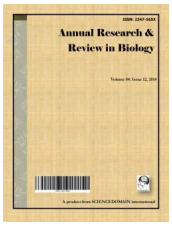

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Characterization and Anti-diabetic Activity of Dihydrophenantherene Isolated from *Khaya senegalensis* Stem Bark

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Authors' contributions

This work was carried out in collaboration between all authors. Authors IUM, AJA, A. Muhammad and AI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MSS, AMW, AAI, A. Mohammed, IA and AN managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Khaya senegalensis A. Juss (Meliaceae), is regarded as the most popular medicinal meliaceous plant. The present study was conducted to evaluate anti-diabetic potentials of column chromatography fractions (FI-FVII) from ethyl acetate extract of *Khaya senegalensis* stem bark and detect the bioactive compounds present in the fractions using spectroscopic techniques. Anti-

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diabetic potential of the fractions (FI-FVII) were tested at dose of 50 mg/kg on wistar albino rats. Fraction (VI) and metformin treated diabetic groups showed significant decrease in fasting blood glucose (FBS), ameliorate hepatic and renal damages by decreasing the level of AST, ALT, ALP, Urea and creatinine compared to untreated diabetic rats and stimulate insulin secretion by β cells. After alloxan administration, the levels of hepatic and renal tissues antioxidant enzymes such as glutathione peroxidase (Gpx), superoxide dismutase (SOD) and catalase (CAT) were decreased whereas the level of hepatic and renal tissues lipid peroxidation (LPO) was elevated. The levels of these antioxidant enzymes were also brought to normalcy by Fraction (VI). Histological studies supported the biochemical findings, and treatment with Fraction (VI) was found to be effective in restoring alloxan-induced pancreatic toxicity in rats. FTIR, GCMS and NMR analysis was conducted for the detection of bioactive compound(s) in Fraction (VI), and the result revealed the presence of methyl 6-ethenyl-7-hydroxy-7,8-dihydrophenanthrene-2-carboxylate. The study concludes that; the anti-diabetic property of *Khaya senegalensis* stem bark is mediated by the bioactive compound "methyl 6-ethenyl-7-hydroxy-7,8-dihydrophenanthrene-2-carboxylate" through its antioxidant properties and stimulation of damaged pancreas to produce more insulin.

Keywords: Anti-diabetic activity; anti-oxidant; column chromatography; characterization; isolation and Khaya senegalensis.

1. INTRODUCTION

Diabetes mellitus, a chronic non-communicable disease, is ranked 7th killer disease in the world characterized by persistent high glucose level in the blood over a prolonged period [1]. According to the International Diabetes Federation, about 366 million people are living with diabetes and this figure is projected to increase to 552 million by the year 2030 [2]. Diabetes mellitus is due to either pancreatic β cells not producing enough insulin, or the cells of the body are not responding properly to the insulin produced. Insulin is the principal hormone that regulates the uptake of glucose from the blood into most cells of the body, especially liver, muscles, and adipose tissues. Therefore, deficiency of insulin and/or insensitivity of its receptors play a central role in all forms of diabetes mellitus [3]. The classic symptoms of untreated diabetes are weight loss, polyuria (frequent urination), polydipsia (increased thirst), and polyphagia (increased hunger). Other symptoms include blurry vision, headache, fatigue, slow healing of cuts, and itchy skin [4]. Conventional drugs used in the management of diabetes are sometimes inadequate, unaffordable and can have serious side effects. It is therefore imperative to search for alternative drugs of higher efficacy and safety to replace and/or support the currently used drugs. The world health organization has also recommended the evaluation of the effectiveness and safety of plants used in traditional and complementary medicines [5].

Khaya senegalensis A. Juss (Meliaceae), commonly called the dry zone mahogany or African mahogany is regarded as the most popular medicinal meliaceous plant in African traditional remedies [6]. The bitter stem bark aqueous extract have been used as a folk medicine for management of diabetes [7], hypertension [8], jaundice [9] and malaria [10]. Roots are applied topically against stomach-ache, oedema and amenorrhoea [11].

Almost all bioactive compounds isolated from different parts and extracts of *Khaya senegalensis* are limonoids. The first limonoid isolated in 1996 was khayanolide limonoids [12] which was followed by the isolation of a novel limonoid, 2,6-dihydroxyfissinolide and two known limonoids, fissinolide and methyl 3b-acetoxy-6-hydroxy-1-oxomeliac-14-enoate from stem bark of the plant [13]. Another research group identified the presence of two mexicanolide-type limonoids named khayanone and 2-hydroxyseneganolide as well as one rearranged phragmalin limonoid 1-O- acetylkhayanolide A [14]. Apart from limonoids, the only group of phytochemicals isolated from *Khaya senegalensis* are dimeric proanthocyanidins fisetinidol-(4α,6)-catechin (proanthocyanidin B3) and catechin-(4α,8)-catechin [15].

Due to its reported anti-diabetic properties, it is imperative to characterize the active compound(s) responsible for the anti-diabetic activity of the stem bark. In preliminary studies, the stem bark was subjected to extraction using solvents of varying polarity (hexane, chloroform, ethyl acetate and water). The extracts were then screened for anti-diabetic activity using alloxan induced diabetic rat model. The ethyl acetate extract was found to possess the highest anti-diabetic activity among all other extracts. Hence,

in the present study, the ethyl acetate extract of the stem bark was further fractionated using column chromatography and the fractions obtained were subjected to comprehensive anti-diabetic study.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Study animals

Male and female albino rats weighing between 100 - 200 g were purchased from animal house of Biological Science Department; Bayero University, Kano. The animals were housed in well-ventilated cages in the animal house of Biological Science Department of Bayero University Kano. The rats were allowed to acclimatize for one week prior to the experiment and had free access to food and clean water.

2.1.2 Plant material

Khaya senegalensis stem barks were collected from Bayero University Kano (located in the ancient city of Kano, North West of Nigeria) on Monday 2nd January 2017. The plant was identified and authenticated at the Herbarium of Plant Biology Department; Bayero University, Kano and was given a voucher number of (BUK/HAN/0116). The stem bark was shade dried and ground to powder.

2.2 Methods

2.2.1 Preparation of alloxan

One gram of Alloxan Hydrate was dissolved in 10 mL of distilled water and to give a concentration of 100 mg/mL

2.2.2 Induction of diabetes mellitus with alloxan

Rats induced with Diabetes mellitus were fasted overnight for a period of 12 hours before induction of the diabetes by injecting alloxan hydrate intraperitoneally at dose of 100 mg/kg using a sterile 1 ml syringe. The volume of the solution containing 100 mg/kg given to each experimental albino rat was determined by the following relationship [16].

$$Volume(ml) = \frac{Dose(mg/kg) \times weight of rat(kg)}{Concentration of extract(mg/ml)}$$

Animals with fasting blood glucose ≥200 mg/dl after 48 hours of alloxan administration were considered to be diabetic and used for the study [17].

2.2.3 Packing of the column

Silica gel of mesh size of 60-120G was used as the stationary phase while varying solvents combinations were used as the eluent. Wet packing method as describe by Jerry et al. [18] was used in preparing the silica column, a slurry was formed by mixing 200 g of the silica gel in 500 ml of hexane and poured down quickly and carefully into the column, the tap was left open during packing to allow free flow of the solvent into a beaker below. At the end of the packing, the tap was closed and left to stand undisturbed for 24 hours, after which the clear solvent on top of the silica gel was allowed to drain down to the silica gel meniscus. The flow rate of the column was noted.

