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Evaluation of acute toxicity, *In-vitro* α-Amylase and α-glucosidase inhibitory and hypoglycaemic effects of the hydro-ethanolic leaf extract of *Bridelia ferruginea* Benth in Alloxan–induced Diabetes in Rats

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Abstract
Background: The discovery that plants could serve as indispensable therapeutics weapons against various pathogenic and physiological ailments has made them a sine qua non to human and animal lives. *Bridelia ferrugihea* is one of such plants extensively employed by traditional herbalists in Nigeria.
Objectives: This study investigated the oral acute toxicity and *in vitro* inhibitory effects of different solvents leaf extracts of *B. ferrugihea*. Also evaluated were the antioxidant activities, postprandial and hypoglycaemic effects of *B. ferruginea* leaf extract.
Materials and Methods: Acute toxicity of the extract was evaluated in Swiss albino mice, fed with extract doses of 1.0 to 20.0 g/kg body weight, observed for 72hr (Acute toxicity study). The crude extract and fractions of *Bridelia ferruginea* leaf were tested for enzyme inhibitory effects at different concentrations. Forty Wister rats were induced diabetes with saline alloxan monohydrate (150mg/kgbwt) and were randomly assigned to eight groups and treated with the extract doses the mixture and reference drugs for 30days. The effects of the extract on weight, plasma glucose and other biochemical parameters were evaluated using standard procedures.
Results: Acute toxicity studies showed that animals treated with different doses survived beyond 24hrs.Significant difference ($p \leq 0.05$) in IC_{50} of reference drug, and fractions were observed. Methanol and n-hexane extracts inhibited α-glucosidase significantly ($p \leq 0.05$).
Conclusion: In **t**he study *Bridelia ferruginea* leaf extract demonstrated to have potential antidiabetic activity supporting the traditional claim. It also demonstrated *in vitro* the inhibition of enzymes of α-amylase and α-glucosidase involved in carbohydrate metabolism.

Keywords: α-amylase, α-glucosidase. Acute toxicity, postprandial and hypoglycercemia

Introduction
Plants and their derived products from the onset have served as veritable sources of food for humans and animals and the discovery since prehistoric era that plants products, in addition to their food and nutritive values, could serve as potential therapeutic weapons against various human, animal and even plant diseases has made plants a sine qua non to human and animal lives (Ogbonnia *et al*., 2008 [27]; 2011 [32]). Plant derived medicine popularly known as "Herbal drug" or "phytomedicine" is currently renowned and is recognized as the most common form of alternative medicine. It is used by about 60% of the world population both in the developing and in the developed countries where modern medicines are predominantly used (Rickert *et al*., 1999 [40]; Ogbonnia *et al*., 2009 [28]). The use of herbal remedies especially in the form of teas or extracts for the treatment of various diseases is gaining increasing popularity, making them the main stay of health care system, especially among the rural populace in the developing countries. This increasing popularity could be attributed to their advantages of being efficacious and cheap with fewer side effects and could be employed to manage such diseases that orthodox medicines could not easily proffer solutions to and one of such diseases is diabetes mellitus.
Diabetes mellitus (DM) has been described as a chronic metabolic disorder resulting from defects of insulin secretion, or increased cellular resistance to insulin or both. (WHO, 1999; Ahmed and Goldstein, 2006, Andrade-Cetto *et al*., 2007) [51, 2, 4].

It is now recognized as one of the leading causes of death in developing countries, where the high prevalence of the disease can be attributed to improved nutritional status coupled with a gross lack of modern facilities for early diagnosis of the disease (Ogbonnia *et al*., 2010 [30]). Diabetes mellitus is often characterized by chronic elevation of glucose in the blood known as hyperglycemia which could lead to renal failure, vision impairment and a range of other types of tissue damages (Ogbonnia *et al*., 2010 [30]).

The major goals in the treatment of diabetes, therefore, have been to keep both short-term and long-term glucose levels within acceptable limits, thereby reducing the risk of long term complications (Ogbonnia *et al*., 2016 [33]). This could be achieved by optimizing both fasting blood glucose and postprandial glucose levels found to be very important in achieving near normal glucose levels. Postprandial glucose levels have been reported to serve as a better maker of glycaemic control than fasting blood sugar levels (Ogbonnia el al., 2016 [33]), and some drugs acting by inhibiting α-glucosidase activity, have been developed to improve postprandial hyperglycaemia.

Several drugs such as biguanides, sulfonylureas, thiazolidinediones are currently being used for the management of diabetes. However, they are associated with serious side-effects such as hypoglycaemia, flatulence, diarrhoea, weight gain and are often unable to lower glucose concentrations to within the normal range, or to reinstate a normal pattern of glucose homeostasis (Senthilvel *et al.*, 2006 [51]; Ogbonnia *et al*., 2011 [32]). Even when effective glycaemic control is achieved, the use of these drugs is restricted by their pharmacokinetic properties, secondary failure rates and accompanied undesirable effects (El.Nagar *et al.*, 2005 [11]; Ogbonnia *et al*., 2009 [28]). For these reasons, there is apparent need to intensify search for an acceptable, cheap and safe blood sugar lowering oral hypoglycaemic agents that would be more effective in the treatment of diabetes and devoid of serious side effects that is associated with the currently used oral hypoglycaemic agents. Therefore, alternative therapy, with plants and plant derived products are being considered as the best options. (Pari and Saravanan, 2004 [36]; Ogbonnia *et al.*, 2010 [30]).

Currently, many researches are working on the possibilities of decreasing postprandial hyperglycemia by delaying the absorption of carbohydrates/glucose through the inhibition of carbohydrate hydrolysing enzymes, α-amylase and α-glucosidase enzymes using natural product compounds (Etxeberria *et al*., 2012 [12], Swanston-Flatt, 1990 [48]; Puls *et al*., 1977 [37]; Lebovitz, 1998 [22]; Singh *et al*., 2001 [42]; Ojewole, 2002 [36]). Inhibitors of both α-amylase and α-glucosidase delay digestion and subsequent absorption of carbohydrates thereby lowering postprandial glucose levels (Puls *et al*., 1977 [37]). Studies with inhibitors of enzymes involved in both glycogenolysis and gluconeogenesis suggest that inhibition of these enzymes could be one of the mechanisms whereby some anti-diabetic agents exert their blood glucose lowering effect (Marles and Farnsworth, 1995 [24]). Hyperglycemia also has been found to contribute to the development oxidative stress.

Oxidative stress has been considered as a major contributing factor associated with the β-cell damage (Quilliot *et al*. 2005 [39]). Oxidative stress emerges from the existence of free radicals (molecules possessing an unpaired electron) and reactive oxygen species (ROS), formed in normal physiological processes but become deleterious when they are not quenched by a cascade of antioxidant systems. In type 2 diabetes, resulting hyperglycemia induces generation of free radicals, including ROS, hydroxyl and nitric oxide (NO) radicals (Ceriello, 2006 [9]) which are responsible for oxidative stress leading to pancreatic β-cell destruction as well as the activation of all major pathways underlying the different components of chronic vascular diabetic complications such as glycation and sorbitol pathways among others. Plant based drugs are being investigated for the management of diabetes and accompanied oxidative stress. *Bridelia ferruginea* Benth is one of such plants.

