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Research Article

In vivo and *in vitro* Anti-diabetic Activity of Extracts of *Anthoclestia Vogelii* Planch and Isolation of Decussatin, a New α-amylase Inhibitor from its Stem Bark and Leaves

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ABSTRACT

The aqueous methanol crude extracts of *Anthocleista vogelii*, Planch were investigated for their anti-diabetic activities in normoglycaemic and alloxan-induced diabetic rats. Bioassay guided fractionation in vitro was used to investigate the α-amylase inhibitory activities. The leaves and stem bark crude extracts at 1 g/kg body weight (BW) gave 60.35 % and 69.68 % ($p < 0.05$) peak reduction in blood glucose levels on day-7 and day-5 respectively, which was higher than the 58.43 % for the reference group, glibenclamide given on day-7. Decussatin, a xanthone at 1 mg/mL with a 78.0 % ($p < 0.001$) α-amylase inhibition was isolated with comparable anti-diabetic activity to acarbose, a known synthetic α-glucosidase drug, which gave a 54.9 % inhibition at the same concentration. Spectral characterization of the isolated compound using IR, GC-MS, and NMR established the structure of the compound to be decussatin. In addition, computational calculations further confirmed the structure as decussatin with spectroscopic data that matched the experiment. The anti-diabetic activity as revealed by the results further validates, scientifically, the traditional use of the plant in managing diabetes. As such, we provide evidence of a probable mode of operation by α-amylase inhibition due to the presence of decussatin isolated from the leaves and stem bark.

Keywords: *Anthocleista vogelii*, Hyperglycaemia, Anti-diabetic, Alpha-amylase, Decussatin.

INTRODUCTION

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (ADA, 2010). The increase in the number of cases of diabetes mellitus is making the disease to become a public health burden worldwide with the number expected to rise to another 200 million by 2040 (Zheng *et al.*, 2018). Chronic hyperglycemia in cooperation with other metabolic anomalies in patients with diabetes mellitus can cause damage to various organ systems, leading to the development of serious health complications, chief among which are microvascular (neuropathy, retinopathy and nephropathy) and macrovascular complications resulting in a 2-4-fold increased risk of cardiovascular diseases (Zheng *et al.*, 2018).

Type 2 diabetes (formerly called non-insulin-dependent or adult-onset) is the commonest form of diabetes mellitus characterized by hyperglycemia, insulin resistance, and comparative insulin deficiency (Maitra and Abbas, 2005). This type of diabetes also results from interaction between hereditary, environmental and behavioral risk factors (Chen *et al.*, 2011).

A report in 2017 showed that about 425 million people suffer from diabetes all over the world and 3.1% of this number, representing about 15.9 million people, are domiciled in Africa (IDF, 2017). The aim of therapy in diabetes management is to maintain blood glucose at normal levels. One of the ways this can be achieved is by drug therapy focusing glucose-lowering agents such as *α*-glucosidase and *α*-amylase to decrease the reabsorption of glucose in the intestine as well as medications to treat or prevent the secondary complications of the disease (Muhammed and Lakshmi, 2005; Sim *et al.,* 2010). An effective approach for the management of type 2 diabetes is strong inhibition of intestinal α-glucosidases and mild inhibition of pancreatic α-amylase (Kwon *et al.,* 2007).

Postprandial hyperglycaemia plays an important role in the development of type 2 diabetes and has been proposed as an independent risk factor for cardiovascular disease (Li *et al.,* 2005). However, the existing α-amylase and α-glucosidase inhibitors in clinical use have attendant side effects such as diarrhea, hypoglycemia, bowel distending and flatulence which limit their use in the management of diabetes and its complications (Evans and Rushakoff, 2007).

