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A Diterpene Isolated from *Ficus sur* Forssk (Moraceae) and it's Alpha-Amylase Inhibition Activity

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Abstract

The prevalence of diabetes mellitus is increasing worldwide. The objective is to investigate and characterize the alpha-amylase inhibition principles of the leaves of *Ficus sur* Forssk (Moraceae) used in ethnomedicine for management of diabetes mellitus. Powdered leaves were successively macerated with N-hexane, chloroform and 70% ethanol respectively for three consecutive days. The extracts were assessed for phytochemicals and inhibition of alpha-amylase. Alpha-amylase inhibition was assessed using porcine α-amylase. Bioactive n-hexane extract was fractionated on column chromatography packed with Silica Gel G (mesh 60-120) and eluted with gradient mixture of hexane, ethyl acetate and ethanol. Active fractions (F1 - F5) were purified on preparative thin layer chromatography. Active pure compounds were assessed for bioactivity and identified by spectroscopy (UV, IR, MS and NMR). The n-hexane extracts of the plant exhibited highest significant ($p < 0.05$) inhibition. Fraction F4 was the most active and was and compound FB was characterized from it as a novel abietane-type diterpene (4,7,10-b-trimethyl-benzofuro[c]-6,6-a,8,9,10,10-a-hexahydroisochromene-7-carboxylic acid) with 55% inhibition of alpha-amylase at 50µg/ml. This work reports for the first time a novel diterpenoid from *Ficus sur* with alpha-amylase inhibition activity.

Keywords: Ficus sur, Moraceae, anti-hyperglycaemia, diterpenoids.

INTRODUCTION

Diabetes treatment has been faced with imminent discoveries in the plant kingdom. Some of the plant metabolites have a known mechanism of action while others are yet to be known. The understanding of the mechanism of action of diabetic drugs usually start from the type of the diabetes. Type II diabetes has been the most common type of diabetes due to its prevalence in adult across the globe with serious concern on its economic burden and life shortening. One of the treatment approaches is the inhibition of the major enzymes (alpha-amylase and alpha-glucosidase) involved in the digestion of carbohydrates in the body system. Alpha-amylase catalyzes the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds (Sales *et al.*, 2012). Neither terminal glucose residues nor α-1,6-linkages can be cleaved y α-amylase. The end products of the α-amylase reaction are oligosaccharides with a varying length and with an α-configuration and α-limit dextrins (van der Maarel *et al.*, 2002), that constitute a mixture of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units which contain both α-1,4 and α-1,6 linkages (Whitecomb and Lowe, 2007). Other amylolytic enzymes participate in the process of starch breakdown, but the contribution of α-amylase is a prerequisite for the initiation of this process (Tangphatsornruang *et al.*, 2005). Alpha-amylase inhibitors, therefore, inhibit or slow down the digestion of starches to oligosaccharides causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise. This has become one of the mechanisms of action of some antidiabetic drugs and thereby makes them relevant in the management of type 2 diabetes. A variety of compounds isolated from plants and microorganism had been reported to show α-amylase inhibitory. Sequel to our earlier report on the antidiabetic activity of the extracts and fractions that emanated from the separation of the most active extract of *F. sur* in animal studies (Suleiman and Kio, 2018), we were prompted to understand the mechanism of its action and isolate the active principle that is responsible

for the activity. This study, therefore, investigated the alpha-amylase inhibitory activity of the active extracts and fractions and also isolates the bioactive principle.

MATERIALS AND METHODS

Plant Materials

Ficus sur Forssk (Moraceae) leaves were collected from Ahoada Local Government Area of Rivers State respectively. The leaves were identified and authenticated by Dr A.T. Oladele and Herbarium Specimens with voucher numbers UPF300 was deposited in the Herbarium Section of the Department of Forestry, University of Port Harcourt and in the Herbarium of Department of Pharmacognosy and Phytotherapy, University of Port Harcourt. The leaves were air-dried at room temperature, pulverized and preserved for further use.

Extraction and column chromatography

The powdered leaves of *Ficus sur* (1kg) were successively macerated with 2 Litres each of *N-h*exane, chloroform, and 70% ethanol respectively. The filtrates from each solvent were combined and concentrated using rotary evaporator *en vacuo* at 40^0C. The combined N-hexane, chloroform and 70% ethanol extracts were later assessed for alpha-amylase inhibition as described below. The bioactive N-hexane extract was fractionated on column chromatography packed with silica gelG (60-120) eluting with a gradient mixture of N-hexane, dichloromethane, and ethanol. Fractions were monitored on analytical thin-layer chromatography (TLC) as earlier described by Suleiman and Kio (2018) and pooled to Fractions F1 to F5 were and then assessed for alpha-amylase inhibition.