Bridelia ferruginea Benth. (Fam. Euphorbiaceae) bark extract has been reported to have other activities including trypanocidal, molluscidal (Adeoye *et al*, 1988 [1]) antimicrobial (Ndukwe *et al*. 2005 [26]) and anti-inflammatory (Olajide *et al*. 1999 [36]). However, detailed investigation of α- glucosidase or α- amylase inhibitory activity of the extracts of the plants has not been carried out to date. In this study a comprehensive and systematic investigations of *α*-glucosidase and *α*-amylase inhibitory activities of the solvent fraction of *Bridelia ferruginea*, along with identification of some chemical compounds in most solvent fractions were carried out as well as establishing the mechanisms of *α* -glucosidase and *α* -amylase inhibitions *via* the enzyme kinetics approach.

Materials and Methods

Preparation of Plant Extracts

Fresh leaves of *Bridelia ferruginea* Benth (Euphobiaceae) were obtained from a local market in Abeokuta. The plant was identified and authenticated by O.O. Oyebanji, a taxonomist at Department of Botany, University of Lagos. A voucher specimen with voucher number LUH 7550 was deposited at the herbarium of the Department of Botany for reference purposes. They were dried under room temperature and ground to coarse powder. The powdered leaves were extracted using cold macerations in 90% ethanol for two weeks. A rotary evaporator was used to concentrate the extract. The crude extract obtained was further lyophilized at -4 ^{0}C to obtain 88.20g dry mass (7.35% yield)

Reagents and Chemicals

General chemicals used include organic solvents (n-Hexane, Ethyl acetate and Methanol). Other chemicals used were 3, 5-Dinitrosalicilate reagent (DNS) and sodium carbonate. All were obtained from Merck and were of analytical grade. Substrates used for α-amylase include soluble starch. Substrate for α-glucosidase was *p*-Nitrophenyl glycopyranoside and all were purchased from Sigma-Aldrich Chemical Company.

Preliminary Phytochemical Screening

Phytochemical screenings of the extract for the presence of primary and secondary metabolites were performed with standard methods as described by Farnsworth [13], (1966); Harborne [17], (1998) and, Sofowora [45] (1993). The presence or absence of anthraquinones, reducing sugars, saponins, tannins, terpenoids. flavonoids, steroids, cardiac glycosides, coumarins, alkaloids, carbohydrates and cyanogenetic glycosides was, therefore, established.

Alpha(α)- Amylase and Alpha (α)-Glucosidase Assay

α-Amylase Inhibitory Assay

This assay was carried out using a modified procedure of McCue and Shetty (2004) [24]. In a 96-well plate, reaction mixture containing 250µl of 0.02 M sodium phosphate buffer (pH 6.9), 10 µl α-amylase (0.5 mg/mL), 250 μL of varying concentrations of extract and fractions (40µg/ml, 80µg/ml, 120µg/ml, 160µg/ml, and 200µg/ml) was pre-incubated at 37°C for 20 min. after which 250 μL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals and then further incubated at 37°C for 20 min. The reaction was terminated by adding 500 μL of dinitrosalicylic acid (DNS) reagent and the absorbance was measured at 540 nm using spectrophotometer. Acarbose at various concentrations was used as a standard. A control was prepared using the same procedure replacing the extract with distilled water. The α-amylase inhibitory activity was calculated as percentage inhibition:

$$\%\text{Inhibition}=\left[\frac{\text{Abscontrol}-\text{Absextract}}{\text{Abscontrol}}\right] \text{X } 100$$

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically.

Mode of α-Amylase Inhibition

The mode of inhibition of α-amylase by the leaf extract was conducted using the extract with the lowest IC_{50} according to the modified method described by Ali *et al*. (2006) [3]. Briefly, 250 μL of the extract (40µg/ml) was pre-incubated with 250 μL of α-amylase solution for 10 min at 37°C in one set of tubes. In another set of tubes α-amylase was pre-incubated with 250 μL of phosphate buffer (pH 6.9). 250 μL of starch solution at increasing concentrations (0.25-5mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 25°C and then boiled for 5 min after the addition of 500 μL of DNS to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using a maltose standard curve and converted to reaction velocities. A double reciprocal plot ($1/v$ versus $1/(S)$) where v is reaction velocity and (S) is substrate concentration was plotted. The type (mode) of inhibition of the crude extract on α-amylase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

α-Glucosidase Inhibitory Assay

The effect of the plant extracts on α-glucosidase activity was determined according to the method described by Kim *et al*. using α-glucosidase from *Saccharomyces cerevisiae*. The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 50µl of 20 mM phosphate buffer (pH 6.9). 100 μL of α-glucosidase (1.0 U/mL) was pre incubated with 50 μL of the different concentrations of the extracts (40, 80, 120, 160 and 200 µg/ml) for 10 min. Then 50 μL of 3.0 mM (pNPG) as a substrate dissolved in 20 mM phosphate buffer (pH 6.9) was then added to start the reaction. The reaction mixture was incubated at 37°C for 20 min and stopped by adding 2 mL of 0.1 M Na_2CO_3. The α-glucosidase activity was determined by measuring the yellow-coloured paranitrophenol released from pNPG at 405 nm. Acarbose at various concentrations (40-200 µg/ml) was included as a standard. The results were expressed as percentage of the blank control.

Percentage inhibition is calculated as

$$\%\text{Inhibition}=\left[\frac{\text{Abs control}-\text{Abs extract}}{\text{Abs control}}\right] \text{x } 100$$

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically. Percent inhibition obtained in each sample was processed in form of graph and compared to the percent inhibition of acarbose as a positive control.

Mode of α-Glucosidase Inhibition

The mode of inhibition of α-glucosidase by the leaf extract was determined using the extract with the lowest IC_{50} according to the modified method described by Ali *et al*. Briefly, 50 μL of the (5mg/mL) extract was preincubated with 100 μL of α- glucosidase solution for 10 min at 25^0C in one set of tubes. In another set of tubes α-glucosidase was preincubated with 50 μL of phosphate buffer (pH 6.9). 50 μL of pNPG at increasing concentrations (0.63–2.0mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10min at 25^0C, and 500 μL of Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using a paranitrophenol standard curve and converted to reaction velocities. A double reciprocal plot (1/V versus 1/[S]) where V is reaction velocity and [S] is substrate concentration was plotted. The type (mode) of inhibition of the crude extract on α-glucosidase activity was determined by analysis of the double reciprocal (Lineweaver-Burk) plot using Michaelis-Menten kinetics.

Laboratory Animals

Swiss albino mice (22.5±2.5g) and Wistar rats (120±10g) of both sexes were obtained from the Laboratory Animal House of the College of Medicine, University of Lagos, Idi-Araba and kept under standard environmental conditions (12/12hr light/dark cycle). They were housed in cages (5 animals per cage), maintained on standard animal pellets (Pfizer Feeds Plc, Nigeria), and provided with water *ad libitum.* They were allowed to acclimatize for seven days to the laboratory conditions before the experiment. The use and care of the animals were in strict compliance with the National Research Council guidelines on the care and use of laboratory animals (NRC, 2011).

