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# Justifying Antidiabetic Ethnomedicinal Claim of *Senecio biafrae* through Its Antihyperglycemic and Anti-Oxidant Activities

***Marcus Durojaye Ayoola[1]*, Adeleke Clement Adebajo[1,2], Francis B Zotor[3] and Martha Gelemete Pinkoane[4]***

*[1]Department of Pharmacognosy, Obafemi Awolowo University, Nigeria*

*[2]Department of Pharmacognosy and Herbal Medicine, University of Health and Allied Sciences, Ghana*

*[3]School of Public Health, University of Health and Allied Sciences, Ghana*

*[4]Department of Applied and Computer Sciences, Vaal University of Technology, South Africa*

## Abstract

**Objectives:** Increasing trend of urbanization and lifestyle changes, especially a "Western-style" diet are projected to give astronomical leap in world prevalence of diabetes. Anti-hyperglycemic and antioxidant activities of *Senecio biafrae* was investigated in justifying its folkloric antidiabetic claims in western-Nigeria and Ghana.

**Methods:** Methanolic extract (100, 200, 400 mg/kg) of the whole plant was investigated for anti-hyperglycemic activity, using normal, glucose-loaded and alloxan induced hyperglycemic rats, while its *in vitro* antioxidant effects were evaluated using 1,1-dipheny l-2-dipicrylhydrazyl radical scavenging, ferric reducing antioxidant power, total antioxidant capacity and hydroxyl radical scavenging activity assays. Glibenclamide (5 mg/kg) and appropriate anti-oxidant standard drugs were used as positive controls. Anti-hyperglycemic activity-directed purification of the methanolic extract, using glucose-loaded rats, led to the isolation and characterisation of β-stigmasterol, by comparing its spectral data with those in the literature.

**Results:** Extract (100 to 400 mg/kg) gave significantly ($p<0.05$) lower hypoglycemic activity than Glibenclamide (5 mg/kg) in normal rats, comparable ($p>0.05$) time and dose dependent activity in glucose-loaded rats and a significantly higher activity than Glibenclamide in sub chronic alloxanised rats. Anti-hyperglycemic activity of isolated β-stigmasterol, extract and Glibenclamide were comparable, indicating that β-stigmasterol is one of its anti-hyperglycemic constituents.

**Conclusion:** The demonstrated significant anti-hyperglycemic activity and additional anti-oxidant property of *S. biafrae,* justified its anti-diabetic ethnomedicinal use, β-stigmasterol, operating through both extra pancreatic and insulin stimulating mechanisms of action, is one of its anti-hyperglycemic constituents, and an additional insulinotropic mechanism of action was suggested for β-stigmasterol.

**Keywords: Diabetes mellitus; *Senecio biafrae*; Anti-hyperglycemic activity; Antioxidant activity; β-Stigma sterol**

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****Correspondence:***
*Marcus Durojaye Ayoola, Department of Pharmacognosy, Obafemi Awolowo University, Ile Ife, Nigeria, Tel: +2347030949601;*
*E-mail: ayosther@yahoo.com*

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## Introduction

Diabetes mellitus has been defined as a chronic hereditary disease of impairment of metabolism of fats, carbohydrates and proteins, which is occasioned by a relative or absolute deficiency of insulin secretion, and/or its resistance and results in hyperglycaemia [1-4]. Presently, it affects about 285 million worldwide and 16 million people in the United States. Its prevalence is projected to rise to about 400 million in 2030, especially in urban populations of the developing countries due to increasing trend of urbanization and lifestyle changes, such as increased sedentary life styles and perhaps most importantly, a "Western-style" diet [3,5]. Since the disease is only managed and has no cure, patients take life-long medicaments. Complications and serious adverse effects of the available synthetic hypoglycemic drugs justify the increased investigations of plants with anti-diabetic ethno medicinal use [1-3,6-10]. In diabetes mellitus, hyperglycaemia Generates Reactive Oxygen Species (ROS), such as superoxide ($O_2^{\bullet-}$), Hydroxyl (OH), Peroxyl ($RO_2$) and hydroperoxyl ($HO_2$) radicals,

that play an important role in secondary complications of diabetes mellitus, including those of the kidney, eye, blood vessel and nerves [11]. Several studies have shown that generated ROS are involved in the pathogenesis of diabetes and their scavengers are effective in preventing experimental diabetes in animal models as well as reducing the severity of types 1 and 2 diabetic complications [12]. *Senecio biafrae* Oliver & Hiern (Asteraceae), syn *Crassocephalum biafrae,* is a leaf that is commonly eaten as vegetable in Sierra Leone, Ghana, Benin, and South-Western Nigeria. In these communities, it is cooked together with pepper, tomato and onions. Its leaves are used fresh on wounds, sores and cuts in Ghana [13]. In some districts of Lagos, Nigeria, *S. biafrae,* in combination with the seeds of *Aframomum melegueta* and *Carica papaya* leaves, is used in the management of diabetes [14]. The hypoglycemic, hypolipidemic and antioxidant activities of its aqueous leaf extract have been reported [15,16]. Biafrae coumarins A, B and C, and triterpenes have been isolated from the stem while the leaves have been found to contain essential fatty acids, amino acids and vitamins, such as vitamins A, C, E, K and B complex [16,17]. Carcinogenic pyrrolidizine alkaloids have been reported in *Senecio longilobus, S. glabellus* and *S. jacobaea*, which are related species of *S. biafrae*. However, these hepatotoxic alkaloids have so far not been reported in *S. biafrae* [18,19]. Also, the anti-hyperglycemic constituent of the plant has not been identified or isolated, a condition necessary for the conclusive justification of the antidiabetic folkloric claim of the plant. Therefore, to justify the antidiabetic ethnomedicinal claims of *S. biafrae*, hyperglycaemia-lowering activity of the extract was determined using normoglycemic, glucose and alloxan induced hyperglycemic rats, while glucose-loaded rats were used to monitor the anti-hyperglycemic activity-directed purification of the extract. The isolated compound was tested to establish its glucose-lowering effect and its structural formula was elucidated using spectroscopic analytical techniques.

