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ORIGINAL ARTICLE

Ethyl acetate Fraction of Bark of *Bridelia ferruginea* inhibits Carbohydrate Hydrolyzing Enzymes associated with type 2 Diabetes (α-glucosidase and α-amylase)

Oluwafemi Adeleke Ojo[1*], Bashir Ajiboye[1], Israel Olayide[1], Adewale Fadaka[1] and Olasehinde, O.R[2]
[1]Department of Biochemistry, Afe Babalola University, Ado-Ekiti, Nigeria.
[2] Department of Medical Biochemistry, Afe Babalola University, Ado-Ekiti, Nigeria.
Email: oluwafemiadeleke08@gmail.com

ABSTRACT
In West Africa, various preparations of extracts of Bridelia ferruginea Bth. stem bark are used for management of diabetes mellitus and other metabolic disorders.The present study evaluated the inhibitory effect of ethyl acetate fraction on the key enzymes linked to type-2 diabetes (α-glucosidase and α-amylase) activities. The stem bark of the plant was sequentially extracted with ethanol and ethyl acetate and the tested for their inhibitory effect on α-glucosidase and α-amylase activities as well as determination of mode of inhibition was performed. Our findings revealed that the total phenolic content of the extract yielded 3.66 ± 0.05 mg GAE/100g. Furthermore, the results revealed that ethyl acetate fraction of B. ferruginea had α-glucosidase and α-amylase inhibitory activities with IC_{50} values of 4.52 ± 0.50 mg/mL and 5.42 ± 1.10 mg/mL respectively. Enzymes kinetics studies indicated that the ethyl acetate fraction is a non-competitive inhibitor for α-glucosidase and a competitive inhibitor for α-amylase. GC-MS analysis also revealed that the ethyl acetate fraction contained 2, 2'-Oxydiphenol, p-hydroxyphenyl ether, di-1H-pyrrol-1-yl-isopropenylsilane and carbonic acid, phenyl undec-10-enyl ester. It can be inferred from this study that ethyl acetate fraction of B. ferruginea stem bark possessed α-glucosidase and α-amylase inhibitory activities which may be due to the presence of phytochemicals such as phenols. 2, 2' oxydiphenol, and p-hydroxyphenyl ether, could be the main bioactive compounds responsible for the observed activities.
Key words: *α-glucosidase, α-amylase, Bridelia ferruginea,*

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INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. Enzyme inhibitors have potential value in many areas of disease control and treatment. Type-2 diabetes is a heterogeneous disorder characterized by a progressive decline in insulin action (insulin resistance), followed by the inability of pancreatic β-cells to compensate for insulin resistance (β -cell dysfunction) leading to hyperglycemia [2] while oxidative stress is a major contributor to the β -cell damage [3]. Oxidative stress refers to the existence of products called free radicals (molecules possessing an unpaired electron) and reactive oxygen species (ROS), which are formed in normal physiological processes but become deleterious when they are not quenched by a cascade of antioxidant systems. This can result either from an overproduction of ROS or from the inactivation of the antioxidants, thereby shifting the balance in favor of oxidative stress. In type 2 diabetes, hyperglycemia induces generation of free radicals, including