Acute Toxicity Assay

The toxicity study was carried out using twenty five (25) (male and female) Swiss albino mice weighing between 22.5± 2.5g. The animals were randomly distributed into a control group and four treated groups, consisting of five animals per group. After fasting the animals overnight, the control group was given 0.3ml Acacia (2%) suspension orally. while each of the treatment groups received oral solution of the extract prepared with 2% acacia in the doses of 1.0, 2.5, 5.0, 10.0, and 15.0 and 20.0 g/kg body weight respectively. The animals were observed continuously for the first 4 hours and then hourly for the next 24hours and at 6 hourly interval for the next 48 hours after administering the extract to observe any death or changes in general

behaviour and other physiological activities (Shah *et al*., 1997 [43], Bürger *et al.*, 2005 [8]). The dose that results in 50% mortality (LD_{50}) was then determined.

In vivo antidiabetic study of *Bridelia ferruginea* extract

Post -prandial Study

Normal rats male and female were fasted overnight and divided into eight groups of five rats each. Blood samples were collected from the tail veins of the rats to estimate the fasting blood glucose levels. The control, was given 2% Acacia and group 2, 3, 4 and 5 and 6 were given 100, 250, 5000mg/kg of extract, 250mg/kg extract + metronidazole and 5mg/kg of glibenclamide respectively. Group 7 received distilled water while group eight was given metronidazole only. Thirty minutes after, the rats in each group were administered 40% (w/v) glucose at a dose of 1ml/100g body weight orally (Ogbonnia *et al*., 2016 [33]). Blood glucose levels monitored at 30minutes, 60minutes and 120 minutes intervals and reported as the average glucose level of each group.

Induction of experimental diabetes

Healthy Wistar adult rats of both sexes weighing 120 ±10 g were used. The animals were fed on animal cubes (Feeds Nigeria Ltd) and provided with water ad libitium. Diabetes was experimentally induced after fasting animals overnight by administering intraperitoneally (i.p) alloxan monohydrate dissolved in normal saline in the dose of 150 mg/kg body weight. After 72hrs the plasma blood sugar levels were monitored with a glucometer (ACCU-CHEK, Roche Diagnostics) and the rats with plasma glucose >200 mg/dl were classified as diabetic and were included in the study.

Experimental design

The animals were randomly distributed into a total of eight groups containing five animals per group were used. Seven groups were diabetic while the remaining group was used as normal control and were treated daily for 30 days as follows:

Group

I. Induced but treated with 2% acacia solution (Diabetic control)
II. Induced treated with 100mg/kg of extract (E100)
III. Induced treated with 250mg/kg of extract
IV. Induced treated with 500mg/kg of extract
V. Induced treated with 250mg/kg extract +3mg metronidazole
VI. Induced treated with 0.6mg/kg Glibenclamide (Mahdi *et al*., 2003)
VII. Not induced not treated (NINT) (Normal control)
VIII. Induced treated with metronidazole only

Biochemical Studies

The animals were initially weighed and then weighed every four days from the starting of the treatment. On the 30th day, they were anaesthetised with warm urethane and chloralose (25%:1%v/v) at a dose of 5 ml/ kg and blood obtained via cardiac puncture into heparinized container. The blood was centrifuged within 5 min of collection at 4000 g for 10 min to obtain plasma which was analysed for glucose level, total cholesterol, total triglyceride, HDL cholesterol levels by precipitation and modified enzymatic procedures from Sigma Diagnostics (Wasan *et al*., 2001) [50]. LDL cholesterol levels were calculated using Friedwald equation (Crook, 2006) [10]. Plasma was analysed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinines by standard enzymatic assay analysis and the plasma protein and glucose contents were determined using enzymatic spectroscopic methods (Hussain and Eshrat, 2002) [18].

Tissue histology

The pancreatic tissue harvested from each group was fixed in 10% buffered formalin for 7 days before subjecting the tissues to routine histological processing techniques as described by Grizzle *et al*. (2008) [15] and staining with Haematoxylin and Eosin (H and E). Each section was examined under light microscope at high power magnification (×100 and x400) for structural changes. Photomicrographs were taken using an attached digital camera.

Fractionation the crude extract

Vacuum Liquid Chromatography

The VLC column was dry packed with silica gel (Kiesegel 60G, Merck, Germany). Care was taken so that the packing was kept uniform and making an evenly flat surface. The crude extract to be worked on was first mixed with some silica gel and applied as dry powder onto the top of the well packed VLC. Elution was done using various proportions of a solvent gradient of increasing polarity, starting with 100 % *n*-hexane (1L) then EtOAc (1L) and finally MeOH (1L). The fractions collected were concentrated and the percentage yield of each fraction determined

Statistics analysis

Data analysis was done using Graph Pad Prism 7. One way analysis of variance (ANOVA) was used to compare means. One–way ANOVA was done followed by Dunnett's multiple comparisons test of treated groups with control. The results were expressed as Mean ± SEM. Level of significance was set at $p < 0.05$ and $p < 0.01$.

Results

Preliminary phytochemical screening

Results of the preliminary phytochemical screening of the crude extract is as shown in the table below

Table 1: Phytochemical Constituents of *Bridelia ferruginea* Benth

Phytochemical Constituent	Amount Present
Alkaloids	+
Flavonoids	++
Phenols	+
Tannins	++
Saponin Glycosides	++
Cyanogenetic glycosides	-
Cardiac Glycosides	++
Reducing sugars	+
Steroids	_
Terpenoids	++
Anthraquinones	-

++ = abundantly present; + =slightly present - = absent

Screening of *B. ferruginea* leaf crude extract and fractions for enzyme inhibitory activities

The crude extract, and methanol, ethyl acetate and n-hexane fractions of *Bridelia ferruginea* were screened for enzyme inhibitory activities against α-amylase and α-glucosidase

respectively. The results were shown in Figures 1(a) and (b); 2 (a) and (b) below.

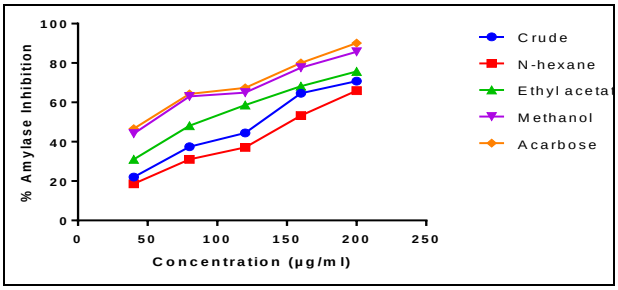


Fig 1(a): Graph of Amylase Inhibition by the extracts of *Bridelia ferruginea*.

For the α-amylase activity (Fig. 1a), Acarbose, the standard drug displayed strong inhibition towards α-amylase enzyme with an IC_{50} value of 50µg/ml. Methanol fraction of the extract showed the highest inhibition potential for amylase enzyme with an IC_{50} value of 55.0µg/ml followed by ethyl acetate fraction with an IC_{50} value of 88.8µg/ml. The crude extract displayed minimal inhibition with an IC_{50} value of 133.0µg/ml. The least inhibition was displayed by the n-hexane fraction with an IC_{50} value of 148µg/ml.

