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Kinetics of inhibitory effect of ethyl acetate leaf fraction of *Lecaniodiscus cupanoides* on enzymes linked to carbohydrate metabolism: An *in vitro* assessment

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ABSTRACT

Background and aims: This study investigates the kinetics of inhibitory activities of ethyl acetate leaf fraction of *Lecanodiscus cupanoides* on α-amylase and α-glucosidase.
Methods: This was done using α-amylase and α-glucosidase enzymes from *Aspergillus oryzae* and *Saccharomyces cerevisiae* respectively, active component and antioxidants capabilities of the fraction were also analyzed.
Results: The fraction scavenged DPPH, OH*, and $ABTS^{+}$ and chelated Fe^{2+} with the IC_{50} values of 0.46, 0.70, 0.75 and 0.97 mg/mL respectively. However, significant and mild inhibitory effect was also observed in α- amylase and glucosidase with IC_{50} values (0.73 and 0.58 mg/mL) in concentration dependent pattern. Kinetic analysis of the fraction revealed an uncompetitive and non-competitive mode of inhibition for α-amylase and α-glucosidase respectively.
Conclusion: This study suggests that ethylacetate fraction of this plant possesses antidiabetic property as a result of its ability to inhibit the metabolism of carbohydrate hydrolyzing enzymes. The elicited activities of the ethyl acetate fraction *Lecaniodiscus cupanoides* may be due to the presence of phytochemicals of utmost pharmacological importance present in the fraction.

Keywords: Acarbose, Diabetes, Hyperglycemia, Postprandial Hyperglycemia, Radical Scavenging.

Original article

INTRODUCTION

Diabetes mellitus (DM) connotes a metabolic disorder characterized by chronic hyperglycemia which arises as a result of disturbance in carbohydrate metabolism caused by the deficiency of β-cells in the pancreas.[1] The global prevalence of diabetes is about 7.8% and if it is not checked, about 438 million people will be suffering from

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this disorder by 2030.[2] In Africa, the prevalence of DM is very high in South Africa and Nigeria with 8.30% and 4.50%, respectively.[3] Reduction of postprandial (PP) hyperglycemia is one of the therapeutic interventions for the treatment of DM. This can be done by retarding the uptake of glucose in the digestive tract though the inhibition of carbohydrate hydrolyzing enzymes (α-amylase and glucosidase).[4]

α-amylase inhibitors are often called starch blockers as a result of their ability to delay the absorption of dietary starch by the body which eventually lower blood glucose through the inhibition of an enzyme such as salivary and pancreatic amylase.[5] However, α-glucosidase inhibitors suppress PH in the intestine by completely and reversibly inhibiting the enzyme (α-glucosidase), which in turn minimizes glucose absorption through delayed carbohydrate catabolism and extended digestion time.[6]

Reactive oxygen species (ROS) is a key to the development of DM.[7] Excessive activity of ROS is physiologically related to the development of postprandial hyperglycemia.[8] The rise in ROS in DM patients occurs through glucose auto-oxidation which leads to production of free radicals, imbalance cellular reduction/oxidation and inhibition of scavenging activity of the enzymes against free radicals.[9,10]

The management of DM with little or no side effects is still a challenge to the pharmacologist. The use of herbs derived drugs in the treatment of this disorder has increased significantly in the recent time. The efficacy and lesser therapeutic side effects pose by herbal drugs has established a novel anti-diabetic therapy.[11] However, it has been established that botanicals with radical scavenging ability could be useful in the management of DM. In view of the foregoing assertions, *Lecaniodiscus cupanioides* (LC) is one of the botanicals with antidiabetic potential owing to its effectiveness in radical scavenging.

LC belongs to the family of sapindaceae, it's popularly used in South West Nigeria for the treatment of array of ailments such as tooth ache, fever, wounds, boils, burns and bruises.[12,13] Pharmacological studies have revealed LC to possess activities such as antimicrobial, hepatoprotective property, and radical scavenging activity on kidney, liver and brain enzymes.[14-17] However, no study has been conducted to investigate the antidiabetic activity of the leaves of LC. Therefore, this study was carried out to elucidate the inhibitory effect of ethyl acetate fraction of *Lecaniodiscus cupanioides* (EFLC) on α-amylase and glucosidase which are two key enzymes that is linked to carbohydrate metabolism in the body and its mechanism of action using *in vitro* model.

METHODS

Fresh leaves of LC were collected around Esindale area, Ile-Ife, Osun State Nigeria, authenticated by Mr Ademoriyo G.A of the IFE herbarium with voucher specimen (IFE7492) deposited to the herbarium. The leaf sample was air-dried at room temperature until constant weight and ground. The powered sample (750 g) was soaked and extracted in methanol/ distilled water (3: 2 v/v) for four days with regular agitation at intervals. This was thereafter filtered and the filtrate was concentrated *in vacuo* and lyophilized. The

yield obtained was 130 g. A portion (20 g) of the leaves extract was reconstituted in distilled water (150 mL) and then partitioned into ethyl acetate fraction. The dried fraction was reconstituted in dimethyl sulphoxide (1 mg/mL) and used for all the analyses. Similarly, a stock concentration of 1 mg/mL was also prepared for acarbose and garlic acid.

