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Total Phenolic Content, Antioxidant Activity and *in vitro* Inhibitory Potential against Key Enzymes Relevant for Hyperglycemia of *Bridelia ferruginea* Extracts

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ABSTRACT

Herbal medicine has been used for many years by different cultures around the world for the treatment of diabetes. *Bridelia ferruginea* is one of the traditional remedies used for diabetes in Togo. In this study, we determined phenolic profiles, antioxidant activity and *in vitro* inhibitory potential against key enzymes relevant for hyperglycemia of hydroethanolic extract, acetonic fraction and ethyl acetate fraction. The inhibitory activity against α-amylase, α-glucosidase was carried out. Antioxidant activity was evaluated using AAPH radical scavenging assay. Ethyl acetate fraction possessing high content of phenolics and antioxidant activity showed also high activities on key enzyme (α-glucosidase $IC_{50} = 0.19$ mg mL^{-1}, α-amylase ($IC_{50} = 0.24$ mg mL^{-1}) than acetonic fraction and hydroethanolic extract which have $IC_{50} > 0.25$ mg mL^{-1}. These results suggest that *Bridelia ferruginea* has effective enzyme inhibitory activity useful for the management of postprandial hyperglycemia. Our study provided a strong rationale for further clinical studies and showed that anti-hyperglycemia compound of *Bridelia ferruginea* may be concentrated in the ethyl acetate fraction.

Key words: *Bridelia ferruginea*, α-Glucosidase/α-amylase inhibitor, antioxidant activity, phenolic phytochemicals

INTRODUCTION

Diabetes is increasing rapidly in every part of the world. The prevalence of the disease will grow to 552 million people affected by 2030 (IDF, 2011). About 90% of the total diabetics have type 2 diabetes (American Diabetes Association, 2005). Type 2 diabetes is characterized by absolute or relative deficiency in insulin secretion and/or insulin action associated with chronic hyperglycemia and disturbances of carbohydrate, lipid and protein metabolism (WHO, 1999). The chronic hyperglycemia in type 2 diabetes can be expressed by fasting and postprandial blood glucose levels. Recent studies have indicated that high postprandial blood glucose level is a major factor in the onset and development of type 2 diabetes (Chang *et al.*, 2004). Fast glucose uptake in the intestine in which α-amylase and α-glucosidase play important role due to hydrolysis of starch and oligosaccharide is an important factor responsible of postprandial hyperglycemia (Gray, 1995). It

is believed that inhibition of these enzymes can effectively control the postprandial elevation of blood glucose level. α-Glucosidase inhibitors, such as acarbose, are clinically used to control blood glucose levels, especially postprandial blood glucose levels in type 2 diabetes (De Melo *et al.*, 2006). This synthetic drug has a strong inhibitory activity against both α -glucosidase and α-amylase, but presents side effects (Bischoff *et al.*, 1985).

The use of herbal medicine by chronic disease victims is encouraged because of concern about the adverse effects of synthetic drugs while treatment using medicines of natural origin appears to offer more gentle means of managing such disease (Klepser and Klepser, 1999; Hamdan and Afifi, 2004).

The chronic hyperglycemia in diabetes causes oxidative stress (Brownlee, 2005). Oxidative stress plays an important role in the chronic complications of diabetes (Dicarli *et al.*, 2003). Therefore, alleviation of oxidative stress is essential for preventing or reversing diabetic complications (DeFronzo, 1999). A compound with antioxidant activity combined with inhibitory activities against α-glucosidase and α-amylase may be a more effective anti-diabetic agent.

Bridelia ferruginea is a shrub commonly growing of sub-Saharan Africa. The root bark of the plant is used in Togolese traditional medicine to treat diabetes mellitus. In our previous study, significant anti-diabetic effects of *B. ferruginea* hydro ethanolic extract were proven in high fructose diet fed Wistar rats (Bakoma *et al.*, 2011).

The objective of this research was to investigate *B. ferruginea* hydroethanolic extract and its ethyl acetate and acetone fraction phenolic profiles, antioxidant activity and potential for managing early stages of Type 2 diabetes such as hyperglycemia relevant to α-glucosidase and α-amylase enzyme using *in vitro* models.