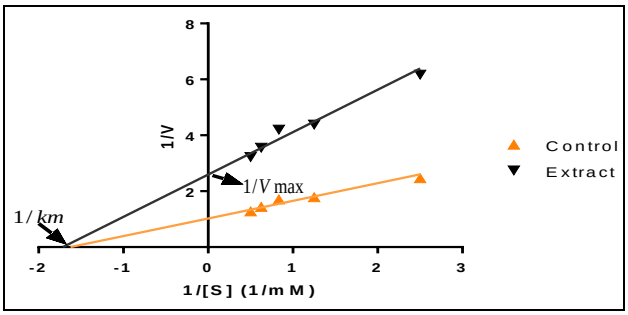


Fig 1(b): Mode of inhibition of α- amylase (Line weaver Burk Plot showing 1/v against 1/s (mM)

A line weaver-Burk plot (Double reciprocal plot) of the fraction with the least IC_{50} value was done. The Line weaver-Burk plot (Fig.1b) showed that the methanol fraction of the extract of this plant displayed a non-competitive inhibition against α-amylase enzyme as the K_M (Michaelis-Menten constant) remained unaffected during the reaction while the V_{Max} increased.

Fig 2(a): Inhibition of α-Glucosidase by *Bridelia ferruginea*

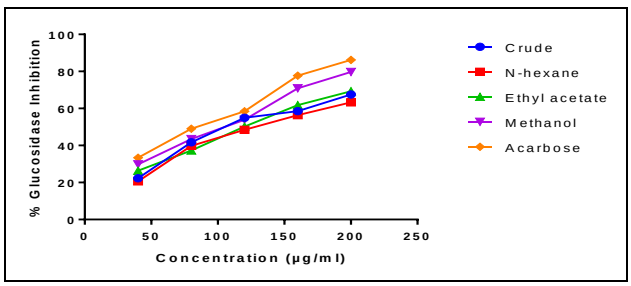


Fig 2(a): Graph of % Inhibiton of α-Glucosidase by *Bridelia ferruginea*

For α-glucosidase inhibitory effects (Fig.2b), the n-hexane fraction of the extract exhibited the strongest inhibition potential towards the enzyme with an IC50 value of 25.5µg/ml. The crude fraction had an IC50 value of 27.5µg/ml, ethyl acetate fraction, 28.5µg/ml and methanol 33.5µg/ml.

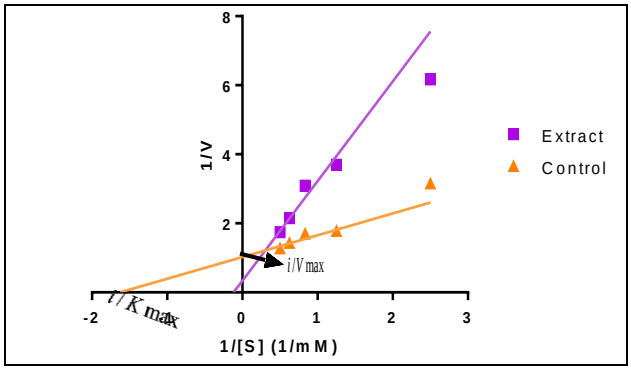


Fig 2(b): Mode of Inhibition of α- Glucosidase (Lineweaver-Burk Plot)

Table 2: Oral acute toxicity of the leaf extract of *Bridelia ferruginea*

Group	No of mice	Dose (mg/kg)	Log dose	No of Deaths	% Cumulative Death
1.	5	Control	-	0	-
2.	5	5,000	3.69	0	0
3.	5	10,000	4.00	0	0
4.	5	15,000	4.18	0	0
5.	5	20,000	4.30	0	0

Control group received 0.3 ml each of 2% acacia solution. Acute toxicity studies revealed that *Bridelia feruginea* was nontoxic in nature since no death, no lethality or toxic signs were observed. The animals that received a very high dose of 20g/kgbwt survived beyond 72hr. The median lethal dose (LD_{50}) value of the extract could be assumed to be higher than 20g/kg bwt. According to Ghosh (1984) and Klaasen *et al.*, (1995), the mixture can be classified as being non-toxic, since the LD_{50} by oral route was found to be much higher than WHO toxicity index of 2 g/kg.

The weight variation effects of animals treated with various doses of *B. ferruginea* extract and glibenclamide

The summary of the effects on the body weight of rats induced with diabetes and treated with various doses of the extract, standard drug, glibenclamide, and induced but untreated animals were shown in Fig 6. There was a significant ($p< 0.05$) weight reduction in diabetic rats treated with only acacia on the 28th day when compared with rats treated with glibenclamide and 500mg/kg extract.

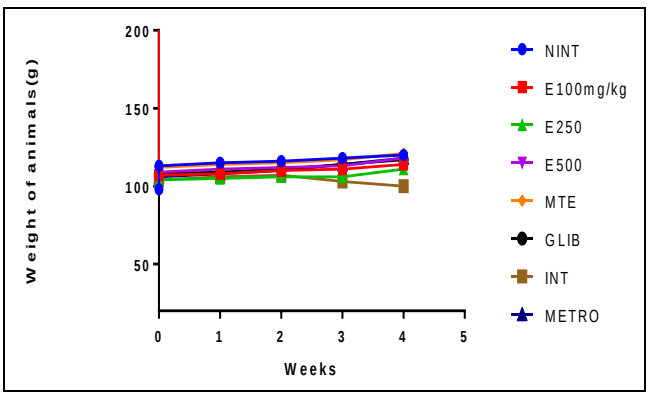


Fig 6: Effect of Bridelia ferruginea extract on the weight of animals

INT= Induced not treated (Diabetic control), NINT= Not induced not treated (Normal control), E100 = Induced treated with 100mg/kg extract, E250 = diabetic rats treated with 250mg/kg extract. E500 = Diabetic treated with 500mg/kg extract, GLIB= Diabetic rats treated with Glibenclamide (Standard Control), MTE= 250mg/kg extract + Metronidazole, Metro =Diabetic treated with metronidazole

The effect of *B. ferruginea* extract on postprandial blood glucose level

Figure 7: is the summary of the effect of various doses of *B. ferruginea* extract and standard drug glibenclamide on postprandial glucose level in diabetic rats. There was a significant reduction (p>0.05) in blood glucose levels after 90minutes in treated animals when compared with the diabetic control with the most significant being those treated with glibenclamide.

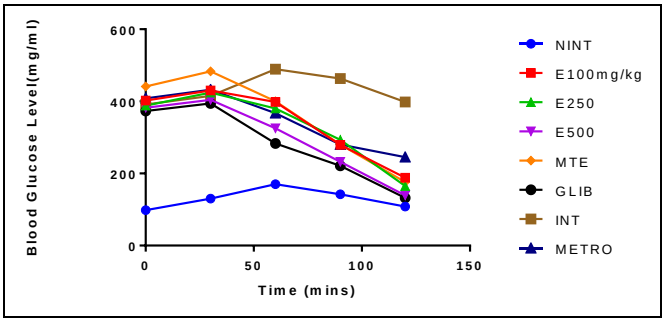


Fig 7: Post Prandial Test of *Bridelia ferruginea* extract

Figure 8: Weekly Blood Glucose Level of Diabetic Rats But Treated With the Extracts, Normal Untreated, Glibenclamide and Diabetic but Treated with Acacia Solution (Control)

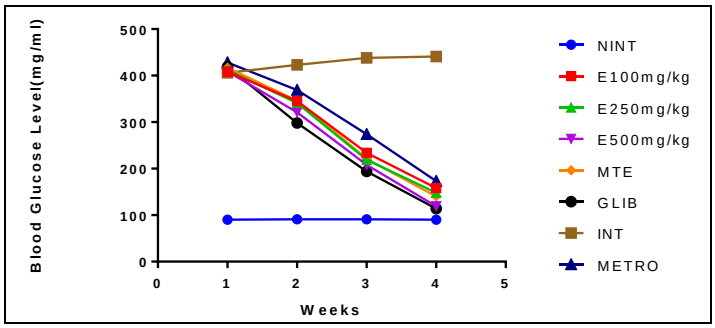


Fig 8: Weekly Blood Glucose Levels of Rats Treated with *Bridelia ferruginea* extract, Glibenclamide and Untreated rats.