EFLC was then subjected to qualitative phytochemical screening for detection of alkaloids, anthraquinones, glycosides, flavonoids, phenols, saponins, triterpenes, phytosterols and sterols.[18,19] For quantitative phytochemical analysis,total phenolic content was done by mixing 1 mL of the EFLC (1 mg/mL) with Folin-Ciocalteu reagent (5 mL) and 75g/1000 mL of sodium carbonate (4 mL) in a tube. For colour development, the resulting mixture was vortexed (15 s) and subsequently incubated (40 °C, 30 min). Afterwards, the absorbance of the mixture was spectrophotometrically taken (Biochrom WPA Biowave II, Cambridge) at 765 nm. The determinations were triplicated in each case. Using garlic acid as standard, the phenolic content of the EFLC was estimated from its calibration curve and expressed as %w/w garlic acid equivalent.[20]

To determine flavonoid content, equal portion (0.5 mL each) of alcoholic aluminum solution (2%) and the EFLC (1 mg/mL) were mixed in a test tube prior to incubation (25 °C, 1 h). Subsequently, the absorbance was taken at 420 nm and the flavonoid content was expressed as quercetin equivalent using its (quercetin) standard calibration curve.[21] For the estimation total flavanol, the rutin calibration curve was prepared by mixing 2000 µL of different concentrations (200-1000 µg/mL) of rutin with 2000 µL (20 g/L) aluminum chloride and 6000 µL (50 g/L) sodium acetate in test tubes. Following incubation (20 °C, 2.5 h), the absorbance was read at 440 nm. The determinations were carried out in triplicates and the protocol was also performed with 2000 µL of the EFLC (100/ 1000 µg/mL) instead of rutin solution. The total flavonols in the EFLC was thereafter estimated from rutin standard curve.[22]

To determine DPPH radical scavenging potential of the fraction, different concentrations (200-1000 µg/mL) of EFLC and methanolic garlic acid solutions were mixed with methanolic solution of DPPH (0.2 M). Following 30 min of incubation (25 °C), the absorbance of the resulting solutions were colorimetrically determined against blank at 517 nm.[23] For Metal chelating assay, 0.1 mL of either garlic acid or the samples (0.2-1.0 mg/mL) was added to 0.2 mM ferrous chloride solution (0.5 mL). To initiate the reaction, 5 Mm Ferro zine (0.2 mL) was added and the mixture incubated (25 °C, 10 min). The absorbance of the resulting solution was then taken against blank at 562 nm.[24]

For ABTS radical determination, 50 mL each of 7 M ABTS and 2.45 mM potassium persulphate were mixed and incubated for 16 h in the dark. Subsequently, the concentration of solution was spectrophotometrically (734 nm) adjusted to 0.700 using ethanol. A portion (20 µL) of the EFLC (0.2-1.0 mg/mL) was thereafter reacted with the resulting ABTS solution (200 µL) in a 96-well microtiter plate prior to incubation (25 °C, 15 min) and final absorbance reading (734 nm) in a microplate reader (Model 680 BIO-RAD, USA).[25]

Hydroxyl radical scavenging activity (OH*) was determine by adding 0.1 mL of EFLC (0.2-1.0 mg/mL) to a reaction medium containing 20 mM deoxyribose (0.12 mL), 0.1 M phosphate buffer (0.4 mL), 20 mM hydrogen peroxide (0.04 mL) and 500 µM ferrous sulphate (0.04 mL). Following this, sterile distilled water (0.1 mL) was added and the resulting solution incubated (37 °C, 30 min). To bring the reaction to a halt, 0.5 mL of 2.8% trichloroacetic acid and 0.4 mL of 0.6% thiobarbituric acid solutions were added. Thereafter, a portion (0.3 mL) was taken and dispensed into a 96-well microtiter plate, boiled for 20 min prior to absorbance reading at 532 nm.[26]

For each of the antioxidant assay, the experiment was conducted in triplicate, inhibition/ scavenging rate (I %) was calculated using the expression:

Percentage inhibition (I %)= ($A_{control}$-$A_{fraction}$) /$A_{control}$] ×100

Where $A_{control}$ is the absorbance of the control, $A_{fraction}$ is the absorbance of the fraction. The concentration of the fraction and garlic acid causing 50% inhibition (IC_{50}) for all the assays was estimated using standard calibration curve.

