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ANTIHYPERGLYCAEMIC ACTIVITY OF ETHANOLIC EXTRACT OF THE STEM OF *ADENIA LOBATA* ENGL (PASSIFLORACEAE)

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ABSTRACT: *Adenia lobata* Engl (Passifloraceae) is a woody climber which grows in most African countries mainly the coastal belt. It is an important medicinal plant used to treat haemorrhoids, malaria, fever, diabetes and gonorrhea. In our attempt to find out the constituents of this medicinal plant, the dried stem powder of *A. lobata* was successively extracted by Soxhlet with petroleum ether and 70% ethanol to obtain the crude petroleum ether (PEAL: yield =1.1w/w %) and ethanol (EEAL: yield = 5.4 w/w %) extracts. The antihyperglycaemic activity of PEAL and EEAL were determined in streptozotocin-induced diabetic rats (70 mg/kg body weight). The ethanol extract was most active and was subjected to chromatographic separation to isolate its chemical constituents. The isolated compounds were identified using NMR spectroscopic namely ^{1}H, ^{13}C NMR, COSY, HSQC and HMBC Spectroscopy with reference to literature. Two compounds isolated from the extract were found to be, palmitic acid and γ-hydroxy-δ-valerolactone. To the best of our knowledge, this is the first time these compounds are reported from this medicinal plant.

INTRODUCTION: Diabetes mellitus is a chronic metabolic disorder in which the pancreas does not produce enough insulin to meet the body's needs or the body cannot properly use the insulin that is produced [1].

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In Ghana, the prevalence rate is estimated to be 6.3%, with women at higher risk of having the disease [2] whereas worldwide projection is estimated to be 6.0% [3-4]. The cost of controlling a diabetic case in Ghana annually has increased from $180-420 in 2001 to $1276-7660 in 2007 [5] and the economic cost of controlling diabetes using orthodox drugs in the world is estimated to be $232 billion and will cost $302.5 billion by 2025 [3-4]. Diabetes has become a public health problem globally and most of these medicinal plants used in the treatment of this disease in traditional medicine have no scientific documented information.

It is therefore imperative to investigate such medicinal plants used in traditional treatment of various diseases [6]. These medicinal plants which show promising activity can then be standardised and formulated. This will facilitate the promotion of rational use of quality, safe and efficacious traditional remedies. This work seeks to provide the scientific knowledge of antihyperglycaemic activity on the stems of *A. lobata* used in the traditional treatment of diabetes, fever and malaria [7].

MATERIALS AND METHODS:

Plant material: The stem of *Adenia lobata* Engl was collected from Bobiri forest in December 2009 by Mr Osafo Asare, a herbalist. Authentication was done at the Department of Botany, University of Ghana, Legon by Mr. Amponsah. Voucher specimen (KNUST/HM1/2010/S-005) has been deposited at the Faculty of Pharmacy and Pharmaceutical Science Herbarium.

Extraction: The stem of *A. lobata* was air-dried for ten days at room temperature and milled into a coarse powder. The powder (650.0 g) was Soxhlet extracted sequentially using petroleum ether (PEAL) and 70% ethanol (EEAL). The extracts were concentrated to viscous liquids under reduced pressure using a rotary evaporator. The viscous liquids were further evaporated to solvent-free, semi-solid masses over water bath (yield; PEAL: 1.1 $^{w}/_{w}$ %, EEAL: 5.4 $^{w}/_{w}$ %) and kept in a desiccator until needed for use. Large scale Soxhlet extraction by 70% $^{v}/_{v}$ ethanol was done for 3 kg of the powder.

Phytochemical Screening: The preliminary phytochemical evaluation of part of powdered stem of *A. lobata* was done using standard methods as described by [8].

Animals: Adult male sprague-dowley rats (280-295 g) were housed at a standard condition of room temperature and supplied with standard pellet food with tap water *ad libitum* in animal house at Department of Pharmacology. The rats were obtained from the Centre for Scientific Research into Plant Medicine at Mampong-Akwapim, Ghana. All rats were treated in accordance with the National Institute of Health Guidelines for the care and use of laboratory animals [9].

The research protocol was approved by the College of Health Sciences Ethics Committee.