Effects of *bridelia feruginea* on lipid profile

A significant decrease (p<0.05) in the plasma total cholesterol (TC) level was observed in all the diabetic animals treated with the extract or glibenclamide while a significant increase in TC level was observed in the untreated diabetic rats. There was no significant difference in the LDL of treated rats when compared with the diabetic control.

Table 3: Effects of *Bridelia feruginea* on the Lipid Profile of Diabetic Rats Treated with the Extracts, Normal Untreated, Glibenclamide and Diabetic but Treated with Acacia Solution (Control).

	INT	NINT	E100 mg/kg	E250 mg/kg	E500 mg/kg	MTE	GLIB	MT
Triglyceride (mmol/L)	138.7±1.9	94.6±1.3*	87.5±2,6*	83.26±0.7*	86.9±1.4*	91.2±3.1*	78.1±2.7*	88.9±0.8*
Cholesterol (mmol/L)	148.3±1.5	84.6±1.4*	86.2±2.4*	79.1±1.5*	86.4±2.6*	69.4±1.3*	63.7±0.9*	72.3±1.8*
HDL(mmol/L)	41.4±1.3	70.3±1.6*	67.2±3.2	56.7±1.9	63.7±1.4	57.9±0.8	66.0±1.5	69.4±0.6
LDL(mmol/L)	126.4±1.7	96.4±1.9*	131.3±2.4	108.6±1.4	106.5±1.8	103.0±1.5*	91.0±2.4*	111.9±3.1

N=5, Value= Mean±SEM * p< 0.05, ** p< 0.01 vs control group (The difference is significant). INT= Induced not treated (Diabetic control), NINT= Not induced not treated (Normal control), E100= Induced treated with 100mg/kg extract, E250= diabetic rats treated with 250mg/kg extract. E500= Diabetic treated with 500mg/kg extract, GLIB= Diabetic rats treated with Glibenclamide (Standard Control), MTE= 250mg/kg extract + Metronidazole, METRO=Diabetic treated with metronidazole only.

Table 4: The effects various doses of the extract and glibenclamide on biochemical parameters of diabetic rats and induced but untreated rats

Table 4 summarized the result of the effects of various doses of *Bridelia ferruginea* extract and glibenclamide on the biochemical parameters. The extract and glibenclimade significantly (p<0.05) reduced the plasma glucose levels of the diabetic rats and astronomical increase in the plasma glucose level of the untreated alloxan-induced diabetic compared with the control. There was no significant difference (p < 0.05) in the plasma protein levels of the diabetic rats treated with 500 mg/kg 250mg/kg, 100mg/kg of the extract and the diabetic rats untreated. The plasma albumin levels of the diabetic rats treated with glibenclamide and the extracts showed no significant decrease (p < 0.05) compared with the control. There was a significant increase (p < 0.05) in the plasma creatinine levels of the untreated diabetic rats compared with the treated. The plasma urea levels of the diabetic rats treated with glibenclamide, 500 mg/kg and 250 mg/kg of the extracts were significantly increased (p < 0.05) compared with the control. There was no significant change in the plasma AST, ALT and ALP of the diabetic rats treated with the extract compared with the control. However, there was a significant increase (p < 0.05) in the plasma AST, ALT and ALP of the diabetic rats treated with insulin compared with the control. There was also a significant increase in (p < 0.05) the plasma AST and ALT levels of the diabetic rats untreated compared with the control.

Table 4: Plasma glucose level and other biochemical parameters of diabetic rats treated with the extracts, normal untreated, glibenclamide and diabetic but treated with acacia solution (Control)

Parameter	INT	NINT	E100 mg/kg	E250 mg/kg	E500 mg/kg	MTE	GLIB	MT
Glu (mmol/L)	406±1.2	92.3±2.1*	139.4±0.8*	131.8±1.9*	119.4±2.1*	124.3±0.6*	108.2±1.9*	159.8±10*
T.Protein (g/L)	4.0±0.6	6.9±1.0	6.9±0.5	5.5±0/8	6.1±0.1	7.4±0.9	6.8±0.08	5.7±0.60
ALB(g/L)	45.3±1.3	43.7±2.5	41.3±3.2	44.5±2.3	46.9±1.4	46.7±1.3	39.7±0.9	40.5±1.3
ASTI.U/L)	107±1.7	56.24±1.4*	57.18±2.8*	59.12±1.8*	58.12±3.4*	63.47±1.7*	61.63±2.8*	62.19±3.1*
ALT(I.U/L)	34.17±2.	28.20±1.9	24.51±3.4*	28.13±1.8	23.19±4.9*	27.26±3.8	29.14±2.4	30.19±3.1
Creatinine (mg/dl)	2.1±0.8	0.8*±0.02	0.8±0.02*	1.2±0.05	0.7*±0.01	0.8±0.03	0.6*±0.02	1.4±0.06
Bilirubin(µmol/L))	0.8±0.02	0.7±01.	0.6±0.01	0.5±±01.	0.5±02.	0.6±03	0.6±0.03	0.5±01.
ALP(µmol/L)	29.6±02.	71.4±1.*	46.2±3.2	49.7±3.2	52.1±3.1	44.7±2.1	76.3±3.2*	48.3±2.3
Urea(µmol/L)	27.7±1.5	31.9±2.1	34.4±2.7	34.2±3.3	36.4±2.2	34.4±2.4	33. ±3.2	33.8±2.7

N=5, Value= Mean±sem * $p< 0.05$, ** $p< 0.01$ vs control group (The difference is significant). INT= Induced not treated (Diabetic control), NINT= Not induced not treated (Normal control), E100= Induced treated with 100mg/kg extract, E250= diabetic rats treated with 250mg/kg extract. E500= Diabetic treated with 500mg/kg extract, GLIB= Diabetic rats treated with Glibenclamide (Standard Control), MTE= 250mg/kg extract + Metronidazole, METRO= Diabetic treated with metronidazole only.

