Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2015; 7(2); 74-79

ISSN: 0975-5160

Research Article

In vitro α-Amylase and α-Glucosidase Enzymes Inhibitory Effects of the Aqueous Extract of *Combretum molle* Twigs

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Available Online: 2nd March, 2015

ABSTRACT
The present study is carried out to evaluate the *in vitro* α-amylase and α-glucosidase enzymes inhibition studies together with *in vivo* studies to confirm the activity of *Combretum molle* twigs extract to control postprandial blood glucose level in starch and sucrose loaded normal rats. The inhibitory effect of the extract on α-amylase and α-glucosidase was determined in *vitro* studies. In *in vivo* studies, oral tolerance tests were done by inducing hyperglycemic state via administration of starch (4g/kg) and sucrose (3 g/kg). Thereafter, hyperglycemic rats groups received 0, 125, 250 and 500 mg/kg of the extract. The glycemia was evaluated at 0, 30, 60, 90 and 120 minutes after the administration of carbohydrates. *In vitro* studies had indicated dose-dependent inhibitory activity of the extract against α-amylase and α-glucosidase with the IC_{50} values of 31.25 ± 5.95 and 50.60 ± 2.81 µg/mL, respectively. In *in vivo* studies, the extract alone was administered to starch and sucrose loaded normal fasting rats which produced a significant decrease in AUC and in postprandial glycemia at 90 and 120 minutes at 500 mg/kg. However, at 250 mg/kg, the extract induced a significant decrease in AUC and in blood glucose concentration at 60 and 120 minute after carbohydrates loading. The current study demonstrates one of the mechanisms in which *C. molle* twigs extract effectively inhibits α-amylase and α-glucosidase leading to suppression of postprandial hyperglycemia in rats loaded with starch and sucrose. The extract seems to be promising in the treatment of type-2 diabetes mellitus by reducing postprandial hyperglycaemia.
Key words: α-glucosidase, α-amylase, *Combretum molle* twigs, aqueous extract, postprandial hyperglycemia, acarbose.

INTRODUCTION

Diabetes mellitus (DM) is a chronic disorder characterized by both postprandial and fasting hyperglycemia with disturbances in carbohydrate, fat and protein metabolism[1]. Diabetic hyperglycemia results either from an absolute deficiency in insulin secretion (type 1 DM) or insulin action (type 2 DM) or both[2]. Diabetes causes about 5% of all deaths globally each year and 80% of the people with diabetes live in low and middle income countries[3]. If not controlled, hyperglycemia can lead to the development of macrovascular and microvascular complications and it is the major independent risk factor for cardiovascular diseases[4].

One therapeutic approach to prevent postprandial hyperglycemia is to retard the digestion and absorption of carbohydrates in the gastrointestinal tract through inhibition of enzymes such as α-amylase and α-glucosidase[5,6]. Alpha-amylases hydrolyze complex polysaccharides to produce oligosaccharides and disaccharides which are then hydrolyzed by alpha-glucosidase to monosaccharides which are absorbed through the small intestines into the hepatic portal vein[7]. Acarbose, voglibose and miglitol are commerical α-glucosidase and α-amylase inhibitors that are considered as first line treatment for diabetic individuals with postprandial hyperglycemia. On giving them along with other oral hypoglycemic agents like metformin, sulfonylurea improves glycemic control (reduced HbA1c). However, these α-amylase and α-glucosidase inhibitors have prominent gastrointestinal side effects like flatulence, diarrhoea, and abdominal discomfort[8]. Therefore, it becomes necessary to identify the amylase and glucosidase inhibitors from natural sources having fewer side effects. Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. Ethno botanical information indicates that more than 1200 plants species are used for the treatment of diabetes throughout the world but still there is an insufficient scientific proof of their antidiabetic activity[9].

Combretum molle (Combretaceae) have long been used to control diabetes mellitus all around the tropical Africa, especially in Cameroon. The hypoglycemic effects of leaves and twigs of this plant have been supported by various scientific studies[10,11]. However, there are no previous reports of any *in vitro* α-glucosidase and α-amylase inhibitory activity of *C. molle*. Hence *in vitro* α-glucosidase and α-amylase enzyme inhibition studies together with *in vivo* studies to confirm the activity in live animals were carried out to evaluate the possible

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antihyperglycemic potential of aqueous extract of *C. molle.*

MATERIALS AND METHODS

Drugs and chemicals: Acarbose, α-glucosidase, α-amylase, 3,5-dinitrosalicylic acid and pnitrophenyl-D-glucoside (pNPG) were purchased from Sigma-Aldrich, St. Louis, USA. Starch, D-glucose and sucrose were purchased from Edu-Lab Biology Kit, Bexwell, Norfolk PE38 9GA, UK. All chemicals and drugs were obtained commercially and were of analytical grade.