Induction of diabetes in rats using streptozotocin (STZ): The bioassay model employed was according to the description of [10] with some modification. The normal blood glucose level of the rats was taken and was found to be in the range of (3.8-4.9 mmol/l). Rats were injected intraperitoneally with a single dose of 70 mg STZ per kg body weight after an overnight fast. After injection, rats were given free access to feed and water. After a rest period of 48 hours, diabetes was confirmed by determing the fasting blood glucose levels. Rats with blood glucose level above 10.0 mmol/l were selected for the experiment.

Assessment of antihyperglycaemic activities of *A. lobata* extracts on diabetic rats: The diabetic rats were randomly divided into five groups of five animals each; Groups A, B, C, D and E. Groups A, B and C were given oral doses of 150, 300 and 600 mg/kg body weight of EEAL respectively. Group D was given an oral dose of 5 mg/kg body weight of glibenclamide, a standard hypoglycaemic agent, as positive control and Group E was given 5 ml/kg body weight distilled water as normal control.

Fractionation of EEAL: Ethanol extract of *Adenia lobata*, EEAL (54.0 g) was flash chromatographed over silica gel (60-120 mesh) using petroleum ether (40-60 ^{o}C), petroleum ether – ethylacetate mixtures, ethylacetate and ethylacetate – ethanol mixtures.

Aliquots (500 ml) were collected at a time, and 28 fractions were collected. They were bulked into two fractions I (19.6 g) and II (24.3 g) as shown in **Fig. 1**, after monitoring the chemical profiles of the 28 fractions with analytical tlc.

Fraction I was further column chromatographed using petroleum ether and ethylacetate mixtures (85:15, 75:25, 60:40, 50:50, 30:70 and 10:90) as eluting solvents and silica gel (60-120 mesh) as stationery phase. In all, 40 fractions of 10 ml aliquots were collected. Four sub-fractions A (7.00 g), B (3.30 g), C (1.80 g) and D (1.42 g) were obtained after monitoring the chemical profiles of the 40 fractions with tlc analysis. Fraction A was further column chromatographed over silica gel using petroleum ether and ethylacetate mixtures in the ratios (80:20, 70:30). This led to isolation of compound AL 1 (512 mg) which appeared as single blue spot on tlc analysis. The R_f value for AL 1 was 0.82 in petroleum ether: ethylacetate (1:4).

Fraction II was column chromatographed using petroleum ether and ethylacetate (30:70, 25:75, 15:85, 10:90) followed by ethylacetate and ethanol (90:10, 70:30, 50:50) as eluting solvents and silica gel (60-120 mesh) as stationery phase. In all 50 fractions of 25 ml aliquots were collected. They were bulked to five sub-fractions A1 (2.70 g), B1 (8.45 g), C1 (1.93g), D1 (4.25 g) and E1 (3.62 g) based on the tlc analysis as described above.

B1 was further column chromatographed using petroleum ether and ethyl acetate (20:80). This led to the isolation of compound AL 2 (810 mg) which appeared as single pink spot. The R_f value for AL 2 was 0.64 in petroleum ether: ethylacetate: methanol (0.5: 4.5: 0.5). Schematic representation of the fractionation of the stem of *A. lobata* is shown in Figure 1.

