Bioassay-Guided Isolation and Structural Elucidation of Antidiabetic Principle of Methanol Leaf Extract of *Newbouldia laevis* (P.Beauv)

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Abstract: NLE (*Newbouldia laevis* (P.Beauv)) is used in folk medicine to treat diabetes. The aim of the study was to isolate and characterize the active compound responsible for the antidiabetic activity. This was carried out using standard *in vivo* and *in vitro* models in rats. The antidiabetic activities were evaluated using alloxan-induced diabetes in male and female albino rats after an overnight fast and various doses of NLE and glibenclamide, the reference drug 2.0 mg/kg. Bioassay-guided isolation/fractionation techniques were used to isolate the active compound. Characterization of the active compound was carried out including molecular and structural elucidation using NMR (nuclear magnetic resonance) and Gas-Chromatography Mass spectroscopy. The extract caused 60.2% reduction in the FBS (fasting blood sugar) of diabetic rats. Bioassay-guided fractionation of NLE yielded ten (10) fractions with F9 (fraction nine) as the active fraction, which caused 66.0% reduction of FBS in alloxan-induced diabetic rats. Further purification using preparative TLC (thin layer chromatography), gave sub-fraction 9.2 as the active compound. Sub-fraction 9.2 reduced the FBS by 61.4%. The characterization of F9:2 using nuclear magnetic resonance and MS (mass spectroscopy) confirmed it to be a polyunsaturated fatty acid with a molecular weight of 358.56 g.

Key words: Antidiabetic principle, bioassay, elucidation, isolation, methanol, *Newbouldia laevis*.

1. Introduction

Apart from being the primary source of food for animals including humans, a great proportion of plant species have shown over times to have wonderful medicinal values [1, 2]. Some of the plants have shown to have some antidiabetic effects over the years [1, 3, 4]. *Newbouldia laevis* (P. Beauv) Seemann ex. Bureau is one of such plants. It is a tree of the family Bignoniaceae in the order Bignoniae. It is a genus of one species. It is a medium size angiosperm tree that grows up to 7-8 m. It can also be a shrub of about 3 m and widely distributed in the tropics. It is shrubby or erect with vertically ascending branches. The leaves are oblanceolate to broadly elliptic with serrated or entire margins. The flowers are pink or whitish. The

plant originates from tropical Africa and extends from Senegal to Zaire (Congo Democratic Republic). It is usually found in old and new settlements where it is being planted to demarcate boundaries of plots of land hence the name border or boundary tree [5-7]. It is also known by various ethnic names in various places such as “Aduruku” or “Bareshi” in Hausa, “Ogirisi” in Igbo, “Akoko” in Yoruba, “Kontor” in Tiv, “Ikhimi” in Effik, “Oboti” in Edo. In Ivory Coast it is called “Sokunde” by the Fula-Fulfulde and it is known as “Sesemasa” by the Akan-Asante in Ghana [5-9]. *Newbouldia laevis* is used in folkloric medicine to treat a number of diseases. Some of which include the following: the leaves and roots are boiled and used to treat earaches, sore foot, chest pain, fever, convulsion and epilepsy in children [6, 10], diarrhoea [11]. The roots are used to treat arthritis, malaria and general malady and worms [7]. The leaves are used as decoction for eye wash in

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conjunctivitis. The leaves are also used as chieftaincy leaf in Yoruba land [7]. The stem bark is used for toothache, febrifuge, stomach and skin infections [6, 7]. Recently, the flowers and leaves have been used in the treatment of diabetes [10] and [11-13] respectively. It is also used to stop vaginal bleeding in threatened abortion [7] and had shown strong antioxidant activity [8]. The aim of the study was to isolate and characterize the active compound responsible for the antidiabetic activity. This becomes imperative because there is paucity of information on bioassay-guided isolation of African plants constituents. Most of the research deals purely with phytochemicals [2]. This was carried out using standard *in vivo* and *in vitro* models in rats.

