 mg/kg) gained weight 11.98% ($P < 0.05$), and 12.92 %, respectively compared to the diabetic vehicle control group after 28 days. Serum total cholesterol levels were significantly lower (P<001) following the administration of EAcf and glibenclamide (Fig. 5A). The trend was the same for triglycerides (Fig. 5B), total protein (Fig. 5C), creatinine (Fig. 5D), LDL (Fig. 6A), urea (Fig. 6C) and uric acid (Fig. 6D) levels. In contrast, HDL levels were higher (Fig. 6B) compared with the diabetic vehicle control group.

4. DISCUSSION

The treatment of DM is managed with oral hypoglycaemic and anti-hyperglycaemic agents and/or insulin. Anti-diabetic plants used by traditional medicine practitioners in West Africa could be an important source of such agents. The aim of the present study was to evaluate the effects of ethyl acetate fraction of the *H. floribunda* ethanolic leaf extract (EAcf) on blood glucose and on some metabolic disorders related to diabetes mellitus.

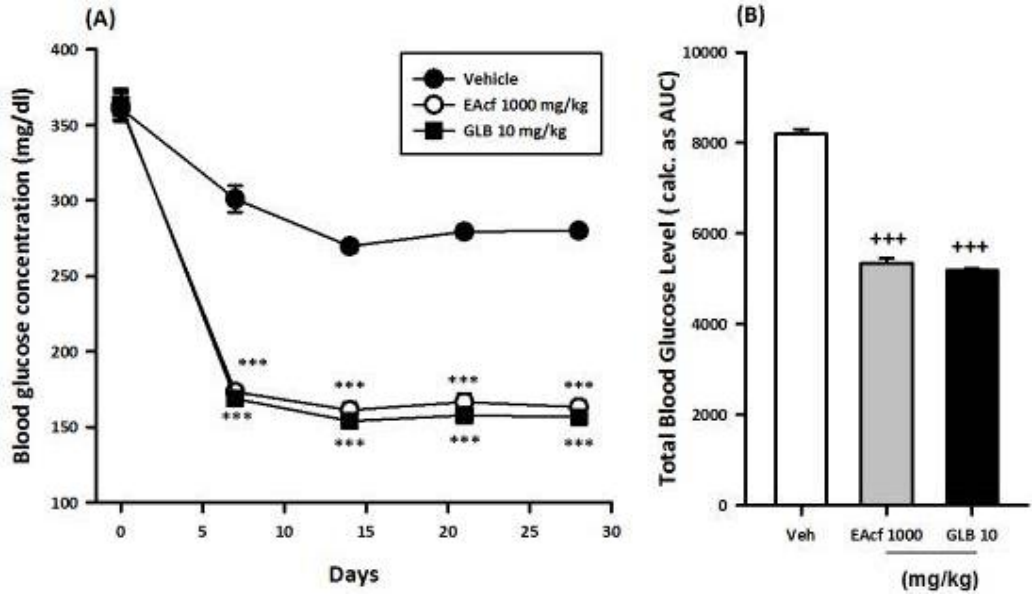

Fig. 4. The effect of EAcf (1000 mg/kg) and GLB (10 mg/kg) (A and B) on blood glucose kinetics of STZ-induced diabetic rats. Panel A shows the time course effects over twenty height (28) day-period and Panel B shows the total blood glucose level from AUCs over the twenty height day-period

Data are Means±SEM (n = 6). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle treated group (two-way ANOVA followed by a Bonferroni's post hoc test), $^{+++}P < 0.001$ compared to vehicle treated group (one-way ANOVA followed by a Dunnett's Multiple Comparison Test)

Table 1. Alteration in different body weight on treatment of diabetic rats with *Holarrhena floribunda* (leaves ethyl acetate fraction) and glibenclamide (n= 6)

Groups	Body weight(g) before and after treatment				
	Day 0	Day 7	Day 14	Day 21	Day 28
Normal control	173.17±2.15	177.83±3.19	181.33±2.56^{b*}	186±3,08^{b***}	190,67±2,29^{b***}
Diabetic control	175.33±3.08	171±2.73^{b*}	159.67±0.84 ^{a***b**}	151.17±1.83 ^{a***b***}	146.17±1.96 ^{a***b***}
EAcf 1000	169,67±3,76	170.83±4.34	174.5±4.33^{c*}	183±3.51^{c***}	192±2.68 ^{b**c***}
Glibenclamide	170.33±2.99	172.67±3.58^{b*}	177.67±3.58^{b*c**}	186.5±3.13^{b*c***}	190±3.61^{b*c***}