2.2.4 Loading of the sample

Twenty gram (20 g) of the dried extract was mixed thoroughly with 20 g of silica gel and then gently layered on top of the column. Elution of the column was done with various solvent combination of varying polarity. The following solvents systems were used in the elution process; hexane: ethyl acetate 100:0, 80:20, 60:40, 40:60, 20:80, 0:100; chloroform: methanol 100:0, 80:20, 60:40, 40:60, 20:80, 0:100. For each solvent combination, the elution was done until each solvent ratio becomes clear. The eluted fraction were collected in an aliquots volume of 100 ml.

2.2.5 Pooling of the fractions using analytical TLC

Each fraction was spotted in a pre-coated aluminum silica gel plate and developed in a chromatographic tank in the appropriate solvent systems. With the aid of a capillary tube, a spot of the sample was applied on the plate at 1.0 cm distance from the base of the plate, the plate was allowed to dry at room temperature, and lowered in a chromatographic tank containing the solvent system saturated with the solvent vapour. The solvent was allowed to ascend the plate until the solvent front reaches about ¾ of the length of the plate. The plate was removed and allowed to dry at room temperature. The relative retention factor (R_f) was calculated

$$Rf = \frac{\text{Distance travelled by compound from origin}}{\text{Distance travelled by solvent from origin}}$$

2.2.6 Screening of column chromatography fractions for hypoglycemic activities

Fifty (50) rats were used and grouped into ten (10) groups of five (5) rats each.

Group I : Normal control
Group II : Diabetic control
Group III : Standard drug (metformin, 100 mg/kg body weight)
Group IV : Diabetic, administered with 50 mg/kg body weight of fraction I
Group V : Diabetic, administered with 50 mg/kg body weight of fraction II
Group VI : Diabetic, administered with 50 mg/kg body weight of fraction III
Group VII : Diabetic, administered with 50 mg/kg body weight of fraction IV
Group VIII : Diabetic, administered with 50 mg/kg body weight of fraction V
Group IX : Diabetic, administered with 50 mg/kg body weight of fraction VI
Group X : Diabetic, administered with 50 mg/kg body weight of fraction VII

Fasting blood glucose concentrations of rats was measured at an interval of three days for a period of two weeks. On 15th day after 24 h of treatment, blood from all animals was collected by retro-orbital puncture and after that the animals were euthanized. Blood was allowed to clot and centrifugation was performed at 3500 rpm for 10 min at 4℃ to separate the serum which was used for the assay of biochemical marker enzyme. Liver and kidney tissue samples were taken and homogenized for antioxidant assay, while pancreas was fixed in 10% formalin solution for 24 h for pathological examination.

2.2.7 Statistical analysis

Results were expressed as mean ± standard deviation. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test after investigating the data for normality using Shapiro-Wilk test and for variances homogeneity to be sure that the data are normally distributed and variances would be homogenous using GraphPad Instat3 Software version 3.05 Differences of $P < 0.05$ were considered to be significant [19].

3. RESULTS

3.1 Effect of Column Chromatography Fractions (FI-FVII) on Fasting Blood Glucose

Fig. 1 shows the fasting blood glucose concentrations of diabetic rats administered with column chromatography fractions (FI-FVII) taken at the interval of three (3) days over two weeks of administration. After 48th hours of alloxan injection, the blood glucose levels of group II (diabetic control group) and test groups (groups III-X) increases significantly ($p<0.01$) compared to normal control (group I). A significant decrease ($p<0.05$) in fasting blood glucose of diabetic rats administered with fraction I, II, III, IV and VI, with fractions VI possessing the highest hypoglycemic activity. Contrary to this however, there is a significant increase ($p>0.05$) in blood glucose concentration of diabetic rats administered with fractions V and VII.

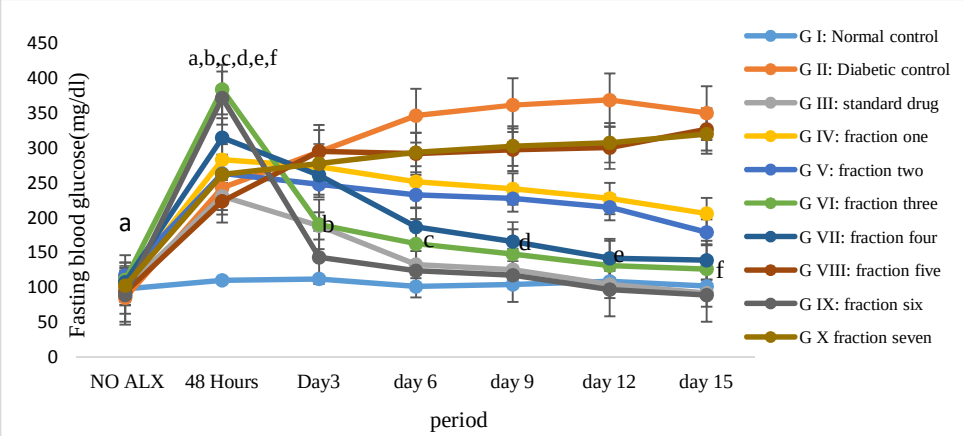


Fig. 1. Blood glucose level (mg/dl) of rats before and after alloxan administration and at interval of three days on oral administration with column chromatography fractions

3.2 Effect of Column Chromatography Fractions (FI-FVII) on Feed Intake, Water Intake and Body Weight of Diabetic Rats

Figs. 2 and 3 shows the feed and water intake of diabetic rats administered with column chromatography fractions (FI-FVII). There was a significant ($p<0.05$) increase in both feed and water intake in diabetic control compared to normal control. Administration of the fractions lead to significant ($p<0.05$) decrease in both feed and water intake in groups administered with standard drug, fraction VI respectively compared to diabetic control. While Fig. 4 shows the initial and final weight of the rats administered with the fractions. No significant ($p>0.05$) difference was observed between the groups.

3.3 Effect of Column Chromatography Fractions (FI-FVII) on Liver Function Indices

Table 1 Present the liver function indices (AST, ALT, ALP, DB, TB, TP and ALB) of diabetic rats administered with column chromatography fraction (FI-FVII). There was a significant ($p<0.01$) increase in serum AST, ALT, ALP and TP level in diabetic control group, fraction V and VII administered groups compared to the normal control. A significant decrease ($p<0.05$) in serum AST, ALT, ALP and TP level was observed in standard drug and fraction VI administered groups compared with diabetic control group.

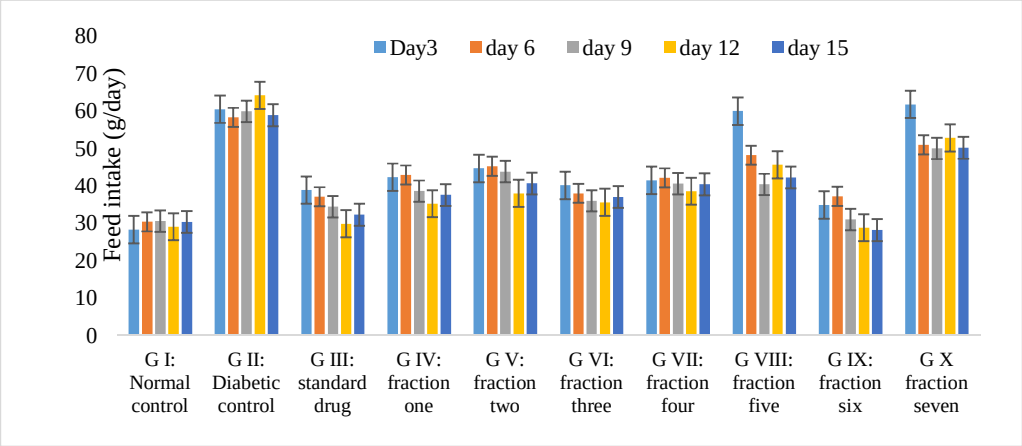


Fig. 2. Feed intake of alloxan induced diabetic rats at interval of three days on oral administration of column chromatography fractions (F1-F7) for two weeks