2. Materials and Methods

The animals used for this experiment were albino wistar rats of both sexes. They were obtained from the animal breeding unit of the College of Health Sciences, Benue State University Makurdi, Benue State Nigeria. They were housed in steel cages in houses with ambient temperature of 27-35°C and a lighting period of about 12 hours day and night and a relative humidity of 40-60 %. They were supplied with clean drinking tap water and fed *ad-libitum* with standard commercial pelleted feed (Vital feed®, Nigeria) except when their fasting blood sugar was required. All ethical conditions governing the use of animals for experiments were observed as stipulated [14-16]. The male and female rats were kept in separate cages during the period of the experiment. One kilogramme (1 kg) of the leaves of *Newbouldia laevis* “Aduruku” “Ogirisi” “Akoko” or “Kontor” was collected in Makurdi, Benue State after the plant was identified by Mr. Terry Waya of Botany Department, Benue State University, Makurdi and a voucher specimen UAM/FHM/205 was deposited in the herbarium of forestry department of the University of Agriculture Makurdi. The leaves were grinded in a mill in the Department of Animal Science University of Agriculture, Makurdi and extracted by cold maceration using hydromethanolic (80% methanol 20% water)

solution as used by the traditional practitioners. Rotary evaporator was used to concentrate and dry the extract and the percentage yield was calculated.

The selected albino rats were fasted between 15-18 hours. Their fasting blood sugar levels were determined with the blood from their tail vein using autoanalyzer (Accu-Check Advantage II® glucose kits). Diabetes was induced by single intraperitoneal administration of alloxan monohydrate at 150 mg/kg body weight as described by Szudelski [17]. The FBS (fasting blood sugar) of the rats was monitored every other day until diabetes was established about the sixth day. Rats with FBS ≥ 7.0 mMol/L were considered diabetic [3, 18] and were selected for subsequent experiments.

2.1 Effect of the Crude Extract of NLE on Alloxan-Induced Diabetes in Rats.

In this experiment, twenty-five (25) alloxan-induced diabetic rats were used. The rats were randomly divided into 5 groups of 5 rats per group. Their initial fasting blood sugar was measured at 0 h after which they were treated as follows. Group 1 rats received 10 ml/kg body weight of distilled water and served as negative control. Group 2 rats received 2 mg/kg body weight of glibenclamide, a standard antidiabetic drug and served as positive control while groups 3-5 rats served as NLE treatment groups and were given 62.5, 125.0 and 250.0 mg/kg of the extract respectively. All the treatments were given orally through intragastric tube. The FBS of the rats were measured at 0, 1, 3, 6 and 24 hours post drug/extract administration using an autoanalyzer (Accu-Check Advantage II® kits). The blood used for FBS measurement was collected from the tail vein. The percentage reduction in the FBS at each time was calculated.

2.2 Bioassay Guided Fractionation/Determination of Active Compound

Column chromatography was carried out as described by Harbourn [19] using Silica gel (F₂₅₄)

mesh 200 as the stationary phase while the gradient solvent system comprising hexane, chloroform, ethylacetate and methanol was used as the mobile phase. The column was allowed 24 hours to stabilize. Ten (10 g) of the NLE was introduced into the column and eluted at the rate of 45 drops per minute and were collected in test tubes. The content of each test tube eluted from the Column Chromatography was spotted on a precoated silica gel GF₂₅₄ plate and was eluted with the solvent system : chloroform : ethylacetate : methanol (3:2:1) as described by Stahl [20]. Based on their relative mobilities and colour reactions with the ultra-violet light the individual eluents were identified, pooled into different fractions and concentrated using rotary evaporator at 40°C and 210 milibar. Their yields were determined as well as their resolution fronts (R_f) by spotting them on TLC plates. The R_f values were calculated using the following formula.

$$R_f = \frac{\text{Distance travelled by spot from starting point}}{\text{Distance travelled by solvent front}}$$

The fractions were stored in a refrigerator at 4 °C until needed.

2.3 Screening of Fractions of NLE for Bioactivity/Selection of Active Compound

Thirty six rats were used in this experiment. Diabetes was induced as described earlier and the rats were randomly divided into 12 groups of 3 rats per group. They were fasted for 15 hours and treated as follows;

Group 1 served as negative control and received 10 ml/kg distilled water. Group 2 served as positive control and received 2 mg/kg glibenclamide while groups 3-12 received 50 mg/kg of the various fractions of NLE (Fractions 1-10) respectively through intragastric tube orally. The FBS of the rats were measured at 0, 1, 3, 6 and 24 hours after treatment using Accu-Check Advantage II® kits. The percentage reduction for each fraction was calculated and the fraction with the highest percentage reduction of blood glucose level was selected as the active fraction for

further work.