Alpha-amylase inhibition assay

The alpha-amylase inhibition assay was performed as previously described by Suleiman *et al.*, (2019). A 0.5% w/v Porcine α-amylase (EC 3.2.1.1, type VI, Sigma) was prepared in Ice cold distilled water. Potato starch (0.5%w /v) in 20mM phosphate buffer (pH 6.9) containing 6.7mM Sodium Chloride was used as a substrate solution. Varying concentrations (1µg - 5µg/ml) of column fractions F1 to F5, (10µg/ml - 50µg/ml) of FB and 5mg/ml of Acarbose (1ml) were mixed with 1ml of potato starch. This was followed by adding 1ml of the alpha-amylase solution and allowed to incubate for 5 minutes at 25^0C. This reaction was stopped by adding 1ml DNS reagent and boiled at 90^0C in a water bath for 15 minutes. The cooled reaction mixture was diluted with 1ml of deionized water and the absorbance of the test, acarbose, blank (without α-amylase), control (without extract) mixtures were immediately measured at 540nm using UV-spectrophotometer. Percentage inhibition was calculated using the formula:

Percentage inhibition = Mean Absorbance (control) – Mean Absorbance (test sample) ×100%/Absorbance (control)

Isolation, Purification and Characterization of Active Compound from *Ficus sur*

Active column Fraction F4 was further separated on preparative TLC on silica gel GF_{254} (0.5mm thickness) developing in hexane: ethyl acetate (3:7) which separates as two red and blue UV fluorescent bands. Each band was then scrapped off from the plates, extracted with dichloromethane and filtered. The filtrate was allowed to evaporate and re-dissolved in dichloromethane and then spotted on the TLC plate to ascertain its purity. They were assayed for inhibition. The active blue band was labelled FB. It has an R_f of 8.53 (Silica Gel GF_{254} developed in a mobile phase of hexane: ethyl acetate (3:7)). It was an amorphous compound (9mg). It exhibited a molecular mass of 316 with a molecular formula of $C_{19}H_{24}O_4$. Details of its spectra data are shown in Table 1.

RESULTS AND DISCUSSION

One of the common drugs in the market that inhibits the breakdown of glucose in the body is acarbose. It inhibits glycoside hydrolases (enzymes) that are needed to digest carbohydrates, precisely, alpha-glucosidase enzymes in the brush border of the small intestines, and pancreatic alpha-amylase. Unfortunately, its side effects which include flatulence, diarrhoea and in some cases hepatitis also demand a new drug candidate with potent and undesired side effect. In Figure 1 only fraction F4 exhibited a dose-dependent inhibition of alpha-amylase activity with 75.43% inhibition at 4ug/ml. however, this correlates with the activity reported in antidiabetic studies by Suleiman and Kio (2018) which also suggests that inhibition of alpha amylase is its mechanism of action. Further isolation and purification of fraction F4 yielded compound FB which also showed a dose-dependent inhibition of alpha-amylase activity with highest inhibition of 55% at 50µg/ml in Figure 2. The percentage inhibition at 50µg/ml was comparable to the standard drug that exhibited 61% inhibition though with apparent difference in their IC_{50} values in Figure 3. This, however, revealed that compound FB can delay the breakdown of carbohydrates by inhibiting the activity of alpha-amylase, hence could be a good candidate in the management of diabetes mellitus.

Compound FB has an R_f value of 0.85 on TLC and fluoresces blue colour under a UV lamp at 365nm. It was an amorphous compound. In its UV spectrum, an intense absorption at (λ_{max} 230nm, ε_{max} 528.44) and a moderate absorption at (λ_{max} 224nm, ε_{max} 347.71) were observed.