## Materials and Methods

### Chemicals, equipment and instrumentation

UV Spectrophotometer (Model M 107, Spectronic Camspec Ltd, UK), Vortex Genie rota mixer (K-550-GE model, Vortex-Genie accessories, USA), Care Sens™ N Glucometer (model PGA 1E3028 REV3, i- SENS, Inc, Korea) with Care Sens™ test strips (i- SENS, Inc, Korea), ammonium molybdate, ascorbic acid, sodium acetate, 2,4,6-tripyridyl-*s*-triazine (TPTZ), trolox, alloxan monohydrate and 1,1,-diphenyl-1-picrylhydrazyl radical (Sigma-Aldrich Co. LLC, USA). Vacuum liquid chromatographic (dimension: 9 cm × 12 cm, silica gel HR60 size 10 µm to 40 µm), column chromatographic (dimension: 60 cm × 4 cm, silica gel mesh 70 to 230) apparatuses were used. Others were aluminium plated thin-layer chromatographic (silica gel 60 F254, 0.25 mm) and glass plated preparative thin-layer chromatographic (silica gel 60 F254, 0.25 mm, 0.5 mm, 1 mm, 2 mm, Whatman Inc, USA), silica gel (70 to 230 mesh, Merck & Co, Inc, USA). Nuclear Magnetic Resonance (NMR) spectra (400, 500, and 600 MHz) were obtained with Bruker AMX 400, Varian Nova 500, and Varian Unity plus 600 NMR instruments. High-Resolution Mass Spectra (HRESIMS) were obtained through determination of the [M+Na]+[2M+Na]+ ions in a Micro T of a mass spectrophotometer (Bruker Daltonics, Germany) with loop injection and Electro Spray Ionisation (ESI) technique. Mass Spectra (ESIMS) were obtained with a GCQ (Finnegan MAT, Germany) spectrometer (70 eV, EI +ve) using the ESI technique. All solvents used were of analytical grade.

### Animals

Healthy Wistaralbino rats of either sex (210 g, average weight) that were used for the experiments, were those bred under standard conditions (temp 27 ± 3°C, relative humidity 65%, natural 12 h day-night) and housed in different cages in the animal house, Department of Pharmacology, Faculty of Pharmacy, OAU, Ile-Ife, Nigeria, based on their sex. They were acclimatized for at least 5 days before commencement of the experiments and fed on a standard pellet diet (Bendel Feeds, Benin, Nigeria), with water given ad libitum. Groups of five rats were fasted for 24 h before administration of either glucose, extract, fractions, drugs or vehicle [1-3,6-8]. All animal experiments conformed to the guide for the care and use of laboratory animals published by the national academies press [20].

### Plant material, extraction and its partition fractions

*Senecio biafrae* Oliver & Hiern (*Asteraceae*) was collected from the Campus of Obafemi Awolowo University (OAU), Ile-Ife, Nigeria after authentication by Prof HC Illoh, Botany department, Faculty of Science, OAU, Ile-Ife. Its voucher specimen, IFE 16518 was deposited in IFE Herbarium, Department of Botany, OAU, Ile-Ife. The whole plant was air dried, powdered and 1 kg of the powdered material was extracted with methanol at room temperature and concentrated *in vacuo* to give 10.2% w/w yield. The methanolic extract (A) was suspended in water, successively partitioned with *n*-hexane, dichloromethane and ethyl acetate and concentrated *in vacuo* to obtain their corresponding *n*-hexane, dichloromethane, ethyl acetate and aqueous partition fractions, coded $B_1$-$B_4$, respectively.

### Acute toxicity study of the extract

The biological safety of *S.biafrae* was evaluated by determining $LD_{50}$ of its methanolic extract, using the 1983 Lorke's method [21]. Animals (120 g to 170 g) that were bred as given in section 2.2 above were fasted overnight before doses (10 to 5000 mg/kg) of the extract were administered orally. In phase 1 of the study, nine rats were divided into 3 groups of 3 rats each and were *p.o.* administered with the extract, using three geometrically increasing doses of 10,100 and 1000 mg/kg. These animals were observed for mortality and or toxicity signs within each group over a 24 h period. Due to the results obtained from phase 1, phase 2 studies was carried out, using eight rats that were divided into 4 groups of 2 rats each, which were given 1000, 1600, 2900 and 5000 mg/kg of the extract, respectively. The animals were observed for mortality and/or toxicity signs for 24 h. The $LD_{50}$ was calculated as the geometric mean of the dose that resulted in 100% lethality and that which caused no lethality at all [21].

$$LD_{50} = \sqrt{D_0} \times D_1$$

Where,

$D_0$= highest dose that gave no mortality

$D_1$= lowest dose that produced mortality

### Anti-hyperglycemic studies of the extract

**Effects of the extract in normal rats:** Groups of five 24 h fasted normoglycemic rats were ingested (*p.o.*) with either 1% Tween 80 in normal saline (negative control), or *S. biafrae* methanolic extract (100, 200, 400 mg/kg), or Glibenclamide (5 mg/kg, positive control). At 0.0, 0.5, 1.0, 2.0 and 4.0 h after administration of the test agents, a drop of blood that was taken from the tip of the tail of each rat was dropped onto a glucometer strip and the blood glucose level was directly read off the glucometer. The blood glucose levels at 0.0 h ($T_o$) be taken as

100%, while those at other times were expressed as percentages of these values [1-3,6-8].

**Assay of the extract using glucose-loaded rats:** Glucose (10 g/kg, *p.o.*) was given to 24 h fasted normal rats and those that were hyperglycemic [blood glucose level ≥ 7.0 mmol/l (126 mg/dl)] after 0.5 h ($T_o$) were selected and divided into groups of five and administered (*p.o.*) with extract (100, 200, 400 mg/kg), or 1% Tween 80 in normal saline (negative control), or Glibenclamide (5 mg/kg, positive control). Their blood glucose levels were determined as given in section 2.5.1. Also, glucose-lowering activities of the partition fractions were similarly assayed at 400 mg/kg, the highest active dose of the extract [1-3,6].

**Assay of the extract using alloxan-induced diabetic rats:** Groups of rats were injected (IP.) with 150 mg/kg alloxan monohydrate dissolved in normal saline [1,2,7,8,22]. For the next 6 days, they were fed and water was given ad libitum. The rats with fasting (24 h) blood glucose (FBG) levels ≥ 11.0 mmol/l (200 mg/dl) were considered diabetic, selected and divided into groups of 5 rats each. They were administered daily for 14 days with 1% Tween 80 in normal saline (negative control), or extract (400 mg/kg) or Glibenclamide (5 mg/kg) (positive control) dissolved in the vehicle. Their FBG levels were determined and recorded on 1, 4, 7, 10 and 14 days after administration of test agents, as given in 2.5.1 above [1,2,7,8,22].

### Antioxidant assays

**1-diphenyl-2-dipicrylhydrazyl (DPPH) radical scavenging assay:** The DPPH radical scavenging activity was determined using the standard method earlier reported and l-ascorbic acid was the reference standard [1,2,23].