2.4 Purification of the Active Fraction/Isolation of Pure Compound

This was carried out through preparative thin-layer chromatography as described by Stahl [20]. The prepared plates were activated in the oven at a temperature of 110 °C for 1 hour before they were used. The active fraction was dissolved in methanol and a modified capillary tube was used to streak the solution on the activated plate from one side to the other in a straight line band form and allowed to dry on the plate. Meanwhile a standard chromatographic tank that can contain the 20 x 20 cm plates was saturated with predetermined solvent system (Chloroform : Ethylacetate : Methanol: Formic acid in the ratio: 3:1:2:0.05). Two plates were introduced into the tank at the same time inclined at an angle of 30° from the edge of the tank. The eluting solvent was allowed to run a distance of ≥ 15 cm starting from the streaked end after which the plates were removed and dried. Each plate was observed under ultra-violet lamp at 254 and 365 nM wavelength in a dark corner and the separated zones or bands were marked with a pin. This process was repeated several times on many plates.

The separated zones or bands were scraped into different centrifuge tubes with the aid of a spatula. They were dissolved in methanol and centrifuged at 3000 rpm for 10 minutes. This was to ensure proper separation of the eluent from the adsorbent (silica gel). The collected eluent in methanol was pooled for a particular band (based on R_f and colour reaction) and evaporated to dryness using a hot air oven at 40°C. The resulting compound was weighed, labeled, sealed and stored in the refrigerator until when needed.

2.5 Determination of the Active Fraction

Twenty-four (24) albino diabetic rats were used. Diabetes was induced as earlier described. The rats were randomly divided in 8 groups of 3 rats each and treated as follows: group 1 was given 10 ml distilled

water/kg, group 2 received 2 mg/kg of glibenclamide while groups 3-8 received 5 mg/kg of the compounds from the active fraction (F9: 1-6). The FBS of the rats were measured at 0, 1, 3, 6 and 24 hours. The compound with the highest antihyperglycaemic activity was selected as the active compound.

2.6 Thin-Layer Chromatography of the Active Fraction

Analytical TLC was carried out on the active compound using precoated silica gel (G_{f254}) and sprayed with vanillin-sulphuric acid prepared by dissolving 0.16 g of vanillin in 30 ml of concentrated tetraoxosulphate (VI) acid (H_2SO_4) in an atomizer. The plate was viewed under the UV (ultraviolet) lamp at 254 and 365 nm wavelengths. The colour reaction was observed and R_f value calculated using the formula described earlier. This test is usually carried out to check those compounds that may not be visualized due to lack of noticeable colour reaction on the TLC plate.

2.7 Characterization and Structural Elucidation of the Pure Compound Using NMR (Nuclear Magnetic Resonance) and Mass Spectroscopy

Structural elucidation and characterization of the pure active compound was carried out using ^{13}C (thirteen carbon) and hydrogen or proton (1H) NMR (nuclear magnetic resonance) and GC-MS (gas chromatography-mass spectroscopy) at the SIPBS (strathclyde institute of pharmacy and biomedical sciences) Laboratory, University of Strathclyde, Glasgow, United Kingdom. Before running the NMR, deuterated acetone was used to check the purity of the sample or to identify the type of sample. Later the sample was dissolved in DMSO (dimethylsulfoxide) before running the NMR. This enables the instrument to achieve field frequency stabilization. The dissolved sample was then transferred to the NMR tube and then run with a Bruker TopSpin 450 Hz programme. ^{13}C NMR was used to obtain information on the number of carbon atoms in the molecule and 1H NMR

spectroscopy for information on the number of protons. There after GC-MS was used to obtain detailed information on bound connectivity and assignments of chemical shifts from which the structure of the pure compound was elucidated [21]. The initial temperature of the GC-MS was 120 °C for 2 min which was programmed at 5 °C/min up to 200 °C for ramp 1 and 280 °C for ramp 2 with a holding time of 5 and 9 min respectively using electrospray and m/z positive mode.

2.8 Data Analysis

The data obtained were presented as mean $\pm$ SEM (standard error of mean). One way ANOVA (analysis of variance) was carried out using SPSS 16 and LSD (least square difference) post hoc test. The level of significance was accepted at 0.05%. Results were presented in tables, figures and plates.