In dealing with diabetic complications which includes heart failure, cardiovascular diseases, adverse drug reactions, infections, and early mortality amongst others (Thomas *et al.,* 2015), there is the need to search for complementary and alternative therapies with minimal side effects in the management of diabetes mellitus. Traditionally, in different parts of Africa, *Anthocleista vogelii* (*A. vogelii*) Planch is used in the treatment of diabetes, cardiovascular diseases, abdominal pains, jaundice, malaria, alleviation of fever, and as an antipyretic, haemostatic, a purgative and a snake bite anti-venom (Ampofo, 1977; Burkill, 1995; Madingou *et al.,* 2012). In Ghana a root decoction of *A. vogelii* and *Combretum mucronatum* with pepper and ashes is taken to treat chest pain (Jegede *et al.,* 2011). Research works carried out on the antidiabetic effect of *A. vogelii* aqueous root extract and the ethanolic extract revealed that the extracts exerted significant decrease in fasting blood glucose level (FBGL) in diabetes induced albino rats using alloxan and streptozotocin (Sunday *et al.,* 2015; Sunday *et al.,* 2016). We therefore set out to investigate the plant scientifically for its anti-diabetic activities and probable mechanism of action.

MATERIALS AND METHOD

Equipment

A rotary evaporator Buchi® was used to concentrate the extracts in vacuo at 37 ℃, freeze drying of the extracts was on a Labconco freeze zone 6 Freeze drier (Labconco, USA). Absorbance was measured on a UV-VIS 916 Spectrophotometer. NMR spectra, 1D and 2D, 1H and 13C were acquired in CDCL3/TMS on Bruker Avance (600 MHz). IR spectra of the crystalline compound was examined neat and acquired on a Perkin Elmer 400 FT-IR spectrophotometer, GC-MS analysis of the isolated compound was performed on an Agilent 6890N GC, 5973 MS detector at 70 eV, the column was HP 5 medium polarity, 30 m in length with an internal diameter of 250 microns and film thickness of 0.25 microns. The simulated spectroscopic parameters were obtained with Gaussian-09 software package.

Plant collection

The leaves and stem bark of *A. vogelii* were collected during the rainy season at J4 (a farm settlement) along Ijebu Ode - Benin road, Nigeria and were authenticated at the Forest Herbarium Ibadan (FHI) of the Forestry Research Institute of Nigeria (FRIN), where a voucher specimen with number FHI 10906 was deposited. The plants specimens were washed, chopped, oven-dried at 40 ^{o}C, powdered using a Hammer mill (with a 5-KVA motor) and kept in amber-coloured bottles until ready for extraction.

Extraction

2.4 kg and 1.2 kg of the leaves and stem bark were each macerated for 72 h using 80% aqueous methanol. The solvent was decanted every 24 h, filtered and replaced with fresh 80% aqueous methanol for the duration of the extraction period (Handa *et al.,* 2008). The filtrate collected each day was pooled and concentrated in vacuo at 37 ^{o}C using a Buchi® rotary evaporator and then freeze dried on a Labconco freeze zone 6 Freeze drier (Labconco, USA). The resulting crude aqueous methanol extracts (leaf 60 g and stem bark 65 g) were further partitioned into hexane and ethyl acetate (EtOAc) and stored in a refrigerator until when required for the bioassays.

Animal Studies

Adult albino Wistar rats of both sexes between 150 - 200 g were obtained from the animal house, University of Ibadan, Nigeria housed in metal cages in a well-ventilated room and were cared for under the ethical guidelines and care of laboratory animals of the University of Ibadan. The animals were fed on standard pelletized feed (Ladokun feeds) and allowed water ad libitum. They were divided into five (5) groups A, B, C, D, E, of five (5) animals each. Rats in groups B - E were fasted overnight, given a single intraperitoneal injection of 80 mg/kg of alloxan monohydrate (Abdel-Barry *et al.,* 1997) in isotonic saline and allowed to rest for three days to stabilize their blood glucose levels. The rats in group A were not induced and were allowed food and water *ad libitum*. Rats in group B were untreated while those in groups C, D, and E were

treated with 1 g/kg BW of the crude aqueous methanol extracts of the leaves and stem bark of *A. vogelii*, and 250 mg/mL of glibenclamide-the standard reference drug. The animals were observed for 7 days.

Alpha-amylase Inhibitory Assay

The α-amylase inhibitory activity was determined by slightly modifying the dinitrosalicylic acid method described by Benfield (1951) and Miller (1959). The total assay mixture containing 200 µL of 0.02 M sodium phosphate buffer, 200 µL of enzyme (α-amylase E.C. 3.2.1.1 Sigma-Aldrich), and the plant extracts (crude and fractions) in the concentration range 0.1 - 1.0 mg/mL were incubated for 20 min at 37 ºC, followed by addition of 200 µL of 1 % starch in all the test tubes. The reaction ended with addition of 400 µL of 3, 5-dinitrosalycylic acid colour reagent, placed in boiling water bath for 5 min, cooled to room temperature and diluted with 5 mL of distilled water. The absorbance was measured at 540 nm, using a UV-VIS 916 Spectrophotometer. The control samples were also prepared accordingly without any plant extracts.