**Ferric reducing antioxidant power (FRAP) assay:** This assay was performed by slight modifications of the method described and the antioxidant activity was presented as Trolox equivalents as earlier given [1,2,24].

**Total antioxidant capacity (TAC) assay:** This was done following the prescribed method and the results were expressed as Ascorbic Acid Equivalents (AAE) (μmol/g) [1,2,25].

**Hydroxyl radical scavenging activity (HRSA) assay:** The HRSA of the test extract was evaluated by modification of a described method [26]. The experiments were carried out in triplicates and all reagents were freshly prepared. A 10 μl aliquot of test sample or standard was mixed with 990 μl of reaction buffer containing 100 mm phosphate buffer (pH 7.4), 3.6 mm sodium benzoate, 145 μm EDTA, 140 μm $Fe(NH_4)_2SO_4$, and 3.6 mm $H_2O_2$. The reaction mixture was incubated at 37ºC for 1 h after which 1 ml of 20% acetic acid and 1 ml of 0.8% thiobarbituric acid, dissolved in 50 mm NaOH, was added. The new mixture was thereafter incubated for 30 min at 80ºC and cooled rapidly in an ice bath. The absorbance of the sample was measured at 532 nm and the percent HRSA was calculated. Trolox in the concentration range of 50 μm to 250 μm was used as standard for the calibration curve and from the linearity equation, concentration of sample that produced same absorbance as 1 mm of Trolox (mm Trolox equivalent) was determined [26].

**Bio-activity directed fractionation of the extract using glucose-loaded rats:** Fraction $B_1$ with the highest anti-hyperglycemic (hyperglycemic-lowering) ability was further fractionated, as summarized in the flow chart (Figure 1), and the successive column and PTLC sub fractions, and isolated compound were tested for anti-hyperglycemic activity using glucose-loaded rat model given in 5.2.

**Column chromatography of n-hexane fraction ($B_1$):** Fraction $B_1$ (70.0 g) was adsorbed on silica gel and subjected to Column Chromatography (CC), eluted with gradient mixtures of n-hexane, $CHCl_3$, and MeOH, to obtain fractions that were bulked into sub fractions $C_1$-$C_{12}$, based on TLC (Figure 1).

**Column chromatography of sub fraction $C_7$:** Anti-hyperglycemic sub fraction $C_7$ (800 mg) was adsorbed on silica gel, subjected to CC and eluted with gradient mixtures of solvents, and the resulting fractions were bulked by TLC as $D_1$ (n-hexane: $CHCl_3$ 4:6, 700 ml), $D_2$ (n-hexane:$CHCl_3$ 4:6, 100 ml), $D_3$ (n-hexane:$CHCl_3$ 4:6, 120 ml), $D_4$ (n-hexane:$CHCl_3$ 4:6, 220 ml), $D_5$ ($CHCl_3$ 100%, 40 ml), $D_6$ ($CHCl_3$ 100%, 140 ml).

**Further purification of sub fraction $D_4$:** A PTLC (0.5 mm, $CHCl_3$ 100%) of sub fraction $D_4$ (170 mg) yielded $D_{4a}$ (40.2 mg), $D_{4b}$ (35.6 mg), $D_{4c}$ (20.0 mg), $D_{4d}$ (40.3 mg) and $D_{4e}$ (10.2 mg). Methanol wash of $D_{4c}$, $D_{4d}$ and $D_{4e}$ gave residues that were coded $D_{4c2}$ (10.0 mg), $D_{4d2}$ (20.0 mg) and $D_{4e2}$ (10.0 mg), respectively. They were all shown by TLC ($CHCl_3$ 100%, n-hexane:$CHCl_3$ 2:8) to be the same and pure.

**Column chromatography of sub fractions $C_8$ and $C_9$:** The $C_8$ and $C_9$ (2.0 g) with similar TLC pattern, were adsorbed on silica gel, subjected to CC and the sub fractions were bulked by TLC into $E_1$ (n-hexane:$CHCl_3$ 2:8, 175 ml, 270 mg), E2 (n-hexane:$CHCl_3$ 2:8, 100 ml, 670 mg), $E_3$ (n-hexane:$CHCl_3$ 2:8, 340 ml, 840 mg), $E_4$ (n-hexane:$CHCl_3$ 2:8, 180 ml; $CHCl_3$ 100% 20 ml, 210 mg), E5 ($CHCl_3$ 100%, 220 ml, 310 mg), E6 ($CHCl_3$ 100%, 180 ml, 70 mg), $E_7$ ($CHCl_3$:MeOH 95:5, 200 ml, 10 mg).

**Further purification of sub fractions $E_2$, $E_4$ and $E_5$:** Similar to 2.7.3, $E_2$, $E_4$ and $E_5$ were subjected to PTLC (0.5 mm, n-hexane:$CHCl_3$ 2:8), followed by methanol solvent wash to produce $E_{2d1}$ (20 mg), $E_{4b1}$ (20 mg), $E_{4c1}$ (30 mg) and $E_{5d1}$ (40 mg) PTLC sub fractions, respectively. They were all shown by TLC ($CHCl_3$ 100%, n-hexane:$CHCl_3$ 2:8) to be same and pure.

### Identification of the isolates

1H/1H-NMR- Homonuclear Correlated Spectroscopy (COSY), IH/13C-NMR- Heteronuclear Multiple Bond Correlation (HMBC), Heteronuclear Single Quantum Correlation (HSQC), Total Correlation Spectroscopy (TOCSY), and Electro-Spray Ionisation Mass Spectrometry (ESIMS) data of the isolates, $D_{4c2}$, $D_{4d2}$, $D_{4e2}$, $E_{2d1}$, $E_{4b1}$, $E_{4c1}$ and $E_{5d1}$, were compared with information in the literature and their identity was confirmed as one and same compound, β-stigmasterol [27-29].

**Anti-hyperglycemic assay of the isolated β-stigmasterol:** The isolated β-stigmasterol was assayed for anti-hyperglycemic activity as given in section 2.5.2.

### Statistical analysis

Data represent mean ± SEM and n=5 and 3 for the animals in the groups and antioxidant assays, respectively. They were analyzed with One Way Analysis of Variance (ANOVA), followed by Bonferroni t-test or Student-Newman-Keuls post hoc tests, using Graph Pad® Instat, version 5.0 (Graph Pad Software Inc, San Diego, USA). P<0.05 was considered significant.

## Results and Discussion

Early extra-pancreatic and late insulin stimulating effects have been given as the mechanisms of action of Glibenclamide [29,30].