ROS, hydroxyl and nitric oxide (NO) radicals [4], which are responsible for oxidative stress induced 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 [5]. However, concept of oxidative stress as an important trigger, and postprandial hyperglycemia at the onset and progression of diabetes, offer a unique therapeutic strategy for the treatment of type-2 diabetes and for reducing chronic vascular complications [6].
Some antidiabetic agents such as acarbose, exert their blood glucose lowering effects through the inhibition of gastrointestinal carbohydrate hydrolyzing enzymes such as the salivary and pancreatic α-amylase as well as intestinal α- glucosidase enzymes [7]. Inhibition of these enzymes may delay the digestion and absorption of carbohydrates and consequently suppress the rise in postprandial blood glucose level [8]. Acarbose is an inhibitor of both α -amylase and α -glucosidase [9]. In contrast to acarbose, plant based agents were reported to be more acceptable source of amylase and glucosidase inhibitors due to their low cost and relatively better safety levels, including lower incidence of serious gastrointestinal side effects [10]. At least to the knowledge of the authors, there are no previous report on both the α -amylase and α -glucosidase inhibitory effects of the *Bridelia ferruginea* extracts, thereby providing a holistic avenue to control hyperglycemia and other diabetic complications resulting from oxidative stress, of the utmost importance.
Bridelia ferruginea Bth. (Euphorbiaceae) is commonly found in Savannah regions [11]. The bark is dark grey, rough and often marked scaly [12]. A decoction of the leaves has been used to treat diabetes. It is also used as purgative and a vermifuge [13]. The bark extract has been used for the coagulation of milk and also lime juice for the formulation of a traditional gargle "egun efu". It is also reported of having potential for water treatment [14] and chemopreventive potentials [15]. Other reported activities of the bark extract include typanocidal, molluscidal [16], antimicrobial [17] and anti-inflammatory [18]. However, detailed investigation of α- glucosidase or α- amylase inhibitory activity of the extracts from this part of these plants has not been conducted to date. Hence, in this study, we conducted a comprehensive and systematic investigation of *α*-glucosidase and *α*-amylase inhibitory activities of the solvent fraction of *Bridelia ferruginea*, along with identification of active compounds in the most solvent fraction as well as establishing the mechanisms of *α* -glucosidase and *α* -amylase inhibitions *via* the enzyme kinetics approach.

MATERIALS AND METHODS

Chemicals and reagents

α- Glucosidase from *Saccharomyces cerevisiae*, porcine pancreatic amylase, *p*-nitrophenyl-*a*-D-glucopyranoside (pNPG), *p*-nitrophenol, gallic acid, and potassium ferricyanide were obtained from Sigma-Aldrich, Germany. Starch, dinitrosalicylic acid (DNS), maltose, ethanol, ethyl acetate, trichloroacetic acid reagents were obtained from Merck Chemical Company, Germany.

Plant material

Fresh stem bark peelings of *Bridelia ferruginea* were collected March 25th, 2015 at a farm in the suburbs of Ado Ekiti, Nigeria. The plant was identified and authenticated by a plant scientist in the Department of Plant Science, Ekiti State University, Ado-Ekiti, Nigeria and a voucher specimen number (UHAE 46) was deposited accordingly at the herbarium of the Department of Plant Science, Ekiti State University, Ado-Ekiti, Nigeria.

Preparation of crude extracts and solvent partitioning of the crude extract

The stem bark were air-dried in the laboratory at ambient temperature (30 ± 2°C), pulverized using a laboratory mechanical grinder (Christy and Norris limited, machine type 8) and the fine powders obtained stored until further use. The powdered sample (120 g) was extracted with solvent combination of 70% ethanol for 48 h. The filterate measured was evaporated to dryness using a freeze dryer to obtain 40% yield ethanol residue. Crude ethanol extract (30 g) was weighed and reconstituted in distilled water and successively partitioned with ethyl acetate (2 x 200 mL). The resulting fractions were evaporated to dryness at 40 °C under reduced pressure while the remaining aqueous fraction was dried on a water bath. The crude extracts and fractions in each case were weighed, transferred to micro tubes and stored at 4 °C until further analysis.

$$\%\ yield = \frac{Weight\ of\ the\ dry\ extract}{Weight\ of\ powdered\ leaves} \times 100\%$$

Determination of α -glucosidase inhibitory activity of fractions of the bark ethyl acetate extract