The percentage inhibition was calculated using the formula:

$$\% \, Inhibition = \left[{}^{(A_{control} - A_{test})}/_{A_{control}} \right] \times 100$$

Where, $A_{control}$ = Absorbance of Control, A_{test} = Absorbance of inhibitor (plant extract or acarbose)

Column Chromatography

Column chromatography was performed in a glass column of internal diameter 3.5 cm, column height 28 cm packed with activated silica gel (Kieselgel G60, 70-230 mesh, ASTM) for each of the ethyl acetate fractions of the leaves and stem bark of *A. vogelii*. Ethyl acetate fraction (5 g each) was loaded in the column which was eluted with a gradient solvent system of hexane and ethyl acetate starting with 100 % hexane 200 mL, and ending with 100 % ethyl acetate 200 mL. The column was then washed with 100 % methanol. A total of 18 - 20 fractions were collected for each ethyl acetate fraction, which were then spotted on analytical TLC plates (silica gel 60 F254) using appropriate solvent systems for development.

Spectral Studies

NMR spectra, 1D and 2D, ^{1}H and ^{13}C were acquired in $CDCL_3$/TMS for the crystals isolated from the leaves and stem bark of *A.vogelii*. IR spectra of the crystalline compound was examined neat and acquired on a Perkin Elmer 400 FT-IR spectrophotometer. GC-MS analysis of the isolated compound was performed on an Agilent 6890N GC, 5973 MS detector at 70 eV, the column was HP 5 medium polarity, 30 m in length with internal diameter of 250 microns and film thickness of 0.25 microns. The conditions: initial oven temperature was 40 ºC, Ramp 1, 15 ºC/min to 200 ºC for 5 min, Ramp 2, 20 ºC/min to 24 ºC for 5 min. Injector: 280 ºC Splitless 110.72 kPa. Detector temperatures: 150 ºC Quad, 230 ºC Source. Helium was used as the carrier gas.

Computational Method

To further validate the identification of the compound, we performed computational calculations using DFT method with Gaussian-09 (Frisch *et al.*, 2013). The atomic coordinates of decussatin and tetramethylsilane (TMS) were obtained from the online chemical database PubChem (PubChem, 2019). Initial geometry optimizations were done using unrestricted hybrid density functional at the B3LYP/BS level of theory whereby BS is a triple-zeta split-valence basis set, 6-311++G**. This was followed by vibrational frequency and NMR calculations performed at the same level of theory with B3LYP/BS.

Statistical Analysis

Analysis of variance (ANOVA) was used to evaluate the level of significance between groups at $p < 0.05$ and $p < 0.001$.

RESULTS AND DISCUSSION

Reduction in blood glucose levels

Blood glucose level (mg/dL) reduction by the different crude extracts and the reference drug, glibenclamide are shown in Table 1. Peak reduction in blood glucose levels were observed on day-7 (117 mg/dL) and day-5 (86.2 mg/Dl) for the leaf and stem bark extracts respectively, while glibenclamide gave (122.7 mg/dL) on day-7.

Peak reduction in blood glucose level of 69.68 % was observed on day-5 for the *A. vogelii* stem bark extract while the leaves extract had peak reduction of 60.35 % on day-7 which were higher than the peak reduction of 58.43 % given by glibenclamide on day-7 as shown in Figure 1.