Table 5: Haematology and blood chemistry parameters of diabetic rats treated with the extracts, normal untreated, glibenclamide and diabetic but treated with acacia solution (Control)

Parameter	INT (Control)	NINT	E100 mg/kg	E250 mg/kg	E500 mg/kg	MTE	GLIB	MT
RBC ($x10^6$)	4.20±1.1	5.93±1.2	5.4±1.3	4.9±0.9	5.8±1.5	6.1±0.9	5.2±1.4	5.32±2.7
Hb (g/dl)	9.3±1.1	11.7±1.2	11.4±0.6	12.8±0.8	12.4±1.3	12.9±2.4	13.2±1.7	11.9±2.2
WBC($x10^3$)	5.3±1.2	5.7±1.1	5.2±0.9	5.8±0.3	6.1±0.4	5.9±2.1	5.40±0.9.	5.0±2.3
MCV (FL)	56.3±3.6	55.4±3.2	52.8±2.1	57.9±1.9	60.3±3.2	59.4±2.9	54.2±1.4	55.2±2.1
MCH (pg)	18.4±3.2	19.70±4.1	19.0±1.4	19.2±3.8	18.9±1.7	20.2±1.7	18.3±2.6	19.23±2.3
MCHC(g/dl)	33.1±2.3	34.6±1.4	30.1±2.4	32.0±4.1	39.4±2.1	31.4±3.5	38.4±2.9	33.1±2.7
PCV (%)	23.4±1.1	31.4±3.3*	31.8±2.7*	28.9±1.9	33.4±3.7*	32.7±3.3	32.3±4.1*	30.7±2.6

N=5, Value= Mean±SEM * $p< 0.05$, ** $p< 0.01$ vs control group (The difference is significant).

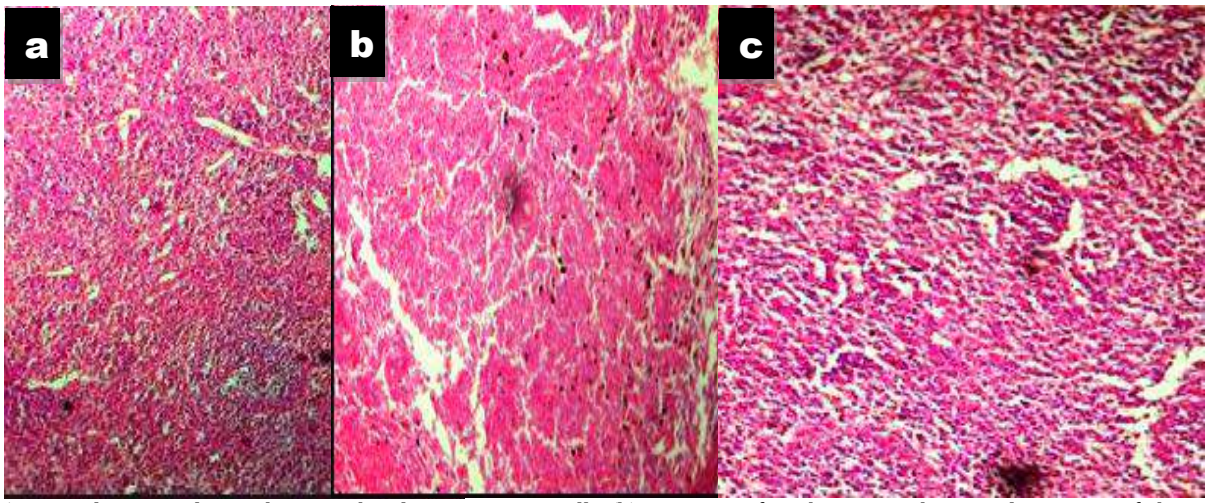
N=5, Value= Mean±sem * $p< 0.05$, ** $p< 0.01$ vs control group (The difference is significant). INT= Induced not treated (Diabetic control), NINT= Not induced not treated (Normal control), E100= Induced treated with 100mg/kg extract, E250= diabetic rats treated with 250mg/kg extract. E500= Diabetic treated with 500mg/kg extract, GLIB= Diabetic rats treated with Glibenclamide (Standard Control), MTE= 250mg/kg extract + Metronidazole, METRO= Diabetic treated with metronidazole only.

Table 6: Organs weights variation of diabetic rats treated with the extracts, normal untreated, glibenclamide and diabetic but treated with acacia solution (Control).

Organ weight/ 100g	INT (Diabetic control)	NINT (Normal control)	E100 mg/kg	E250 mg/kg	E500 mg/kg	MTE	GLIB	MT
Heart	0.41±0.08	0.49±0.03	0.44±0.04	0.46±0.13	0.46±0.06	0.49±0.06	0.48±0.02	0.41±0.01
Liver	3.62±0.19	5.10±0.92	3.86±0.72	3.91±0.91	4.28±0.83	4.46±0.65	4.91±0.62	4.73±0.81
Kidney	0.72±0.04	0.91±0.06	0.93±0.07	0.72±0.05	0.84±0.01	0.88±0.13	0.83±0.09	0.86±0.04
Pancreas	0.86±0.09	1.13±0.04	1.12±0.11	0.98±0.06	1.03±0.09	1.10±0.13	1.02±0.01	0.87±0.2

N=5, Value= Mean±sem * $p< 0.05$, ** $p< 0.01$ vs control group (The difference is significant). INT= Induced not treated (Diabetic control), NINT= Not induced not treated (Normal control), E100= Induced treated with 100mg/kg extract, E250= diabetic rats treated with 250mg/kg extract. E500= Diabetic treated with 500mg/kg extract, GLIB= Diabetic rats treated with Glibenclamide (Standard Control), MTE= 250mg/kg extract + Metronidazole, METRO= Diabetic treated with metronidazole only.

Histopathological studies



(a) Normal untreated rats showing abundant pancreatic cells (b) Pancreas of Diabetic rats showing destruction of the pancreatic cells (c) Pancreas of diabetic rats after treatment with glibenclamide (d) Pancreas of diabetic rats after treatment with 500mg/kg of extract (e) Pancreas of diabetic rats after
(b) Treatment with 250mg/kg of extract (f) Pancreas of diabetic rats after treatment with 100mg/kg of extract. (All figures are in x10 magnification
(a) Normal untreated rats showing abundant pancreatic cells (b) Pancreas of Diabetic rats showing destruction of the pancreatic cells (c) Pancreas of diabetic rats after treatment with 500mg/kg of extract

Fig 9: Histological Examination of Rat Pancreas after 30 Days Treatment:

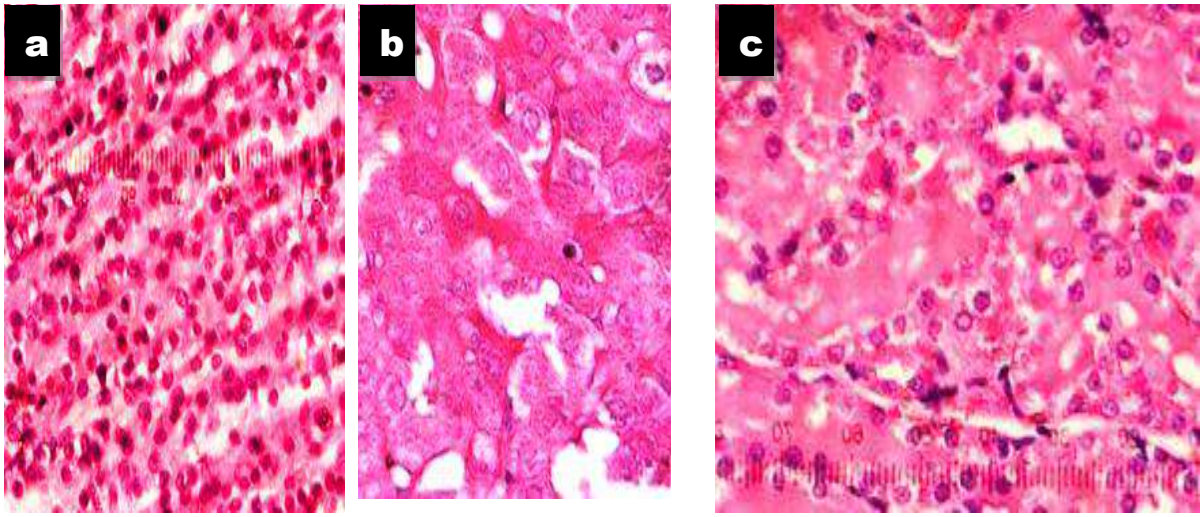


Fig 4.8.2 (a): Liver of normal rats (Positive control) b. Liver of diabetic rat showing necrotic lesions C. Liver of diabetic rats after treatment with 500mg/kg of *B. ferruginea* extract

