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In vitro antioxidant and α-glucosidase inhibitory activities of aqueous extract and ethyl acetate fraction of *hallea stipulosa* (rubiaceae)

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ABSTRACT

Antioxidant potential of plants is useful in the management of diabetes mellitus. Aim of this study was to determine the *in vitro* antioxidant and α-glucosidase inhibitory activities of aqueous extract and ethyl acetate fraction of *Hallea stipulosa*. *In vitro* antioxidant assays were used DPPH (1,1-Diphenyl-2-Picrylhydrazyl) scavenging assay, nitric oxide (NO) inhibition assay, ferric reducing antioxidant power (FRAP), determination of total phenol and flavonoid contents. Aqueous extract and ethyl acetate fraction exhibited a scavenging activity by inhibiting DPPH and NO radicals and showed good reducing power capability. They contain a significant amount of phenols and flavonoids. Ethyl acetate fraction showed a considerable α-glucosidase inhibitory activity with an IC_{50} of 39.19 µg/ml. Results suggest that antioxidant potential and α-glucosidase inhibitory activity of *H. stipulosa* could be due to phenols and flavonoids and could be a part of the mechanism by which it manages diabetes.

Keywords: Hallea stipulosa, antioxidant, α-glucosidase, flavonoids, phenols

INTRODUCTION

Diabetes has become a major health problem in the world. It is a metabolic disease characterized by a high blood glucose level and can cause other health complications, such as cardiovascular disease, neuropathy, retinopathy, nephropathy and other dysfunctions[1]. The priority in the management of diabetes is to decrease the postprandial hyperglycemia[2,3]. Alpha glucosidase inhibition helps in reducing the rate of digestion of carbohydrates thereby reducing absorption of glucose and consequently resulting in decrease in postprandial blood glucose level[4]. Acarbose is the most widely used α-glucosidase inhibitor, but it also has gastrointestinal side effects[5]. Because of side effect of synthetic drugs, plants have long been used for the treatment of diabetes, particularly in developing countries where most people have limited resources and do not have access to modern treatment. Increasing evidence has shown that prolonged exposure to elevated glucose induces the production of free radicals, particularly reactive oxygen species (ROS) through glucose autooxidation and protein glycation[6,7] leading to oxidative stress. ROS cause lipid peroxydation and membrane damage and thus, plays an important role in the production of secondary complications such as kidney, eye, blood vessel, and nerve damages[8].

Recent investigations have shown that antioxidant properties of plants could be correlated with oxidative stress defense[9]. Moreover, antioxidants

have been shown to prevent the destruction of β cells by inhibiting the peroxydation chain reaction and thus they may provide protection against the development of diabetes[8].

Hallea stipulosa is a plant of the family Rubiaceae that is found mainly in marshy areas, and occasionally in forests[10]. It has been reported that *H. stipulosa* contains betulinic acid, tannins, flavonoids and polyphenolic compounds[11,12]. Betulinic acid is known to inhibit α-glucosidase enzyme[13] whereas flavonoids and phenols are known to possess antioxidant properties. Therefore, in view of phytochemical composition and based on the previous studies which shown the antihyperglycemic activities of aqueous extract of *H. stipulosa* in dexamethasone induced insulin resistance in rats[14], the aim of the present study was to evaluate the α-glucosidase inhibitory activity and antioxidant properties of aqueous extract and ethyl acetate fraction of *Hallea stipulosa* stem bark by *in vitro* method.

MATERIALS AND METHODS

Plant material

Collection and identification: *H. stipulosa* stem barks were collected in Foumban (West Cameroon) in June 2013 and a voucher specimen was authenticated by comparison to specimen No. 21076/ SRF/ CAM of the Cameroon herbarium.

Preparation of the aqueous extract and ethyl acetate fraction of stem barks of *Hallea stipulosa*

Aqueous extract: A decoction was prepared by boiling 100 g of a dried powder with 500 ml of distilled water for 20 min. The decoction once cooled at room temperature was filtered. Then the filtrate was concentrated by evaporating water at 45°C an oven for 48 h.