MATERIALS AND METHODS

Porcine pancreatic α-amylase (PPA) (EC 3.2.1.1) and baker's yeast α-glucosidase (EC 3.2.1.20) were purchased from Sigma Chemical (France). Unless noted, all other chemicals were also purchased from Sigma Chemical (France).

Plant material: The roots of *B. ferruginea* were collected in August 2010 from Tsévié area, 35 km North East of Lomé (Togo). Botanical authentication was confirmed at the Department of Botany, University of Lomé, where a voucher specimen of *B. ferruginea* was deposited at the herbarium (No. 83, 2010).

Preparation of crude extract and fractions: The root barks of *B. ferruginea* were sliced, shade dried and coarsely powdered. The powder was macerated at room temperature with ethanol-water (8:2 v/v) three times during 72 h. The filtrate was concentrated to dryness under vacuum in a rotary evaporator at 45°C to obtain a residue (yield, 17.5%). A part of hydroethanolic extract was dissolved in distilled water and extracted with equal volume of ethyl acetate. The aqueous residue was concentrated to dryness and extracted with acetone.

α-glucosidase inhibition assay: α-Glucosidase inhibitory activity was determined using a method described by Ranilla *et al.* (2010) with some modifications. A volume of 50 μL of sample extract diluted with 50 μL of 0.1 M potassium phosphate buffer (pH 6.9) and 100 μL of 0.1 M potassium phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 IU mL^{-1}) was incubated in 96-well plates at 25°C for 15 min. After preincubation, 50 μL of 5 mM p-nitrophenyl-

α-D-glucopyranoside solution in 0.1 M potassium phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 min. Before and after incubation, absorbance readings (A420 extract) were recorded at 420 nm using a microplate reader (Thermomax; France) and compared to a control which had 50 µL of buffer solution in place of the extract (A420 control). The α-glucosidase inhibitory activity was expressed as percentage of inhibition and was calculated as follows:

$$\frac{\text{A420 control-A420 extract}}{\text{A420 control}} \times 100$$

α-amylase inhibition assay: PPA (Porcine pancreatic α-amylase) inhibitory activity was determined using the method of Bhandari *et al.* (2008) with a slight modification. Starch azure (2 mg) used as a substrate was suspended in 0.5 M Tris-HCl buffer (pH 6.9) containing 0.01 M $CaCl_2$ and heated in boiling water for 5 min. Then, the starch azure solution was preincubated at 37°C for 5 min. The test samples (0.4 mL) in 50% DMSO and 0.1 mL of PPA solution (2.189 IU mL^{-1}, α-amylase from Porcine Pancreases, EC-3.2.1.1, Sigma Chemicals Co.) were added into each assay sample. Whereas 0.1 mL 0.5 M Tris-HCl buffer was used in place of the plant extract for the blank sample. After thoroughly mixing, both the sample and the blank test tubes were incubated at 37°C for 15 min and the reaction was stopped by adding 0.1 mL of 50% acetic acid. The reaction mixture was then centrifuged (2000 g, 4°C) for 10 min. The absorbance of the supernatant, at 540 nm was measured and the inhibitory activity was calculated using following formula:

$$\frac{\text{A540 control-A540 extract}}{\text{A540 control}} \times 100$$

Total phenolic assay: The total phenolic content was determined by the Folin-Ciocalteu method modified by Shetty *et al.* (1995). Briefly, 1 mL of the extract (0,05 mg mL^{-1}) was transferred into a test tube. To each sample, 5 mL of 10% (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 4 mL of Na_2CO_3 (145 g L^{-1}) was added to the reaction mixture and allowed to stand for 30 min. The absorbance was read at 725 nm.

The standard curve was established using various concentrations of gallic acid and results were expressed as mg of gallic acid per gram of sample in dried weight (dw).

Antioxidant activity: This study has the approval of institute's ethical committee on animal experimentation (N°011; October 2010). Principles of laboratory animal care as described in the European Community guidelines were followed (http://eur-lex.europa.eu/).

