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In Vitro and *In Vivo* Evaluation of Antidiabetic Potential of Extracts of Selected Medicinal Plant Preparations Collected from Nigerian Traditional Medical Practitioners

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Diabetes mellitus is one of the main chronic diseases worldwide. It is characterized by persistent hyperglycaemia. Individuals with persistent hyperglycaemia are at high risk of developing complications (blindness, leg amputations and kidney failure). Nigeria is 1 of 4 highly populated countries in Africa with the largest number of people with diabetes mellitus majority of who use plant extracts given to them by traditional medical practitioners (TMPs).
Objectives: To assess the antidiabetic potential of extracts of selected Nigerian medicinal plant recipes collected from TMPS.
Materials and Methods: Eighteen recipes (maximum 2 plants) were selected after an ethnobotanical survey of medicinal plant recipes used by TMPs in the management of diabetes mellitus in the 6 geopolitical zones of Nigeria. Recipes were extracted in water according to traditional usage and screened *in vitro* to assess glucose uptake in C2C12 muscle cells and glucose production by the H4IIE liver cells (through inhibition of glucose-6-phosphatase, the rate limiting enzyme) and *in vivo* through the oral glucose tolerance test in normal mice (2 g/kg glucose).
Results: Two extract recipes (NC01 and NC09E) stimulated glucose uptake in C2C12 cells, 5 (NW14, NE15, NC01, SE01 and NW01) reduced glucose production in H4IIE cells and 4 (SE04, SE11, NC01 and NC09E) prevented hyperglycaemia in glucose-loaded normal mice. **Conclusion:** Only a few of the extracts from the recipes collected from the TMPs showed antidiabetic activity by increasing glucose uptake in the muscle (11%), reducing glucose production in the liver (28%) and/or preventing hyperglycaemia (22%).

Keywords: Diabetes mellitus, Recipe extracts, Glucose uptake, Glucose production, Oral glucose tolerance test

INTRODUCTION

Diabetes mellitus is one of the main chronic, non-communicable diseases worldwide. It is characterized by persistent hyperglycaemia due to decreased insulin secretion, insulin sensitivity or both. Several organs (such as the pancreas, the liver, the muscle and the adipose tissue) are involved in glycemic control. Post-prandial glucose uptake in skeletal muscle and adipose tissue accounts for 85% of glucose disposal, while the liver regulates fasting glycaemia (DeFronzo, 2004). Therefore, regulation of glucose utilization and production in these tissues is crucial for diabetes management. In Sub-Saharan Africa, the prevalence and the burden of type 2 diabetes mellitus are increasing (Nyenwe *et al*., 2003; Mbanya *et al*., 2010). In 2013, Nigeria had the largest number of persons living with diabetes mellitus in Africa with at least 63% undiagnosed and asymptomatic (International Diabetes Federation, 2013). A wide range of antidiabetic medicines (insulin, sulphonyl ureas, biguanides, thiazolidinediones, inhibitors of α-glucosidase, DPPIV and SGLT2) are available worldwide, but a majority of Nigerian diabetic population lacks access to

these medicines, cannot afford meaningful treatment and do not store these medicines properly (International Diabetes Federation, 2013; Ezuruike and Prieto, 2014). Many Nigerian diabetics also have drug therapy problems with these medicines (Odili *et al*., 2011) and therefore use herbal remedies from traditional medical practitioners, herb sellers and herbalists (Abo *et al*., 2008; Gbolade, 2009; Jegede *et al*., 2011). Although some of these products are registered by the Nigerian National Agency for Food and Drugs Administration and Control (NAFDAC), none of them is standardized and only a few have been tested for efficacy and safety (Akah & Okafor; 1992, Ojewole & Adewunmi, 2004; Chika & Bello, 2010). The need to provide culturally adaptable, cheap, proven and readily available treatments for diabetes mellitus is therefore pertinent in Africa's most populated country. In our previous work, we established that most Nigerian diabetics visit TMPs either as first line treatment or when orthodox medicines fail (Jegede *et al*., 2011). As part of an on-going project with the overall aim of verifying the efficacy and safety of Nigerian medicinal plants traditionally used for the treatment of diabetes mellitus, standardizing them based on content of active ingredients or on biological activity and subsequently developing phytomedicines for managing diabetes mellitus, our team collected about 90 anti-diabetic medicinal plant preparations from TMPs in Nigeria (Jegede *et al*., 2011). The TMPs gave only the number of plants in the preparations; the names of the plants were not given to protect their Intellectual Property Rights. For ease of extraction and phytochemical screening, we selected 18 of the preparations with a maximum of 2 plants composition and evaluated their antidiabetic potential using cell-based *in vitro* and *in vivo* assays.