Ethyl acetate fraction: Methanol extract was first prepared by maceration of 200 g of dried powder with 1000 ml of methanol. After 72 h the mixture was filtered; then the filtrate was concentrated at 65 ° C in a rotary evaporator. Thereafter, the fraction was purified by flash chromatography using methanol extract.

Determination of total phenolic content: The total phenolic content was determined by the Folin-Ciocalteu reagent method described by Vinson *et al*.[15]. Twenty (20) µl of sample (AE or EAF) was added to 200 µl of Folin-Ciocalteu reagent 2 N diluted to 1/5th. The preparation was mixed and incubated at room temperature for 15 min. Then, 400 µl of a sodium carbonate solution (20%) was added and the mixture was incubated at room temperature for 30 min. The absorbance was measured at 760 nm using a spectrophotometer. Quercetin was used as standard for the calibration curve and total phenolic content was expressed as mg quercetin equivalents per gram of sample (mg QE/g).

Determination of total flavonoids: The flavonoids of AE and EAF of *H. stipulosa* were determined using the aluminum trichloride colorimetric method described by Kim *et al*.[16] . Distilled water (0.4 ml) was added to 0.1 ml of extract or fraction. Then 0.03 ml of sodium nitrite solution (5%) was added, followed by 0.03 ml of an aluminum chloride solution (10%). Test tubes were incubated at room temperature for 5 min. After incubation, 0.2 ml of sodium hydroxide solution (1M) was added to the mixture and then the volume of the reaction mixture was supplemented to 1 ml with distilled water. The preparation was stirred with the aid of the vortex and the absorbance was read at 510 nm. A calibration curve was prepared with catechin and the results were expressed as mg catechin equivalents per gram of sample (mg CE/g).

Antioxidant tests

DPPH free radical scavenging assay: The free radical scavenging activity of the extract and fraction, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca *et al*.[17]. Twenty (20) µl of test substance (AE, EAF or ascorbic acid) at different concentrations (12.5, 25, 50, 100, 200 µg/ml) were mixed with 2 ml of a methanol solution of DPPH (0.3 mM). After 30 minutes of incubation at room temperature in dark place, the absorbance was measured at 517 nm against methanol as blank by spectrophotemeter. The percentage inhibition of radical was calculated using the following formula:

Inhibition (%) = 100 x $(A_0 – A_1) / A_0$

Where A_0 is the absorbance of control and A_1 is the absorbance of sample.

Ferric-reducing antioxidant power assay: The ferric-reducing antioxidant power (FRAP) test was performed according to the method described by Bensie and Strain[18]. Briefly, the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride in proportions of 10:1:1 (V/V/V) respectively. The FRAP reagent was freshly prepared and warmed to 37 ° C prior to use. Fifty (50) µl of sample (AE, EAF or ascorbic acid) at different concentrations (12.5, 25,

50, 100, 200 µg / ml) were mixed with 1.5 ml of FRAP reagent. The tubes were stirred and incubated at 37 ° C for 10 min. The absorbance of the reaction was read at 593 nm against the white (1.5 ml of FRAP reagent + 50 µl of distilled water). The results are expressed in mM (Fe2 +) / per g of sample.

Nitric oxide scavenging assay: Nitric oxide scavenging activity was determined according to the method described by Sreejayan et Rao[19]. Two (2) ml of sodium nitroprusside (10 mM) dissolved in 0.5 ml of phosphate buffer saline (pH 7.4) were mixed with 0.5 ml of sample (AE, EAF or ascorbic acid) at different concentrations (12.5, 25, 50, 100, 200 µg/ml). The mixture was incubated at 25 ° C for 60 min. After incubation, 0.5 ml of the incubated solution was taken and mixed with 0.5 ml of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). The absorbance of the chromophore formed was read at 540 nm. Ascorbic acid was used as a positive control. The percentage inhibition of the NO radical was calculated using the following formula:
Inhibition (%) = 100 x ($A_0 – A_1$) / A_0
Where A_0 is the absorbance of control and A_1 is the absorbance of sample.