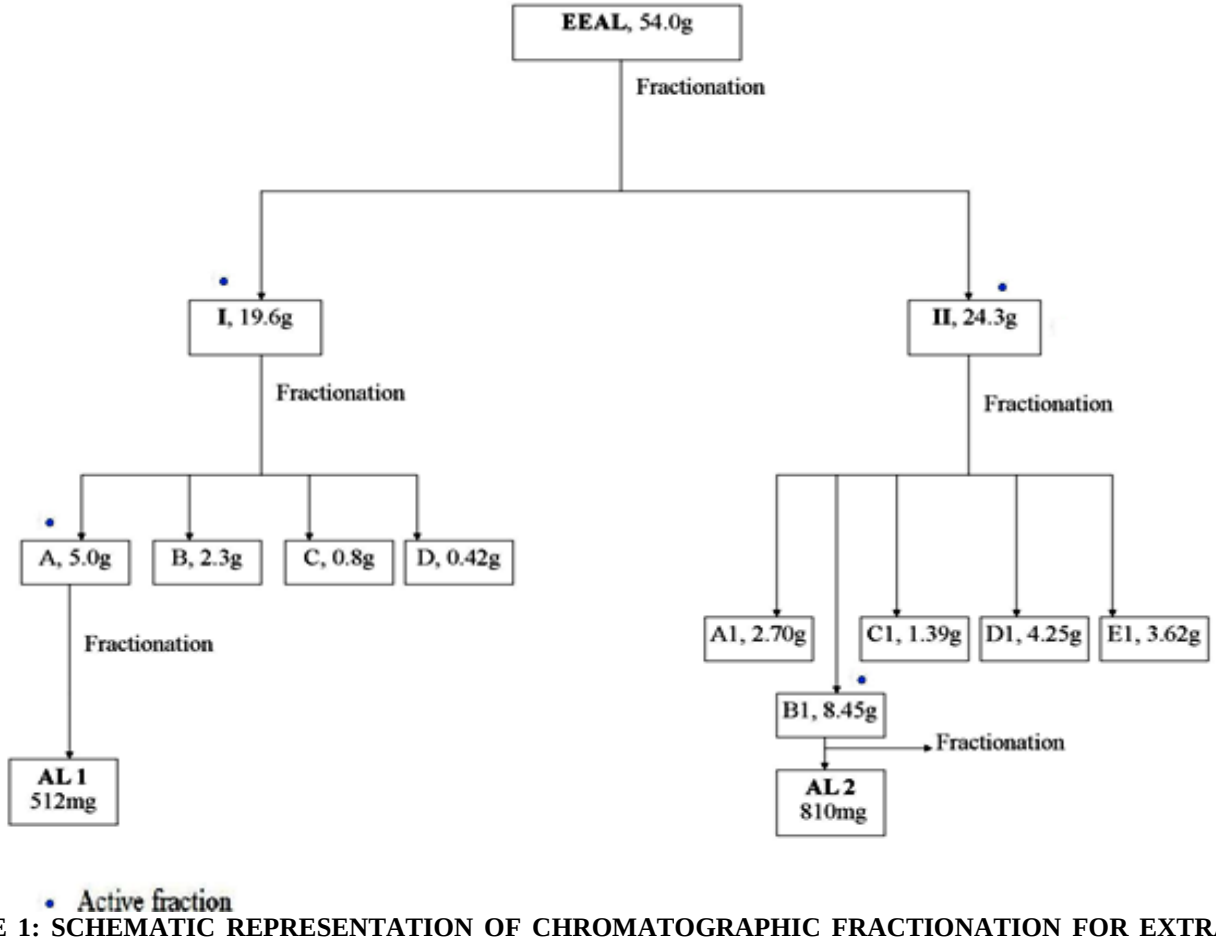

FIGURE 1: SCHEMATIC REPRESENTATION OF CHROMATOGRAPHIC FRACTIONATION FOR EXTRACTING THE POWDERED STEM OF *A. LOBATA*

Assessment of palmitic acid (AL 1) on STZ diabetic rats: A set of diabetic rats were grouped into five groups of five animals. AL 1 was suspended in 2% tragacanth solution. Groups A, B and C were given oral doses of 30, 60 and 180 mg/kg body weight of AL 1 respectively. Group D was given an oral dose of 5 mg/kg body weight of the reference drug, glibenclamide as positive control. Group E, the normal control group was given an oral dose of 5 ml/kg body weight of distilled water.

Blood glucose measurement: Blood glucose levels were initially monitored hourly over six hours after drug administration starting at (0 hour) to see the immediate effect.

The glucose level was subsequently monitored at three days interval basis over the next 24 days. Blood samples were collected from tail vein of the rats and Accu-Chek® glucometer (Roche Diagnostics GmbH, Mannheim Germany) was used to measure the glucose level. Throughout the study period, rats received unrestricted access to standard feed and water.

Statistical Analysis: All the data provided in this study represents means ± S.E.M. The results were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test to establish significance ($p < 0.001$) between the treated and the control groups.

RESULTS AND DISCUSSION:

Phytochemical Screening: The results of the phytochemical screening on the powdered *A. lobata* are shown in **Table 1**.