The structure of compound FB was further elucidated using FTIR, NMR (1-D and 2-D) and Mass spectroscopy. Confirmatory evidence are in the ^{1}H-NMR spectrum showing signals of de-shielded protons with chemical shifts at δ_H: 3.5, 4.2, 5.2, 7.5 and 7.7 ppm corresponding to H-4-a of the secondary carbinol, H-6 of the primary carbinol, and the three olefinic (sp^2)

protons H-11, H-3 and H-2 respectively. Also evident are three methyl protons singlets at δ_H: 0.75, 0.9 and 1.3 ppm for H-7-Me, H-10-b-Me and H-4-Me respectively. Their unambiguous assignment was rationalized based on 2-Dimensional one bond proton to carbon correlation (HSQC). Other proton chemical shift signals were also similarly rationalized as in Table 1.

The ^{13}C-NMR spectrum of compound FB showed a total of nineteen carbon signals indicating a diterpenoid which were rationalized based on 2-Dimensional one bond proton to carbon correlation (HSQC) as three methyl groups (–CH_3) at δ_C ppm: 11.0 (C-7-Me), 14.0 (C-10-Me), and 29 (C-4-Me); four methylene (CH_2) groups at δ_C ppm: δ24.0 (C-8), 34.0 (C-9), 42.0 (C-10) and 69.0 (C-6), due to the primary carbinol or oxymethylene ((-CH_2O-) at ring B); six methine groups (-CH) that resonated at δ_C: 39.0 (C-10-a), 58.0 (C-6-a), 69.0 (C-4-a due to the secondary carbinol or oxymethine (>CHO-) of ring B), in addition to the olefinic methine (=CH-):122.0 (C-11), 129.0 (C-3) and 131.0 (C-2); and the six quartenary carbon (>C<) signals at δ_C ppm 30.0 (C-10-b), 43.1 (C-7), 139.0 (C-4), 140.0 (C-11-a), 149.0 (C-3-a) and 168.0 (C-7-COOH) as presented in Table 1.

The H-H COSY spectrum of the compound FB showed the coupling correlation between the protons: H-6 (δ_H4.2 ppm) and H-6-a (δH1.3 ppm), and also H-3 (δ_H 7.5 ppm) and H-2 (δ_H 7.7 ppm) of the furanoid ring D. Further confirmatory evidence of this unambiguous assignment is supported by the HMBC experiment which confirmed that: H-6 (δ_H 4.2 ppm) showed a $^4J_{HC}$ correlation with carbon signals at position: C-10-b-Me (δ_C 14 ppm), C-10-b (δ_C 30 ppm), C-8 (δ_C 24 ppm), in addition to the $^3J_{HC}$ correlation with carbon signals at position C-10-a (δ_C 39 ppm). Similarly, other correlations were evident in the HMBC as rationalized in Table 1. The carbon, hydrogen and oxygen number from the one and two dimensional NMR experiments were therefore rationalized with the mass spectrum obtained for Compound FB which showed a peak at m/z 317 correspondings to M+1equivalent to a molecular mass of 316 ($C_{19}H_{24}O_4$) that is supporting the information from the NMR. Compound FB though found to be a novel compound with alpha-amylase inhibition activity has the same structural skeleton with abietane-type diterpenoids reported by Landucci and Zinkel (1991) and a generic name: 4,7,10-b-trimethyl-benzofuro[c]-6,6-a,8,9,10,10-a-hexahydroisochromene-7-carboxylic acid.

Figure 1. Alpha-amylase inhibition assay of extract and column fractions obtained from *N*-Hexane extract of *F. sur*

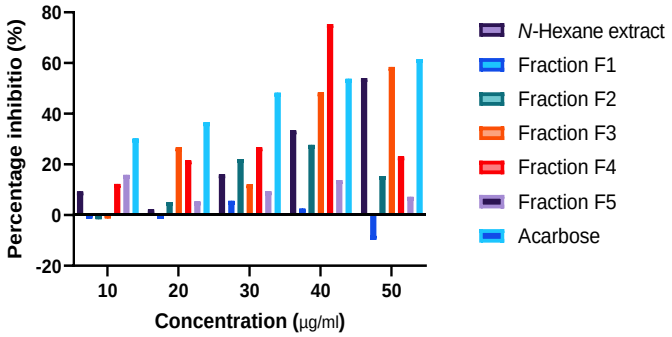


Figure 2. Alpha-amylase inhibition assay of Compound FB obtained from column Fraction F4 obtained from *F. sur*