The *α*-glucosidase inhibitory activity was determined according to the method described by [6], with slight modifications. Briefly, 250 µL of ethyl acetate fraction, at different concentrations (1–5 mg mL^{-1}), was incubated with 500 µL of 1.0 U mL^{-1} *α*-glucosidase solution in 100 mmol L^{-1} phosphate buffer (pH 6.8) at 37 °C for 15 min. Thereafter, 250 µL of pNPG solution (5 mmol L^{-1}) in 100 mmol L^{-1} phosphate buffer (pH 6.8) was added and the mixture was further incubated at 37 °C for 20 min. The absorbance of the

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released *p*-nitro phenol was measured at 405 nm and the inhibitory activity was expressed as percentage of a control sample without inhibitors.

$$\alpha\text{- glucosidase inhibition (\%)} = \frac{A_{405}\text{control} - A_{405}\text{sample}}{A_{405}\text{control}} \times 100$$

Determination of α-amylase inhibitory activity of fractions of the bark ethyl acetate extract

The *α*-amylase inhibitory activity was determined according to the method described by [19], with slight modifications. A volume of 250 µL of ethyl acetate fraction at different concentrations (1-5 mg mL^{-1}) was incubated with 500 µL of porcine pancreatic amylase (2 U mL^{-1}) in 100 mmol L-1 phosphate buffer (pH 6.8) at 37 °C for 20 min. Two hundred and fifty µL of 1% starch dissolved in 100 mmol L^{-1} phosphate buffer (pH 6.8) was then added to the reaction mixture and incubated at 37 °C for 1 h. One mL of DNS color reagent was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm and the inhibitory activity was expressed as percentage of a control sample without inhibitors. All assays were carried out in triplicate.

$$\alpha\text{- amylase inhibition (\%)} = \frac{A_{540}\text{control} - A_{540}\text{sample}}{A_{540}\text{control}} \times 100$$

Mechanism of α-glucosidase and α-amylase inhibitions

The ethyl acetate fraction was subjected to kinetic experiments to determine the type of inhibition exerted on *α*-glucosidase and *α*-amylase. The experiment was conducted according to the protocols as described above at a constant concentration of sample fraction (5 mg mL^{-1}) with a variable concentration of substrate. For the *α*-glucosidase inhibition assay, 0.625-5 mmol L^{-1} of *p*NPG was used and 0.125-1 % of starch was used for the *α*-amylase inhibition assay. The initial rates of reactions were determined from calibration curves constructed using varying concentrations of *p*-nitrophenol and maltose for the *α*-glucosidase and *α*-amylase inhibition assays, respectively. The initial velocity data obtained were used to construct Lineweaver-Burke's plot to determine the *K*M (Michaelis constant) and *v* max (maximum velocity) of the enzyme as well as the *K*i (inhibition binding constant as a measure of affinity of the inhibitor to the enzyme) and the type of inhibition for both enzymes.

$$\text{Reaction rate (v) (mg mL}^{-1}\text{)} = \frac{\text{Amount of product liberated (mg mL}^{-1}\text{)}}{1200\text{ (s)}}$$

Gas chromatography-mass spectrometric (GC-MS) analysis

The ethyl acetate fraction was further subjected to GC-MS analysis. The analysis was conducted with an Agilent Technologies 6890 GC coupled with an Agilent 5973 mass selective detector and driven by Agilent Chemstation software (Agilent Technologies, USA). A DB-5SIL MS capillary column was used (30 m x 0.25 mm i.d., x 0.25 µm film thickness). The carrier gas was ultra-pure helium at a flow rate of 0.7 mL min^{-1} and a linear velocity of 37 cm s^{-1}. The injector temperature was set at 250 °C. The initial oven temperature was 60 °C, which was programmed to 280 °C at the rate of 10 °C min^{-1} with a hold time of 3 min. Injections of 2 µL were made in the splitless mode with a manual split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 °C, quadrupole temperature 150 °C, solvent delay 4 min and scan range 50-700 amu. Compounds were identified by direct comparison of the retention times and mass fragmentation pattern with those from the National Institute of Standards and Technology (NIST) library.