3. Results

3.1 The Effect of Crude Extract of NLE on FBS Level of Alloxan- Induced Diabetic Rats

The effect of graded-doses of NLE on the FBS of alloxan-induced hyperglycaemic rats after 24 hours is presented in Table 1. The result indicated that there was an increase in the mean FBS of rats in the negative control group from 25.3 ± 4.12 mMol/l to 28.3 ± 2.45 mMol/l from the 0 to 24 hours respectively representing 11.9 % increase. The various doses of the extract and the reference drug (glibenclamide) produced significant ($P < 0.01-0.001$) dose and time-dependent decreases in FBS levels of treated rats when compared to the negative control especially at the 6th and 24th hours. The extract doses of 62.5, 125.0 and 250.0 mg/kg induced a decrease in FBS of the rats by 24%, 25% and 33.6% at the 6th hour and 27.8% 55.3% and 60.2% at the 24th hour respectively as against 40% and 51.5% reduction of the FBS in rats by the reference drug at the same time frame of 6th and 24th hours respectively. The highest FBS reductions by all doses of the extract and the reference drug (glibenclamide) were

Table 1 Graded dose response effect of NLE on FBS of alloxan-induced hyperglycaemic rats.

Group	Treatment	Mean fasting blood glucose levels $\pm$ SEM (mMol/L)								
		0h	1h	% Reduction	3h	% Reduction	6h	% Reduction	24h	% Reduction
1	Distilled water (10ml/kg)	25.3 $\pm$ 4.12	25.8 $\pm$ 3.80	2.0 $\uparrow$	25.9 $\pm$ 4.05	2.4 $\uparrow$	26.3 $\pm$ 3.5	4 $\uparrow$	28.3 $\pm$ 2.49	11 $\uparrow$
2	Glibenclamide (2mg/kg)	33.4 $\pm$ 4.02	28.5 $\pm$ 3.18	14.7	20.9 $\pm$ 3.12*	37	20.0 $\pm$ 1.4*	40	16.2 $\pm$ 1.40*	51.5
3	NLE (62.5mg/kg)	33.4 $\pm$ 1.22	30.5 $\pm$ 2.75	8.7	31.5 $\pm$ 2.7	5.7	25.3 $\pm$ 1.8	24	24.1 $\pm$ 1.55	27.8
4	NLE (125mg/kg)	29.5 $\pm$ 1.48	26.2 $\pm$ 2.31	11.1	25.3 $\pm$ 1.7	14.0	22.0 $\pm$ 1.6	25	13.2 $\pm$ 1.42**	55.3
5	NLE (250mg/kg)	22.6 $\pm$ 2.02	28.5 $\pm$ 2.62	26 $\uparrow$	22.0 $\pm$ 3.15	2.7	15.0 $\pm$ 2.11**	33.6	9.0 $\pm$ 2.25***	60.2***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared to negative control.

$\uparrow$ represents increase in blood sugar level.

Table 2 The yields and RF (resolution fronts) values of the different NLE fractions.

	Yields (g)	RF Values
1	0.05	0.014
2	1.018	0.583
3	0.381	0.749
4	1.397	0.857
5	1.793	0.929
6	3.033	0.875
7	2.385	0.764
8	6.306	0.821
9	0.248	0.786
10	0.508	0.714

obtained at the 24th hour. Also at the 24th hour, there was no significant difference ($P > 0.05$) between the extract (125.0 and 250.0 mg/kg) and the reference drug glibenclamide.

3.2 Bioassay Guided Fractionation of NLE and Isolation of Active Compound Column and thin-Layer Chromatography

A total of seven hundred and thirty four (734) 10ml aliquots were collected in test tubes after eluting the crude extract of NLE from column. A trial of various solvent systems showed that the combination of Chloroform, Ethylacetate and Methanol in the ratio (3:2:1) gave the best separation and this solvent system (3:2:1) was used to spot all the eluents in each test tube. Based on their colour reactions under U.V (ultraviolet) light and eluting patterns, the various fractions were pulled into ten (10) fractions of NLE (Fraction 1-10).

The various properties of the fractions such as R_f (resolution front) values and their yields are presented in Table 2.