Animals: Albinos males rats aged 3-4 months, average weight 250 g were used. These animals were raised in the animal house of the Department of Animal Biology, Faculty of Sciences at the University of Dschang, Cameroon. The animals were housed in plastic cages and maintained in ambient temperature of 24 ± 1^0C, relative humidity of 55- 65% and normal light/dark cycle. They were fed standard laboratory diet and water *ad libitum*. The rats were acclimatized to laboratory condition for one week before commencement of experiments. The Ethical clearance for the usage of rats was obtained from the Institutional Animal Ethical Committee (IAEC) prior to the beginning of the study[12].

Plant material: The plant material was constituted of the twigs of *Combretum molle*. These last were collected in December 2012 in the locality of Moutourwa, zone located at a distance of approximately 55 km of the Maroua city, Far North Region-Cameroun. The collected specie was authenticated in the National Herbarium located in Yaoundé-Cameroon where voucher specimen was deposited. After authentication, the collected fresh samples were cleansed thoroughly under running tap water, dried under shade and ground into fine powder using a milling machine.

Extraction procedure: Mixed powder material was extracted using water. Dried powder (200 g) of twigs mixed with 500 mL of distilled water were boiled for 15 min and then cooled for 15 min. Afterwards, the mixture was filtered using Whatman filter paper No.1 and the filtrate obtained was dried at a temperature of 45ºC to produce 19.69 g of the crude aqueous extract, with a yield of 9.64 %. The extract was preserved in refrigerator till further use.

Phytochemistry essay: It was performed to determined chemical compounds content of extract qualitatively. Based on an established standard procedures, was conducted to explore the secondary metabolites such as, alkaloids (Dragendoff reagent), saponins (frothing test), tannins ($FeCl_3$), glycosides (Legal test), flavonoids (NaCl and HCl), reducing sugars (Fehling liquor), steroids (chloroforme and H_2SO_4 concentrate), terpenoïdes (chloroforme and H_2SO_4 concentrate) and phenols ($FeCl_3$ and $K_3Fe\ (CN)_6$)[13].

In vitro α-amylase inhibition study: A total of 500 µL of test samples and standard drug (1, 3, 10, 30, 100, 300 µg/mL) were added to 500 µL of 0.20 mM tris buffer (pH 7) containing 500 µL of α-amylase (0.5mg/mL) solution and were incubated at 25°C for 20 min. After these, 250 µL of a starch solution in 0.02 M sodium tris buffer (pH 7) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 2 mL of 3,5-dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath at 100°C for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 mL distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way by replacing extract with vehicle[14].

In vitro α-glucosidase inhibition study: The α-glucosidase inhibitory effect of plant extract was determined according to the method described by Kim et al.[15]. A reaction mixture containing 50 µL of tris buffer (50 mM; pH 6.8), 100 µL of alpha-glucosidase (1 U/mL) and 50 µL of plant extract of varying concentrations (1, 3, 10, 30, 100, 300 µg/mL) was pre-incubated for 10 min at 25°C, and then 50 µL of 5 mM of p-nitrophenyl-α-d-glucopyranoside was added to the mixture as a substrate. After further incubation at 37°C for 15 min, the reaction was stopped by adding 2 mL of sodium carbonate (500 mM). All the enzyme, inhibitor and substrate solutions were made using the same buffer. Acarbose was used as a positive control and distilled water as control. The yellow colour produced (due to *p*-nitrophenol formation) was quantitated by colorimetric analysis and reading the absorbance at 400 nm.

Calculation of 50% inhibitory concentration (IC_{50}): The concentration of the plant extract required to inhibit 50% of the enzyme (IC_{50}) was calculated by using the percentage scavenging activities at six different concentrations of the extract. Percentage inhibition (I %) was calculated as follows:

I % = (Ac-As)/Ac x 100

Where

Ac is the absorbance of the control

As is the absorbance of the sample

Oral starch tolerance test[16]: Rats were divided into five groups consisting of six rats (n = 6) in each group. The rats were fasted overnight for 16 hours but had free access to water.