In the α-amylase inhibitory assay, varying concentrations (0.2-1.0 mg/mL) of the fraction and acarbose (control) were prepared and 500 mL of each was mixed with 500 mL of 0.02 M sodium phosphate buffer (pH 6.9) containing 0.5 mg/mL of α-amylase solution and incubated (25 °C, 10 min). After initial incubation, 500 mL of 1% starch solution in 0.02 M sodium phosphate buffer (pH=6.9) was added to each tube at timed intervals. The reaction mixtures were incubated (25 °C, 10 min) and stopped with 1.0 mL of dinitrosalycyclic acid colour reagent. The tubes were then incubated in a boiling water bath for 5 min and subsequently cooled to room temperature. The reaction mixtures were then mixed with distilled water (15 mL), and the absorbance readings were taken (540 nm) using a spectrophotometer (Biochrom WPA Biowave II, Cambridge) and the values compared with a control which contained 500 mL of the buffer instead of the fraction.[27,28]

For α-amylase kinetics study, EFLC was incubated with 5 mg/mL α-amylase with the starch concentration varied from 0.016-0.200 mg/mL. The reaction was then allowed to proceed as highlighted above. Maltose standard curve was used to determine the amount of reducing sugars released spectrophotometrically, before it is converted to reaction velocities (v). Line- weaver-Burk double reciprocal plot (1/v versus 1/[S]) was constructed and the kinetics of α-amylase inhibition by the extract was determined (Lineweaver and Burk 1934).[29]

α-glucosidase glucosidase inhibitory potential of EFLC was determine by adding 50 mL from the stock solution with 100 mL of 0.1 M phosphate buffer (pH=6.9) containing 1.0 m of α-glucosidase solution. The mixtures were then incubated in 96-well plates at 25 °C for 10min. Following this, 50 mL of 5mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 phosphate buffer (pH=6.9) was added to each well at timed intervals. The reaction mixtures were

incubated (25 °C, 5 min) before the absorbance was taken using microplate reader (405 nm). The absorbances were compared with a control which contained 50 mL of the buffer. Acarbose was prepared in distilled water at same concentrations as the fraction and used as control.[28,30] For the enzyme kinetics of α-glucosidase activity by EFLC, 50 µL of 5mg/mL EFLC was initially incubated with100 µL of α-glucosidase solution (10min, 25 °C) in one group of tubes. In another separate group of tubes, α-glucosidase was pre-incubated with 50 µL of phosphate buffer (pH=6.9). 50 mL of pNPG at varying concentrations (0.016-0.2 mg/mL) was added to both groups of reaction mixtures to start the reaction. The mixture was then incubated (10 min, 25 °C) and 500 mL of Na_2CO_3 was added to halt the reaction. P-nitrophenol standard curve was used to evaluate the amount of reducing sugar released. Reaction rates (v) were then calculated and double reciprocal plots (1/v versus 1/[S]) of enzyme kinetics were constructed to study the nature of inhibition (Lineweaver and Burk, 1934).[31] Km and Vmax values were also calculated from the curve.

The experiments were conducted in triplicate and the α-amylase inhibitory activity was expressed as % inhibition. Using the expression:

% inhibition= [(ΔAcontrol - ΔAfraction)/ ΔAcontrol] ×100, where Δ Acontrol and ΔAfraction are the changes in absorbances of the control and fraction respectively. Using standard calibration curve, the concentration of EFLC causing 50% inhibition (IC_{50}) of the activity was estimated.

Antioxidant potentials were presented as percentage (%). The remaining data were presented as mean ± standard deviation (SD) of replicate experiments. The data were analyzed using analysis of variance (one- way) complemented with Duncan multiple range test (Statistical Analysis System (SAS) Model 9.13) to detect significant differences between mean values. Differences were considered significant at $P<0.05$ confidence interval value.

RESULTS

The preliminary phytochemical screening of EFLC revealed the presence of Alkaloid, phenol, saponin, tannin, cardiac glycosides, steroids, phytosterol and flavonoids (Table 1).

Table 1: Phytochemical constituents of EFLC

Phytochemicals	Remark
Alkaloid	Detected
Flavanoid	Detected
Cardiac glycoside	Detected
Phenol	Detected
Anthraquinone	Not detected
Tannin	Not Detected
Saponin	Detected
Triterpene	Not Detected
Phytosterol	Detected
Steroids	Detected

The total phenol, flavonoid and flavonol were 62.41 ± 5.01 %w/w, 47.68 ± 3.97

%w/w and 20.49 ± 1.62 %w/w respectively (Table 2).

Table 2: Total phenolic, flavonoid, and flavonol contents EFLC

Phytochemicals	EFLC
Phenol (%w/w)	62.41 ± 5.01
Flavonoid (%w/w)	47.68 ± 3.97
Flavonol (%w/w)	20.49 ± 1.62

Values were expressed in % w/w of plant extracts and are means of triplicate determinations ± Standard deviation.

The *in vitro* activity of the ethyl acetate fraction is presented in Figure 1-4. The fraction exerts its antioxidant activities in dose dependent manner. For the $ABTS^{+}$, the fraction demonstrated a very strong scavenging ability against 3-ethylbenzothiazoline-6-sulphonic acid cation chromophore when compared with garlic acid judging from its IC_{50} value (0.75 mg/mL) which is not significantly different from that of the standard. However, DPPH scavenging ability of the fraction is significantly potential when compared with garlic acid (Table 3).