3.3 Screening of Fractions of NLE for Bioactivity/Selection of Active Fraction

The various Fractions (1-10) of NLE (50 mg/kg each) and glibenclamide (2 mg/kg) were tested in alloxan-induced hyperglycaemic rats as described earlier in experiment 3:2:3:1. The result is presented in Table 3. Glibenclamide (2 mg/kg) and Fractions 1,5,6,7,8,9 and 10 produced various levels of percentage decreases in the FBS of the alloxan-induced diabetic rats at various times with most of them having their peak activity at the 6th hour except fraction 1 which had its peak activity of 30% reduction at the 24th hour. Meanwhile Fractions 2, 3, 4 and the negative control caused various levels of percentage increase in the FBS of the diabetic rats over the 24 hour period. Among the Fractions that caused decrease in the FBS were Fractions 7 and 9 which demonstrated consistent and higher level of decrease in the FBS. Fraction 7 caused low but consistent decrease of FBS over the 24 hour period while Fraction 9 caused 34.7, 40.9 and 66.0 % decreases in FBS at 1, 3 and 6th hours but could not sustain the effect up to 24 hours. Glibenclamide the reference drug showed 8.8, 23.8, 51.9 and 16.3% decreases in FBS at 1, 3, 6 and 24th hour. Fraction 9 was selected as the active fraction for further studies.

Table 3 Effect of different fractions of NLE on FBS (mMol/l) of alloxan-induced diabetic rats.

Fraction	Dose	0h	1h	% Change	3h	% Change	6h	% Change	24h	% Change
Distilled H ₂ O	10ml/kg	13.4	14.7	9.7↑	16.5	23.1↑	18.4	37.3↑	19.2	43.2↑
Glibenclamide	2mg/kg	23.9	21.8	8.8↓	18.2	23.8↓	11.5	51.9↓	20.0	16.3↓
Fraction 1	50mg/kg	13.9	13.2	5.0↓	13.3	4.3↓	10.4	25.2↓	9.6	30.9↓
Fraction 2	50mg/kg	13.6	15.4	13.2↑	16.9	24.3↑	11.9	12.5↓	11.3	16.9↓
Fraction 3	50mg/kg	17.0	19.3	13.5↑	16.9	0.6↓	14.0	17.6↓	15.8	7.1↓
Fraction 4	50mg/kg	22.1	24.5	10.9↑	22.6	2.3↑	22.1	0.0	20.1	9.0↓
Fraction 5	50mg/kg	11.8	10.5	11.0↓	9.4	20.3↓	7.8	33.9↓	12.2	3.4↑
Fraction 6	50mg/kg	11.6	10.3	11.2↓	9.0	22.4↓	8.1	30.2↓	11.5	0.9↓
Fraction 7	50mg/kg	13.0	11.1	14.6↓	11.1	14.6↓	7.4	43.1↓	9.8	24.6↓
Fraction 8	50mg/kg	28.8	23.9	17.0↓	23.4	18.8↓	21.3	26.0↓	27.3	5.2↓
Fraction 9	50mg/kg	14.4	9.4	34.7↓	8.5	40.9↓	4.9	66.0↓	15.9	10.4↑
Fraction 10	50mg/kg	13.6	13.7	0.7↑	13.2	2.9↓	9.8	27.9↓	23.3	71.3↑

↑ Mean increase in FBS.

↓ Mean decrease in FBS.

Table 4 Effect of subfractions of fraction 9 on FBS of alloxan-induced diabetic rats.

Percentage change of FBS (mMol/L)					
Sub-fraction	0 hr	1 hr	3 hr	6 hr	24 hr
F 9:1	15.5	5.763↑	17.09↑	24.5↑	3.6
F 9:2	11.4	37.5↑	1.2↑	1.3	61.4
F 9:3	11.5	37.8↑	46.5↑	34.9↑	19.3
F 9:4	10.5	27.9↑	58.4↑	55.5↑	8.6↑
F 9:5	17.8	19.1↑	19.1↑	4.5↑	23
F 9:6	11.4	7.5↑	15.9	4.3↑	21.2

↑ Mean % increase in FBS.

Table 5 Yields and R_f values of the subfractions of fraction 9.