Group I rats were treated orally at 10 mL/kg of distilled water and considered as normal control group. Group II rats were treated orally with acarbose at the dose of 10 mg/kg b.wt. and considered as positive control group. Groups III, IV and V animals received the extract at doses of 125, 250 and 500 mg/kg b.wt. of each, and considered as treated groups.

After 10 min, all rats were given starch 3 g/kg body weight orally and the tail was snipped for blood glucose estimation before (0 min) and at 30, 60, 90 and 120 minutes after starch administration. Blood glucose concentrations were recorded and area under the curve (AUC) was determined.

Oral sucrose tolerance test: The oral sucrose tolerance test was carried out with distilled water, acarbose and different doses of extract, in the same way as above, but in this test, sucrose at the dose of 4 g/kg body weight was used.

Statistical analysis : Statistical analysis was performed using the statistical functions of the Graph pad Prism version 4.1. All the results were expressed as mean ± SEM. The significance of difference between mean values for the

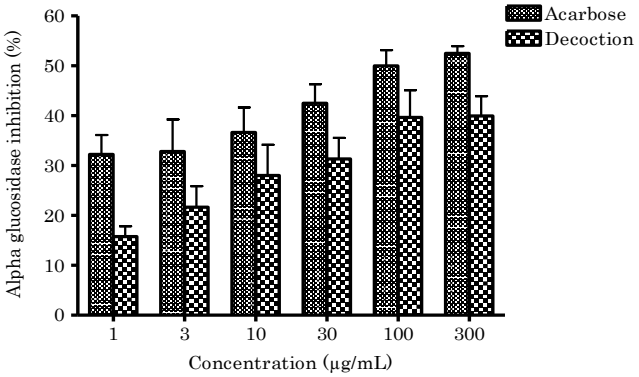

Figure 1. Inhibitory activity of aqueous extract of *Combretum molle* twigs against α-amylase. Values are expressed as mean ± SE. SE: Standard error.

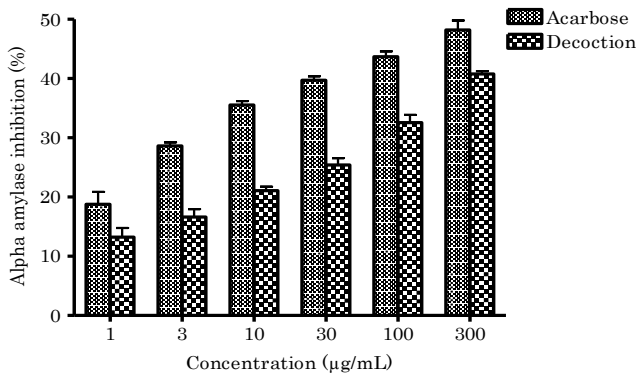

Figure 2. Inhibitory activity of the aqueous extract of *Combretum molle* twigs against α-glucosidase. Values are expressed as mean ± SE. SE: Standard error.

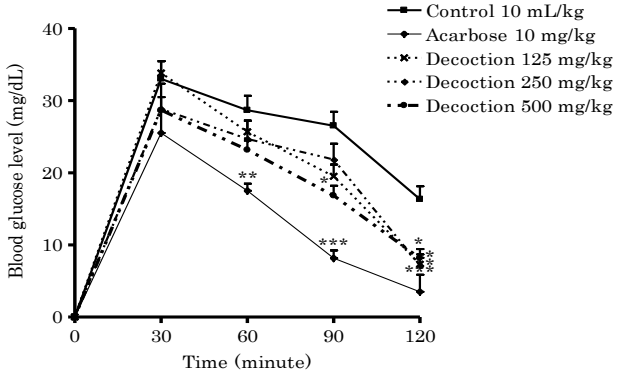

Figure 3: Effect of the aqueous extract of *Combretum molle* twigs on oral starch tolerance test in normal rats. Values are expressed as mean ± SE of 6 rats in each group. *P < 0.05; ***P < 0.001 compared to control. SE: Standard error.