Statistical analysis

Data were analyzed using a statistical software package (SPSS for Windows, version 20, IBM Corporation, USA) using Duncan multiple range *post- hoc* test. Values were considered significantly different at $p < 0.05$.

RESULTS

The ethyl acetate extract of *B. ferruginea* bark has 12% as its yield. The total phenolic content of ethyl acetate extract of *B. ferruginea* bark is 3.66 ± 0.05 mg GAE/100g.

Figure 1 shows the percentage inhibition of α-glucosidase by ethyl acetate fraction of *B. ferruginea* bark. The fraction inhibited α- glucosidase activities *in vitro* in a centration dependent manner. The concentration of the extract required to cause 50% inhibition (IC_{50}) against α-glucosidase was 4.52 ± 0.50 mg mL^{-1} (Table 1). Figure 5 depicts that the mode of inhibition of α-glucosidase by the ethyl acetate fraction of *B. ferruginea* is a non competitive type of inhibition.

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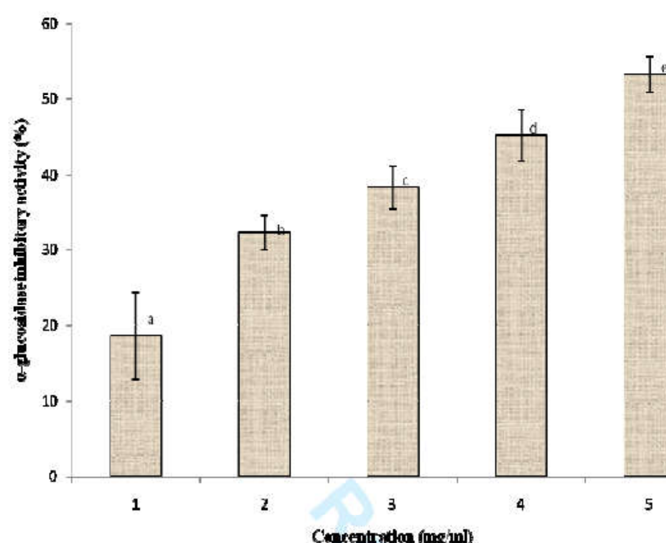

Figure 1: *α*-glucosidase inhibitory activities of ethyl acetate fractions of *Bridelia ferruginea* bark. Data are presented as mean ± SD of duplicate determinations. a-e Different letters over the bars for a given concentration for each extract indicate a significant difference from each other (Duncan multiple range *post hoc* test, $p < 0.05$).

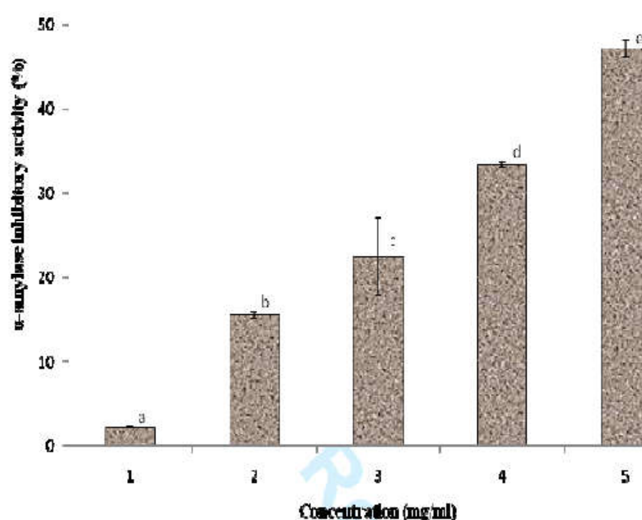

Figure 2: *α*-amylase inhibitory activities of ethyl acetate fractions of *Bridelia ferruginea* bark. Data are presented as mean ± SD of duplicate determinations. a-e Different letters over the bars for a given concentration for each extract indicate a significant difference from each other (Duncan multiple range *post hoc* test, $p < 0.05$).