TABLE 1: RESULTS OF THE PHYTOCHEMICAL SCREENING OF POWDERED *A. LOBATA*

Plant secondary metabolites	*Adenia lobata*
Reducing sugars	+
Steroids	+
Phenolic compounds	+
Alkaloids	-

+ = present; - = absent

Assessment of Antihyperglycaemic activity on diabetic rats: The blood glucose levels of the untreated diabetic rats (diabetic control) after 6 hours increased by 16.4 % (**Table 2**). The blood glucose levels of diabetic rats treated with EEAL at doses of 150, 300 and 600 mg/kg, had their blood glucose levels lowered by 36.7, 41.1 and 51.7 % respectively within the same period. Glibenclamide, the standard antidiabetic drug (5 mg/kg) lowered the blood glucose levels by 54.7 %. The extracts at the selected doses and glibenclamide showed significant antihyperglycaemic activities ($p < 0.001$) as compared to the diabetic control group 6 hours after treatment. On the 24th day, untreated diabetic (diabetic control) rats had 34.0 % increase in blood sugar level. EEAL at 150, 300, and 600 mg/kg body weight decreased the blood glucose levels by 71.4, 78.2 and 82.8 % respectively (**Table 3**). Glibenclamide (5 mg/kg body weight) lowered the blood glucose by 79.7 %. The antihyperglycaemic activity of EEAL at 600 mg/kg body weight was comparable to that of the standard drug, glibenclamide. The differences in activities of the extracts may be due to the type of constituents that are present in the extracts. EEAL having been obtained using a relatively polar solvent principally contained polar constituents.

TABLE 2: EFFECT OF EEAL IN STREPTOZOTOCIN-INDUCED DIABETIC RATS AFTER 6 HOURS

TREATMENT AND DOSE	BLOOD GLUCOSE LEVEL (mmol/l)						
	TIME (hours)						
	0	1	2	3	4	5	6
EEAL (150 mg/kg)	25.5±1.5	21.4±1.0	19.6±1.4	18.2±1.0	16.9±1.0	16.4±1.0	15.9±1.1 (36.7)
EEAL (300 mg/kg)	27.5±1.2	22.9±1.1	20.6±0.8	19.4±1.2	18.9±1.3	17.6±0.7	16.2±0.5 (41.1)
EEAL (600 mg/kg)	30.2±0.7	21.5±1.9	19.5±1.4	17.8±0.9	16.8±0.7	16.2±0.7	14.6±0.6 (51.7)
GB (5 mg/kg)	23.2±3.0	18.7±2.4	15.9±2.1	13.3±2.2	12.7±2.1	12.1±2.0	10.5±1.5 (54.7)
Control (5 ml/kg)	24.4±0.9	25.6±1.4	25.3±1.7	26.7±0.8	27.3±1.1	28.0±0.9	28.4±1.0 (-16.4)

All values are expressed as mean ± S.E.M. (N = 5); Number in the parentheses denotes percentage reduction (blood glucose level) after 6 hours; EEAL = 70% Ethanol extract of the stem bark of *A. lobata*; GB = Glibenclamide ; EEAL treated groups were compared with control group ($p < 0.001$)

TABLE 3 EFFECT OF EEAL IN STREPTOZOTOCIN-INDUCED DIABETIC RATS AFTER 24 DAYS

TIME (days)	TREATMENT AND DOSE				
	EEAL (150 mg/kg)	EEAL (300 mg/kg)	EEAL (600 mg/kg)	GB (5 mg/kg)	Control (5 ml/kg)
	Blood	Glucose	Level	(mmol/l)	
0.0	25.5±1.5	27.5±1.2	30.2±0.7	23.2±3.0	24.4±0.9
3.0	15.1±1.2	13.0±1.5	12.2±1.1	14.7±0.2	31.8±0.4
6.0	13.5±1.1	11.6±1.5	10.7±0.9	12.5±0.4	31.9±0.4
9.0	12.0±1.0	10.5±1.2	9.6±0.7	10.4±0.3	32.3±0.3
12.0	11.0±0.9	9.1±1.1	8.5±0.3	9.7±0.3	32.2±0.3
15.0	9.8±0.6	7.8±0.8	7.2±0.1	7.6±0.2	32.4±0.4
18.0	9.3±0.5	7.6±0.7	6.3±0.1	7.5±0.2	32.1±0.3
21.0	7.9±0.4	7.1±0.7	5.9±0.1	6.1±0.2	32.3±0.3
24.0	7.3±0.2	6.0±0.4	5.2±0.1	4.7±0.4	32.7±0.2
	(71.4)	(78.2)	(82.8)	(79.7)	(-34.0)

All values are expressed as mean ± S.E.M. (N=5); Number in the parentheses denotes percentage reduction (blood glucose level) after 24 days; EEAL = 70% ethanol extract of the stem bark of *A. lobata;* GB = Glibenclamide ; EEAL treated groups were compared with control group ($p < 0.001$).