Data are Means±SEM (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Statistical comparison: (a) Normal control vs (EAcf 1000 or Diabetic control); (b) Day 0 vs (Day 7 or Day 14 or Day 21 or Day 28); (c) Diabetic control vs (EAcf 1000 or Glibenclamide); one-way ANOVA followed by a Fisher-Snedecor multiple comparison test

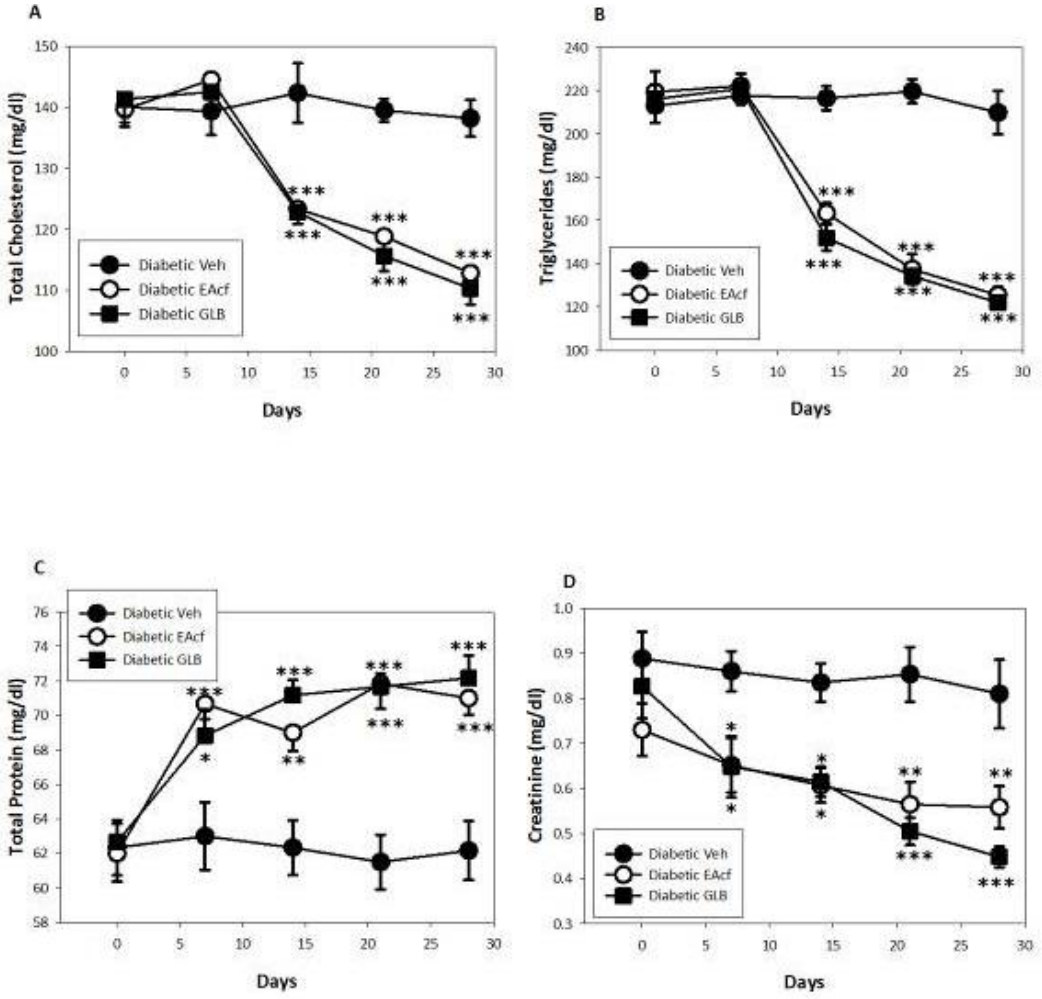

Fig. 5. The effect of EAcf (1000 mg/kg) [Diabetic EAcf] and GLB (10 mg/kg) [Diabetic GLB] on the levels of cholesterol (A), triglycerides (B), total proteins (C) and creatinine (D) in STZ-induced diabetic rats. The graphs represent the time course effects over twenty height (28) day-period

Data are Means±SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle treated group [Diabetic Veh] (two-way ANOVA followed by a Bonferroni's post hoc test)

The hypoglycaemic effect of glibenclamide, a sulphonylureas, also decreased fasting blood glucose levels due to its facilitation of endogenous insulin release from pancreatic β-cells and the promotion and facilitation of peripheral glucose uptake and utilisation [30,31].