MATERIALS AND METHODS

Collection and authentication of clinical isolates

Collection of medicinal plant preparations and preparation of extracts

Many medicinal plant preparations used for managing diabetes mellitus were collected in situ from TMPs through an ethnonobotanical survey (Jegede et al., 2011). The plant preparations were packed in glass containers and coded according to the region from where they were collected. For the purpose of this study, 18 of the plant preparations with one or two plant materials were selected (Figure 1).

Each plant preparation was pounded into powder using a mortar and pestle. According the instructions of the TMPs, 10-65 g of each powdered plant material was extracted in distilled water overnight by decoction, hot infusion or cold maceration. The resulting extracts were filtered using a mesh cloth and freeze-dried prior to storing in desiccators at room temperature**.** The phytochemical compositions of the extracts were determined by standard methods (Harborne, 1998; Evans, 2002; Sofowora, 2008) (Table 1).

In vitro cell-based assays

Cell culture

H4IIE rat hepatoma cell line cells (American Type Culture Collection, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v Fetal Bovine Serum (FBS) and 0.5% Penicillin/streptomycin in a humidified incubator at 37^oC with 5% CO_2 and 95% air atmosphere. H4IIE cells were proliferated in 12 well plates until they reached 90% confluence then treated with different conditions. C2C12 murine skeletal myoblasts (ATCC) were cultured in DMEM containing 10% FBS, 10% horse serum (HS) and 0.5% Penicillin/streptomycin until 75-80% confluence, then differentiation was initiated by culturing the cells DMEM containing 2% HS for 6 days. Media was changed every 2 days. Cell culture reagents were obtained from Wisent Inc. (St-Bruno, Quebec).

Determination of maximum non-toxic extract concentrations

Plant extracts were solubilized in DMSO, filter sterilized, aliquoted and stored at -20^0C. Aliquots were thawed at room temperature and dissolved in experimental medium at 1:1000 for a final DMSO concentration of 0.1%. The maximum non-toxic concentrations of extracts was determined by treating H4IIE cells or C2C12 myotubes (on 6^{th} day of differentiation) with media containing either vehicle (0.1% DMSO) or extract at concentrations ranging from 6.25 to 100 µg/ml for 18 h. Toxicity was assessed by using cytoxicity detection kit (Roche Diagnostics, GmbH, Mannheim, Germany) according to manufacturer's specifications. This assay is based on the release of lactate dehydrogenase (LDH) in media when cells are exposed to toxic agents. Briefly, after collecting the supernatant, cell layer was treated with 1 % triton-x 100 for 30 min and scrapped off (cell lysates). Supernatants and lysates were then centrifuged at 250 g for 10 min at 4^0C. Quantitation of LDH levels in either supernatant or lysates was obtained by combining each sample (50 µl) with LDH solution (100 µl), incubating for 30 min at room temperature in the dark and reading absorbance at 492 nm. Toxicity was calculated as a ratio of LDH released into the supernatant to the total intracellular LDH (supernatant + lysate). Three independent experiments of 3 replicates each were performed for each extract in C2C12 and H4IIE cell lines.

Determination of glucose uptake

On day 6, fully differentiated myotubes grown in 12 well plates were treated with either vehicle (DMSO 0.1%) or the maximum non-toxic concentration of extracts for 18 h. Insulin (100 nM) was used as positive control. After 18h, cells were rinsed twice with Krebs-phosphate buffer (KRB; 136 mM NaCL, 20 mM HEPES, 4.7 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 4.05 mM Na_2HPO_4, 0.95mM NaH_2PO_4), containing 5 mM D-glucose, pH 7.4 at 37^0C. They were then incubated with KRB alone (vehicle) or extracts or KRB containing insulin 100 nM (positive control) for 30 min at 37^0C. After 2 washes with warm glucose-free KRB, glucose uptake was initiated by adding assay buffer (glucose-free KRB containing 0.5 µCi/mL 2-

deoxy-D-[1-^{3}H]-glucose) and incubating for 10 min at 37 ^{0}C. Background non-specific glucose transport was assessed in certain wells by adding cytochalasin B (10 µM) in assay buffer. At the end of the assay, cells were placed on ice, immediately washed thrice with ice-cold glucose-free KRB, lysed in 0.1N NaOH at room temperature for 30 min then scraped. Lysates were transferred to a tube containing 4 mL Ready-Gel liquid scintillating cocktail and incorporated radioactivity was measured in a scintillating counter. Three separate experiments of two replicates each were performed for each extract. Cell protein content was measured with bicinchoninic acid using the Pierce^{R} (BCA) protein assay kit. Glucose uptake was normalized with the cell protein amount and results are expressed as percentage glucose uptake versus vehicle.