Gallic acid showed significant effect on metal chelating and hydroxyl radical scavenging, though that of the fraction was also commendable judging from its IC_{50} values (Table 3).

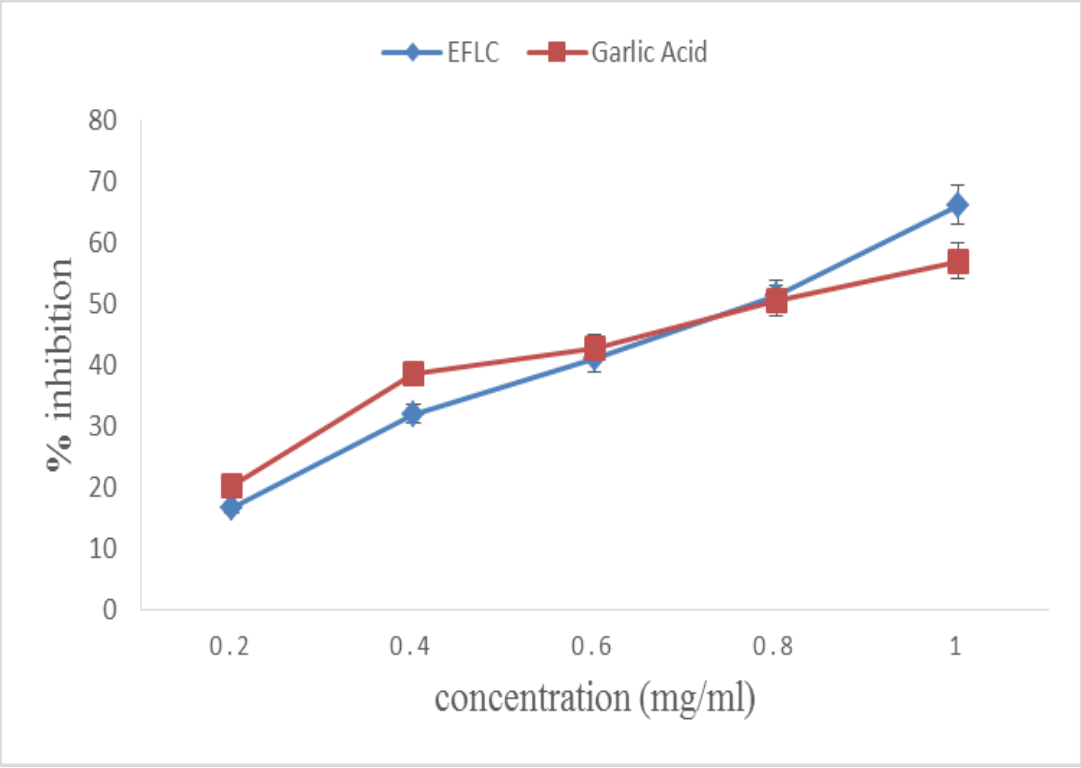

Figure 1: ABTS scavenging effect of EFLC

Values are mean ± standard deviation (SD) of triplicate determinations.