The maximum decrease in glucose tolerance was noted for the tested doses of 500 and 1000 mg/kg only after 90 min, respectively 17.71 and 31.60% compared with control. This effect could be accounted for at least in part by a decrease in intestinal glucose absorption achieved by an extra pancreatic action that includes the stimulation of peripheral glucose utilisation or the enhancement of glycolytic and glycogenic processes with a concomitant decrease in glycogenolysis and glyconeogenesis [32]. In addition, the maximum reductions in the blood glucose levels of the fasted and glucose given rats occurred at the dose of 1000 mg/kg.

In this study a single administration of EAcf (1000 mg/kg b.w.) significantly decreased blood glucose level in STZ-induced diabetes in rats (Fig. 3). The mechanisms by which streptozotocin brings about its diabetic state include selective destruction of pancreatic insulin secreting beta cells, which make cells less active [33,34] and lead to poor glucose utilisation by

tissues [11]. The hypoglycaemic effect of EAcf in this model of experimental diabetes in rats suggests that the extract may possess an insulin-like effect on peripheral tissues by either promoting glucose uptake and metabolism, by inhibiting hepatic gluconeogenesis [35] or absorption of glucose into the muscles and adipose tissues [36] by the stimulation of a regeneration process and revitalisation of the remaining β cells [37-39]. After 28 days treatment, EAcf significantly reduced the high fasting glucose level in streptozotocin-induced diabetic rats (Fig. 4). This extract showed antihyperglycaemic effect after the prolonged treatment.

In this study, streptozotocin-induced diabetic untreated rats were characterized by severe loss in body weight of untreated rats, which might be due to increased muscle wasting in diabetes [40]. Moreover, when EAcf was administered to diabetic rats for a period of 28 days, there were differences in weights of the rats. The change in body weight showed that the rats given the extract had a significant effect in controlling the loss of body weight (Table 1). The weight gains seem to be as a result of the ability of the extracts to reduce hyperglycaemia within the period of this study [41]. An increase in body weight of diabetic rats might be due to an improvement in insulin secretion and glycaemic control [42].

Diabetes mellitus usually affects carbohydrate, fat and protein metabolisms, followed by multi organs regression in the later period and hyperlipidaemias are associated with hyperglycaemia [42,43].