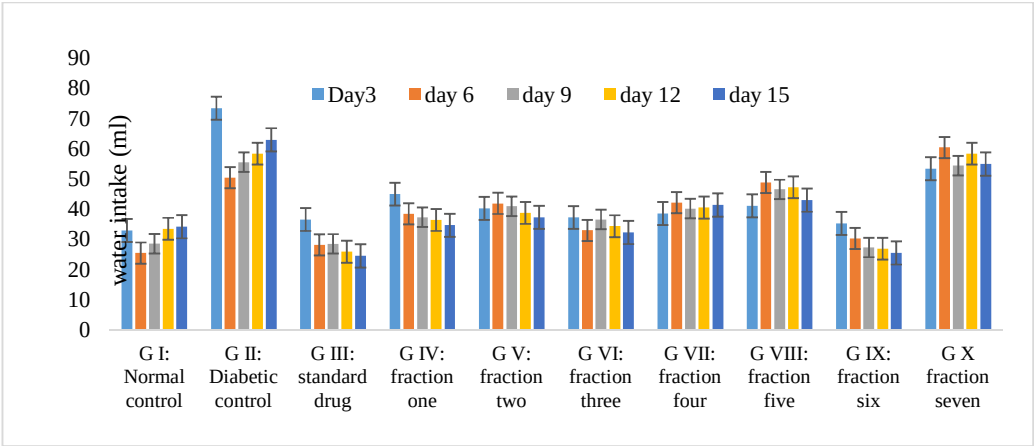


Fig. 3. Water intake of alloxan induced diabetic rats at interval of three days on oral administration of column chromatography fractions (F1-F7) for two weeks

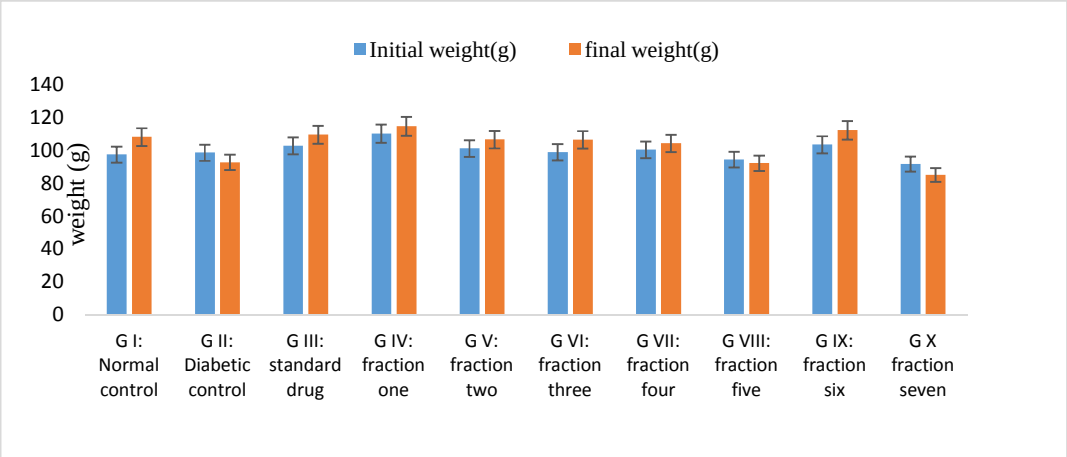


Fig. 4. Initial and final body weight of diabetic rats administered with column chromatography fractions (FI-FVII) for two weeks

Table 1. Liver function indices (AST, ALT, ALP, DB, TB, TP and ALB) of rats administered with column chromatography fractions (FI-FVII) for two weeks

Groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	DB (mg/dl)	TB (mg/dl)	TP (g/dl)	ALB (g/dl)
I	27.25±5.21^{a}	29.70±3.02 ^{a}	78.60±5.26^{a}	0.30±0.10	1.86±0.52	7.82± 1.28 ^{a}	4.80±1.07
II	61.10±7.10^{a,b,c}	78.67±5.20 ^{a,b,c}	182.44±4.02^{a,b,c}	0.42±0.20	2.82±0.67	12.17±2.09^{a,b,c}	3.50±1.07
III	26.07±3.27^{b}	28.00±2.80^{b}	59.54±2.80^{b}	0.31±0.08	1.09±0.16	6.86±1.39^{b}	5.06±1.98
IV	46.23±2.10	47.70±2.50	103.60±3.30	0.36±0.22	2.00±0.55	9.06±1.35	4.20±1.16
V	40.40±3.10	38.33±4.28	82.70±5.22	0.34±0.15	1.84±0.42	8.64±2.01	4.48±1.46
VI	34.77±2.43	38.10±3.20	72.30±4.80	0.31±0.25	2.05±0.16	8.06±1.63	4.09±2.98
VII	37.07±3.03	37.00±2.55	73.40±3.06	0.30±0.12	1.68±0.18	8.06±2.32	4.27±2.12
VIII	53.00±4.22	62.20±4.10	121.50±4.22	0.32±0.02	2.24±0.35	9.80±1.82	3.84±1.82
IX	26.32±3.21^{c}	29.33±5.03^{c}	50.44±5.05^{c}	0.28±0.12	1.53±0.44	7.10±2.10^{c}	4.90±2.02
X	60.32±4.50	67.32±4.44	130.0±2.90	0.38±0.10	2.50±0. 52	10.2±2.35	3.71±2.20

Values are presented as Mean ± standard deviation, (n=5). Value with the same superscripts in a column are significantly different compared to each other (P<0.05). Key: Group I: Normal control, Group II: Diabetic control, Group III: standard drug, Group IV: fraction one, Group V: fraction two, Group VI: fraction three, Group VII: fraction four, Group VIII: fraction five, Group IX: fraction six, Group X fraction seven

3.4 Effect of Column Chromatography Fractions (FI-FVII) on Kidney Function Indices

Table 2 shows serum concentrations of urea, creatinine and electrolytes (Na^+, Cl^-, K^+, HCO_3^-) of diabetic rats administered with column chromatography fractions (FI-FVII). A significant increase ($p<0.01$) was observed in mean serum urea, creatinine, K^+ and Cl^- with a significant decrease ($p<0.05$) in mean serum of Na^+ and HCO_3^- in diabetic control group compared to the normal control. There was a significant decrease ($p<0.01$) in urea, creatinine, K^+ and Cl^- in groups VI and IX administered with standard drug, and fraction six respectively, with a significant increase in Na^+ and HCO_3^-.

3.5 Effect of Column Chromatography Fractions (FI-FVII) on Oxidative Stress Markers (MDA, SOD, CAT and GSHpx)

Table 3 shows tissues (liver and kidney) concentrations of oxidative stress markers (MDA, SOD, CAT and GSHpx) of diabetic rats administered with column chromatography fractions (FI-FVII). There is a significant ($p<0.01$) increase in MDA in diabetic control compared to normal control, with a significant decrease ($p<0.01$) in mean serum of SOD, CAT and GSHpx in diabetic control compared to normal control. Administration of the fractions shows a significant ($p<0.05$) decrease in tissues level of MDA with a concomitant increase in level of SOD, CAT and GSHpx in groups administered with standard drug and fraction VI respectively.