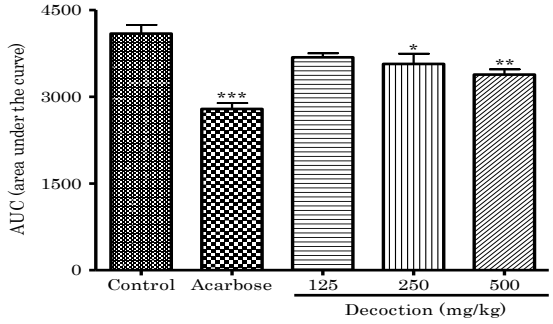

Figure 4. Effect of aqueous extract of *Combretum molle* twigs on area under the curve after starch loading in normal rats. Values are expressed as mean ± SE of 6 rats in each group. *P < 0.05; **P < 0.001; ***P < 0.001 compared to control. SE: Standard error.

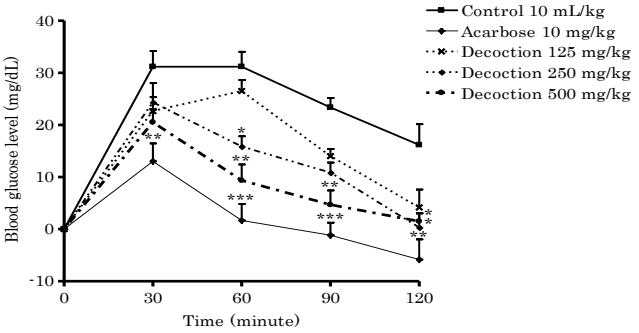

Figure 5. Effect of the aqueous extract of *Combretum molle* twigs on oral sucrose tolerance test in normal rat. Values are expressed as mean ± SE of 6 rats in each group. *P < 0.05; **P < 0.001; ***P < 0.001 compared to control. SE: Standard error.

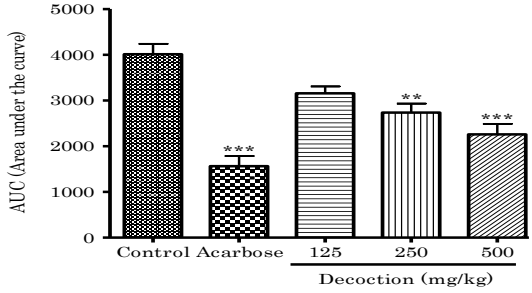

Figure 6. Effect of aqueous extract of *Combretum molle* twigs on area under the curve after sucrose loading in normal rats. Values are expressed as mean ± SE of 6 rats in each group. **P < 0.001; ***P < 0.001 compared to control. SE: Standard error.

various treatments were tested using one-way analysis of variance (ANOVA) followed by Turkey test and two-way analysis of variance followed by Bonferroni test. Statistical significance was considered at $p < 0.05$; $p < 0.01$ and $p < 0.001$.

RESULTS

Preliminary phytochemical screening: The preliminary phytochemical screening of the aqueous extract of *C. molle* twigs revealed the presence of reducing sugar, glycosides, saponins, flavonoids, phenols, triterpenoids and tannins.

In vitro α-amylase inhibition study: The results obtained at the end of the *in vitro* studies showed that aqueous extract of *C. molle* efficiently inhibits α-amylase (Figure 1). There was a concentration dependent increase in percentage inhibition activity against α- amylase by the extract. This last produced a maximum inhibition of about 39.95 ± 3.95% at a concentration of 300 µg/mL. At the lowest concentrations of 1µg/mL, there was about 15.76 ± 2.09% inhibition. The standard drug acarbose showed a strong inhibiting potential with the percentages of inhibition varying between 32.24 ± 3.89% and 52,51 ± 1,43%

Table 1. Effect of aqueous extract concentrations of *Combretum molle* twigs on percentage reduction α-amylase and α-glucosidase enzymes *in vitro*.

	IC_{50} (µg/mL)	
	α- amylase	α- glucosidase
Decoction	31.25 ± 5.95	50.60 ± 2.81
Acarbose	9.35 ± 2.69	4.71 ± 0.84

Data are expressed as mean ± SE. IC_{50} : Concentration of inhibitor to inhibit 50% of his activity under the assayed conditions. SE: Standard error.

corresponding with concentrations ranging between 1 and 300 µg/mL. However, The IC_{50} values for decoction and acarbose are 31.25 ± 5.95 µg/mL and 9.35 ± 2.69 µg/mL, respectively (Table 1).