Determination of glucose-6-phosphatase activity

Glucose-6-Phosphatase (G-6Pase) activity was measured in H4IIE rat hepatoma cells. Briefly, cells (90% confluent in 12 wells/plate) were treated for 18 h with 0.1% DMSO (which was the vehicle used), insulin (100 nM), or the maximum non-toxic concentration of extracts in serum free media. After 18h, cells were washed, then lysed in 15 mM Phosphate buffer containing 0.05% Triton and 1.3 mM Phenol (pH = 6.5). Cell lysates were incubated in glucose-6-phosphate-containing buffer (200 mM) for 40 min at 37°C, where the G6P served as a substrate for endogenous Glucose-6-phosphatase to yield glucose. Quantification of the glucose generated in this reaction was measured using Wako AutoKit Glucose (Wako Chemicals USA Inc, Richmond, VA), according to manufacturers' recommendations. Briefly, using a colorimetric method, samples were incubated for 5 min at 37°C with Wako kit colour reagent (composed of mutarotase, glucose oxidase, peroxidase, 4-aminoantipyrine and ascorbate oxidase), then absorbance was recorded at 505 nm). Protein content was determined using the BSA method. Glucose production was normalized to protein content and results were presented as percentage of vehicle control activity (DMSO 0.1%).

***In vivo* assay**

Animals, diets and treatments

Male and female Swiss albino mice (aged 6-8 weeks; 19-25 g) were bred at the Animal house of National Institute for Pharmaceutical Research and Development (NIPRD) and fed a standard pellet diet. Animals with fasting blood glucose concentration (BGC) of 54-108 mg/dL were selected to form groups of matched BGC and placed in one cage (4-6 mice/group). Plant extracts and metformin (Sigma^{R}, Germany) were dissolved in distilled water at concentrations which ensured that a 30 g mouse received a maximum of 0.3 ml solution or vehicle and administered orally (by cannula). For the plant extracts, a dose of 800 mg/kg body weight was selected based on preliminary results in which 200 and 400 mg/kg bodyweight doses did not lower BGC significantly (data not shown). Metformin (400 mg/kg bodyweight) was used as positive control while distilled water was used as vehicle control). All experiments were conducted in accordance with guidelines for the care and use of laboratory animals (Olfert *et al*., 1993).

Oral glucose tolerance test (OGTT)

OGTT was performed 45 min following administration of vehicle, metformin or extracts. All mice received glucose (Sigma, USA) at 2 g/kg then BGC was measured 15, 30, 60 and 90 min (i.e. 60, 75, 105 and 135 min after treatments). Mice tails were pricked and glycemia was measured using Accu-Check^{R} glucometers (Roche Diagnostics, Germany), which were calibrated after every 10 readings.

Analysis of data

In vitro results are presented as mean % of control ±SEM. One way analysis of variance (ANOVA) followed by Dunnet's post hoc test was used for comparisons between the vehicle control and the treatment or positive control, with $p<0.05$ considered as statistically significant.

From preliminary experiments, it was estimated that a sample size of 4-6 mice per group would be sufficient to detect a change of 58.5 mg/dL BGC between vehicle and treatment groups with 80% statistical power using a 5% significance level. $P<0.05$ was considered statistically significant (One way ANOVA followed by Dunnet's post hoc test). *In vivo* results are presented as means (± SEM) of BGC and % glycaemia.

% glycaemia was calculated as a function of time (t) by using the formula:

$$\% \text{ glycaemia} = \frac{(G_0 - G_t)}{G_0} * 100 \qquad (1)$$

Where G_0 is fasting BGC and G_t are BGC at 15, 30, 60 or 90 min after oral glucose administration.

RESULTS

Phytochemical composition of extracts

All the extracts with the exception of NE03, NE07A, NC01, NC09E, SE01, SE11 and SS03 had over 5% yields. The phytochemical tests revealed the presence of various secondary metabolites including alkaloids, flavonoids, tannins, terpenes, sterols, saponins, carbohydrates and anthraquinones. NE05 and NE07D did not contain carbohydrates while NE03 and NE15 were the only ones not containing phenols (Table 1).