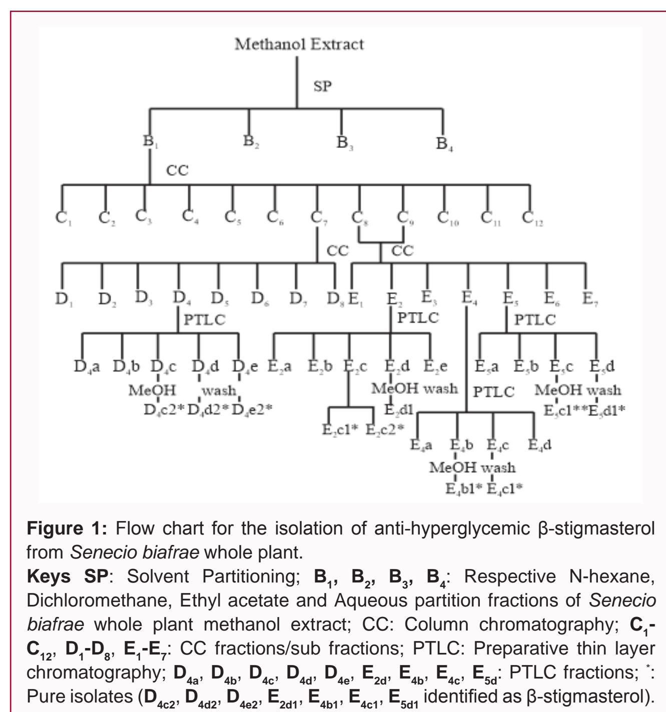


**Figure 1:** Flow chart for the isolation of anti-hyperglycemic β-stigmasterol from *Senecio biafrae* whole plant.

**Keys SP**: Solvent Partitioning; $B_1$, $B_2$, $B_3$, $B_4$: Respective N-hexane, Dichloromethane, Ethyl acetate and Aqueous partition fractions of *Senecio biafrae* whole plant methanol extract; CC: Column chromatography; $C_1$-$C_{12}$, $D_1$-$D_8$, $E_1$-$E_7$: CC fractions/sub fractions; PTLC: Preparative thin layer chromatography; $D_{4a}$, $D_{4b}$, $D_{4c}$, $D_{4d}$, $D_{4e}$, $E_{2d}$, $E_{4b}$, $E_{4c}$, $E_{5d}$: PTLC fractions; $^*$: Pure isolates ($D_{4c2}$, $D_{4d2}$, $D_{4e2}$, $E_{2d1}$, $E_{4b1}$, $E_{4c1}$, $E_{5d1}$ identified as β-stigmasterol).

Therefore, using glucose-loaded and alloxan-induced hyperglycemic rats, activity profile similar to that of Glibenclamide or other insulin stimulatory drugs as standards by plant extracts/fractions/test agents in these models had allowed a guess of their extra-pancreatic and insulin stimulating mechanisms of action [1-3,6-8,10,30,31]. Also, experiments using these two diabetic models and appropriate standard drugs had concluded that plant extracts having such anti-hyperglycemic activity may be useful in managing type 2 diabetes occasioned by insulin insufficiency [1-3,6-8,10,31].

## Safety profile of *Senecio biafrae*

Using the Lorke's method, an $LD_{50}$ of >5000 mg/kg was obtained for the methanol extract of *S. biafrae* [20]. No toxicity or mortality was recorded even at the maximum tested dose of 5000 mg/kg. This may confirm that the extract and the plant were safe for consumption. That the hepatotoxic pyrrolidizine alkaloids have not been reported in *S. biafrae* does not necessarily suggest their absence, as only a conscious effort in analyzing [18,19]. S. *Biafrae* for these toxic alkaloids would remove the concern of potential chronic liver toxicity and carcinogenicity for this plant. Hence, this needs to be done to ensure that the continued consumption of *S. biafrae* food and in traditional medicines is safe for the citizens of these African countries. Nevertheless, the generous washing of the minced plant that is followed by an extensive boiling that the plant undergoes while preparing a vegetable soup for consumption by these African citizens may have helped in its de-toxification. Similarly, Comfrey that also contains pyrrolidizine alkaloids is not only used as an herbal medicine in Germany, but its leaves are also boiled before eating in some areas of Germany. Also, these days, licensed preparations of Comfrey contain extracts that are almost free of these alkaloids (personal communication). Therefore, having established the safety of *S. biafrae*, its methanol extract was further investigated to confirm or otherwise its folkloric antidiabetic usage.

## Hypoglycemic effect of the extract

The blood glucose levels of the normoglycemic rats given Tween 80 (negative control group) and 100, 400 mg/kg of *S. biafrae* extract were unchanged during the 4 h period of the experiment. They were also comparable at all time points (0 h to 4.0 h) of the study. However, 200 mg/kg of the extract demonstrated significant (p>0.05) blood glucose lowering ability, especially at 2 h and 4 h. Furthermore, 200 mg/kg of the extract elicited a blood glucose level that was comparable to that given by the standard drug, Glibenclamide (5 mg/kg). However, only Glibenclamide (positive control group) gave 41, 54, 61 and 44% blood glucose reductions at 0.5 h to 4.0 h, respectively and produced glucose levels in the rats that were significantly (p<0.05) lower than those of the negative control and test (100 to 400 mg/kg of the extract) groups (Table 1). Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at 0 h (T0), n=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (p<0.05, one-way analysis of variance followed by the Student–Newman–Keuls' test). Tween 80: <1% of Tween 80 in normal saline (negative control); GLU (10 g/kg): Glucose in <1% of Tween 80 in normal saline administered at 10 g/kg (hyperglycaemic negative control); SBW: *Senecio biafrae* whole plant methanol extract; GLI (5 mg/kg): Glibenclamide (5 mg/kg, positive control). From this result therefore, only Glibenclamide would function as an effective anti-hyperglycemic agent, precipitating hypoglycemia in normal rats, while *S. biafrae* methanolic extract did not have this property. Hence, *S. biafrae* could be regarded safe when consumed as a vegetable by the non-diabetic human subjects and hence its culinary use could therefore be considered justified. Similarly, aqueous extract of *Nauclea latifolia* and methanolic extract of *Eugenia uniflora* leaves that lacked significant hypoglycemic effect in normal rats have also been reported safe [1-3,13,32].