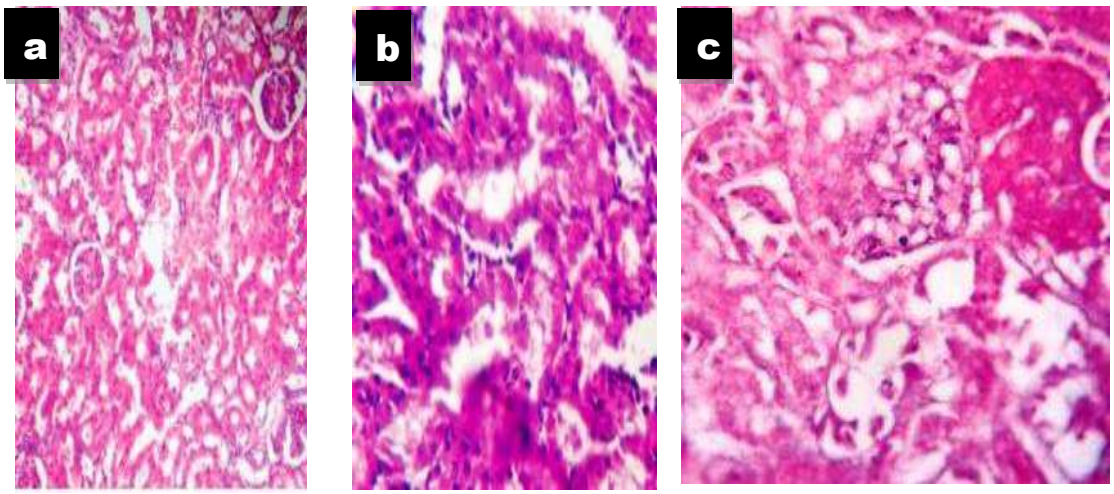


Fig 4.8.3 (a): Kidney tissues of normal untreated rats (b) Kidney tissues of diabetic untreated rats (Negative control). (c) Kidney tissues of diabetic rats after treatment with 500mg/kg of extract

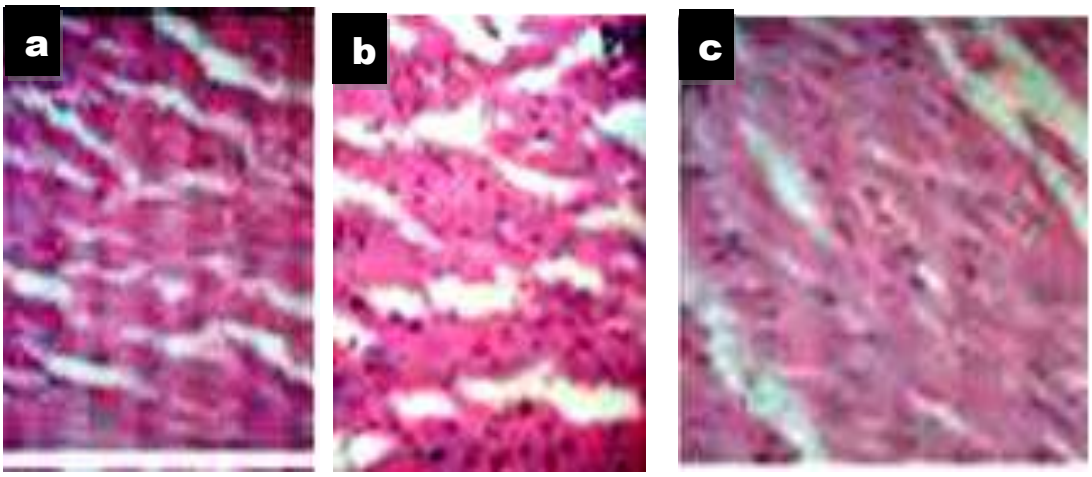


Fig 4.8.4: (a) Heart muscles of normal untreated rats (b) Heart muscles of diabetic untreated rats (c) Heart muscles of diabetic rats after treatment with 500mg/kg of extract. (All images are in x10 magnification)

Discussion

Herbal medicines have received greater attention as alternative to clinical therapy in recent times leading to subsequent increase in their demands (Sushruta *et al.*, 2006 [46]; Ogbonnia *et al.*, 2010 [30]). In rural communities, the exclusive use of herbal drugs, prepared and dispensed by herbalists without formal training, for the treatment of diseases is still very common. Experimental screening method is, therefore, required for the evaluation of the claimed activity, and to ascertain the safety and efficacy of these herbal products as well as to establish their active constituents (Ogbonnia *et al.*, 2008 [27]). Hyperglycemia is the primary clinical manifestation of diabetes and is thought to contribute to diabetic complications by altering vascular cellular metabolism, vascular matrix and circulating lipoproteins (Barnett and O'Gara, 2003 [5]).

The presence of various secondary metabolites such as flavonoids, terpenoids and tannins was observed in the extract. The observed physiological and pharmacological activities such as antidiabetic activity could, therefore, be attributed to the secondary products, hence the use of the plant by the traditional herbalists to manage various ailments.

The study demonstrated that *Bridelia ferruginea* leaf extract and its various fractions exhibited potent inhibitory effects on the activities of carbohydrate digesting enzymes such as *α*-amylase and *α*-glucosidase. This was evidenced in the inhibitory activity of methanol fraction (IC_{50} value of 55µg/ml) which showed no significant difference ($p \geq 0.05$) with the activity of the standard drug acarbose (IC_{50} value of 50µg/ml) while the ethyl acetate fraction had an activity of IC_{50} value of 88.8µg/ml. The crude extract and n-hexane fractions displayed minimal inhibitory activities of IC_{50}, 133.0 and 148 µg/ml values respectively The mode of inhibition was also observed to be non-competitive as the K_M (Michaelis-Menten constant) remained unaffected during the reaction while the V_{Max} increased(Fig.1b). This suggested that the active components in the extract do not compete with the substrates for the active site of the enzyme but may be binding to other sites of the enzymes rather than the active site. Increasing the substrate concentration had no significant effect on the enzyme activity unlike in competitive inhibition.