In vitro α- Glucosidase inhibitory activity: The α-glucosidase inhibitory activity was determined according to Dewi *et al*. [20] with slight modifications. One mg of α-glucosidase (from *Saccharomyces cerevisiae*) was dissolved in 100 ml of Tris-HCl buffer (pH 6.8) containing 200 mg of bovine serum albumin. In test tubes, 200 µl of a sample at different concentrations (3, 10, 30, 100, 300 µg/ml) was pre-incubated with 100 µl of α-glucosidase for 10 min at 37 °C. The reaction was initiated by addition of 100 µl of *p*-nitrophenyl-*α*-D-glucopyranoside (5 mM). After 15 min of incubation at 37 °C, 800 µl of Na_2CO_3 (200 mM) was added to stop the reaction. Acarbose was used as a positive control and distilled water as control. α-Glucosidase activity was determined spectrophotometrically at 400 nm by measuring the quantity of p-nitrophenol release from pNPG. The α-glucosidase inhibitory activity was calculated from the difference in two absorbencies and expressed in percentage of inhibition (% inhibition) as follow:
Inhibition (%) = 100 x (AC – AS) / AC
Where AC is the absorbance of control and AS is the absorbance of sample

Statistical analysis: All the measurements were taken in triplicate and the mean values were calculated. The values are expressed as mean ± SEM. IC_{50} values were calculated using nonlinear regression analysis with the GraphPad Prism version 5.01.

RESULTS

Total phenolics and flavonoids content: Total phenolic and flavonoids contents are presented in Table 1. Results of phenolic content show higher values of this compound in both AE and EAF. The amount of phenols was 124.1 ± 5.55 mg QE/g in AE as it was 18.52 ± 0.00 mg QE/g in EAF. The flavonoids content was found to be 0.30 ± 0.01 mg CE/g in aqueous extract and 0.27 ± 0.02 mg CE/g in ethyl acetate fraction.

Antioxidant activity

DPPH radicals scavenging activity: As shown in Figure 1 and Table 2, AE and ethyl EAF of *H. stipulosa* scavenged DPPH radicals in concentration-dependant manner, with IC_{50} values of 20.41 µg/ml and 66.83 µg/ml respectively. IC_{50} value of ascorbic acid was 17.79 µg/ml.

Ferric reducing antioxidant power: It was observed from Figure 2 and Table 2 that AE showed a higher reducing power (IC_{50} = 40.42 µg/ml) than EAF (IC50 = 79.25 µg/ml). Positive control (ascorbic acid) showed a strongest reducing power with IC50 = 21.58 µg/ml.

Table 1: Total phenolic and flavonoid content of *Hallea stipulosa*

	Phenolics content (mg QE/g)	**Flavonoids content (mg CE/g)**
Aqueous extract	124.1 ± 5.55	0.30 ± 0.01
Ethyl acetate fraction	18.52 ± 0.00	0.27 ± 0.02