## Glucose lowering effects of the extract

The glucose-induced hyperglycemic rats administered with normal saline (negative control) demonstrated significant time dependent reductions in their blood glucose levels up to the 4th h (Table 1) due to the homeostatic regulatory mechanism in the normal animals. The reductions also confirmed that their pancreases were functioning well [1-3,10,31,33]. The *S. biafrae* extract (100 to 400 mg/kg) and Glibenclamide demonstrated an anti-hyperglycemic activity that was both time dependent. At all time points (0.5 h to 4 h), the anti-hyperglycemic activity of Glibenclamide was comparable (p>0.05) to those of 100 and 200 mg/kg of the extract. However, the blood glucose lowering ability of 400 mg/kg of the extract was significantly higher at all time points than that of Glibenclamide and comparable with that of its 200 mg/kg at 2 h and 4 h (Table 1). Also, an activity profile that was similar to that of Glibenclamide (Table 1) may indicate similar stimulation of insulin release as its main mechanism of action [1-3,6-8,30]. Furthermore, based on similar profile of anti-hyperglycemic activity as Glibenclamide, extracts of *Gongronema latifolium, E. uniflora*, *Jatropha tanjorensis* and *Clausena lansium* have been reported to have insulin stimulation as their mechanism of action [1-3,6-8]. Their *in-vitro* and *in-vivo* insulin stimulation confirmed this assumption [1-3,6-8]. However, the 49% and 52% blood glucose lowering effects given by the highest tested dose (400 mg/kg) of *S. biafrae* extract at 0.5 and 1.0 h (Table 1), suggested presence of constituents with extra pancreatic activity in the extract [1-3,6-8].

## Anti-hyperglycemic effects of the extract on alloxan-induced diabetic rats

With the demonstrated anti-hyperglycemic activity of *S. biafrae,*

**Table 1:** Glucose lowering effects of *Senecio biafrae* methanolic extract in normal and glucose loaded rats.

| Extract/Drug doses (mg/kg) | Blood glucose level as percentage of T0 (reduction in blood glucose relative to negative control at Tt) | | | | |
|---|---|---|---|---|---|
| | 0 h | 0.5 h | 1 h | 2 h | 4 h |
| **Normal rats** | | | | | |
| Tween 80 | 100 | 115.64 ± 12.08$^{b}$ | 108.22 ± 8.62$^{b,c}$ | 101.25 ± 10.30$^{c}$ | 102.81 ± 5.23$^{b,c}$ |
| SBW (100) | 100 | 108.22 ± 10.30$^{b}$ (6.38%) | 105.68 ± 6.09$^{b,c}$ (2.35%) | 101.15 ± 6.78$^{c}$ (0.10%) | 91.45 ± 7.42$^{b,c}$ (11.05%) |
| SBW (200) | 100 | 99.37 ± 8.91b (14.07%) | 89.93 ± 11.02b (16.90%) | 79.94 ± 16.53$^{b}$ (21.05%) | 78.70 ± 11.88$^{a, b}$ (23.45%) |
| SBW (400) | 100 | 107.96 ± 16.65$^{b}$ (6.64%) | 100.42 ± 11.68$^{b,c}$ (7.21%) | 93.49 ± 7.64$^{b,c}$ (7.66%) | 86.00 ± 12.65$^{b,c}$ (16.35) |
| GLI (5) | 100 | 68.04 ± 6.88$^{a}$ (41.16%) | 50.22 ± 4.14$^{a}$ (53.59%) | 40.02 ± 2.36$^{a}$ (60.47%) | 57.76 ± 4.41$^{a}$ (43.82%) |
| **Glucose loaded rats (10 g/kg)** | | | | | |
| GLU (10 g/kg) | 0 h | 0.5 h | 1 h | 2 h | 4 h |
| | 100 | 83.79 ± 3.81$^{b}$ | 85.89 ± 0.50c | 76.45 ± 1.71$^{c}$ | 74.18 ± 1.97$^{c}$ |
| SBW (100) | 100 | 77.48 ± 6.35$^{b}$ (7.53%) | 61.71 ± 2.14$^{b}$ (28.15%) | 49.97 ± 3.13$^{a,b}$ (34.87%) | 44.40 ± 3.19$^{b}$ (40.15%) |
| SBW (200) | 100 | 82.91 ± 3.93$^{b}$ (1.05%) | 69.68 ± 6.33$^{b}$ (18.87%) | 48.61 ± 4.70$^{a,b}$ (36.42%) | 33.81 ± 4.05$^{a,b}$ (55.27%) |
| SBW (400) | 100 | 43.02 ± 9.44$^{a}$ (48.66%) | 40.91 ± 9.95$^{a}$ (52.37%) | 36.26 ± 8.56$^{a}$ (52.57%) | 23.34 ± 3.00$^{a}$ (68.54%) |
| GLI (5 mg/kg) | 100 | 75.64 ± 6.73$^{b}$ (9.73%) | 70.68 ± 6.86$^{b}$ (17.71%) | 58.32 ± 6.44$^{b}$ (23.72%) | 45.27 ± 6.88$^{b}$ (38.97%) |

Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at 0 h ($T_0$), *n*=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (*p*<0.05, one-way analysis of variance followed by the Student–Newman–Keuls' test). Tween 80: <1% of Tween 80 in normal saline (negative control); GLU (10 g/kg): Glucose in <1% of Tween 80 in normal saline administered at 10 g/kg (hyperglycaemic negative control); SBW: *Senecio biafrae* whole plant methanol extract; GLI (5 mg/kg): Glibenclamide (5 mg/kg, positive control).

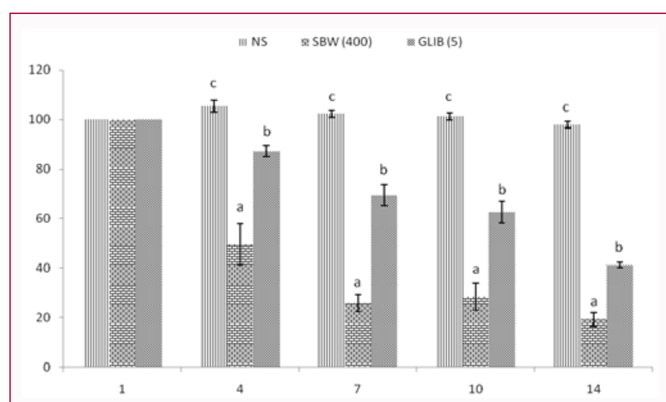


**Figure 2:** Anti-hyperglycemic activities of *Senecio biafrae* using alloxan-induced diabetic rats.
Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at day 1 ($T_0$), *n*=5. Values with different superscripts within each time points are significantly different (*p*<0.05, one-way analysis of variance followed by the Student–Newman–Keuls' test). NS: <1% of Tween-80 in normal saline (negative control); SBW (400): *Senecio biafrae* whole plant methanolic extract at 400 mg/kg; Glib (5): Glibenclamide at 5 mg/kg (positive control).