Figure 2 shows the percentage inhibition of α-amylase by ethyl acetate extract of *B. ferruginea* bark. The fraction had appreciable in vitro inhibitory activity against α-amylase. The IC_{50} values for the ethyl acetate fraction against α- amylase activity were determined from a concentration response curve to be 5.42 ± 1.10 mg mL^{-1} (Table 1). The Lineweaver-Burke plot was generated to determine the mode of inhibition of the enzyme and the result showed that ethyl acetate extract of *B. ferruginea* inhibited α-amylase in a competitive manner.

Table 1: IC50 values for the inhibition of *α*-glucosidase and *α*-amylase by ethyl acetate fraction of the ethanolic extract of *Bridelia ferruginea* bark

Fraction	IC_{50} (mg/ml)	
	α-glucosidase	*α*-amylase
Ethyl acetate	4.52 ± 0.50^a	5.42 ± 1.10^b

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Data are presented as mean ± SD of duplicate determinations. a-b Different letters over the bars for a given concentration for each extract indicate a significant difference from each other (Duncan multiple range *post hoc* test, $p < 0.05$).

The GC-MS chromatogram of the fraction is presented in Figure 3. Thirteen peaks were visible in the chromatogram and the various chemical constituents at those peaks were identified from the NIST library (Table 2). The retention time and molecular mass of the detected compounds are provided in Table 2. The most abundant phytochemicals (>80%) in the fraction as identified by the library were phenolic (2, 2'-oxydiphenol) and phenolic acid (carbonic acid, phenyl undec-10-enyl ester) (Figure 4) and therefore the fraction was considered as a phenolics-rich fraction.

Table 2: Identified compounds of the ethyl acetate fraction of *Bridelia ferruginea* bark ethanolic extract by GC-MS

Peak No	Compound	Retention time	Molecular mass (Mr)
1	Hydroquinone	4.18	110
2	3-Acetyl-2,5-dimethyl furan	5.82	138
3	1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)	6.6	134
4	Ethyl α.-d-glucopyranoside	9.39	208
5	d-Glycero-d-ido-heptose	9.80	210
6	3-O-Methyl-d-glucose	10.83	194
7	Phenol, 2,2'-oxybis-	12.43	202
8	Stigmastan-3-en-6-ol	13.51	414
9	Hexadecane, 1,16-dichloro-	13.75	294
10	1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7	16.87	222
11	Stigmast-4-en-3-one	18.09	412
12	Cyclo propanecarboxylic acid, 3-methylbut-2-enyl ester	18.46	154
13	1,2-Cyclohexanedicarboxylic acid, di(3-fluorophenyl)ether	18.96	360

DISCUSSION

Bridelia ferruginea is used by traditional healers and herbalist to treat and manage non metabolic chronic disorders [13]. It has been reported that *Bridelia ferruginea* bark exhibited good antioxidant activity [20]. Ojo et al. [21] also reported that ethyl acetate fraction of stem bark of other plants exhibited such high level of antioxidant properties. Management of blood glucose level is a strategy in the control of diabetes [22]. The total phenolic content of the ethyl acetate extract of *Bridelia ferruginea* is 3.66 ± 0.05 mg GAE/100g.