Table 2. Kidney function indices of rats administered with column chromatography fractions (FI-FVII) for two weeks

Groups	Urea(mmol/L)	Crea(mmol/L)	Na^+(mmol/L)	K^+(mmol/L)	Cl (mmol/L)	HCO_3 (mmol/L)
I	61.22±5.12[a]	17.80±1.05[a]	128.35±5.02[a]	4.07±1.21[a]	106.45±8.07[a]	28.32±4.21[a]
II	156.08±4.81[a,b,c]	30.66±1.51[a,b,c]	104.24±3.84[a,b,c]	7.46±1.86[a,b,c]	176.78±7.19[a,b,c]	19.40±5.35[a,c]
III	58.34±7.22[b]	19.05±2.04[b]	130.42±4.88[b]	3.84±1.59[b]	98.92±8.74[b]	26.80±6.71[b]
IV	92.80±6.44	22.40±1.80	117.84±5.80	4.80±1.06	124.10±8.21	20.19±4.06
V	89.92±5.22	20.22±2.44	120.05±6.08	4.00±1.86	114.12±7.85	23.50±3.85
VI	84.26±7.24	21.00±1.82	121.40±6.48[c]	4.67±1.25	100.54±9.24	27.08±5.21
VII	86.33±6.28	20.08±.2.33	121.53±5.47	4.26±1.40	110.32±7.36	22.84±4.42
VIII	105.44±3.73	26.34±1.43	115.36±4.28	5.39±2.10	132.76±8.72	20.35±3.31
IX	65.07±4.22[c]	18.34±2.32[c]	127.63±5.09[c]	4.01±1.85[c]	103.47±10.32[c]	26.74±4.32[c]
X	120.43±5.33	27.63±2.61	110.43±7.42	5.36±2.08	142.90±8.25	20.85±3.23

Values are presented as Mean ± standard deviation, (n=5). Value with the same superscripts in a column are significantly different compared to each other (P<0.05). Key: Group I: Normal control, Group II: Diabetic control, Group III: standard drug, Group IV: fraction one, Group V: fraction two, Group VI: fraction three, Group VII: fraction four, Group VIII: fraction five, Group IX: fraction six, Group X: fraction seven

Table 3. Tissues oxidative stress markers (MDA, SOD, CAT and GSHpx) of diabetic rats administered with column chromatography fractions (FI-FVII) for two weeks

Groups	MDA (nmol/mg prt)		SOD (mmol/mg prt)		CAT (mmol/mg prt)		GSHpx (mmol/mg prt)	
	Liver	kidney	Liver	kidney	Liver	kidney	Liver	kidney
I	2.24±0.12[a]	2.11±0.43[a]	9.27±1.35[a]	9.43±0.76[a]	26.65±5.02[a]	28.41±4.24[a]	107.22±10.21[a]	120.43±8.75[a]
II	6.66±0.81[a,b,]	5.92±1.02[a,b,c]	4.52±0.81[a,b,c]	5.98±1.20[a,b,c]	14.44±3.84[a,b,c]	16.08±2.25[a,b,c]	64.63±8.65[a,b,c]	84.78±9.30[a,b,c]
III	3.30±0.72[b]	3.42±.032[b]	7.82±1.04[b]	8.76±0.94[b]	20.42±4.88[b]	23.45±3.53[b]	98.54±12.55[b]	113.45±10.20[b]
IV	5.48±0.44	4.10±0.55	5.10±1.05	5.87±1.32	17.24±2.80	18.43±4.03	76.80±8.56	96.34±9.80
V	4.42±0.52	3.91±1.22	5.22±1.46	6.05±0.95	17.20±3.28	18.78±3.78	84.00±6.87	99.20±8.43
VI	4.72±0.74	4.30±0.32	5.10±0.82	5.75±1.20	16.60±4.08	19.32±3.18	60.32±10.22	91.63±7.63
VII	5.40±0.68	4.30±0.72	5.80±.1.03	5.20±0.83	19.33±2.40	19.52±4.32	90.60±6.44	94.76±6.82
VIII	4.58±0.73	4.11±0.56	5.34±1.33	6.08±1.12	15.6±3 .48	18.43±3.79	68.21±7.20	96.79±6.02
IX	3.35±1.04[c]	3.00±0.44[c]	8.34±1.42[c]	9.31±1.03[c]	21.32±3.08[c]	25.85±4.10[c]	105.12±13.5[c]	114.65±8.82[c]
X	6.60±0.83	6.43±0.92	4.63±1.60	6.20±0.86	15.33±5.4 2	16.55±3.15	70.36±11.84	86.43±6.62

Values are presented as Mean ± standard deviation, (n=5). Value with the same superscripts in a column are significantly different compared to each other (P<0.05). Key: Group I: Normal control, Group II: Diabetic control, Group III: standard drug, Group IV: fraction one, Group V: fraction two, Group VI: fraction three, Group VII: fraction four, Group VIII: fraction five, Group IX: fraction six, Group X fraction seven

3.6 Effect of Column Chromatography Fractions (FI-FVII) on Serum α-amylase, α Glucosidase and Insulin

Table 4 shows serum α-amylase, α glucosidase and insulin of diabetic rats administered with column chromatography fractions (FI-FVII) for two weeks. There is a significant ($p<0.05$) increase in serum α-amylase, α glucosidase with a significant decrease in serum insulin in diabetic control compared normal control. Administration of the fractions shows a significant ($p<0.05$) decrease in levels of α-amylase and α glucosidase in groups administered with fraction VI only with a significant ($p<0.05$) increase in the level of serum insulin.

3.7 Histopathological Examination of Pancreatic Tissues

Plate 1 shows a photomicrograph of Section of pancreas control rats showing no significant pathology. Plate 2 shows area of pancreatic damage and distortion of normal pancreas as a result of alloxan administration. Plates 3, 4, 5, 6, 7, 8 and 10 shows photomicrograph pancreas of diabetic rats administered with fractions I, II, III, IV, V and VII showing area of pancreatic damage. While Plate 9 shows Section of pancreas administered with fraction VI showing no significant pathology.

3.8 Characterization of Bioactive Compound Using Various Analytical Techniques

From the results of anti-diabetic studies, fraction "VI" showed promising results. Hence this fraction was subjected to analytical TLC, which yield a single band.

3.8.1. IR studies of F-VI

The IR spectra exhibit characteristic absorption band at 3376 cm-1 which shows that the compound possesses -OH strecthing and a C=O strecthing is present at 1709 cm-1.

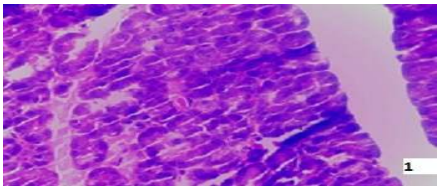


Plate 1. Section of pancreas of normal control rats showing no significant pathology (H and E, mag.×100)

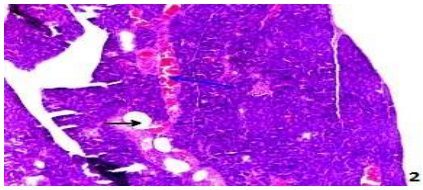


Plate 2. Section of pancreas of diabetic control rats showing area of pancreatic damage (H and E, mag.×100)

Plate 3. Section of pancreas of rats administered with standard drug showing area of pancreatic damage (H and E, mag.×100)

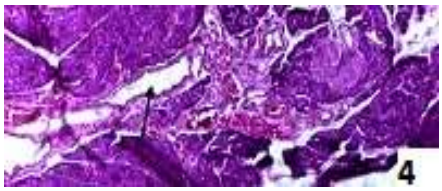


Plate 4. Section of pancreas of rats administered with fraction 1 showing area of pancreatic damage (H and E, mag.×100)

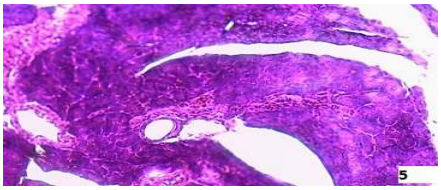


Plate 5. Section of pancreas of rats administered with fraction 2 showing area of pancreatic damage (H and E, mag.×100)