Characterisation of AL 1 and AL 2 isolated from EEAL extract: AL 1 (512 mg) was isolated as a white crystalline compound. It was insoluble in water and had a melting point of 64-65ºC compared to the literature value of 62.9ºC [11]. It gave a blue spot on a silica gel chromatogram when sprayed with anisaldehyde and heated at 110ºC for five minutes. It had an R_f value of 0.82 in pet-ether: EtOAc (1:4).

The ^{1}H NMR spectrum (**Figure 2**) revealed the presence of a methyl triplet at δ_H 0.88 (representing a terminal methyl group), a multiplet at δ_H 1.25 representing an envelope of twelve methylene groups, a multiplet at δ_H 1.63 representing a methylene group and a triplet at δ_H 2.34 representing a deshielded methylene group.

The ^{13}C NMR spectrum (**Figure 3**), showed sixteen carbon atoms including a carbonyl carbon (δc 180.37), two deshielded methylene carbons (δc 34.22, δc 32.07), twelve methylene carbons (δc 22.83- δc 29.84) and a methyl carbon (δc 14.24). These were confirmed in the ^{13}C NMR DEPT 135 spectrum (**Figure 4**).

Using COSY, HSQC and HMBC spectra, partial structure of AL 1 was formed. However, the ^{1}H and ^{13}C data of AL 1 favorably compared with those reported by [12]. This led to the proposed structure of AL 1 as palmitic acid, which was confirmed with an authentic sample by co-tlc chromatography.

AL 2 (810 mg) was isolated from fraction II as white amorphous compound. It was soluble in chloroform and methanol, and gave an R_f value of 0.64 in pet-ether: EtOAc: MeOH (0.5:4.5:0.5) and pink spot when sprayed with anisaldehyde and heated at 110 ºC for five minutes. It melted at 35-37 ºC compared to the literature value of 31.0 ºC [13].

The structure was established as the known γ-hydroxy-δ-valerolactone, by examining the ^{1}H and ^{13}C NMR spectrum and other 2D-NMR spectra such as COSY, HSQC-DEPT, NOESY and HMBC.

The ^{1}H NMR-spectrum (Figure 5) revealed the presence of deshielded proton multiplet δ_H 4.63 (H-3A), two geminal proton doublets at δ_H 3.66 (H-2B_1) and δ_H 3.91 (H-2B_2). It further showed four methylene proton multiplets at δ_H 2.57 (H-4D_1 and H-4D_2) and δ_H 2.15 (H-5E_1 and H-5E_2).

The ^{13}C NMR spectrum (Figure 6), showed five carbon atoms representing a carbonyl (δc 177.49), two oxygenated carbons (δc 64.36, δc 80.66) and two methylenes (δc 23.30, δc 28.82).

The HSQC, COSY and HMBC spectra led to the proposed structure AL 2, which favourably compared with those reported by [14].