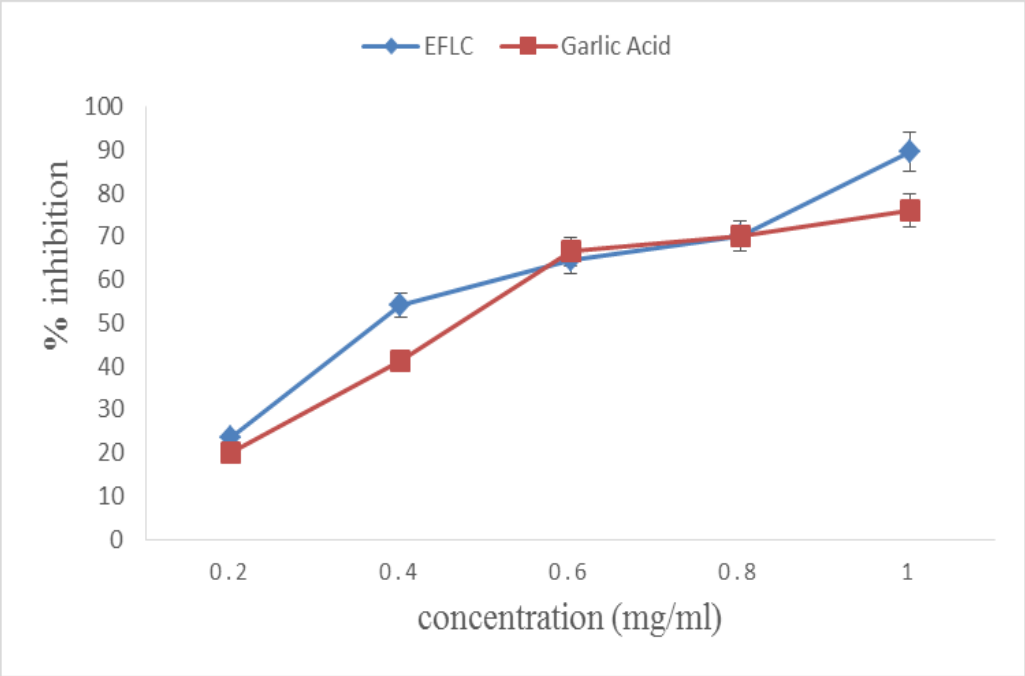

Figure 2: DPPH scavenging effect of EFLC

Values are mean± standard deviation (SD) of triplicate determinations.

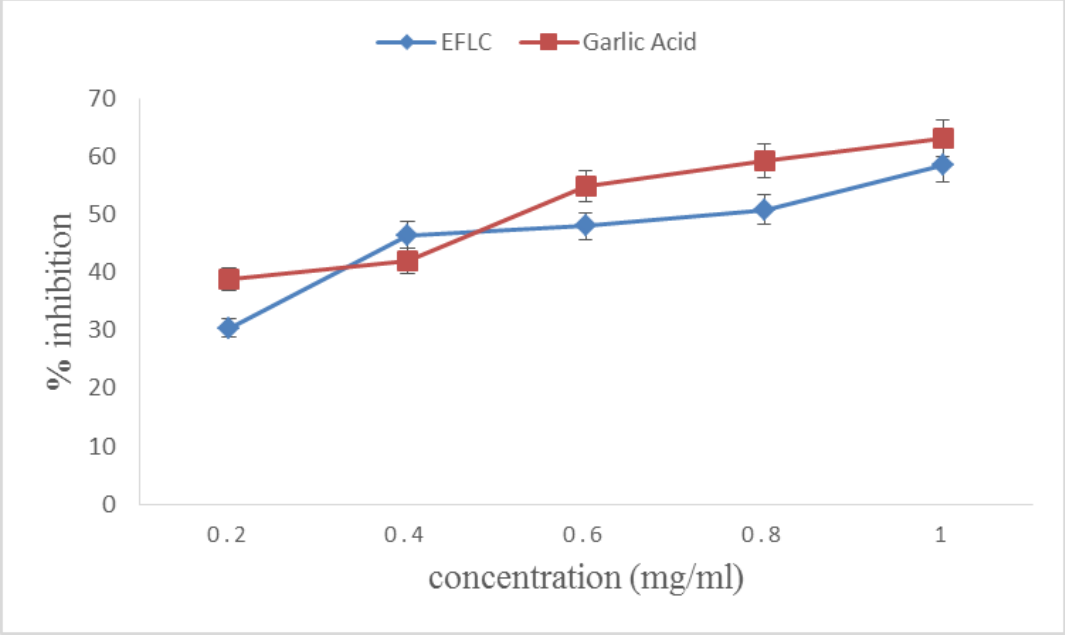

Figure 3: Hydroxyl radical scavenging potential EFLC

Values are mean ± standard deviation (SD) of triplicate determinations.

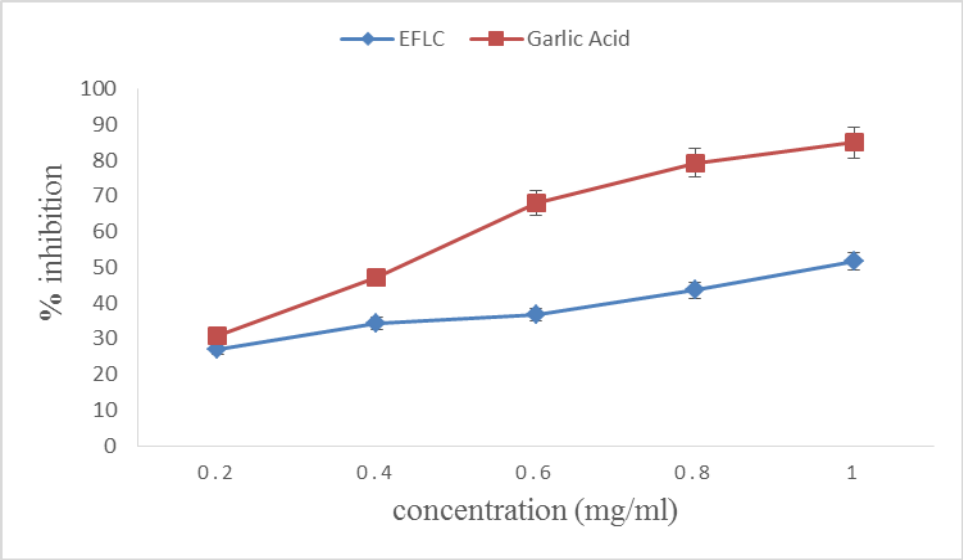

Figure 4: Iron chelation potential of EFLC

Values are mean± standard deviation (SD) of triplicate determinations.