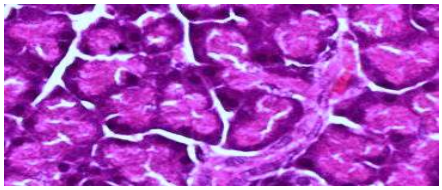


Plate 6. Section of pancreas of rats administered with fraction 3 showing area of pancreatic damage (H and E, mag.×100)

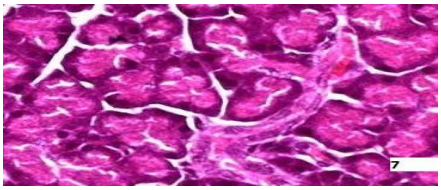


Plate 7. Section of pancreas of rats administered with fraction 4 showing area of pancreatic damage (H and E, mag.×100)

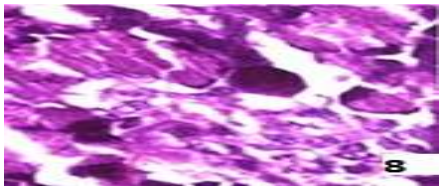


Plate 8. Section of pancreas of rats administered with fraction 5 showing area of pancreatic damage (H and E, mag.×100)

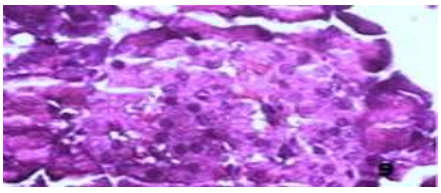


Plate 9. Section of pancreas of rats administered with fraction 6 showing area of pancreatic regeneration (H and E, mag.×100)

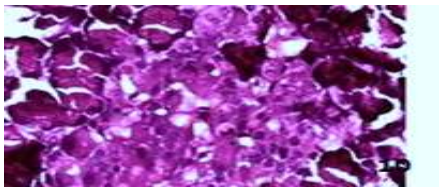


Plate 10. Section of pancreas of rats administered with fraction 7 showing area of pancreatic damage (H and E, mag.×100)

Table 4. Serum α-amylase, α-glucosidase and insulin levels of diabetic rats administered with column chromatography fractions (FI-FVII)

Group	α-amylase (IU/L)	α-glucosidase (IU/L)	Insulin (mIU/L)
I	34.23±4.26[a]	17.80±1.05[a]	13.65±2.10[a]
II	66.44±3.12[a,b]	30.66±1.51[a,b]	7.21±1.64[a,b]
III	43.64±2.72	21.05±2.04	8.56±0.78
IV	53.44±4.14	22.40±1.80	8.44±2.13
V	48.42±3.52	20.22±2.44	8.02±1.90
VI	48.02±3.34	29.00±1.82	9.06±2.06
VII	45.66±4.68	20.08±.2.33	9.81±1.52
VIII	58.10±3.73	26.34±1.43	8.29±1.44
IX	28.24±3.08[b]	18.34±2.32[b]	11.32±1.01[b]
X	62.54±3.54	32.63±2.61	8.07±2.10

Values are presented as Mean ± standard deviation, (n=5). Value with the same superscripts in a column are significantly different compared to each other (P<0.05). Key: Group I: Normal control, Group II: Diabetic control, Group III: standard drug, Group IV: fraction one, Group V: fraction two, Group VI: fraction three, Group VII: fraction four, Group VIII: fraction five, Group IX: fraction six, Group X: fraction seven

A characteristic absorption band at 1611 cm-1 shows that the presence of C=C, stretching at 1376 indicate the presence of CH_3 and 1248 cm-1 shows that C–O stretching. The spectrum of the compound is given in Fig. 5.

3.8.2 GCMS studies of F-VI

From the mass spectrum of the fraction it was observed that a molecular ion (M+2) signal at m/z= 281.10, using the rule of thirteen on the molecular ion, a hydrocarbon formula of $C_{18}H_{16}O_3$ containing the functional groups detected in the IR spectrum (i.e OH, C=O and C-O) was obtained. The fragmentation pattern of the mass spectra shows the presence of aromatic hydrocarbon which usually fragments via the loss of a molecule of ethene (m/z= 28). Fragmentation of ring compounds requires the cleavage of two carbon–carbon bonds, which is a more difficult process than cleavage of one such bond. Therefore, first ethene fragment (C_2H_4) observed at m/z 253 is from the branch at carbon 6 not from the parent aromatic hydrocarbon. Elimination of the OR moiety of the ester account for the observed peak at m/z 206; a characteristics of aromatic ester, while the observed peak at m/z 189 is from loss of OH m/z =17.

3.8.3 ^{13}C-NMR studies of F-VI

From the spectra it was observed that the ^{13}C-NMR showed 18 signals. It revealed that the

chemical shift was observed at δ100.08 ppm and δ=100.21 ppm indicates the presence of an alkene (Aliphatic C=C). The appearance of a peak furthest to the left (δ182.36 ppm) in the spectrum suggest the presence of a carbonyl (−C=O) group while the peak at δ=54.77 ppm is a deshielded carbon atom caused by a neighboring single-bonded oxygen atom (−C−O). Thus confirming the presence of an ester. Fourteen peaks occurring at δ110.96-158.36 ppm shows the presence of an aromatic ring containing fourteen carbon atoms. The spectrum of the compound is given in Fig. 7.