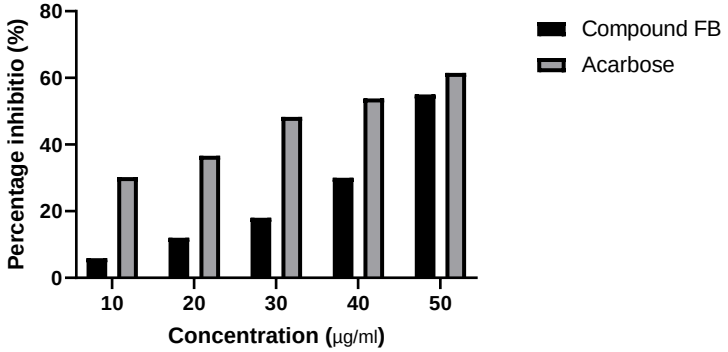


Figure 3. Median inhibitory concentration of Compound FB and Acarbose

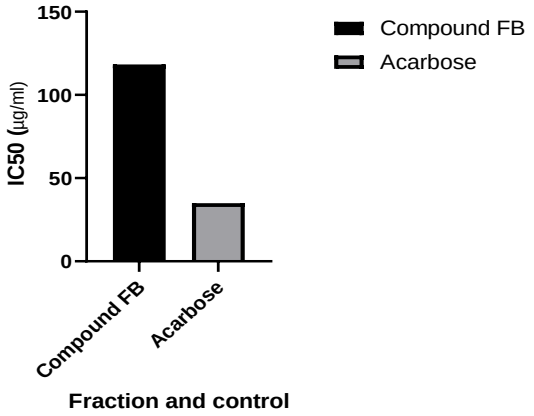
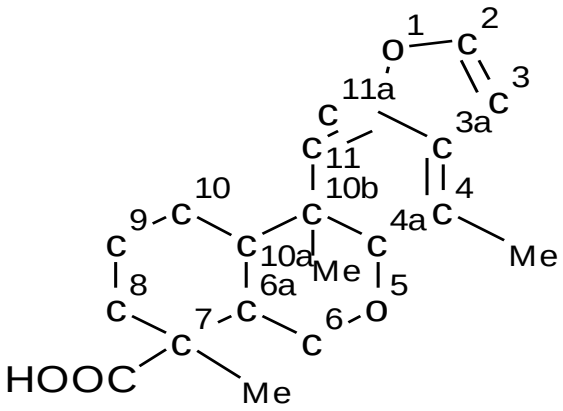


Table 1: Rationalized ^{1}H and $^{13}CNMR$ ($CDCl_3$) spectra data of Compound FB

Assigned position/identity of an atom	δC (ppm)	δHSQC (ppm)	H-H COSY (ppm)	HMBC (ppm) $^{2}J_{HC}$, $^{3}J_{HC}$, $^{4}J_{HC}$
1-O	-	-	-	-
C2	131	7.5	7.7(H_2)	$C_3(^{2}J_{HC})$
C3	129	7.7	7.5(H_3)	
C3a	149	-		
C4	139	-		
C4a	72.9	4.2	1.3(H_5)	
C4-Me	29	3.5		$C_{10b}(^{4}J_{HC})$, $C_{3a}(^{3}J_{HC})$, $C_4(^{2}J_{HC})$
5-O	-	-	-	-
C6	69	1.6		$C_{10b\text{-}Me}(^{4}J_{HC})$, $C_{10\text{-}a}(^{3}J_{HC})$, $C_{10b}(^{4}J_{HC})$, $C_8(^{4}J_{HC})$
C6-a	58	1.3	4.2(H_6)	
C7	43.1	-		
C7-Me	11	0.75		$C_7(^{2}J_{HC})$
C7-COOH	168	-		
C8	24	1.5		
C9	34	1.3		
C10	42	2.4		
C10-a	39	1.6		
C10b	30	-		
C10b-Me	14	0.9		$C_{11a}(^{4}J_{HC})$, $C_{6\text{-}a}(^{4}J_{HC})$, $C_{10b}(^{2}J_{HC})$, $C_{4a}(^{3}J_{HC})$
C11	122	5.2		
C11a	140	-		



Compound FB

CONCLUSION

This study reveals that fraction F4 was the most active fraction with high percentage inhibition of alpha amylase and a novel isolated compound named 4,7,10-b-trimethyl-benzofuro[c]-6,6-a,8,9,10,10-a-hexahydroisochromene-7-carboxylic acid from fraction F4 also inhibits alpha amylase. This suggests that compound FB could be a good lead in the development of anti-hyperglycemic drug.

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