Table 3: IC_{50} (mg/mL) with R^2 and Regression equation of ABTS, DPPH, Hydroxyl radical scavenging and metal chelating abilities of EFLC

		EFLC	Garlic Acid
	IC_{50}	0.75[a]	0.79[a]
ABTS	R^2	0.9990	0.9347
	Regression equation	Y=59.2650x + 5.8390	Y=42.6850x + 16.1730
	IC_{50}	0.46[a]	0.53[b]
DPPH	R^2	0.9272	0.8964
	Regression equation	Y=74.2350x + 15.8230	Y=70.6000x + 12.3700
	IC_{50}	0.70[a]	0.55[b]
Hydroxyl Radical Scavenging	R^2	0.8656	0.9515
	Regression equation	Y=30.2450x + 28.7190	Y=33.6850x + 31.8150
	IC_{50}	0.97[a]	0.42[b]
Metal chelating	R^2	0.9878	0.9562
	Regression equation	29.2800x + 21.6660	69.8550x + 19.9880

The values are expressed as mean of triplicate determinations; Means along the rows not sharing a common superscript are significantly different (P<0.05) from each other; Gallic acid is the standard antioxidant agent.

Figure 5 and 6 shows the inhibitory effects of EFLC on α-amylase and glucosidase. The inhibition of α-amylase and glucosidase was dose dependent, the most potent activity was observed with the fraction at the highest concentration, while the lowest concentration of 0.20 mg/mL revealed minimum inhibitions. In the α-amylase assay, the fraction compete favorably with acarbose, the significant effect was demonstrated by the acarbose at the lower concentrations (0.2, 0.4 mg/mL) and at the highest (1.0 mg/mL) in the percentage inhibition of the enzyme. The IC_{50} values of the fraction and acarbose were also significantly different at ($P<0.05$) (Table 4).

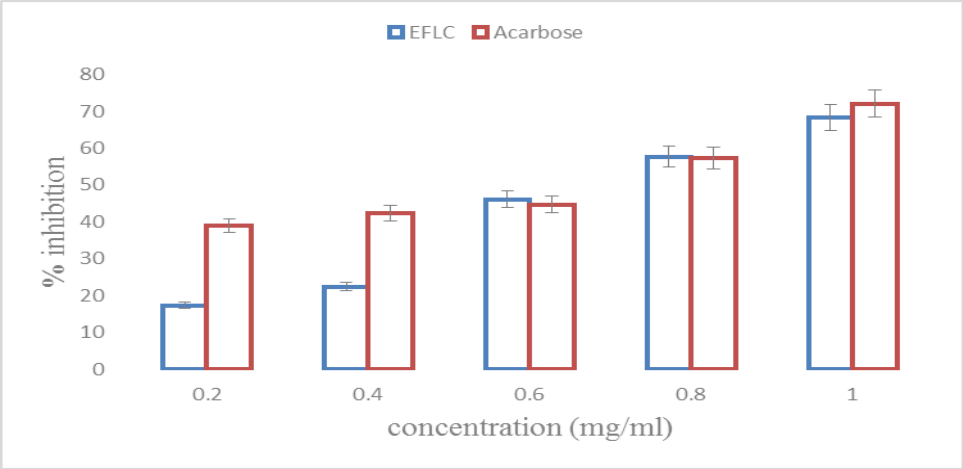

Figure 5: Inhibitory potential of EFLC on α-amylase activity

Values are mean ± standard deviation (SD) of triplicate determinations

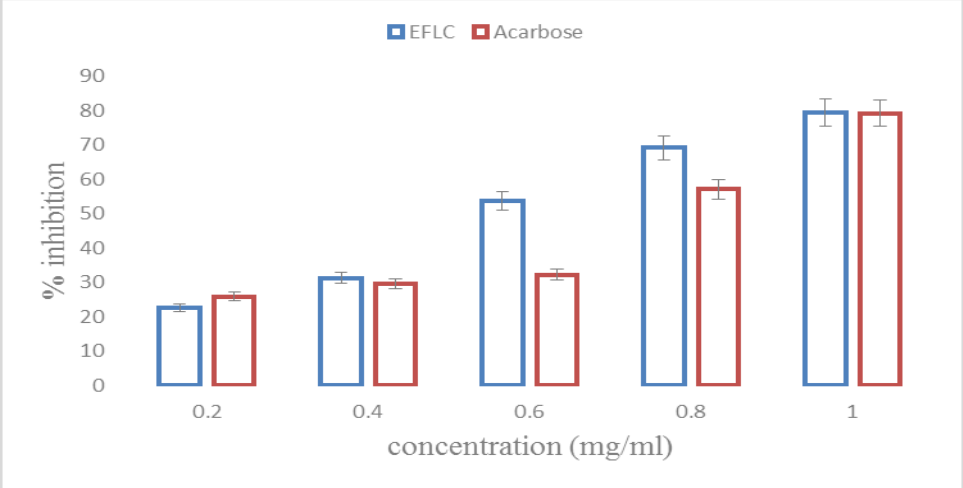

Figure 6: Inhibitory potential of EFLC on α-glucosidase activity

Values are mean ± standard deviation (SD) of triplicate determinations.