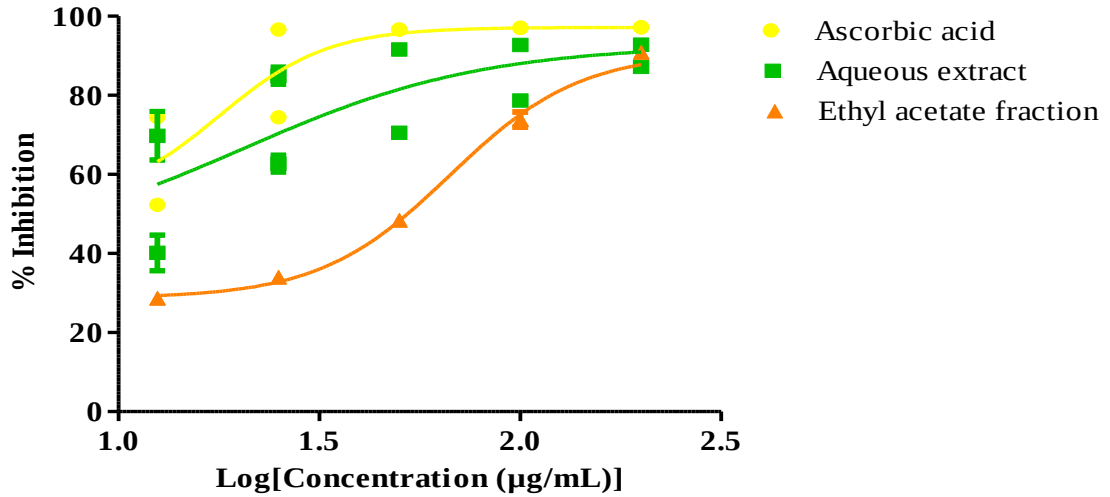

Figure 1: DPPH scavenging activity of aqueous extract and ethyl acetate fraction of *Hallea stipulosa*

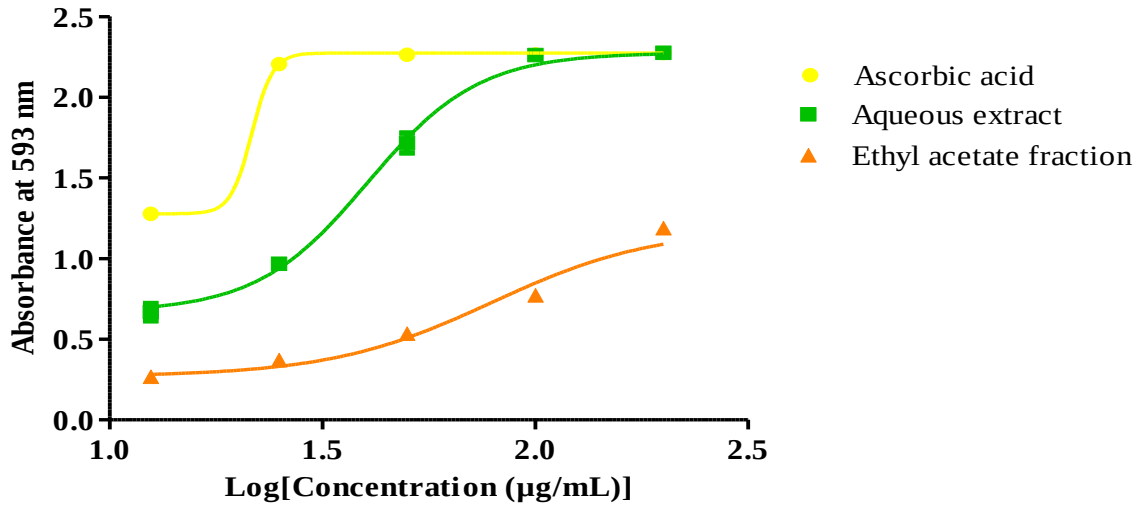

Figure 2: Ferric reducing antioxidant power of aqueous extract and ethyl acetate fraction of *Hallea stipulosa*

Nitric oxide scavenging activity: The scavenging abilities of AE and EAF of *H. stipulosa* on NO radicals are presented in Figure 3 and Table 2. It was observed that only AE and positive control (ascorbic acid) scavenged NO radicals in concentration-dependant manner. The highest activity was observed with ascorbic acid (IC_{50} = 27.71 µg/ml) followed by aqueous extract (IC_{50} = 41.30 µg/ml).

α-glucosidase inhibitory activity: The inhibitory effect of ethyl acetate fraction of *H. stipulosa* against α-glucosidase enzyme compared to acarbose is presented in Figure 4. IC_{50} value of ethyl acetate fraction was 39.19 µg/ml where as the one of acarbose was 24.21 µg/ml.