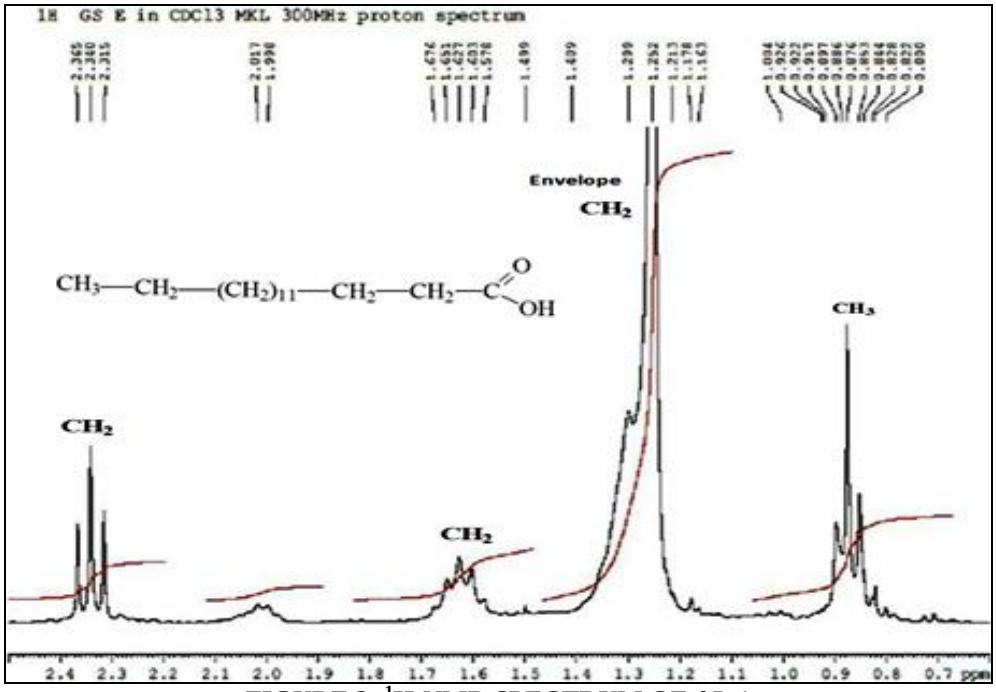

FIGURE 2: ^{1}H-NMR SPECTRUM OF AL 1

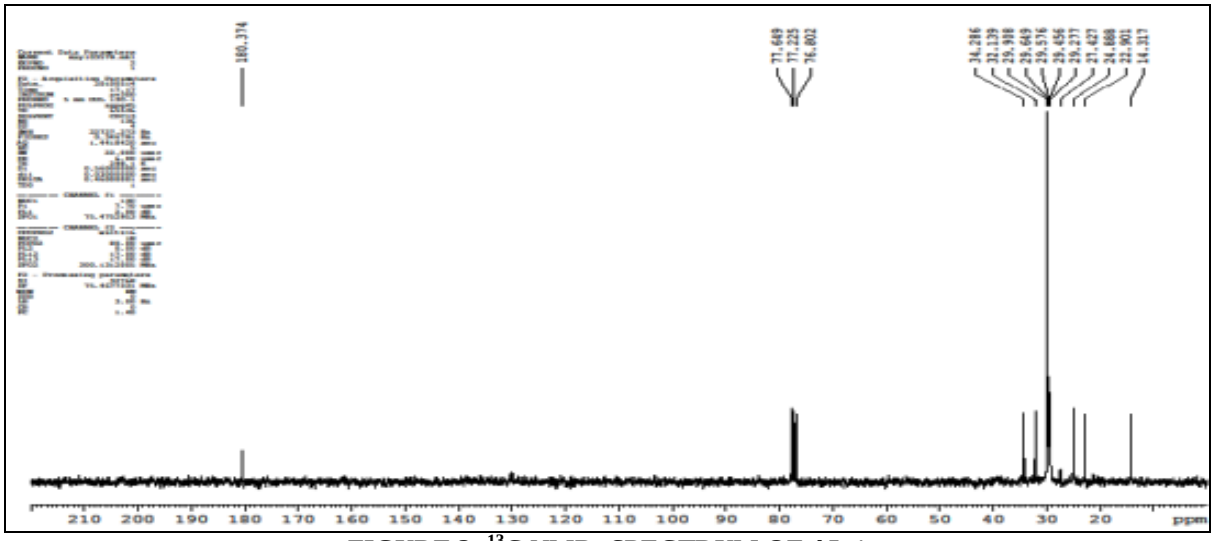

FIGURE 3: ^{13}C NMR- SPECTRUM OF AL 1

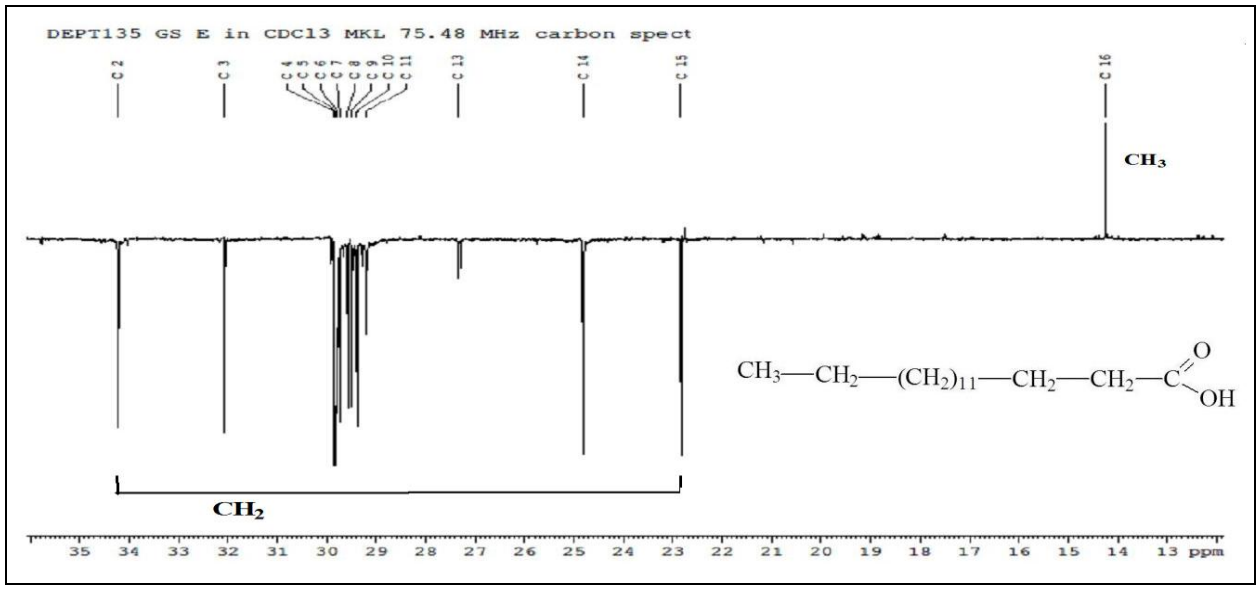

FIGURE 4: DEPT 135 SPECTRUM OF AL 1

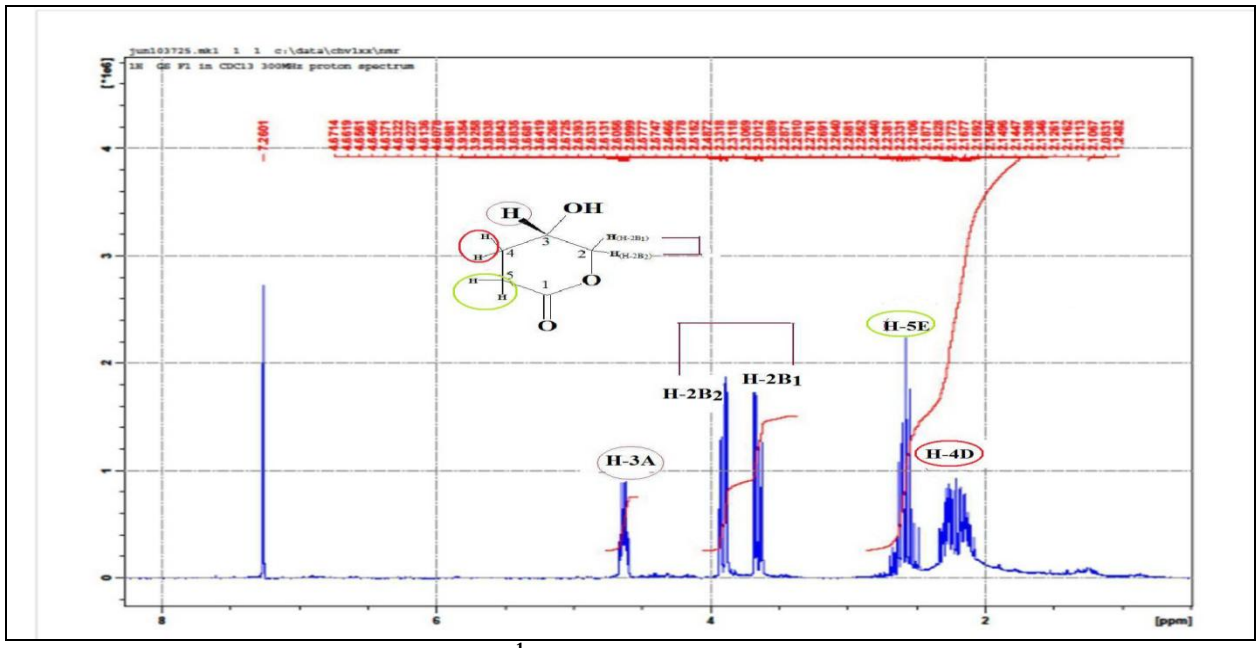

FIGURE 5: ^{1}H-NMR SPECTRUM OF AL 2

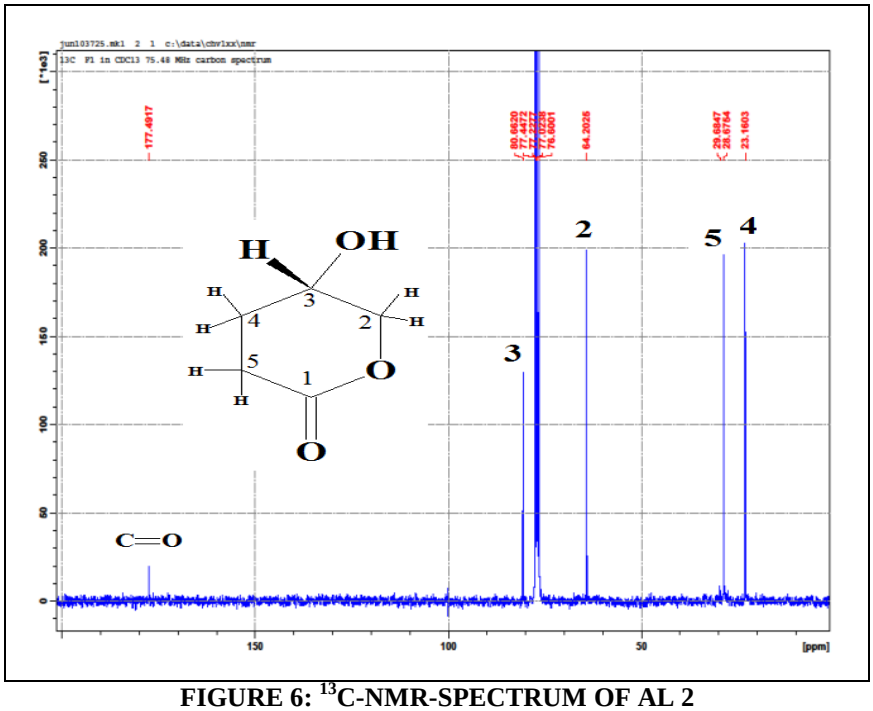

FIGURE 6: ^{13}C-NMR-SPECTRUM OF AL 2

Assessment of antihyperglycaemic activity of palmitic acid (AL 1) on STZ diabetic rat: Palmitic acid (AL 1) was isolated for the first time from *A. lobata* extracts and caused dose-dependent reduction in blood glucose levels of the diabetic rats. At maximum dose of 180 mg/kg body weight, AL 1 caused 37.4% reduction of the