Effect of extracts on glucose uptake in C2C12 mouse muscle cells

Insulin potentiated glucose uptake by 41.7% ± 9.4 as compared to DMSO control ($p \leq 0.01$). Only two extracts (NC01 and NC09E) significantly stimulated glucose uptake in C2C12 muscle cells in comparison to DMSO by 38.7% ± 5.5 and 42.7% ± 11.0, respectively ($p \leq 0.05$ vs DMSO). NE05, NE07A and SE04 decreased glucose uptake although these did not reach statistical significance (Figure 2).

Effect of extracts on glucose production in in H4IIE rat hepatoma cells

Insulin decreased the activity of the G-6Pase enzyme by 58.1% ± 4.6 ($p \leq 0.001$ vs DMSO). Five extracts (NE15, NW01, NW14, NC01 and SE01) significantly diminished the activity of G-6Pase. NE15 and NW01 decreased enzyme activity by 43% ($p \leq 0.05$ vs DMSO), while NW14, NC01 and SE01 lowered it by 45.0% ± 11.6, 41.4% ± 8.7 and 46.2% ± 7.2, respectively ($p \leq 0.01$ vs DMSO). NE08 slightly increased glucose production, but this was not statistically relevant (11.2% ± 3.7 vs DMSO (Figure 3).