Fractions	Yield (mg)	R _f value
F9:1	35	0.081
F9:2	6	0.524
F9:3	19	0.661
F9:4	43	0.833
F9:5	7	0.921
F9:6	28	0.978

Among them, sub-fraction 9 (2) was selected for its higher antihyperglycaemic activity of 61.4%. The result of the sub-fractions is presented in Table 4 and their R_f values are also presented in Table 5. Based on the above results Fraction 9 sub-fraction 2 (F9:2) was selected as a pure compound (Plate 2) with higher antidiabetic activity of 61.4%, a R_f value of 0.524 and a yield of 6 mg.

3.4 Purification of Active Fraction/Isolation of Pure Compound

The selected Fraction (F9) was further purified using preparative TLC. The purification process yielded six (6) compounds (Plate 1). The six compounds were further screened for antihyperglycaemic activity using 5 mg/kg of each of the sub-fractions. The various sub-fractions showed various time dependent reductions of the FBS in alloxan-induced diabetic rats.

3.5 Characterization and Structural Elucidation

Characterization and structural elucidation were carried out using NMR (nuclear magnetic resonance) and GC- MS (gas chromatography-mass spectroscopy). The data is presented in Fig. 1. The compound was identified as a long chain unsaturated fatty acid. The molecular structure is shown in Fig. 2. The chemical name is 9-(4-Nonyl-phenyl)-non-8-enoic acid. The molecular formula and the molecular weight is C₂₄H₃₈O₂.

Plate. 1 Chromatogram of Fraction 9 the active fraction showing various bands.

Plate. 2 Chromatogram of F9:2 showing a single spot with a R_f of 0.524.

Fig. 1 ^1H NMR spectra of F9:2, the active compound.

Fig. 2 Molecular structure of F9:2, the active compound.

and 358.56g respectively.

4. Discussion

The ability of NLE to reduce the FBS (fasting blood sugar) of rats was tested as a means of evaluating its antidiabetic activity through a single intraperitoneal administration of 150 mg/kg of alloxan monohydrate. FBS (fasting blood sugar) is a test of carbohydrate metabolism which measures blood glucose after a fast, which is usually 12-18 h. During this time, the body stimulates the release of the hormone glucagon which in turn releases glucose in to the blood stream through a catabolic process. Under normal circumstances, the body produces and processes insulin to counteract the rise in glucose levels, but in diabetes mellitus this process is impaired, and tested blood glucose levels remain usually high [22]. This procedure was adapted for the bioassay-guided isolation of the antidiabetic principle of NLE (*Newbouldia laevis*).

Experimental diabetes is usually induced using alloxan monohydrate or Streptozotocin [17, 23]. Alloxan mediates its cytotoxic activity through ROS (reactive oxygen species) especially hydrogen peroxide with simultaneous massive increase in cytosolic Ca^{2+} concentration which causes rapid destruction of β -cells [17, 23]. In this present study, diabetes was induced chemically in the rats through a single intraperitoneal

injection of alloxan monohydrate at a dose of 150 mg/kg body weight as described by other workers [17, 23]. The pancreas is especially susceptible to the action of alloxan induced free radical-damage; subsequently, there is a decrease in endogenous insulin and this affects the utilization of glucose by the body tissues. It also leads to elevated blood glucose level, decreased protein content and increased levels of serum cholesterol, triglycerides and other bad lipids [24, 25]. The rats were considered diabetic whenever their FBS was ≥ 7.0 mMol/l and more [18, 26]. In the present study, methanol leaf extract of NLE at doses of 62.5, 125.0 and 250.0 mg/kg body weight caused significant ($p < 0.05-0.01$) dose and time-dependent decreases in the FBS levels of the alloxan-induced diabetic rats. The time of maximum effect was at the 24th h which was 27.8, 55.3 and 60.2% respectively for the above doses compared to 51.5 % of the reference drug glibenclamide 2 mg/kg. Other workers [10-13] have reported the dose dependent reduction in FBS by NLE. Evidence abound to show that antidiabetic drugs bring about their effect on diabetes by lowering the blood glucose level of affected animals through various mechanisms [27-29]. The anti-hyperglycaemic activities of NLE may be due to its ability to restore the functions of pancreatic tissues thereby bringing about an increase in insulin output or insulin utilization just

like many other antidiabetic plants [12, 28].