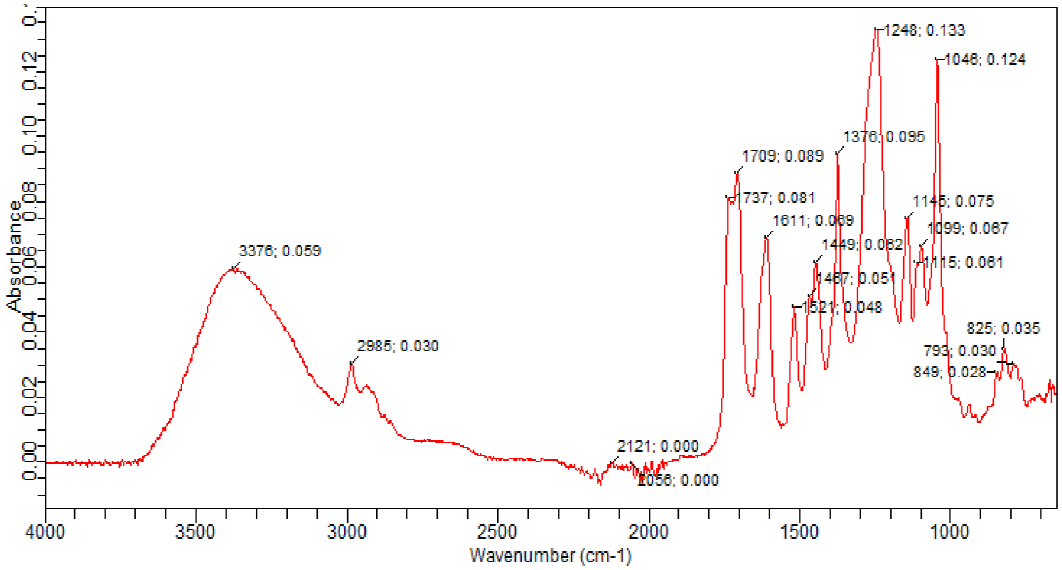


Fig. 5. FTIR spectra of F-VI

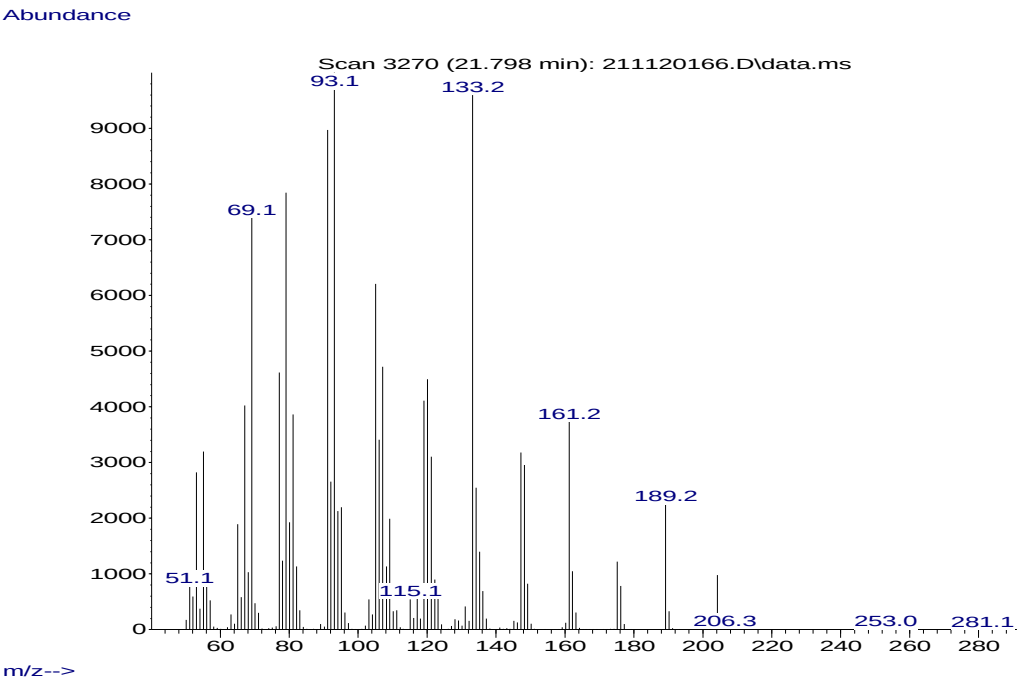


Fig. 6. GC-MS scan spectra of F-VI

Table 5. Analysis of ^{13}C NMR spectra

S/N	Chemical shift (ppm)	Type of carbon
1	182.36	Carbonyl carbon (C=O)
2	110.96 -158.36 (14)	Aromatic carbon
3	100.08	Alkene (C=C)
4	100.21	Alkene (C=C)
5	54.77	Carbon attached to electronegative atom (C-O)

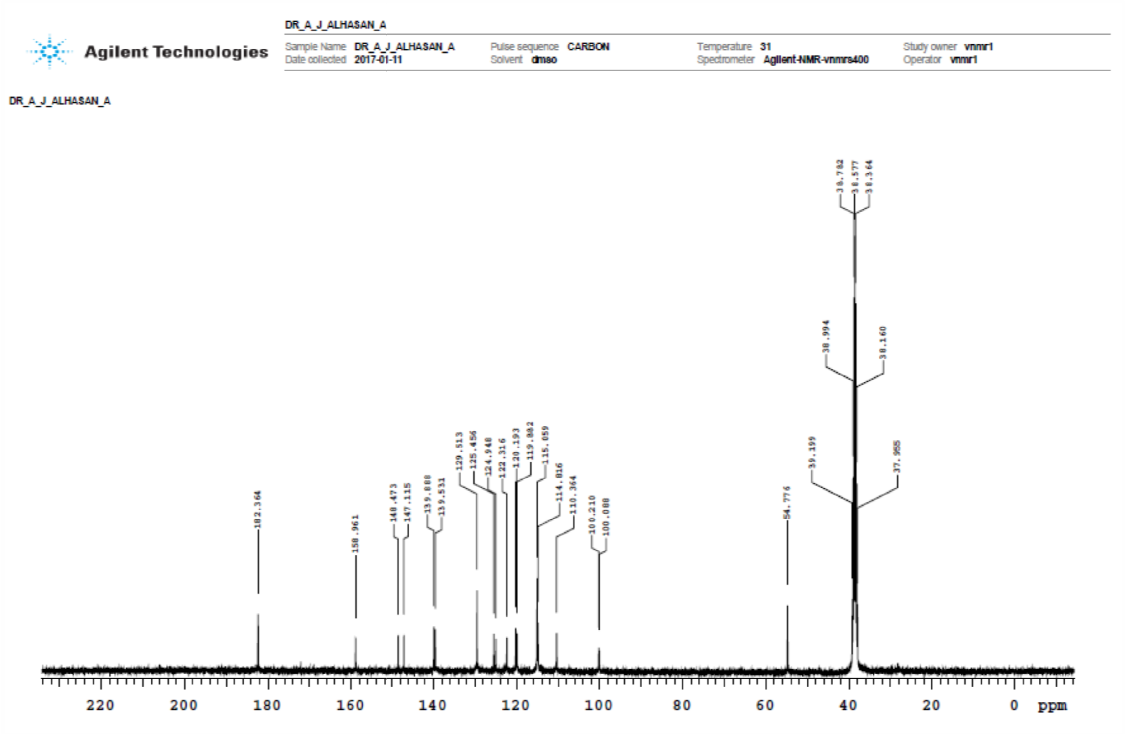


Fig. 7. ^{13}C NMR spectra of F-VI

3.8.4 ^{1}H-NMR studies of F-VI

The proton (^{1}H) NMR spectrum gives information on the numbers and types of hydrogen atoms attached to the carbon skeleton. Peak occurring at δ 5.546 ppm, 5.527 ppm shows protons directly associated with C=C sp^{2} carbon atoms in alkenes (vinyl protons). Peak occurring at δ2.147 ppm represent protons that are directly attached to an electronegative atom (OH), aromatic protons are shown in peak δ6.039 ppm - δ8.811 ppm. Peak occurring at δ4.74 ppm represent protons on carbon directly attached to electronegative atom. The ^{1}H spectrum is shown in Fig. 8.

Table 6. Analysis of ^{1}H-NMR spectra

S/N	Chemical shift (ppm)	Multiplicity	Nature of proton
1	5.546	Doublet	C=C sp^{2} carbon atoms in alkenes (vinyl protons)
2	5.527	Doublet	Same as above
3	2.147	Singlet	Protons attached to an electronegative atom (OH)
4	6.039 - 8.811		Aromatic protons
5	4.74	Doublet	protons directly attached to electronegative atom