In vitro α-glucosidase inhibition study: Figures 2 show the percentage inhibition values of plant extract and acarbose against α-glucosidase. This reveals that the percentage inhibition at 1, 3, 10, 30, 100 and 300 µg/mL concentrations of the extract showed a concentration-dependent increase in percentage inhibition. Thus the highest concentration of 300 µg/mL tested showed a maximum inhibition of 40.77 ± 0.47%. The percentage inhibition varied from 13.22 ± 1.54% to 40.77 ± 0.47% from the highest concentration to the lowest concentration of 1 µg/mL. Acarbose showed a strong inhibitory potential with percentage inhibitions ranging from 18.76 ± 2.12% to 48.21 ± 1.59 % for concentrations ranging from 1-300 µg/mL. The IC_{50} values for decoction and acarbose are 50.60 ± 2.81µg/mL and 4.71 ± 0.84 µg/mL, respectively (Table 1).

Oral starch tolerance test: The results of the oral starch tolerance test in temporary hyperglycemia animals treated with the aqueous extracts of *C. molle* twigs were illustrated by Figures 3 and 4. These reveal that in all the animals groups, the administration *per os* of the starch (3g/kg), after pretreatment with the distilled water, acarbose or various doses of the extract, involved a transitory hyperglycemia with a peak which appears at the end of 30 minutes; thereafter, the hyperglycemia decreases gradually until the end of the experimentation. However, the pretreated animals with the acarbose and the various doses of the extract presented a glycemia less important than that of the control groups.

Indeed, compared to control group rats, acarbose involved a significant reduction in the AUC ($p < 0.001$) and in the blood glucose levels of about 11.17% ($p < 0.01$), 18.33% ($p < 0.001$) and 12.83% ($p < 0.001$) at 60, 90 and 120 minutes, respectively. Moreover, the decoction managed to reduce significantly the AUC level ($p < 0.01$) at the dose of 500 mg/kg, and also the postprandial glycemia ($p < 0.05$) nearly 9.67% and 8.17% at 90 and 120 minutes, respectively. Nevertheless, a considerable reduction ($p < 0.05$) in blood sugar rate of about 9% and 9.17%, were noted at 120 minute in the pretreated subjects at the doses of 125 and 250 mg/kg of aqueous extract, respectively. But, only the dose 250 mg/kg significantly ($p < 0.05$) reduced the AUC level.

Figures 5 and 6 below show the effects of the aqueous extracts of *C. molle* twigs on the postprandial glycemia during oral sucrose tolerance test. This reveals that the regulation of glucose was more efficient in the animals treated with the acarbose and the extract. When compared to normal control rats, decoction at the dose of 125 mg/kg did not demonstrate any significant decrease in blood glucose level and AUC, whereas the dose of 500 mg/kg induced a significant reduction in AUC ($p < 0.001$) and in blood sugar rate nearly 21.83% ($p < 0.01$), 18.67 % ($p < 0.01$) and 14.67% ($p < 0.05$) at 60, 90 and 120 minutes, respectively. However, the plant extract at the dose of 250 mg/kg induced a significant decrease in AUC level ($p < 0.01$) and in blood glucose lowering response ($p < 0.05$) of 15.33% and 15.83% at 60 and 120 minute, respectively. In a similar way, acarbose managed to lower AUC significantly ($p < 0,001$) and blood glucose concentration of about 18.17% ($p < 0.01$), 29.50% ($p < 0.001$), 24.50% ($p < 0.001$) and 22% ($p < 0.01$) at 30, 60, 90 and 120 minutes, respectively.

DISCUSSION

Antidiabetic medicinal plants are reported to exert their blood glucose lowering effects through a variety of mechanisms[17,18]. These mechanisms are more or less similar to those of the synthetic oral hypoglycemic drugs and include : stimulation of insulin synthesis and/or secretion from pancreatic beta-cells, regeneration/revitalization of damaged pancreatic beta cells, improvement of insulin sensitivity (enhancement of glucose uptake by fat and muscle cells), mimicking the action of insulin (acting like insulin), alteration of the activity of some enzymes that are involved in glucose metabolism and slowing down the absorption of sugars from the gut[17].