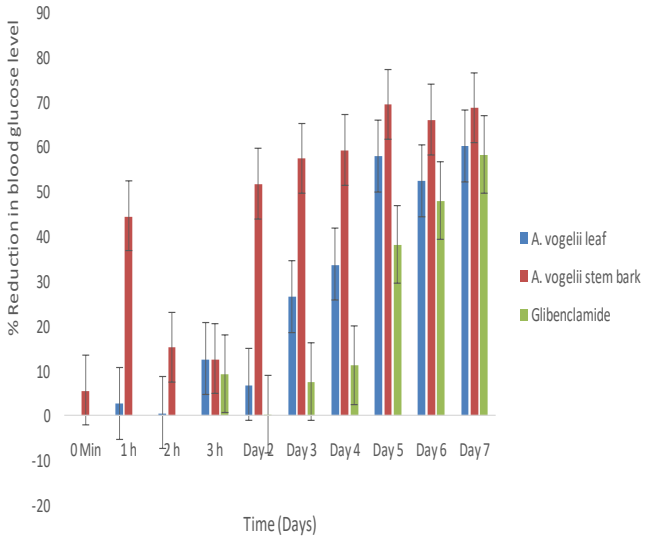

Figure 1. Percentage Reduction in Blood Glucose Levels (mg/dL) of Rats Treated with *A. vogelii* Crude Aqueous Methanol Extracts (1 g/kg BW).

In Figure 2, at 1 mg/mL, the stem bark and leaf crude extracts gave α-amylase inhibitions of 39.14 % and 31.45 % respectively compared to acarbose, the standard reference drug, which gave an inhibition of 54.85 % all significantly different from control at $p < 0.05$. In Figure 3, the ethyl acetate fractions of the stem bark and leaf at 1 mg/mL gave α-amylase inhibitions of 27.27 % and 34.53 % respectively which were significantly different from control at $p < 0.05$, while acarbose gave 54.85 % inhibition. The fraction 5 from both the stem bark and leaves were found to have the similar characteristics and colour, yellowish green needle-like crystals, with similar 1H NMR and 13C NMR profiles.

Table 1. Effect of *A. vogelii* Leaves and Stem Bark Aqueous Methanol Crude Extracts (1 g/kg) on Blood Glucose Level (mg/dL) of Alloxan-induced Diabetic Rats.

Group	Day 1				Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
	0 min	1 h	2 h	3h						
A	41.3 ± 0.67	41.3 ± 0.67	43.7 ± 2.33	40.3 ± 0.33	83.7 ± 11.29	56.7 ± 5.61	71.0 ± 5.51	55.3 ± 4.63	50.7 ± 6.69	67.0 ± 3.60
B	255.4 ± 6.60	288.2 ± 16.52	266.3 ± 11.20	273.9 ± 10.65	285.1 ± 28.68	294.5 ± 35.18	274.0 ± 44.39	284.2 ± 45.61	289.8 ± 48.62	295.1 ± 49.74
C	302.6 ± 5.80	280.0 ± 5.77	264.5 ± 11.06	239.0 ± 17.68	265.3 ± 42.17	216.0 ± 55.24	181.3 ± 44.48	119.0 ± 37.64*	137.3 ± 29.72*	117.0 ± 12.22*
D	240.7 ± 12.08	159.5 ± 5.77*	225.3 ± 5.77*	242.6 ± 8.76	137.1 ± 20.61*	125.1 ± 31.00*	111.2 ± 39.42	86.2 ± 12.48*	98.0 ± 10.44*	91.8 ± 10.98*
E	390.4 ± 2.50	339.5 ± 17.16	380.0 ± 5.55	365.9 ± 5.13	284.0 ± 18.15	272.0 ± 34.31	243.0 ± 24.70	175.3 ± 28.35*	150.3 ± 22.58*	122.7 ± 22.70*

Values were mean ± SEM n= 5, * significantly different from control at $p < 0.05$. A= Non-diabetic rats, B= Diabetic untreated rats, C= Diabetic rats treated with 1 g/kg BW crude aqueous methanol leaf extract, D= Diabetic rats treated with 1 g/kg BW crude aqueous methanol stem bark extract and E= Diabetic rats treated with glibenclamide 2.5 mg/kg BW.