Table 4: IC_{50} (mg/mL) values with R^2 and Regression equation of EFLC on activities of alpha amylase and glucosidase

		EFLC	Acarbose
	IC_{50}	0.73 [a]	0.57 [b]
***α*-amylase**	R^2	0.9644	0.8868
	Regression equation	Y=66.1450x +1.6890	Y=40.5750x+ 26.7550
	IC_{50}	0.58 [a]	0.67 [b]
***α*-glucosidase**	R^2	0.9814	0.8654
	Regression equation	Y=75.7000x + 5.7240	Y=67.1850x +4.4890

The values are expressed as mean of triplicate determinations. Means along theRows not sharing a common superscript are significantly different (P<0.05) from each other.Acarbose is the standard antidiabetic agent.

The Line weaver-Burk double reciprocal plot for the mechanism of inhibition showed that ethyl acetate fraction of LC inhibited α-amylase uncompetitively (Figure 7). The Vmax and Km values for the fraction are 0.03 µM/min and 44.56 mg while that of control is 0.08 µM/min and 18.66, respectively.

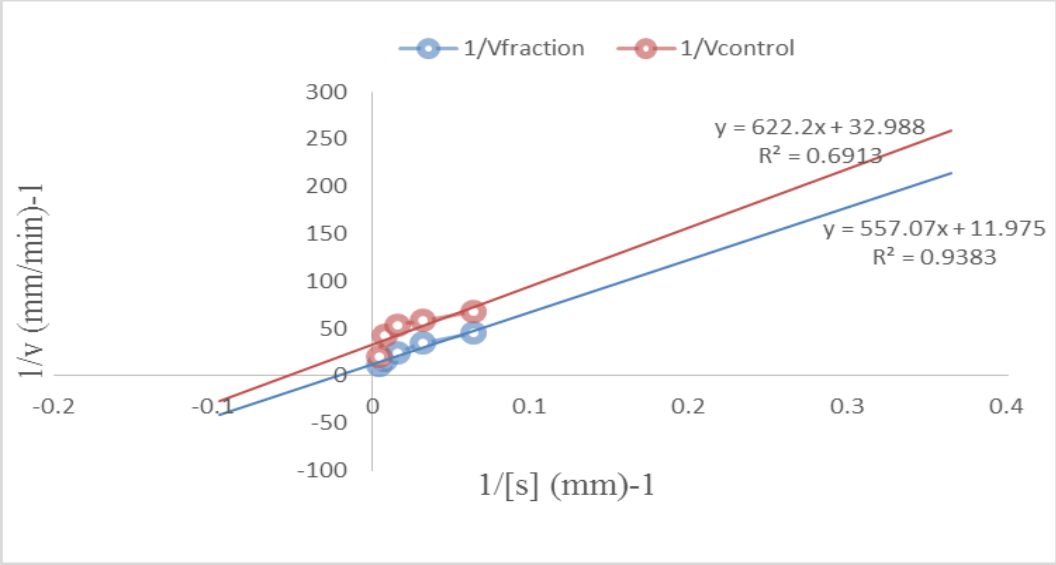

Figure 7: Lineweaver-Burk plot of EFLC eliciting uncompetitive inhibition on α-amylase activity

Results represent mean ± standard deviation; (n=3); (P>0.05).

For α-glucosidase, the fraction exerts a significant dominating inhibitory activity on the enzyme at higher concentrations (0.6-0.8 mg/mL) in the percentage inhibition (Figure 6). Based on the IC_{50} value, EFLC exhibited a significant inhibitory effect on the enzyme when compared with acarbose (Table 4). Further probing into mechanism of inhibition showed that the fraction inhibited α-glucosidase non-competitively (Figure 8). The Vmax and Km values for the fraction are 0.12 µM/min and 31.50 mg while that of control is 0.04 µM/min and 34.86 respectively.