blood glucose level in the diabetic rats (**Table 4**). Glibenclamide (5 mg/kg) caused 39.3% reduction whereas negative control had 18.9% increased in blood glucose level of the diabetic rats.

TABLE 4: EFFECT OF AL 1 IN STREPTOZOTOCIN-INDUCED DIABETIC RATS AFTER 6 HOURS

TREATMENT AND DOSE	BLOOD GLUCOSE LEVEL (mmol/l)						
	TIME (hours)						
	0	1	2	3	4	5	6
AL 1 (30 mg/kg)	24.8±2.2	22.1±3.0	21.8±3.0	21.4±3.0	20.9±3.0	20.3±3.0	19.8±3.1 (20.1)
AL 1 (60 mg/kg)	25.2±0.8	21.5±1.9	20.9±1.9	20.6±1.8	20.1±1.7	19.5±1.8	18.9±1.7 (25.0)
AL 1 (180 mg/kg)	28.9±1.4	23.2±1.9	21.9±2.0	21.1±2.0	20.0±1.8	18.8±1.9	18.1±1.8 (37.4)
GB (5 mg/kg)	22.9±3.0	20.4±2.7	18.5±2.1	17.8±2.2	16.6±2.4	14.5±1.9	13.9±1.7 (39.3)
Control (5 ml/kg)	22.7±3.1	23.6±2.9	24.0±3.0	26.2±2.5	26.9±1.9	27.1±2.1	27.0±1.9 (-18.9)

All values are expressed as mean ± S.E.M. (N=5); Number in the parentheses denotes percentage reduction (blood glucose level) after 6 hours; Compound **AL 1** isolated from the stem of *A. lobata*; GB = Glibenclamide; **AL 1** treated groups were compared with control group ($p < 0.001$)

CONCLUSION: This study has revealed that the ethanol extracts of *A. lobata* and the isolate palmitic acid exhibited promising antihyperglycaemic activity in streptozotocin-induced diabetic rats. This research therefore provides support to the use of *A. lobata* by indigenous people in the management of diabetes in Ghana.

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