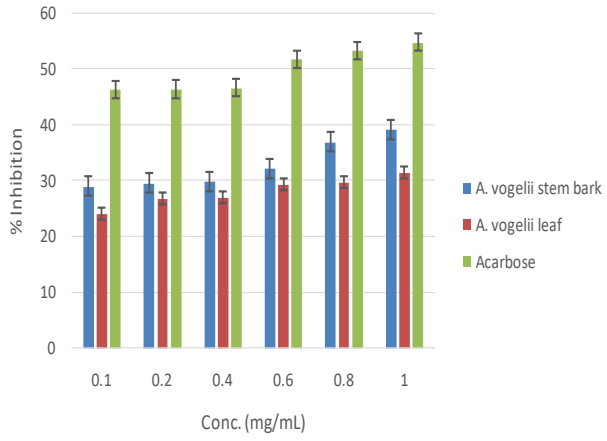

Figure 2. α-amylase Inhibition of *A. vogelii* Crude Aqueous Methanol Extracts and Acarbose.

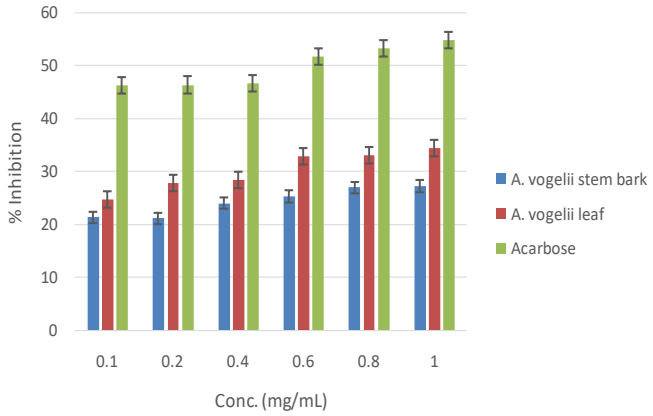

Figure 3. α-amylase Inhibition of *A. vogelii* Ethyl Acetate fractions and Acarbose.

Identification of Crystals from Fraction 5 of *A. vogelii* Leaves and stem bark

The Fraction 5 of *A. vogelii* leaf and stem bark eluted from the column as yellowish green needle-like crystals, and were further purified by re-crystallisation. Its melting point was 152.3 – 153.9 ºC [comparable to that given by Okorie, (1976) as 152 – 154 ºC and Alaribe *et al.* (2012) as 150 – 154 ºC], R_f =0.79 (silica gel, DCM: EtOAc: MeOH 4:5:1). TLC had an intense pink colour under UV 254 nm, faint yellow colour on spraying with methanol solution of DPPH and 78.01 % α-amylase inhibition (p = 0.001) at 1 mg/mL.

The IR *vmax* data showed absorption bands at 3300, 2916, 1603.2, 1655, 1481.65, 826.96 cm^{-1} for major peaks. The peak at 3300 cm^{-1} is consistent with a structure containing a phenolic O-H forming an intramolecular H-bond with the C=O of a xanthone at 1603 cm^{-1}. Also the sharp peak at 1481.65 cm^{-1} is suggestive of aromatic C-H stretching vibrations. These values compared well with those given by Okorie, (1976) and Alaribe *et al.* (2012). The GC-MS spectrum of Fraction 5 (C16H14O6, 302) exhibited ions at m/z 302 [M^+] at run time 32.58 min and fragments at m/z 287 [(M^+ -15)-base peak], 259, 227, 201, 171, 143, 122, 79, 51. These values were in agreement with *Alaribe et al.* (2012) and Silver *et al.* (2013). Comparing the 1H NMR and 13C NMR data (600MHz ($CDCl_3$/TMS) with those in literature further helped in identifying the compound. The presence of the phenolic O-H at δ_H 13.28, the three singlets CH_3 O at δ_H 3.90 - 4.02, the two pairs of aromatic doublets at positions H3, H4, H5 and H7, with the δ_C at 181.17 are consistent with that of a xanthone which confirms the identification of the compound as decussatin (1-hydroxy-2, 7, 8-trimethoxy-9H -xanthene-9-one).