Blood (20 mL) was obtained from 10 male Wistar rats (BW 150-200 g) by retro orbital sinus puncture in tubes containing 0.01% EDTA. Erythrocytes were isolated by centrifugation at 3000 g for 10 min, washed four times with PBS and then re-suspended to the desired hematocrit level using the same buffer. The cells were stored at 4°C. In order to induce free radical chain oxidation in the erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH (2,2-azobis-2-amidinopropane dihydrochloride). in oxygen. An erythrocyte suspension at 5% hematocrit was incubated with PBS (control) and preincubated with extracts (50-250 µg mL^{-1}) or ascorbic acid (50-250 µg mL^{-1}) separately at 37°C for 3 h with 25 mM AAPH in PBS at pH 7.4.

This reaction mixture was shaken gently while being incubated for a fixed interval at 37°C. Two-hundred microliters of the reaction mixture was removed and centrifuged at 3000 g for 2 min and the absorbance of the supernatant was determined at 540 nm (Wang *et al.*, 2009). The inhibition of AAPH radical chain oxidation in erythrocytes was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{A_{AAPH} - A_{sample}}{A_{AAPH}} \times 100$$

Where:
A_{AAPH} = Absorbance of AAPH without extract
A_{sample} = Absorbance of sample

Statistical analysis: The results are expressed as Mean±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test to evaluate significant differences between extract, fraction and acarbose for each concentration. $p < 0.05$ was considered statistically significant. All statistical analyses were carried out using the InStat statistical package (Graph Pad Software Inc., USA).

RESULTS

α-amylase and α-glucosidase inhibition: The inhibitory activities of *B. ferruginea* hydroethanolic extract, acetonic fraction and ethyl acetate fraction against α-glucosidase and α-amylase were measured at 0.1, 0.15, 0.2 and 0.25 mg mL^{-1}. The results were shown in Fig. 1 (α-Glucosidase) and in Fig. 2 (α-amylase). The activity of the extract and the fractions were concentration dependent. Inhibitory activity against α-glucosidase expressed as IC$_{50}$ were 0.13 and 0.19 mg mL^{-1}, respectively for Acarbose and the ethyl acetate fraction. IC$_{50}$ of the acetonic fraction and hydroethanolic extract were higher than 0.25 mg mL^{-1}. We found that inhibitory activity
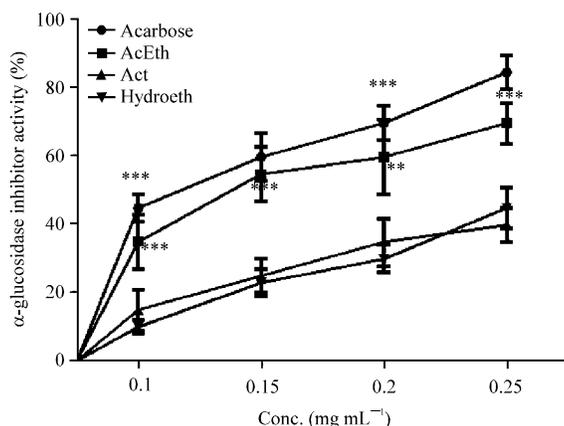

Fig. 1: The inhibition of α-glucosidase activity of *Bridelia ferruginea* hydroethanolic extract (HydroEth), acetonic fraction (Act) and ethyl acetate fraction (AcEth), Values are Means±SD of four replicated samples, **, ***Significant at p<0.01 and p<0.001, respectively by using Tukey's test

Fig. 2: The inhibition of α-amylase activity of *Bridelia ferruginea* hydroethanolic extract HydroEth), acetonic fraction (Act) and ethyl acetate fraction (AcEth), Values are Means±SD of four replicated samples, *, ***Significant at p<0.05 and p<0.001, respectively by using Tukey's test
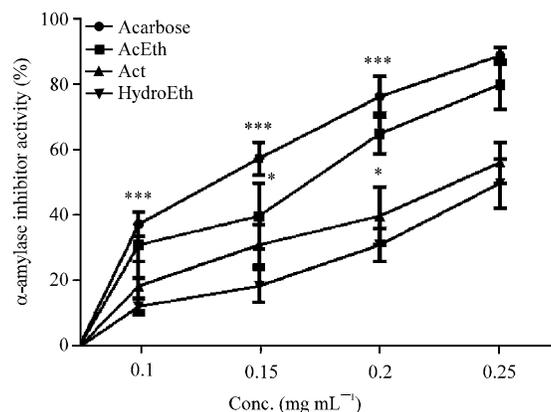
Table 1: Total phenolic content and IC_{50} value of *Bridelia ferruginea* hydroethanolic extract, acetonic fraction and ethyl acetate fraction on AAPH radical oxidation in erythrocytes