The leaves and twigs extracts of *C. molle* has been reported to have antidiabetic activity by causing an increase in glucose utilization, simultaneously lowering plasma glucose in normal and diabetic rats[10,19]. So, perhaps *C. molle* extracts could have an effect on glucose absorption from the gut and may prolong absorption process, suppressing the peak blood glucose levels. Although there are studies of antihyperglycemic and antidiabetic activities of the aqueous extract of *C. molle* there are no previous reports, at least to our knowledge, on the activity of this extract on *in vitro* α-glucosidase activity. In the current study, the effects of *C. molle* twigs extract on the activities of α-amylase and α-glucosidase were investigate *in vitro*. Alpha amylases hydrolyze complex polysaccharides to produce oligosaccharides and disaccharides which are then hydrolyzed by α-glucosidase to monosaccharides which are absorbed through the small intestines into the hepatic portal vein[20]. Inhibitors of both α-amylase and α-glucosidase delay digestion and subsequent absorption of carbohydrates thereby lowering postprandial glucose levels[21].

Our *in vitro* studies demonstrated an appreciable α-glucosidase and α-amylase inhibitory activity of aqueous extract of *C. molle* and of acarbose. The IC_{50} values show that the inhibitor potency of extract is stronger on the α-amylase than on the α-glucosidase. An inhibition dependent concentration was also observed with the acarbose as well on α-glucosidase on α-amylase. Acarbose

seems to show equal preference for both α-glucosidase and α-amylase enzymes. But not always does the *in vitro* inhibitory activity relate to the corresponding *in vivo* activity. Thus proof of concept needs to be demonstrated in preclinical animal studies. For safety and efficacy to be established, it was essential to confirm the *in vivo* action following oral administration to live animals hence the *in vivo* experiments were performed.

In the *in vivo* experiments, the doses of 250 and 500 mg/kg of the decoction produced a significant decrease in AUC and in blood glucose level at 90 and 120 minute after starch loading. Concerning oral sucrose tolerance test, a significant decrease and dose dependent in AUC and in postprandial glycemia was noted with the aqueous extract at 60, 90 and 120 minutes. The extract seems to delay the fast digestion of the starch and the sucrose, and to lengthen the duration of carbohydrates absorption, reducing of this fact the value of the postprandial hyperglycemia and the AUC. Thus, the extract showed a significant inhibitory effect on the α-glucosidase and α-amylase enzymes both *in vitro* and *in vivo*. These findings suggested that the water extract of *C. molle* twigs could decrease the postprandial glycemia level by inhibiting the activity of α-amylase and α-glucosidase, which are important enzymes in the digestion of the complex carbohydrates into absorbable monosaccharides in the food[22]. Recently, some reports showed that flavonoids, glycosides and triterpenoids could effectively inhibit the activity of α-amylase and/or α glucosidase to decrease the absorption of carbohydrates from food[23,24]. So the glycosides and triterpenoids presents in the extract might take the responsibility for the postprandial antihyperglycemic effect of extract. These results confirm those obtained by Sugiwati et al.[25] which showed that the *Mahkota dewa* leaves would contain glycosides made up of sugars having a similar structure to that of monosaccharides, substrates of the α-glucosidases.

Acarbose-like drugs, drugs that inhibit α-glucosidase present in the epithelium of the small intestine, have been demonstrated to decrease postprandial hyperglycaemia[26] and improve impaired glucose metabolism without promoting insulin secretion in type 2 diabetes patients[27]. These medications are most useful for people who have just been diagnosed with type 2 diabetes and who have blood glucose levels only slightly above the level considered serious for diabetes. They also are useful for people taking sulfonylurea medication or metformin, who need an additional medication to keep their blood glucose levels within a safe range. In the present study, acarbose produced results almost similar to those obtained with the plant extract. This corroborates the works realized by Kamiyama et al.[28] which showed that the acarbose would contain compounds having the same structure as disaccharides and monosaccharides resulting from digestion of the glucides, which allowing them to bind to the α-glucosidase and α-amylase, thus inhibiting in a competitive way the pancreatic α-glucosidase.

CONCLUSION

Combretum molle twigs extract shows strong inhibitory activity against key enzymes linked to type-2 diabetes, namely, α-amylase and α-glucosidase. It effectively suppresses the starch and sucrose induced postprandial blood glucose spikes in rats. The extract seems to be promising in the treatment of type 2 diabetes mellitus by reducing postprandial hyperglycemia; it is still early to recommend its use in humans.

ACKNOWLEDGMENTS

This work was carried at the Laboratory of Animal Physiology and Phytopharmacology, University of Dschang. The authors are grateful to Head of this laboratory for providing the facilities and Drs. Dawe A. for the extraction of plant material and Njateng S. for the purchase of the reagents.

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