the effect of the most effective dose (400 mg/kg) of this methanol extract in glucose-loaded rats Table 1 was evaluated in a proper antidiabetic model of alloxanised rats. The negative control group rats showed consistent hyperglycemias throughout the duration (14 days) of the sub chronic experiment. This dose demonstrated significantly higher anti-hyperglycemic activity than Glibenclamide at all times (Figure 2). Also, similar to Glibenclamide, this effect progressively increased from day 4 to day 14 (Figure 2). This result may therefore confirm insulin release as the major mechanism of action of *S. biafrae* extract, as was earlier suggested from its anti-hyperglycemic action with the glucose-loaded rat model (Table 1). Furthermore, the results were in agreement with the reported anti-hyperglycemic activity of the aqueous leaf extract of the plant in alloxanised rats [16]. Hence, *S. biafrae* and its methanolic extract may be useful in the management of acute and prolonged hyperglycemic conditions typified by the alloxan-induced rats, and there by justified its antidiabetic ethnomedicinal use. Similar to Glibenclamide, significant anti-hyperglycemic and insulin releasing activities in glucose-loaded and alloxanised rats have been reported for the methanolic extracts of *S. cayennesis, J. Tanjorensis* and *B monandra* [2,3,6-8].

## Anti-oxidant activity of *S. biafrae* methanolic extract

Using the radical scavenging assay of DDPH, the methanol extract of *S. biafrae* had an $IC_{50}$ value of 1.95 ± 0.02 mg/ml that was significantly higher than the $IC_{50}$ value of 0.01 ± 0.00 mg/ml given by ascorbic acid, the standard drug used. At 0.5 and 1.0 mg/ml, it had a mild antioxidant activity, as measured by the FRAP and TAC values of 0.05 ± 0.00, 0.08 ± 0.00 and 43.13 ± 3.62, 80.87 ± 5.60 µg AAEq/ml, respectively while its HRSA value was 0.86 ± 0.06 mmol/LTE. These results confirmed reported antioxidant activity for the raw and cooked leaves of the plant and suggested that similar to *E. uniflora* and some other Nigerian plants *S. biafrae* has a moderate anti-oxidant activity in addition to its significant anti-hyperglycemic activity (Table 1, Figure 2) [1,2,15]. Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at 0 h (T0), n=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (p<0.05, one-way analysis of variance followed by the Student–Newman–Keuls' test). GLU (10 g/kg): Glucose in <1% of Tween 80 in normal saline administered at 10 g/kg (hyperglycemic negative control); A: Methanol extract of *Senecio biafrae* whole plant; $B_1$: N- hexane fraction; $B_2$: Dichloromethane fraction; $B_3$: Ethyl acetate fraction; $B_4$: Aqueous fraction; GLI (5 mg/kg): Glibenclamide at 5 mg/kg (positive control).

## Glucose lowering activity of partition fractions of *S. biafrae*

The significant anti-hyperglycemic and moderate antioxidant activities (Table 1, Figure 2) demonstrated by this leaf extract stimulated the interest in further purification exercise in order to isolate the active glucose lowering constituent. Generally, only

**Table 2:** Glucose lowering effects of solvent partition fractions (400 mg/kg) of *Senecio biafrae* methanol extract.

| Fraction/Drug | Blood glucose levels as percentages of $T_0$ (% reduction in blood glucose relative to negative control at $T_t$) | | | | |
|---|---|---|---|---|---|
| | 0 h | 0.5 h | 1 h | 2 h | 4 h |
| GLU (10 g/kg) | 100.00 | 83.79 ± 3.81$^a$ | 85.89 ± 0.50$^c$ | 76.45 ± 1.71$^b$ | 74.18 ± 1.97$^{b,c}$ |
| A | 100.00 | 72.03 ± 7.30$^a$ (14.04 %) | 61.65 ± 6.42$^a$ (28.22 %) | 53.04 ± 4.00$^a$ (30.62 %) | 48.17 ± 4.70$^a$ (35.06 %) |
| $B_1$ | 100.00 | 82.62 ± 4.22$^a$ (1.40 %) | 75.50 ± 3.48$^b$ (11.75 %) | 64.31 ± 5.26$^a$ (15.88 %) | 50.92 ± 3.44$^a$ (31.36 %) |
| $B_2$ | 100.00 | 83.69 ± 5.07$^a$ (0.13 %) | 79.62 ± 5.04$^{b,c}$ (7.30 %) | 68.17 ± 5.04$^b$ (10.83 %) | 62.05 ± 3.80$^{a,b}$ (16.35 %) |
| $B_3$ | 100.00 | 87.30 ± 3.75$^a$ (-4.19 %) | 86.29 ± 3.95$^c$ (-0.47 %) | 71.78 ± 5.90$^b$ (6.11 %) | 62.18 ± 6.09$^{a,b}$ (16.18 %) |
| $B_4$ | 100.00 | 87.27 ± 5.17$^a$ (-4.15 %) | 85.42 ± 6.84$^{b,c}$ (0.55 %) | 73.35 ± 4.54$^b$ (4.05 %) | 84.42 ± 9.41$^c$ (-13.80 %) |
| GLI (5 mg/kg) | 100.00 | 75.64 ± 6.73$^a$ (9.73 %) | 70.68 ± 6.86$^a$ (17.71 %) | 58.32 ± 6.44$^a$ (23.71%) | 45.27 ± 6.88$^a$ (38.97 %) |

Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at 0 h ($T_0$), *n*=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (*p*<0.05, one-way analysis of variance followed by the Student–Newman–Keuls' test). GLU (10 g/kg): Glucose in <1% of Tween 80 in normal saline administered at 10 g/kg (hyperglycaemic negative control); **A**: Methanol extract of *Senecio biafrae* whole plant; $\mathbf{B_1}$: N- hexane fraction; $\mathbf{B_2}$: Dichloromethane fraction; $\mathbf{B_3}$: Ethyl acetate fraction; $\mathbf{B_4}$: Aqueous fraction; GLI (5 mg/kg): Glibenclamide at 5 mg/kg (positive control).