The computational simulation of Decussatin gave IR spectrum, 1H and 13C NMR results that are in perfect agreement with the values obtained experimentally on the crystals. The hydrogen of the phenolic O-H became 0.99 Å in the optimized geometry, suggesting an aromatic hydroxyl group, with a vibrational frequency of 3288 cm^{-1} towards the O atom of C1 (atom labels are shown in Figure 4). Since the bonding character of the C1 to some extent differs from C9=O, the vibrational stretch of the latter is more pronounced at about 1655 cm^{-1}. The aromatic C-H vibrational stretches were also observed at 1482 cm^{-1} which perfectly matched the experimentally observed datum. The simulated NMR gave chemical shifts (δ) values within the range 3.68 - 4.94 for the singlet H of the three methoxy groups at, C3, C7, and C8 which was within the ballparks of the values obtained at 600 MHz magnetic field of the NMR machine. The anisotropic property of the phenolic OH at C1 was revealed to be slightly different from the C9=O resulting from weakened intramolecular hydrogen bonding to the two oxygen atoms. As such, the chemical shifts of the two groups have a difference of about 12.11 ppm whereby there is 186.05 at C9 and 173.94 at C1 with respect to the calculated internal standard TMS as computed with gauge-independent atomic orbital (GIAO) method. In fact, many of the simulated spectroscopic parameters confirmed the presence of Decussatin in the plant extract.

There was a gradual increase in the percentage inhibition of the decussatin as the concentration increased, with the highest inhibition of 78.01% recorded at 1 mg/mL which was significantly different from control at $p < 0.001$ and comparable to the 54.85% of acarbose at that concentration as shown in Figure 5.

Discussion

The anti-diabetic activities of *A. vogelii* extracts were investigated *in vivo* using rat models. The reduction in blood glucose levels of the alloxan-induced diabetic rats treated with 1 g/kg BW leaves and stem bark extracts compared with Glibenclamide (2.5 mg/kg BW), a sulphonylurea anti-diabetic drug used as a positive control is seen in Table 1. Significant reduction ($p < 0.05$) in blood glucose level was observed from day-4 of treatment using the leaf extract while the stem bark showed significant reductions from day-1. For glibenclamide, the reduction was from day-5. This is because glibenclamide, like other sulphonylureas, is effective in mild diabetic state and ineffective in severe diabetic animals where pancreatic β-cells are completely destroyed (Qamar, 2011). In Figure 1, the percentage reduction in blood glucose levels receiving *A. vogelii* extracts is shown with peak reduction of 60.35 % and 69.68 % observed for the leaves and stem bark on days 7 and 5 respectively, while glibenclamide had a peak reduction of 58.43 % on day-7 all significantly different from control at $p < 0.05$. The hypoglycaemic effect of the root extracts of *A. vogelii* studied in mice, rats and rabbits at (100, 400 and 800 mg/kg BW) induced significant hypoglycaemic activity in a dose-related manner at 2 h after oral administration in mice and rats respectively, while the extract (800 mg/kg BW) similarly induced lowering of blood glucose levels at 8 h in normoglycaemic rabbits (Abuh *et al.,* 1990). In another related study of the methanol extract (ME) and fractions of *A. vogelii* stem bark, the ME was subjected to gradient chromatographic separation using four solvents as thus, chloroform, ethyl acetate, acetone and water to give the respective fractions - CF, EF, AF and WF. The extract and its fractions exhibited reduction in fasting blood glucose levels of the animals after 6 h ranging from 12.79 % - 64.10 % ($p < 0.05$) compared to 53.77 % for glibenclamide (Osadebe *et al.,* 2014). Our previous work on *Anthocleista djalonensis* had shown that the leaves, stem bark and root possessed anti-diabetic activities (Olubomehin *et al.,* 2013).

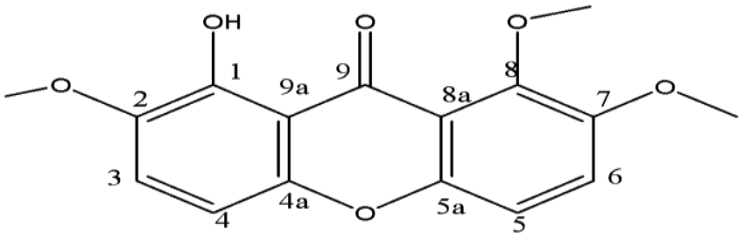

Figure 4. Decussatin (1-hydroxy-2, 7, 8-trimethoxy-9*H*-xanthene-9-one) from *A. vogelii* Leaf and Stem Bark.