α-Glucosidase and α-amylase inhibitors have been useful as oral hypoglycemic drugs for the control of hyperglycemia in patients with type 2 diabetes. Inhibition of these enzymes delay carbohydrate digestion and overall prolong the digestion time causing a reduction in the rate of glucose absorption and consequently reducing postprandial plasma glucose [23]. However, for α- glucosidase inhibition, the strong inhibition of the enzyme displayed by the extract as shown by the IC_{50} values (4.52 ± 0.50 mg mL^{-1}) suggests that the extract contains potent α-glucosidase inhibitor. This is because any drug which a strong inhibitor of α- glucosidase could serve as effective therapy for postprandial hyperglycemia. Thus, the strong inhibition of α-glucosidase by the ethyl acetate fraction would slow down the breakdown of disaccharides to liberate glucose, thereby reducing glucose absorption from the small intestine [24]. This observation might be connected to the high phenolic content recorded in the fraction, because polyphenolic fractions from plants have been shown to inhibit α-glucosidase activity, allowing for tighter control of blood glucose [25]. Further studies to ascertain the mode of inhibition of α-glucosidase by the ethyl acetate fraction showed that the extracts inhibited the enzyme in a non competitive way. This observation could indicate that the fraction bound the α-glucosidase at separates sites(s) of the enzyme causing a conformational changes at the active sites, thereby preventing the binding of the pNPG and consequently reducing the α-glucosidase activity.

The inhibition of α- amylase by the ethyl acetate extract of *B. ferruginea* bark is a pointer to the fact that the plant is an inhibitor of the enzyme, which is desirable in order to prevent some of the side effects by synthetic drugs. This is in agreement with the report of [26]. Conversely, the mechanism of inhibition of α-amylase by the ethyl acetate fraction of *B. ferruginea* bark revealed a competitive inhibition. This observation could indicate that the active sites of the enzymes are directly involved in the inhibitory action and the fraction might contain compounds that could serve as substrate analogues [27].
The possible bioactive compounds in ethyl acetate fraction of *Bridelia ferruginea* bark were detected using GC-MS analysis. However, it has been reported that *Bridelia ferruginea* bark ethyl acetate extract were rich in phenolic compounds [20]. Although, identification of pure compounds have not been recorded. The compounds identified in the ethyl acetate fraction of *Bridelia ferruginea* are shown in (Table 2, Figure 4). Though, the anti-diabetic action of the ethyl acetate fraction of *Bridelia ferruginea* bark in our study could possibly be attributed to the presence of these compounds as well, while not discounting the possible contributions of the other detected phytochemicals.

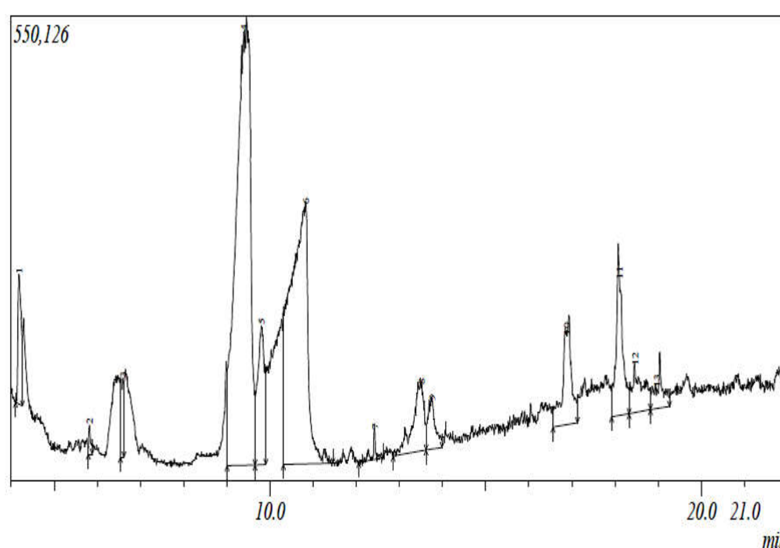
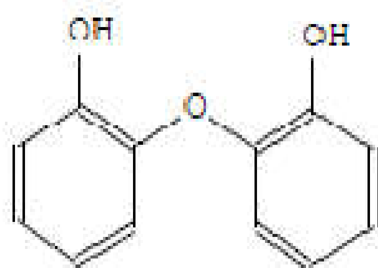
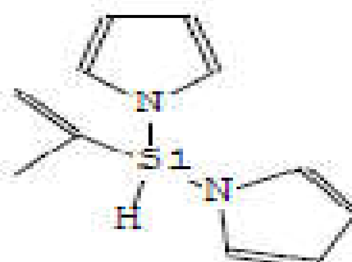