**Table 3:** Glucose lowering effects of $\mathbf{C_1}$-$\mathbf{C_{12}}$ column sub fractions (400 mg/kg) of *Senecio biafrae* methanol extract.

| Extract/Drug (mg/kg) | Blood glucose levels as percentages of $T_o$ (% reduction in blood glucose relative to negative control at $T_t$) | | | | |
|---|---|---|---|---|---|
| | 0 h | 0.5 h | 1 h | 2 h | 4 h |
| GLU (10 g/kg) | 100 | 83.79 ± 3.81$^b$ | 85.89 ± 0.50$^c$ | 76.45 ± 1.71$^c$ | 74.18 ± 1.97$^c$ |
| $\mathbf{B_1}$ | 100 | 82.62 ± 4.22$^b$ (1.40%) | 75.50 ± 3.48$^b$ (12.10%) | 64.31 ± 5.26$^{a,b}$ (15.88%) | 50.92 ± 3.44$^a$ (31.36%) |
| $\mathbf{C_2}$ | 100 | 81.71 ± 8.18$^b$ (2.48%) | 74.08 ± 5.52$^b$ (13.75%) | 63.30± 5.31$^{a,b}$ (17.20%) | 59.68 ± 6.08$^{a,b}$ (19.55%) |
| $\mathbf{C_3}$ | 100 | 84.59 ± 3.50$^b$ (0.95%) | 62.26 ± 6.29$^{a,b}$ (27.51%) | 63.62 ± 6.25$^{a,b}$ (16.78%) | 58.45 ± 5.89$^{a,b}$ (21.21%) |
| $\mathbf{C_4}$ | 100 | 79.98 ± 4.30$^b$ (4.55%) | 71.07 ± 5.03$^b$ (17.25%) | 64.30 ± 2.03$^{a,b}$ (15.89%) | 58.25 ± 3.55$^{a,b}$ (21.47%) |
| $\mathbf{C_5}$ | 100 | 67.75 ± 1.07$^a$ (19.14%) | 62.11 ± 2.43$^a$ (27.69%) | 60.39 ± 91$^{a,b}$ (20.01%) | 62.77 ± 4.34$^b$ (15.38%) |
| $\mathbf{C_6}$ | 100 | 83.25 ± 5.20$^b$ (0.64%) | 66.66 ± 6.67$^{a,b}$ (22.39%) | 65.21 ± 6.31$^{a,b}$ (14.70%) | 57.04 ± 4.67$^{a,b}$ (23.11%) |
| $\mathbf{C_7}$ | 100 | 74.04 ± 4.95$^b$ (11.64%) | 65.12 ± 2.56$^{a,b}$ (24.18%) | 55.13 ± 4.45$^a$ (27.89%) | 55.35 ± 6.70$^{a,b}$ (25.38%) |
| $\mathbf{C_8}$ | 100 | 88.59 ± 5.25$^b$ (-5.73%) | 85.78 ± 6.92$^{b,c}$ (0.13%) | 74.03 ± 5.37$^b$ (4.17%) | 69.79 ± 4.31$^b$ (5.92%) |
| $\mathbf{C_9}$ | 100 | 84.37 ± 1.80$^b$ (-0.69%) | 76.22 ± 3.51$^b$ (11.26%) | 68.27 ± 4.06$^b$ (10.70%) | 64.19 ± 7.42$^b$ (13.47%) |
| $\mathbf{C_{10}}$ | 100 | 76.67 ± 3.53$^b$ 8.50%) | 59.53 ± 6.14$^a$ (30.69%) | 50.62 ± 3.93$^a$ (33.79%) | 42.61 ± 5.16$^a$ (42.56%) |
| $\mathbf{C_{11}}$ | 100 | 80.05 ± 9.18$^b$ (4.46%) | 67.81 ± 10.86$^{a,b}$ (21.0%) | 66.66 ± 7.79$^{a,b}$ (12.81%) | 63.27 ± 8.82$^b$ (14.71%) |
| $\mathbf{C_{12}}$ | 100 | 57.30 ± 7.05$^a$ (31.61%) | 55.26 ± 6.31$^a$ (35.66%) | 51.25 ± 6.18$^a$ (32.96%) | 45.59 ± 5.67$^a$ (38.54%) |
| GLI (5 mg/kg) | 100 | 75.64 ± 6.73$^b$ (9.73%) | 70.68 ± 6.86$^b$ (17.71%) | 58.32 ± 6.44$^{a,b}$ (23.72%) | 45.27 ± 6.88$^a$ (38.97%) |

Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at 0 h ($T_0$), *n*=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (*p*<0.05, one-way analysis of variance followed by the Student–Newman–Keuls' test). GLU (10 g/kg): Glucose in <1% of Tween 80 in normal saline administered at 10 g/kg (hyperglycaemic negative control); **A**: Methanol extract of *Senecio biafrae* whole plant; $\mathbf{B_1}$: N- hexane fraction of S*enecio biafrae* methanol extract; $\mathbf{C_2}$-$\mathbf{C_{12}}$: Column sub fractions of $\mathbf{B_1}$; GLI (5 mg/kg): Glibenclamide at 5 mg/kg (positive control).

400 mg/kg of the methanolic extract of *S. biafrae* and its n-hexane partition fraction, and Glibenclamide (5 mg/kg) elicited comparable blood glucose levels at 1 h to 4 h in the glucose-loaded rats. These glucose levels were significantly lower than those of the negative control group and rats given the other fractions (Table 2). Hence, n-hexane fraction was shown to be the most active fraction, while an activity profile similar to those of Glibenclamide and methanol extract (Table 2) suggested that the main insulinotropic constituents of the extract may be concentrated in this fraction [1-3,7,8,30,31]. Hence, this fraction was chosen for further purification.

## Glucose lowering effects of the column fractions of *S. biafrae*

Of the 12 bulked fractions obtained from the CC of $B_1$, the low yield of $C_1$ prevented its assay for anti-hyperglycemic activity, while $C_8$ and $C_9$ were devoid of glucose lowering activity. The 31%, 34% and 43% blood glucose reductions given by $C_{10}$ were comparable with those elicited by $C_{12}$ at 1.0 h to 4.0 h. They were also comparable with the activity given by Glibenclamide at 2 h and 4 h and significantly higher than that of the standard drug at 1 h. Also, Antihyperglycemic activity of $C_{12}$ was significantly higher than those of $C_{10}$ and Glibenclamide. The 32 and 36% anti-hyperglycemic activity demonstrated by $C_{12}$ at 0.5 and 1 h, respectively and 31% by $C_{10}$ at 1 h (Table 3) showed that the extra pancreatic constituents suggested to be present in the methanol extract when tested at 400 mg/kg (Table 1), were probably concentrated in these CC fractions. Fractions $C_3$, $C_5$ to $C_7$ would similarly contain these active plant constituents. Hence, $C_{10}$ and $C_{12}$ which were obtained by methanol wash of the column, should contain the main Antihyperglycemic constituents of the extract and n-hexane fraction (Tables 1-3), operating through pancreatic and extra-pancreatic actions [1-3,6-8]. The remaining CC fractions demonstrated mild Antihyperglycemic activity. After $C_{10}$ and $C_{12}$ only $C_7$ gave the least blood glucose levels in the rats at 2 h and 4 h (Table 3) indicating that it may contain another set of main insulinotropic agents of the plant. Therefore, $C_7$, which gave the next highest blood glucose reductions of 28% and 25% at 2 h and 4 h,