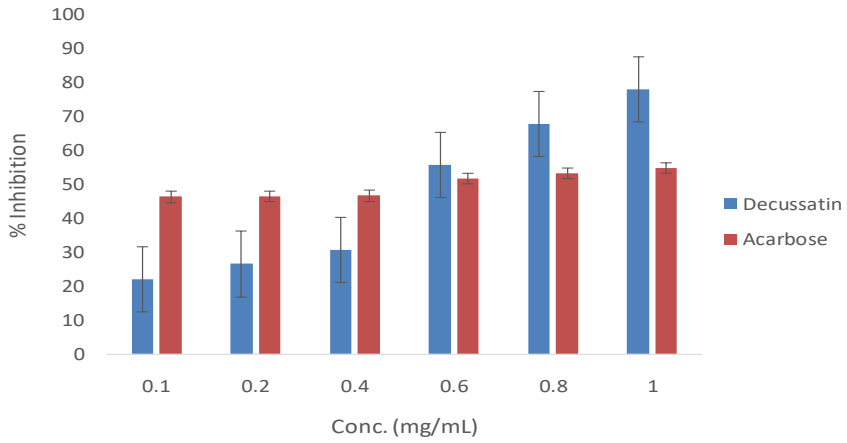

Figure 5. α-amylase Inhibition of Decussatin of *A. vogelii* and Acarbose at Different Concentrations

Column chromatography of the ethyl acetate fractions of both the leaves and stem bark led to the isolation of decussatin which had an α-amylase inhibition of 22.0 % at 0.1 mg/mL and rising gradually to 78.0 % at 1.0 mg/mL statistically significant at $p < 0.001$ as shown in Figure 5. Different plant extracts have been investigated for their α-amylase inhibitory activities with some of them showing very good potential: inhibition of α-amylase and α-glucosidase activities by the ethanolic extract of *Telfairia occidentalis* leaves was in a dose-dependent manner significant at $p < 0.05$ (Oboh *et al.*, 2012). Also, the α-amylase inhibitory activities of *Urticadiocia* and *Juglansregia* extracts were investigated by Rahimzadeh *et al.* (2014) and both plants showed time and concentration dependent inhibition of α-amylase.

Previously, bauerenone and bauerenol found to be highly promising α-glucosidase inhibitors have been isolated from the dichloromethane/methanol extract of the roots of *Anthocleista schweinfurthii* (Mbouangouere, *et al.*, 2007). Decussatin, a known xanthone characterized as 1-hydroxy-2,7,8-trimethoxy-9H-xanthene-9-one was previously isolated by Okorie (1976) and Alaribe *et al.* (2012). Also, Silva and co-workers isolated decussatin from *Tachia grandiflora* and showed that the compound possessed anti-malarial activities (Silva *et al.*, 2013). In a new development, α-amylase inhibitory activity for decussatin from the leaves and stem bark of *A. vogelii* is hereby reported for the first time. The high activity of 78.0 % recorded for it shows that α-amylase inhibitory activity may be the major way by which the plant exerts its anti-diabetic activity. α-amylase (*α*-1,4-glucan-4-glucanohydrolases) is a prominent secretory product of the pancreas and salivary gland in charge of the initial step in the hydrolysis of complex carbohydrate to a mixture of oligosaccharides and disaccharides in the intestinal mucosa and the α-linked polysaccharides such as starch and glycogen hydrolyses (Sim *et al.*, 2010; Thilagam *et al.*, 2013). Their inhibitors such as acarbose and α-glucosidase inhibitors like voglibose reduce the postprandial glucose levels by competitively inhibiting these hydrolase enzymes thereby delaying the absorption of glucose (Kang *et al.*, 2012). Interestingly, this is similar to the glucose inhibitory activity which was attributed to decussatin that was isolated in this study.

CONCLUSION

A. vogelii has been used traditionally to treat diabetes and scientifically this has been further established by our current findings as reported herein. This research work further corroborates that α-amylase inhibition is one of the ways by which anti-diabetic mechanism is elicited by the phyto-active compounds of *A. vogelii*. We isolated decussatin from the plant which was tested and found to be responsible for the observed anti-diabetic activity. Therefore, these findings increase the data available on plants that possess α-amylase inhibition and inhibitors of plant origin.

AUTHORS' CONTRIBUTIONS

Author OOO designed the study and participated in data acquisition and analysis. Author ASF did the computational analysis. Authors ASF, EOA and KAA contributed to the manuscript. All the authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this work.

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