The n-hexane fraction of the extract exhibited the strongest inhibitory potential towards the *α*-glucosidase (Fig. 2b), enzyme with an IC_{50} value of 25.5µg/ml. The crude extract, ethylacetate and methanol fractions had IC_{50} values of 275, and 28.5 µg/ml respectively All the fractions exhibited stronger inhibition for the *α*-glucosidase than standard drug than acarbose. These observations were in accordance with reports of Kwon *et al.* (2006) which noted that natural *α*-glucosidase inhibitors from plants had better inhibitory activities against *α*-glucosidase and therefore could serve as potential and effective therapies for the management of postprandial hyperglycemia and could be associated with minimal side effects. Inhibition of *α*-glucosidase activity lead to delay in the breaking down of carbohydrate in the small intestine and could diminish the postprandial blood glucose excursion in a person suffering from diabetes Kwon *et al.* (2012 [20]).Therefore, one of the strategies and methods being exploited currently for the management of diabetes mellitus might involve the inhibition of carbohydrate digesting enzymes such as *α*-amylase and *α*-glucosidase in the gastrointestinal glucose absorption thereby lowering postprandial glucose level (Kwon *et al.* 2006 [21]). Glucosidase inhibitors are commonly prescribed to diabetics to reduce postprandial hyperglycemia induced by the digestion of starch in the small intestine (Bolen *et al.*, 2007 [7]). These inhibitors are designed to primarily target α-amylase and α-glucosidase, two members of exo-acting glycoside hydrolase enzymes (glucosidases) found in the intestinal tract that are critical for the digestion of carbohydrates. The overall effect of inhibition is to reduce the flow of glucose from complex dietary carbohydrates into the bloodstream, diminishing the postprandial effect of starch consumption on blood glucose levels (Lebovitz, 1997) [22].

The acute toxicity results are shown in Table 2. All the animals that received the highest extract dose of 20 g/kg bwt survived beyond 24hr. The median lethal dose (LD_{50}) value of the extract could be said to be higher dose than 20 g/kg bwt. According to Ghosh [1984 [14]] and Klaasen *et al.* [1995] [19], the extract can be classified as being non toxic, since the LD_{50} extract by oral route was found to be much higher than 15g/kg and also higher than WHO toxicity index of 2 g/kg.

The major goal in the treatment of diabetes has been to keep both short-term and long-term glucose levels within acceptable limits, thereby reducing the risk of long term complications (Park *et al.*, 2009 [37]). The extract was

observed to show good postprandial effects which could be attributed to α-glucosidase inhibitory activities associated with this class of drugs.

There was observed a significant ($p \leq 0.05$) gain in weight of the diabetic animals treated with a sulphonylurea while non significant ($p \geq 0.05$) weight gain was observed in the diabetic animals treated with the various doses of the extract clearly suggesting that the extracts might not have had the obesity forming tendency, which is one of the undesirable side effects normally encountered when treating diabetes with sulphonylureas. The body weight of the induced but not treated animals significantly ($p \leq 0.05$) decrease throughout the experimental period.

The study showed that there was a significant ($p \leq 0.05$) reduction in the plasma glucose levels of all the diabetic rats treated with various doses of the extract compared with the control. The animals that received 100, 250 and 500 mg/kg body weight doses of the extract respectively achieved diabetic control within 14 days and were maintained till the end of the experiment. This marked reduction in plasma glucose concentration may be as a result of increased release of insulin from regenerated beta cells of the pancreas or by inhibition of certain carbohydrate digesting enzymes. Glycemic control is required to be effective in the long-term therapy for individuals with type II diabetes mellitus, so as to reduce the risk of both cardiovascular and neurological complications in the development of the disease (Skyler *et al*., 2009 [44], Blonde, 2012 [6]).

Abnormalities in lipid profile are one of the most common complications in diabetes mellitus. At normal state, insulin activates lipolytic hormone action on the peripheral fat depots, which hydrolyzes triglycerides and prevents the mobilization of free fatty acids. However, insulin deficiency inactivates the lipoprotein lipase, which promotes liver conversion of free fatty acids into phospholipids and cholesterol and their final discharge into blood, resulting in elevated serum phospholipid levels (Guputa *et al*., 2012 [16]). *Bridelia ferruginea* extract at various doses were observed to significantly ($p \leq 0.05$) decrease plasma triglycerides and total cholesterol levels while a significant ($p \leq 0.05$) increase in the HDL levels were observed.

The effects of various doses of *Bridelia ferruginea* extract and glibenclamide on the biochemical parameters were summarized in Table 4. The various extract doses and glibenclimade were observed to significantly ($p<0.05$) reduced the plasma glucose levels of the diabetic rats when compared with the control. There was an astronomical increase in the plasma glucose level of the alloxan-induced diabetic but untreated rats but there was no significant difference ($p \geq 0.05$) in the plasma protein levels of the diabetic rats treated with 500 mg/kg 250mg/kg, 100mg/kg of the extract compared to the control. The plasma albumin levels of the diabetic rats treated with glibenclamide and the various extract doses showed no significant difference ($p \geq 0.05$) compared with the control. There was a significant increase ($p < 0.05$) in the plasma creatinine levels of the diabetic rats untreated compared with the treated. Creatinine is the most commonly used indicator of renal function. A raised plasma level of creatinine is a recognised marker of renal dysfunction (Williams *et al*., 2008 [49]). The significant increase in the plasma creatinine levels of the diabetic rats untreated indicated renal impairment in this group of rats. Therefore, persistent hyperglycemia due to poorly controlled diabetes mellitus may lead to diabetic nephropathy The plasma urea levels of the diabetic rats treated with glibenclamide, 500 mg/kg and 250 mg/kg of the extracts were significantly increased ($p < 0.05$) compared with the control. There was no significant change in the plasma AST, ALT and ALP of the diabetic rats treated with the extract compared with the control. However, there was a significant increase ($p < 0.05$) in the plasma AST, ALT and ALP of the diabetic rats treated with insulin compared with the control. There was also a significant increase in ($p < 0.05$) the plasma AST and ALT levels of the diabetic rats untreated compared with the control. An elevation in plasma concentration of ALT is usually due to liver damage while increase in AST level could be linked to damage to either cardiac or hepatic tissues or damage to both [Wassan *et al*., 2001 [50]; Crook, 2006 [10]].

Haematological studies shown in Table 5 indicated increase in Hb and PVC levels. The increase in their levels following extract treatment may be due to changes in the rate of the RBCs production. The calculated RBC indices, MCH, MCV and MCHC were not significantly altered in the extract treated animals which suggested that the activity of the extracts had minimal effect on the size of RBC and therefore did not cause anaemia which might lead to cardiac failure.

Histopathological studies of pancreatic tissues of the rats were made, and alloxan-induced diabetic rats showed damage in the pancreatic β-cells. Rats treated with *Bridelia ferruginea* extract showed comparable restoration of these cells, which might be due to its antioxidant properties. It was also apparent from pancreatic tissue morphology that the animals treated with the extract showed significant beta cells recovery which implied that the mixture more effectively ameliorated the diabetogenic agent activity. There were also no changes observed in the macroscopic examinations of the organs of the diabetic animals treated with the extracts or glibenclamide

Conclusion

Based on this study it can be postulated that the *Bridelia ferruginea* shows potential antidiabetic activity in alloxan-induced diabetic rats, providing evidence to support the traditional claim. This was demonstrated *in vitro* by the inhibition of enzymes involved in carbohydrate metabolism. *In vivo* diabetic studies also showed significant decrease in plasma glucose levels of treated diabetic rats when compared to untreated ones.

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