Parameter	AA	AcEth	Act	HydroEth
IC_{50} (mg mL^{-1})	0.06±0.008	0.09±0.005	0.13±0.056	>0.25±0.06
Total phenolic (% eq gallic acid)		42.0±3.100	34.0±2.200	24.0±2.500

AA: Ascorbic Acid, AcEth: Ethyl acetate fraction, Act: Acetonic extract, HydroEth: Hydro ethanolic extract

against α-amylase was 0.17 mg mL^{-1} for acarbose, 0.24 mg mL^{-1} for ethyl acetate fraction. IC_{50} values of the acetonic fraction and hydroethanolic extract were also higher than 0.25 mg mL^{-1}.

Total phenol content and antioxidant activity: Total phenolic content is respectively 24±2, 34±2.2 and 42±3.1(%eq gallic acid) for hydroethanolic extract, acetonic fraction and ethyl acetate fraction (Table 1). The AAPH scavenging-linked antioxidant activity of samples was measured while using Vitamin C (IC_{50} = 0.06±0.008 mg mL^{-1}) as reference. As shown in Table 1, the IC_{50} values of *B. ferruginea* hydroethanolic extract, acetonic fraction and ethyl acetate fraction were respectively 0.09±0.005, 0.13±0.056 and >0.25±0.06 mg mL^{-1}.

DISCUSSION

Early effective control of postprandial hyperglycemia is important in early intervention and prevention of diabetic complications of type 2 diabetes management (Ratner, 2001). α-Glucosidase and α-amylase are two key enzymes related to carbohydrate digestion and elevation of levels of fasting blood glucose.

It is now believed that inhibition of these enzymes can be an important strategy in the management of hyperglycemia by retarding the postprandial increase of blood glucose level after a mixed carbohydrate diet (Puls *et al.*, 1977). Currently used inhibitors of α-glucosidase and α-amylase such as acarbose have strong inhibitory activities against both of the two enzymes. These drug therapies are effective but have side effects caused by the abnormal bacterial fermentation of undigested carbohydrates due to an excessive inhibition of α-amylase.

Therefore, strong inhibitors of α-glucosidase with mild inhibitory activity against α-amylase have been sought to overcome this challenge. It was reported (Kwon *et al.*, 2008) that phenolic enriched extracts from several plant have high α-glucosidase inhibitory activity combined with α-amylase inhibitory activity. Bhandari *et al.* (2008) and Shobana *et al.* (2009) evaluated the inhibitory activities of phenolic compounds extracted from several plants against α-glucosidase and α-amylase. They found that the phenolic inhibited both enzymes significantly.

Present results indicated that hydroethanolic extract and acetonic fraction have lower phenolic content with low inhibitor activity and high IC_{50} value. This suggests that the phenolic compounds may be the origin of inhibitor activity against the two enzymes.

Oxidative stress plays important role in initiating beta-cell damage and insulin resistance. Antioxidants may prevent the progressive impairment of pancreatic beta-cell function and thus reduce the occurrence of type 2 diabetes (Song *et al.*, 2005).

Present results indicate that total phenolic content is commensurate to the antioxidant activity and inhibition of α-glucosidase and α-amylase. The high phenolic containing ethyl acetate fraction of *Bridelia ferruginea* showed high enzyme inhibition activity and antioxidant activity. These results are in accord with Bhandari *et al.* (2008) and Shobana *et al.* (2009) who reported that several phenolic compounds with inhibitory activities against α-glucosidase and α-amylase had moderate antioxidant activity. The correlation between phenol content and antioxidant activity were also reported by Janovik *et al.* (2011).

CONCLUSION

The results from this *in vitro* study clearly indicated that *Bridelia ferruginea* have moderate inhibitory activity against α-glucosidase and α-amylase. This activity can be attributed to phenolic compounds of the plant. Studies are ongoing to identify these compounds. The present study provides additional evidence in support for the use of this plant in the traditional treatment of diabetes mellitus in Togo.

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