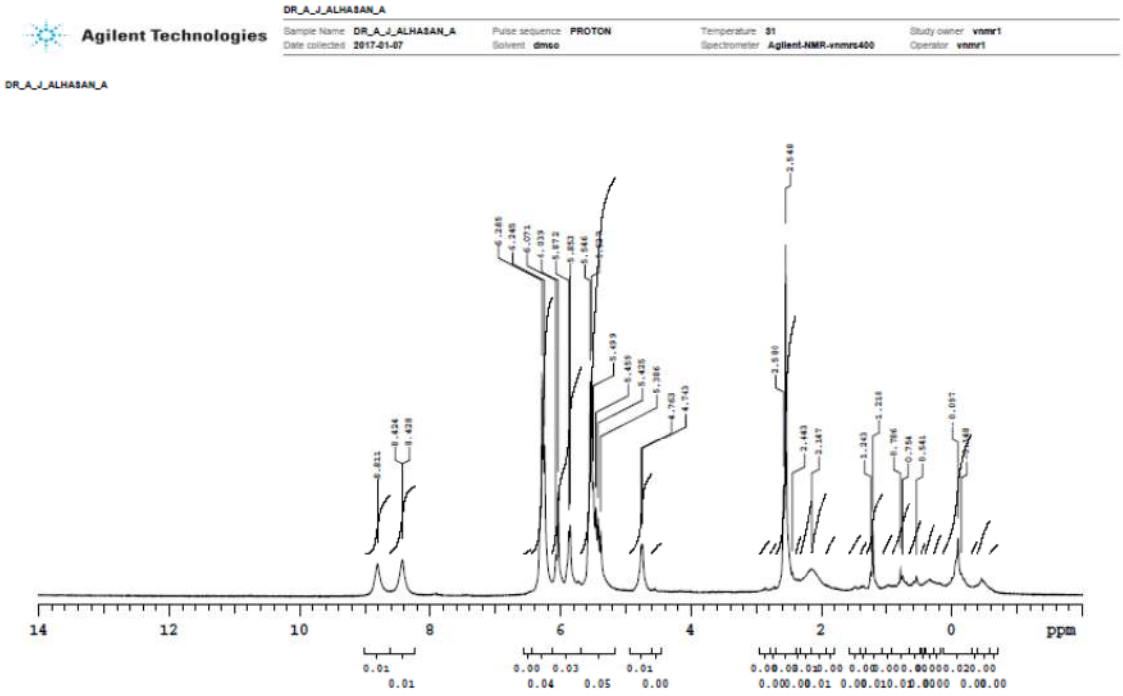


Fig. 8. ^{1}H NMR spectra of F-VI

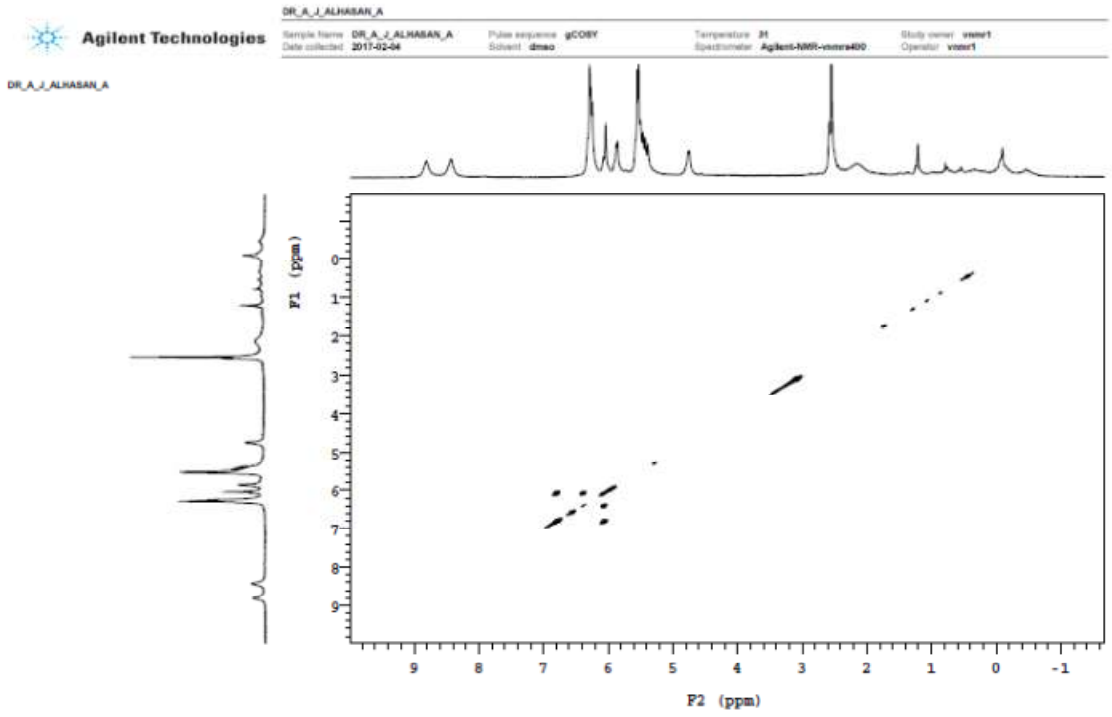


Fig. 9. gCOSY spectra of F-VI

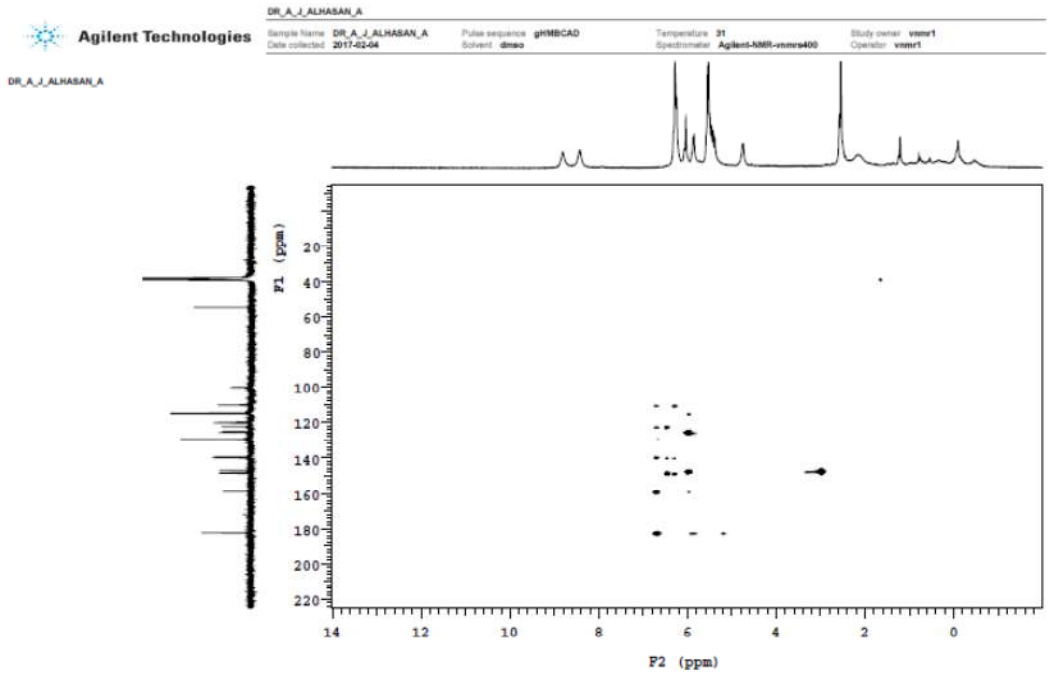
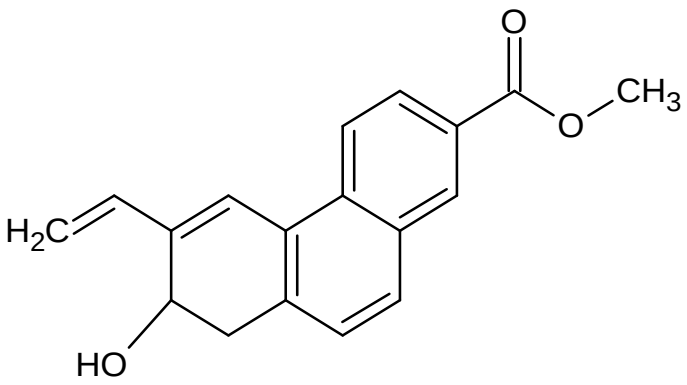


Fig. 10. gHMBCAD spectra of F-VI



methyl 6-ethenyl-7-hydroxy-7,8-dihydrophenanthrene-2-carboxylate

Fig. 11. Structure of isolated compound from fraction VI of ethyl acetate extract of *Khaya senegalensis* stem bark