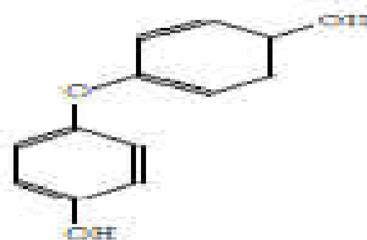
Figure 3: GC-MS Chromatogram of the ethyl acetate fraction of *Bridelia ferruginea* bark ethanolic extract





Name of compound: 2, 2'-Oxydiphenol

Name of compound: Di-1H-pyrrol-1-yl-isopropenylsilane



Name of compound: p-Hydroxyphenyl ether

Name of compound: Carbonic acid, phenyl undec-10-enyl ester

Figure 4: Structures of components identified by GC-MS

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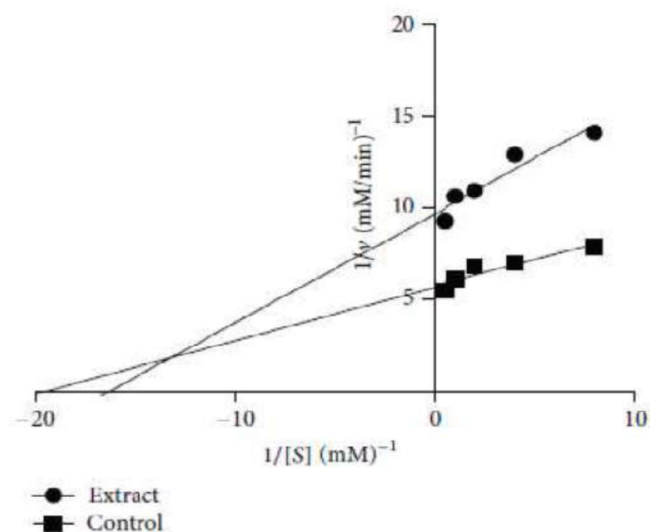

Figure 5: Mode of inhibition of α- glucosidase by ethyl acetate extract of bark of *Bridelia ferruginea*

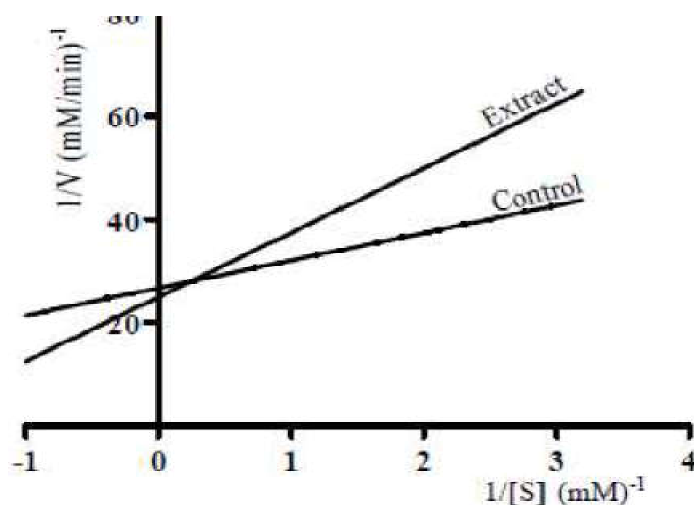

Figure 6: Mode of inhibition of α- amylase by ethyl acetate extract of bark of *Bridelia ferruginea*

CONCLUSION

This study indicates that the ethyl acetate fraction derived from the ethanol extract of *B. ferruginea* stem bark contains potent α- glucosidase and α- amylase inhibitory agents and that the inhibitions were mediated by non-competitive and competitive way for α- glucosidase and α- amylase. Furthermore, the identified phenols could be bioactive agents and further studies can be investigated as anti-diabetic therapeutics.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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