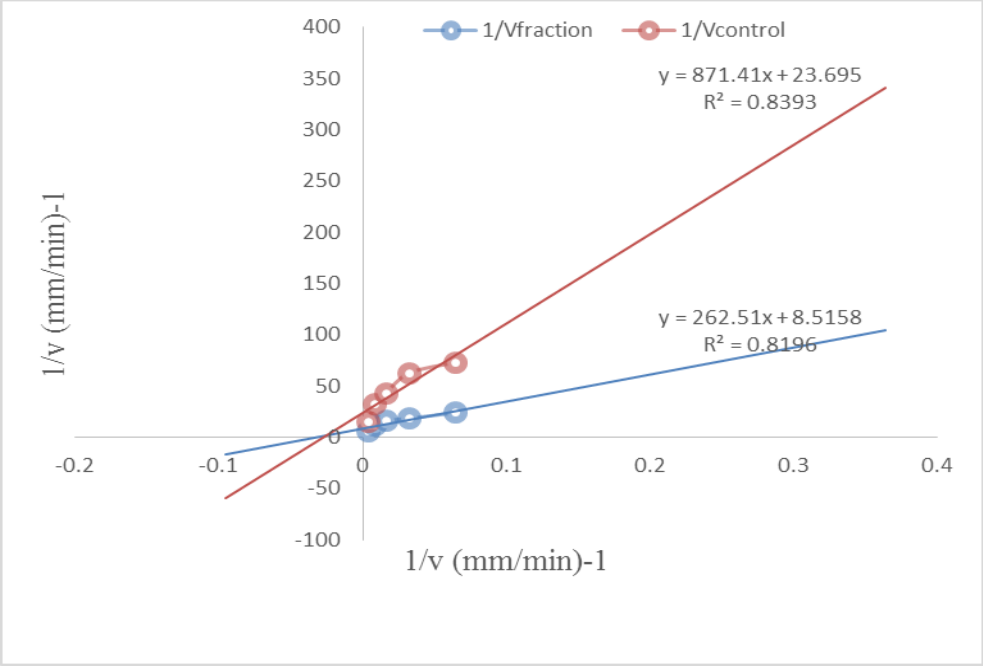

Figure 8: Lineweaver-Burk plot of EFLC eliciting non-competitive inhibition on α-glucosidase activity

Results represent mean ± standard deviation; (n=3); (P>0.05).

DISCUSSION

DM is a metabolic disorder characteristic of carbohydrate metabolism, manifested by hyperglycemia or increased blood glucose level resulting from insulin deficiency.[32] Recently, herbal plants have been employed in the management of DM and are known as important source of novel antidiabetic agents.[33] Antidiabetic herbal plants elicit their activities by inhibiting carbohydrate-hydrolyzing inhibotors (α-amylase and glucosidase). The α-glucosidase inhibitors delay the uptake of absorbed carbohydrates that help to reduce PP sugar and insulin peaks.[34]

In the present study, EFLC displayed a very significant potent and mild inhibitory effect on α-glucosidase and α-amylase respectively judging from its IC_{50} when

compared with the standard. The mild inhibitory activity of the fraction is desirable of good antidiabetic agent. Side effects such as abdominal distention, hypoglycemia and abnormal bacterial fermentation of undigested carbohydrates in the colon is always associated with immoderate inhibition of pancreatic α-amylase.[28,35] Lineweaver- Burk plot also showed that EFLC inhibits α-amylase uncompetitively. This implies that the fraction binds exclusively to the enzyme-substrate complex resulting to an inactive enzyme-substrate-inhibitor complex and this will consequently delay the digestion of carbohydrate and causing reduced glucose absorption rate.[28,36,37]

As for α-glucosidase, the EFLC demonstrates a significant potent inhibition towards the activity of the enzyme when compared with acarbose. Strong inhibition of this enzyme has been known to be a very proactive and effective therapy for control of PP hyperglycemia which is exhibited by this plant. This is due to the fact that α-glucosidase inhibitors have ability to retard sugar absorption speed and the PP blood glucose level. Therefore, its inhibition is an important approach for the regulation of PP hyperglycemia.[38] The non-competitive mechanism of inhibition displayed by EFLC on a-glucosidase implies that components in the fraction do not compete with the substrate for binding to the active site rather the inhibitors bind to a separate site on the enzyme to retard the conversion of disaccharides to monosaccharides.[39,40]

Induction of oxidative stress is a main process in the commencement of diabetic complications and various antioxidants have been developed recently to manage oxidative stress in diabetic patients.[11] In this study, the EFLC displayed a good radical scavenging in all the antioxidant assays studied confirming EFLC as a potential source of antioxidants. Therefore, the antidiabetic potency exhibited by the EFLC may be related to its high antioxidant activity, thereby mopping up free radicals generated by diabetes-induced hyperglycemia.[41] However, it could also play an important role in the prevention of physiological stress generated by free radicals when used to treat DM.

Since the bioactivity of plant products mainly depends on the amount of the major active constituents, the α-amylase and glucosidase inhibitory activities exhibited by this plant may be due to the presence of phytochemicals such as alkaloid, flavonoid, cardiac glycoside, saponin, phenol and phytosterol. Moreover, phyto-constituents such as flavonoids and phenolic derivatives are known to boost the insulin secretion and also to preserve β-cell integrity and by mopping up free radicals in the system.[42,43]

CONCLUSIONS

Conclusively, the EFLC possesses α-amylase and glucosidase modulatory activities which are two key enzymes that is linked to carbohydrate metabolism in the body, these attributes may be due to the presence of phytochemicals of pharmacological importance in EFLC. However, this study has once again demonstrated the efficacy of botanicals in the management of DM.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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