3.8.5 ^{1}H- ^{1}H and ^{1}H -^{13}C NMR studies of F-VI

A gCOSY ^{1}H-^{1}H correlation was detected between protons at δ6.285ppm and δ6.285 ppm with protons at δ5.872 and δ5.546 ppm. The proton at δ6.28 ppm and δ6.245 ppm also showed ^{1}H-^{13}C gHMBCAD interaction with carbon δ110.364 ppm and δ114.816 ppm, a correlation was also seen between the carbonyl carbon at δ182.364 and the neighboring proton at δ6.071 ppm and δ6.039 ppm, supporting the proposed structure of "methyl 6-ethenyl-7-hydroxy-7,8-dihydrophenanthrene-2-carboxylate"

4. DISCUSSION

Alloxan, a toxic glucose analogue causes destruction of pancreatic β-cells when administered to vertebrate. This causes an insulin dependent diabetes mellitus known as alloxan-induced diabetes in the animals. Alloxan is selectively toxic to insulin producing pancreatic β-cells because it preferentially accumulates in β-cells through uptake via glucose transporter-2 (GLUT2) [20]. Alloxan, in the presence of intracellular thiols, generate reactive oxygen species (ROS) in a cyclic reaction with its

reduction product, dialuric acid. The β-cell toxic action of alloxan is initiated by free radicals formed in this redox reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β-cells [21]. Administration of alloxan elevates serum glucose, which signifies induction of diabetes mellitus. This finding is in accordance with the findings of Muhammad et al. [22] who reported induction of diabetes mellitus to experimental rats using 100 mg/kg body weight of alloxan.

Screening of the fractions for anti-hyperglycemic activity reveals a significant decrease in fasting blood glucose of rats administered with standard drug, fractions III and VI with fraction VI possessing the highest anti-hyperglycemic activity (Fig. 1). The observed anti-hyperglycemic effect of the fractions was supported by significant weight gain observed in diabetic rats treated with these fractions (Fig. 4). This can be can be attributed to increase glucose utilization by tissues, thereby decreasing both water and feed intake in fraction VI and standard drug treated groups compared to diabetic control (Figs. 2 and 3). These findings were supported by various studies that during diabetes mellitus, the blood sugar increases and results in lack of sugar in the cells; forcing the cells to use amino acids and fatty acids as a source of energy which eventually leads to loss in bodyweight, polydipsia and polyphagia.

Evaluation of liver function indices shows Hepatocytes damage as a result of alloxan induced diabetes which is characterized by elevation in the level of different hepatic marker enzymes (AST, ALT and ALP) and the levels of total bilirubin and total protein. Administration of fraction VI to diabetic rats significantly ameleriote the hepatic toxicity induced alloxan by decreasing the levels of hepatic marker enzymes (AST, ALT and ALP). This finding is in supports of finding by Muhammad et al. [23] who reported a significant decrease in the activity of liver enzymes (AST, ALT and ALP) in albino rats induced with liver damage after been administered with aqueous extract of *Khaya senegalensis* at doses of 250 mg/kg and 500 mg/kg Body weight for 5 days. Diabetes causes an increase in mean serum urea, creatinine, K^+ and Cl^- of diabetic control group compared to the normal control, while a significant decrease ($p<0.05$) was observed in mean serum of Na^+ and HCO_3^- in diabetic control group compared to the normal control. Hence, indicating kidney damage as a result of induction of diabetes, a finding supporting earlier reports by [24].

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Many studies have shown that diabetes mellitus is associated with increased formation of free radicals and decreased antioxidant potential, leading to oxidative damage of cell components [25]. The raised level of MDA with concomitant fall in level of SOD, CAT and GSHpx observed in diabetic control group support these reports. The reduced activities of anti-oxidant enzymes could be due to their depletion as a result of their involvement to tackle the free radicals produced as a result of alloxan administration [26]. Administration of diabetic rats with fraction VI lead to decrease in MDA and increase in level of SOD, GSHpx and CAT, suggesting that the fraction possess anti-oxidative activity and could scavenge excess free radicals generated by alloxan which may slow down the oxidative damage of the tissues and can increase a protective effect on improving diabetic complications. The exact mechanism by which the fraction increases these enzymes could be at molecular level by increasing the expression of messenger RNA of these enzymes contrary to what was obtainable in the diabetic condition as reported recently by Sindhu et al. [27].

Insulin, a peptide hormone that exerts a broad spectrum of anabolic effects is exclusively synthesized and secreted by pancreatic β – cells [28]. The regulation of whole body fuel homeostasis primarily involves insulin action in skeletal muscle, adipose tissue, and liver where insulin promotes uptake and storage of carbohydrate, fat, and amino acids, while at the same time antagonizing the catabolism of these fuel reserves [29]. The significant increase observed in serum insulin level of diabetic rats administered with fraction VI compared to diabetic control could be linked to high anti-oxidant activity of this fraction there by promoting the regeneration of pancreatic β-cells damaged by alloxan. Thus, stimulating the pancreas to produce more insulin by the damaged pancreas [30].

α-amylase is a key enzyme in the digestive system that catalyze the initial step in hydrolysis of starch to maltose and finally to glucose. Degradation of this dietary starch proceeds

rapidly and leads to elevated post prandial glucose. It has been shown that activity of human pancreatic α-amylase in the small intestine correlates to an increase in post-prandial glucose levels, the control of which is therefore an important aspect in the management of diabetes [31]. α-glucosidase, another important enzyme hydrolyses the terminal glycosidic bonds at the non-reducing end of saccharide polymers to release α-glucose. Much attention has been given to α-glucosidase in the pharmaceutical community because inhibition of its catalytic activity leads to impaired glucose absorption and a decrease in postprandial blood glucose levels [32]. The decrease in serum level of these enzymes observed in fraction VI administered groups compared to diabetic control group supports the observed restoration of pancreatic β cell function by the fraction as well as uptake of glucose by the peripheral tissues, thus reducing the rate of digestion of carbohydrates as well as postprandial blood glucose, probably by reducing expressing the carbohydrates digesting enzymes.

The above biochemical findings were further confirmed by histopathological studies of pancreas. The result of the histopathology showed that alloxan administration caused severe acute pancreatic damage in rats which is characterized by cell necrosis and inflammatory cell infiltration. After treatment with fraction VI pancreatic damage caused by alloxan administration was significantly ameliorated and the structure of pancreas was almost restored to normal.

The active fraction VI afforded one new compounds "methyl 6-ethenyl-7-hydroxy-7,8-dihydrophenanthrene-2-carboxylate" This is the first time a bioactive phenanthrene derivative "methyl 6-ethenyl-7-hydroxy-7,8-dihydrophenanthrene-2-carboxylate" possessing an anti-diabetic activity was isolated from stem bark of *Khaya senegalensis*. Apart from a report on the isolation of dimeric proanthocyanidins from the stem bark of the plant [33], almost all other studies on isolation and characterization of bioactive compound(s) from this plant reported mainly limonoids and Khayanolide [34,35].

5. CONCLUSION

The present study demonstrated that fraction VI obtained from column chromatography possesses significant anti-diabetic activity against alloxan induced diabetes mellitus which is mediated possibly through modulation of β-cell function and or lowering activities of carbohydrates digesting enzymes. Furthermore, the fraction was able to ameliorate some of the diabetes-associated complications and "methyl 6-ethenyl-7-hydroxy-7,8-dihydrophenanthrene-2-carboxylate" is a possible anti-diabetic agent in the fraction. Methyl 6-ethenyl-7-hydroxy-7,8-dihydrophenanthrene-2-carboxylate was established as content of the fraction VI using various spectroscopic techniques. Therefore, the study shows that there is a prospective future in the use of *Khaya senegalensis* stem bark as a source of natural medicine for management of diabetes mellitus.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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