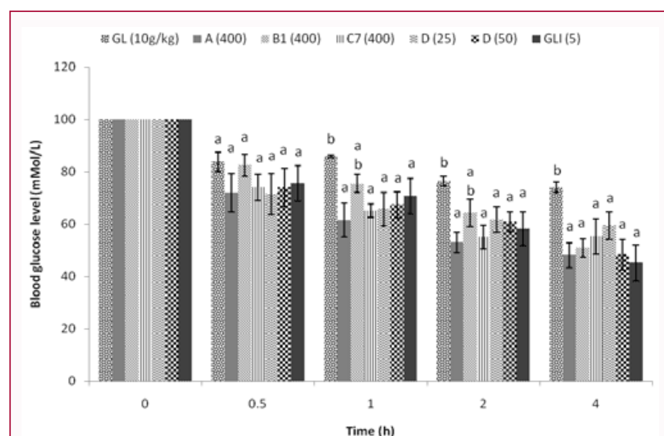


**Figure 3:** Glucose lowering effects of *Senecio biafrae* methanolic extract, its most active partition fraction and column chromatography sub fraction, and isolated β-stigmasterol.
Data show the mean ± SEM blood glucose levels at the different time points expressed as percentage of levels at 0 h ($T_0$), *n*=4. Values with different superscripts for each time point are significantly different (*p*<0.05), One-way analysis of variance followed by the Student–Newman–Keuls' test. **GL (10 g/kg)**: glucose in <1% of Tween-80 in normal saline (negative control); **A (400)**: Methanolic extract of *S. biafrae* at 400 mg/kg; **$B_1$ (400):** N-hexane partition fraction of *S. biafrae* tested at 400 mg/kg; **$C_7$ (400)**: Column fraction of *S. biafrae* at 400 mg/kg; **D (25), D (50)**: Isolated β-stigmasterol from *S. biafrae* at 25 and 50 mg/kg; **Gli (5):** Glibenclamide at 5 mg/kg (positive control).

respectively and blood glucose levels that were comparable to those given by Glibenclamide and its mother n-hexane fraction, was chosen for future investigation in the bid to isolate its insulin stimulating constituent. The CC fractions $C_8$ and $C_9$ with similar TLC pattern as $C_7$ were also investigated. Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at 0 h ($T_0$), n=5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (p<0.05, one-way analysis of variance followed by the Student–Newman–Keuls' test). GLU (10 g/kg): Glucose in <1% of Tween 80 in normal saline administered at 10 g/kg (hyperglycemic negative control); A: Methanol extract of *Senecio biafrae* whole plant; B1: N-hexane fraction of *Senecio biafrae* methanol extract; $C_2$ to $C_{12}$: Column sub fractions of $B_1$; GLI (5 mg/kg): Glibenclamide at 5 mg/kg (positive control).

### Isolation and characterization of β-stigmasterol from *S. biafrae*

The fraction $C_7$ was purified by CC, PTLC and solvent wash (Figure 1) to isolate white crystalline compound that was identified as β-stigmasterol by comparing its NMR and MS spectral data with those in the literature and its TLC characteristics [27-29]. The assignments of all proton and carbon signals of this isolate were supported by COSY, TOCSY, HSQC, and HMBC correlations. The double bond, hydroxyl and other functional groups were confirmed by IR, while ESIMS confirmed their M+ and molecular formulae.

### Glucose lowering effects of the isolated β-stigmasterol

Using glucose-loaded rat model and at all hours, the antihyperglycemic activity demonstrated by 25 and 50 mg/kg of β-stigmasterol isolated from the active *S. biafrae* methanol extract were comparable with themselves and also with those of the standard drug (Glibenclamide, 5 mg/kg), 400 mg/kg of active *S. biafrae* methanol extract (A), n-hexane fraction ($B_1$), and most active CC fractions C7 (Figure 3). This identified β-stigmasterol one of the antihyperglycemic constituents of *S. biafrae*. Therefore, the 49% and 52% glucose lowering effects elicited by methanol extract (A) at 0.5 h and 1.0 h, respectively (Table 1) 12% anti-hyperglycemic activity by n-hexane fraction ($B_1$) at 1.0 h (Table 2), 24 and 31% activity given by CC fractions $C_7$ and $C_{10}$ at 1.0 h, respectively, 32% and 36% activity by $C_{12}$ at 0.5 h and 1.0 h, respectively (Table 3), when tested at 400 mg/kg, as well as the 15% and 23% anti-hyperglycemic activity given by β-stigmasterol (25 mg/kg) isolated from At 0.5 and 1.0 h, respectively (Figure 3), may confirm reported extra-pancreatic action of β-stigmasterol [34]. Anti-hyperglycemic activity, which is operating through increased peripheral utilization of glucose, had been reported as the extra-pancreatic mechanism of action of β-stigmasterol that was isolated from the aerial parts of Bacopamonnieri [34]. Furthermore, the 53% and 69% anti-hyperglycemic activity demonstrated by extract A (Table 1), 16 and 32% by its fraction $B_1$ (Table 2), 28 and 25% activity by $C_7$, 34 and 43% activity by $C_{10}$, and the 33% and 39% activity by its $C_{12}$ sub fractions, respectively at 2 h and 4 h (Table 3), when tested at 400 mg/kg in this present study, as well as the 20 and 35% anti-hyperglycemic activity given by this isolated β-stigmasterol (50 mg/kg) at 2 h and 4 h, respectively (Figure 3), suggested insulin stimulation as an additional and hitherto unreported mechanism of action of β-stigmasterol and *S. biafrae* [1-3,6-8,33].

## Conclusion

The results of this present study confirmed that the plant, *S. biafrae,* is non-toxic and therefore may be safe for human use, has a significant anti-hyperglycemic activity that justified its antidiabetic ethno-medicinal use, has additional anti-oxidant effects, its extract possibly exerts this anti-hyperglycemic effect through both extra pancreatic and insulin stimulating mechanisms of action, β-stigmasterol is one of its active constituents, and reported additional insulinotropic mechanism of action for β-stigmasterol.

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