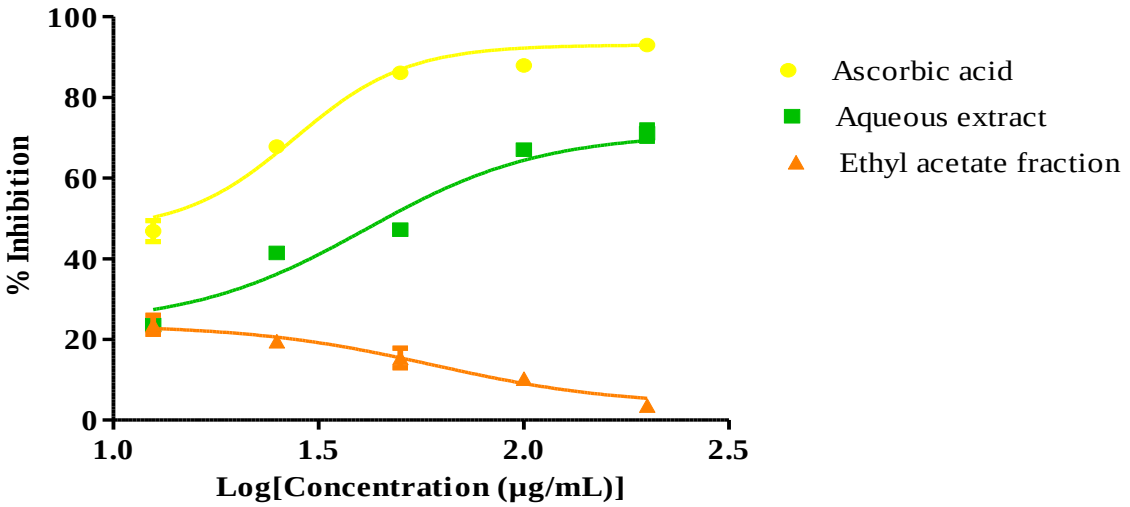

Figure 3: Nitric oxide scavenging activity of aqueous extract and ethyl acetate fraction of *Hallea stipulosa*

Table 2: IC_{50} values of *Hallea stipulosa* aqueous extract and ethyl acetate fraction on ferric reducing antioxidant power, DPPH and NO radicals scavenging activity

	IC_{50} (µg/ml)		
	DPPH	**FRAP**	**NO**
Aqueous extract	20.41	40.42	41.30
Ethyl acetate fraction	66.83	79.25	60.50
Ascorbic acid	17.79	21.58	27.71

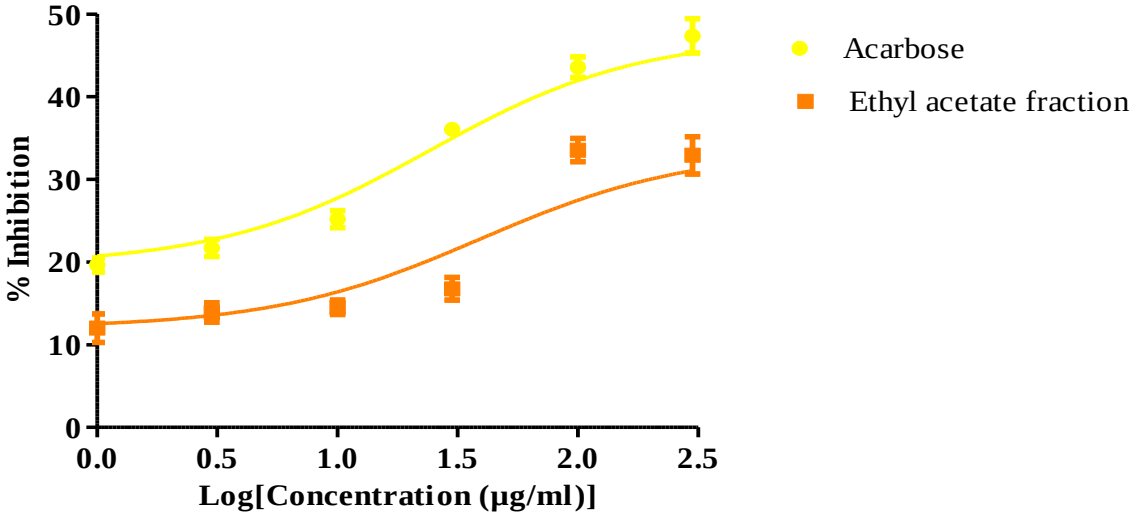

Figure 4: α-glucosidase inhibitory effect of ethyl acetate fraction of *Hallea stipulosa*

DISCUSSION

Increasing evidence in both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus. Free radicals are formed disproportionately in diabetes by glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increasing lipid peroxidation, and development of insulin resistance. These consequences of oxidative can promote the

development of complications of DM[21]. Natural antioxidants offer an alternative to reduce diabetes and its complications. Thus, based upon the previous studies that shown the antihyperglycemic effects of aqueous extract of *H. stipulosa*[11], the present study was carried out to establish the antioxidant potential and α-glucosidase inhibitory activity of aqueous extract and ethyl acetate fraction of this plant.