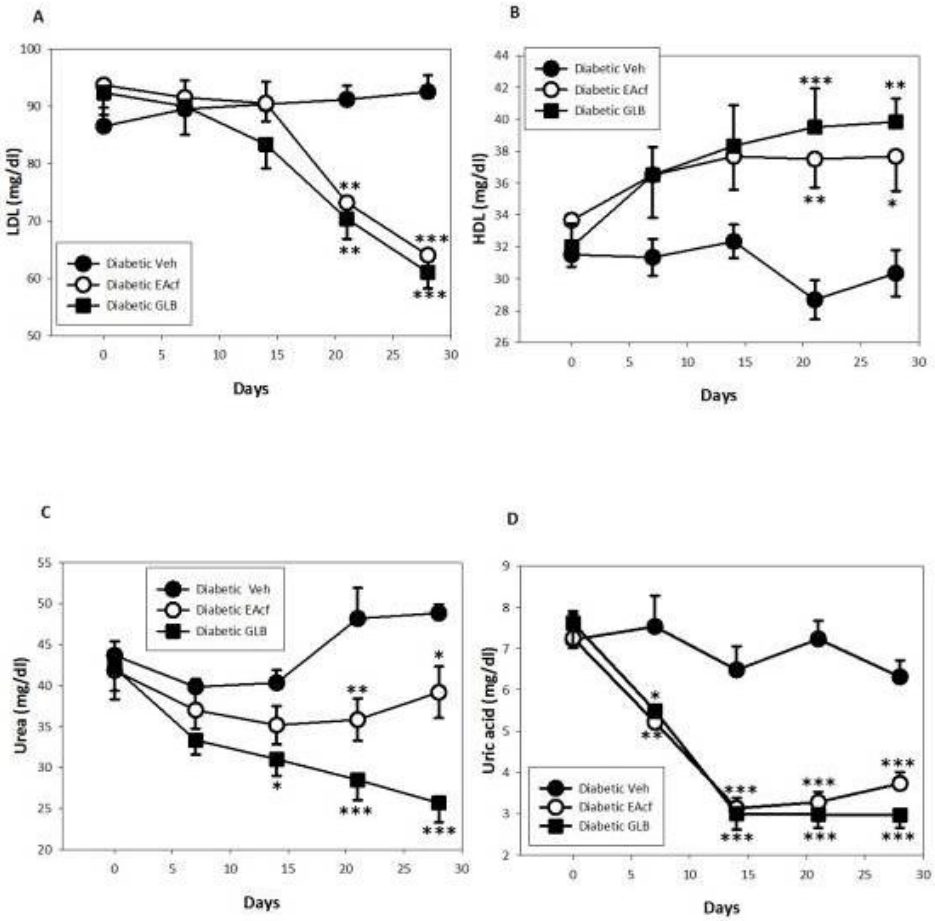

Fig. 6. The effect of EAcf (1000 mg/kg) [Diabetic EAcf] and GLB (10 mg/kg) [Diabetic GLB] on the levels of LDL (A), HDL (B), urea (C) and uric acid (D) in STZ-induced diabetic rats. The graphs represent the time course effects over twenty height (28) day-period

Data are Means±SEM (n = 6); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle treated group [Diabetic Veh] (two-way ANOVA followed by a Bonferroni's post hoc test)

Hypercholesterolemia and hypertriglyceridemia are primary factors involved in the development of arteriosclerosis and coronary heart diseases which are the secondary complications of diabetes [44].

The treatment of the diabetic rats with EAcf markedly decreased triglycerides, total cholesterol and LDL, but increased HDL and total protein (Figs. 5 and 6). The results indicate that EAcf could moderate blood lipid abnormalities, which would be helpful to the prevention of diabetic complications through improving dyslipidemia.

Renal impairment is one of the serious and common diabetic complications. The diabetic rats had increased levels of serum urea, uric acid and creatinine, which are considered as significant markers of renal function impairment [45]. Creatinine is a waste product formed in the muscle by creatine metabolism. Creatine is synthesized in the liver, passes into the circulation and is taken up almost entirely by the skeletal muscle. Its retention in the blood is evidence of kidney impairment [46]. The significant reduction in serum urea, uric acid and creatinine levels in rats treated with EAcf suggests that the extracts improved glycaemic control as well as modulated metabolic disturbances that adversely impacted the renal system.

The toxicity studies indicated that the ethyl acetate leaf fraction is non-toxic up to dose of 3000 mg/kg body weight. According to Schorderet [47], substances with LD50 values greater than 5g/kg body weight are considered to show low toxicity. Thus the EAcf can be said to be of low toxicity. The toxicity of EAcf was nearly equal to the methanolic extract of the Stem bark [48] of *H. floribunda* which LD_{50} values was greater than 5g/kg body weight. These results suggested that ethyl acetate fraction of *Holarrhena floribunda* ethanolic leaf extract contained some biological principle(s) that possess insulin protective or insulin-like activity [49]. However, further experiments are required to elucidate the exact mechanism of action as well as on the isolation of bioactive principles.

The phytochemical screening performed in this study revealed the presence of alkaloids, flavonoids, polyterpens, polyphenols and tannins. In a recent study, the phytochemical screening of *Holarrhena floribunda* leaf revealed the presence of some components such as alkaloid, saponin, tannin and cardiac glycosides in methanolic extract [12]. Thus, the activities of *H. floribunda* could be attributed to some of these active chemical constituents.

Phenolics are found to be effective antihyperglycemic agents [50,51]. Alkaloids and tannins have been reported to possess hypoglycemic activity [52,53]. The saponin is known to elicit serum cholesterol lowering activity and may be classified as a direct hypoglycaemic agent, in contrast to the indirect agents such as the sulphonylureas that act by stimulating the pancreatic beta cells to release more insulin [54,55]. The phenols are reported to inhibit alpha-amylase, sucrase, as well as action of sodium glucose-transporter 1 (SGLUT-1) of the intestinal brush border [56]. Tannins and crude saponins are also reported to possess potent SGLUT-1 mediated inhibition of glucose transport [56]. The flavonoids have been found to be responsible for blood glucose lowering activity in experimental animals [57] and might be responsible for promoting glucose uptake by muscle tissues in streptozotocin-induced diabetic rats [58].

5. CONCLUSION

The present study indicates that treatment with ethyl acetate fraction of *Holarrhena floribunda* ethanolic leaf extract has favourable effects on blood glucose levels, serum lipids and body weight. This indicates that *Holarrhena floribunda* leaves could be a useful antidiabetic medicinal plant. The results also suggest that *Holarrhena floribunda* leaves have potential for use in managing renal impairment associated with diabetic mellitus. Further studies are necessary to isolate the compounds responsible for the observed antihyperglycaemic activity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed. All experiments have been examined and approved by the ethics committee.

The usage and handling of animals were performed in accordance with the European Council legislation 87/609/EEC for the protection

of experimental animals. The protocols for the study were approved by the Departmental Ethics Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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