The process of bioassay-guided fractionation of the NLE through column chromatography yielded 734 of 10 ml aliquots which were pooled into 10 Fractions (F1-10) based on their R_f and colour reactions under UV light (Table 2). This process was used because it makes available information that will predict the effect of drugs in clinical situations. It also provides a true and real clinical situation in which the expected drug entity is going to operate, thereby providing a wholistic environment. This is a process whereby an extract is fractionated and re-fractionated through chromatography in addition to biological testing until a pure biologically active compound is finally isolated. In other words, it is a step by step process of separating extracted compounds based on differences in their physicochemical properties as well as assessing their biological activities [30-32].

The ten (10) fractions obtained were subjected to *in vivo* biological testing. Their effects on the FBS of alloxan-induced diabetic rats were assessed. Based on the percentage reduction in FBS, Fraction 9 was selected as the most active fraction out of the 10 fractions causing the highest reduction in FBS of 34.7%, 40.9% and 66.0% at 50 mg/kg orally in the treated rats as compared to 8.8%, 23.8% and 51.9% reduction by glibenclamide (2 mg/kg), all at 1, 3, and 6th h after treatment respectively (Table 3).

The active fraction (F.9) was then subjected to further purification through the preparative TLC (thin layer chromatography). TLC is a method suitable for separating individual components of mixture/substance by using thin layer of silica gel as a stationary phase and a combination of solvents as the mobile phase [20, 30]. TLC has advantage over other forms of separation because of its selectivity, specificity and rapidity in identifying drug substances over other chemical methods. Other advantages are lack of interference from excipients and can be used semiquantitatively [30, 33]. Further purification of Fraction 9 yielded 6 different compounds (Plate 1). The individual effect of

these 6 compounds on alloxan-induced diabetic rats showed sub-fraction 2 at a dose of 5 mg/kg orally reduced the FBS of the diabetic rats by 61.4% at the 24th h (Tables 4 and 5). Fraction 9 sub-fraction 2 (F.9.2) was suspected to be a pure compound as it yielded one spot on the TLC plate and its total yield was 6 mg and was sent for characterization (Plate 2, Table 5). The process of purification increased the potency of the fraction (F9) by five times and the active compound (F9.2) by fifty times (Tables 3 and 4). Purification process can increase or decrease the activities of pure compounds because some of the activities arise due to synergy of different compounds in the extract [34].

The characterization and structural elucidation of the active compound of NLE were carried out using ^1H and ^{13}C NMR and GC mass spectroscopy nuclear magnetic resonance spectroscopy is a modern tool used in determining the molecular structure, dynamics and characterization of materials at molecular level [35]. The basis for the use of NMR is the distinct difference in resonance frequency of every nuclei in the same magnetic field referred to as chemical shift and the nuclei of neighboring NMR active substances known as spin-spin or J-coupling. J-coupling allows the identification from peak intensities. These information from NMR are used to determine accurately the unique molecular structure of compounds [21]. Gas Chromatography-Mass Spectroscopy was used in combination with the NMR to identify the type of compound and compare its structure with other members of the group. There are other procedures that are also used singly or in combination to identify compounds and their molecular structures. Some of them include IR (infrared) spectroscopy, FAB (fast atom bombardment) spectroscopy etc.

The characterization and structural elucidation of F9:2, showed that the active compound responsible for the antidiabetic activity of *Newbouldia laevis* is a long chain unsaturated fatty acid 9-(4-Nonyl-phenyl)-non-8-enoic acid with the

chemical formula $C_{24}H_{38}O_2$. Polyunsaturated and monounsaturated fats like omega fatty acid are well known to decrease the risk of Type 2 diabetes and other chronic diseases [36].

5. Conclusion

NLE (*Newbouldia laevis* leaf extract) has demonstrated significant antidiabetic activity which is comparable to glibenclamide a standard sulfonylurea, in this study. This establishes the basis for its pharmacological use as an antidiabetic plant in Nigerian folk medicine. The process of purification increased the potency of the fraction by five times and the active compound by fifty times compared to the crude extract. The bioassay-guided isolation procedure led to the identification of a long chain polyunsaturated fatty acid [9-(4-Nonyl-phenyl)-non-8-enoic acid] as the antidiabetic compound. Polyunsaturated compounds are well known for their health benefits in chronic and metabolic diseases.

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