Plant phenolics and flavonoids are reported to have significant antioxidants properties. They possess the ideal chemistry for free radical scavenging activity due to the presence of high reactivity as hydrogen or electron donors and metal chelating activity[22,23]. Our results revealed that aqueous extract and ethyl acetate fraction of *H. stipulosa* contain a significant amount of phenols and flavonoids compound. Therefore, they could be able to interact with radicals and thus neutralize their free-radical character.

In this study, AE and EAF of *H. stipulosa* showed a dose dependant DPPH radical- scavenging activity. According to Moulisha *et al*.[24] this effect may be due to the hydrogen donating capability of compounds present in AE and EAF.

The reducing power of a compound is related to its electron transfer ability, and may therefore served as a significant indicator of it potential antioxidant activity[25]. The obtained results indicated that AE and EAF caused a significant reduction of ferric ions (Fe^{3+}) to the ferrous form (Fe^{2+}), and thus proved their reducing power. It has been shown that, the reducing property is generally associated with the presence of reducing agents which can break free radicals chain by donation of a hydrogen atom or react with certain precursors of peroxide thus preventing peroxide formation[26]. Thus the reducing power of AE and EAF could be attributed to hydrogen donation capacity of phenols and flavonoids present in plants.

NO is a free radical produced in mammalian cells and is involved in the regulation of various physiological processes[27]. However, sustained levels of production of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas a chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions[28]. During hyperglycemic conditions, there is overproduction of superoxide free radicals, which is accompanied by an increased NO generation, resulting in the formation of the stronger oxidant peroxynitrite[29]. The results indicated that AE and EAF strongly scavenged NO radicals. Hence, they may elicit inhibitory action against NO-induced cellular damages.

The results obtained in this study revealed that in all the antioxidant tests performed, AE of *H. stipulosa* and ascorbic acid showed an IC50 <50 µg/ml. According to Omisore *et al*.[30], samples with an IC50 < 50 µg/ml have a high antioxidant capacity while samples with an IC50 > 50 µg/ml have a moderate antioxidant capacity. Therefore, AE *H. stipulosa* and ascorbic acid could be considered to have a high antioxidant capacity.

One therapeutic approach for treating diabetes is to decrease the postprandial hyperglycemia. This is done by reducing or slowing the digestion of carbohydrates. The inhibition of the enzymes involved, such as α-amylase and α-glucosidase enzymes, is a powerful therapeutic target for management the postprandial glycemic response[31]. In this study EAF of *H. stipulosa* showed an inhibitory effect on α-glucosidase enzyme with an IC_{50} of 39.19 µg/ml. It has been shown that disaccharidases are targets of flavonoids in the regulation of glucose absorption[32]. Moreover, Kumar *et al*.[13] showed that betulinic acid isolated from *Dillena indica* inhibits the α-glucosidase enzyme from 52.2%. Therefore, the inhibitory activity of *H. stipulosa* on α-glucosidase could be due to the presence of flavonoids and betulinic acid in the plant. According to the previous study which revealed that AE of *H. stipulosa* inhibited α-glucosidase enzyme with an IC_{50} of 13.14 µg/ml[14], by comparing the effect of AE with that of the ethyl acetate fraction (IC_{50} = 39.19 µg/ml) obtained in this study, it appears that AE have the highest inhibitory effect on α-glucosidase enzyme.

CONCLUSION

From the present study, we can conclude that aqueous extract and ethyl acetate fraction of *H. stipulosa* exhibited higher antioxidant activity as well as inhibitory effect on α-glucosidase enzyme. AE exhibited the highest activity. These effects could be a part of the mechanism by which *H. stipulosa* manages diabetes.

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