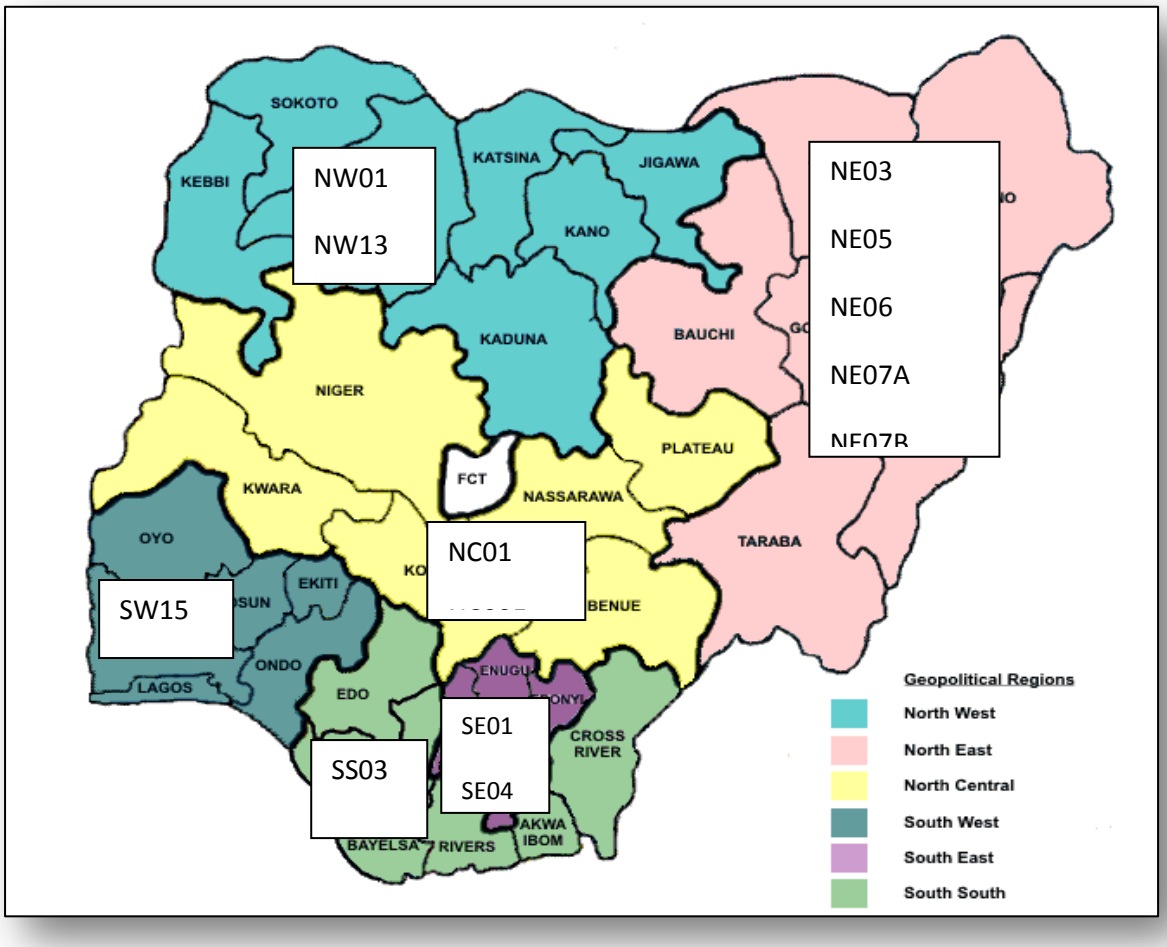

Figure1: The map of Nigeria

Plant preparations were collected from TMPS in the 6 geopolitical zones shown on the map. Eighteen preparations with a maximum of 2 plants were selected (in boxes).

Effect of extracts on BGCs in normal glucose loaded mice

Like metformin, 4 extracts (NC01, NC09E, SE04 and SE11) significantly prevented ($P<0.001$, $P<0.01$ and $P<0.05$ respectively) elevations in BGCs in glucose loaded normal mice. However these reductions in BGCs were not maintained for SE04 after 15 minutes. At the end of the study (90 min after glucose load), the % glycaemia reduced to below 50% in all but the vehicle and 5 extract treated groups (NE05, NE07B, NE07D, NW14 and NC09E) (Table 2).

DISCUSSION

Our team aimed at identifying and evaluating the antidiabetic potential of extracts from selected medicinal plant preparations traditionally used by TMPs from the 6 geopolitical zones of Nigeria. Plant preparations were collected from TMPs as mixtures of dry plant materials to avoid situations where powdered materials would have been adulterated (Schatrz, 2008). Since plant extracts generally contain many complex secondary metabolites acting synergistically or antagonistically to produce biological activity (positive activity or reduced toxicity), we selected plant preparations composed of a maximum of 2 plants to limit interactions and to facilitate future identification of active compounds. Therefore, 18 plant

preparations were selected and their water extracts screened *in vitro* and *in vivo* to address two major components of the multifaceted aetiology of diabetes mellitus: *in vitro* glucose uptake into muscle cells and hepatic glucose production by the liver. The *in vivo* assay addressed hyperglycaemia as a consequence of reduced intestinal glucose absorption, decreased insulin or incretin secretion. It is worthy to note that the extracts were prepared according to traditional usage using water as solvent, which is also consistent with the principle of green chemistry (water is cheap and non-toxic).

The results of the study indicate that aqueous extracts from traditionally used medicinal plant preparations possess insulin like effects *in vitro* and metformin-like effects *in vivo.* It is noteworthy that the effect of these extracts in preventing hyperglycemia when administered prior to glucose load is suggestive of possible involvements of the following mechanisms; inhibition of glucose absorption (probably through α-glucosidase inhibition), stimulation of incretins and/or insulin secretions.

The exact mechanism requires further investigation. Several organs are involved in the development and the aetiology of diabetes mellitus, which complicates screening and validation of traditional medicines (Van de Venter *et al*., 2008). Glucose uptake into muscle cells represent the main pathway (80%) of glucose utilization in humans following a meal, while the liver plays a major role in regulating glycemia at moments of stress, starvation and hunger. In addition, the adipose tissue is targeted by insulin, where it regulates free fatty acid release thus playing an important role in insulin resistance and hyperglycemia. Modulation of pancreatic beta-cell survival and function is crucial for the development and management of diabetes mellitus. Other organs such as the intestines, the kidneys and the brain are also involved (DeFronzo, 2004). In consequence, most of the extracts did not stimulate glucose uptake in C2C12 muscle cells (89%) or decrease glucose production in H4IIE cells (72%) or prevent hyperglycaemia in glucose loaded normal mice (78%, within 60 min). However, a high percentage of the extracts (72%) decreased glycaemia levels to below 50% at 90 min indicating that their onset of action may be longer than 90 minutes and they may exhibit other antidiabetic activities involving other tissues.

These extracts displayed pleiotropic antidiabetic activities which would help manage diabetes at several levels.

A 2nd visit to the TMPs is being planned to intimate them of these findings and to sign memoranda of understanding with the few of them whose plant preparations showed activity. Through this, the local names of the components of the preparations will be established, the herbarium specimens collected, botanical names established. These will facilitate the standardization of the extracts and their subsequent development as culturally adaptable phytomedicines for managing diabetes mellitus**.**

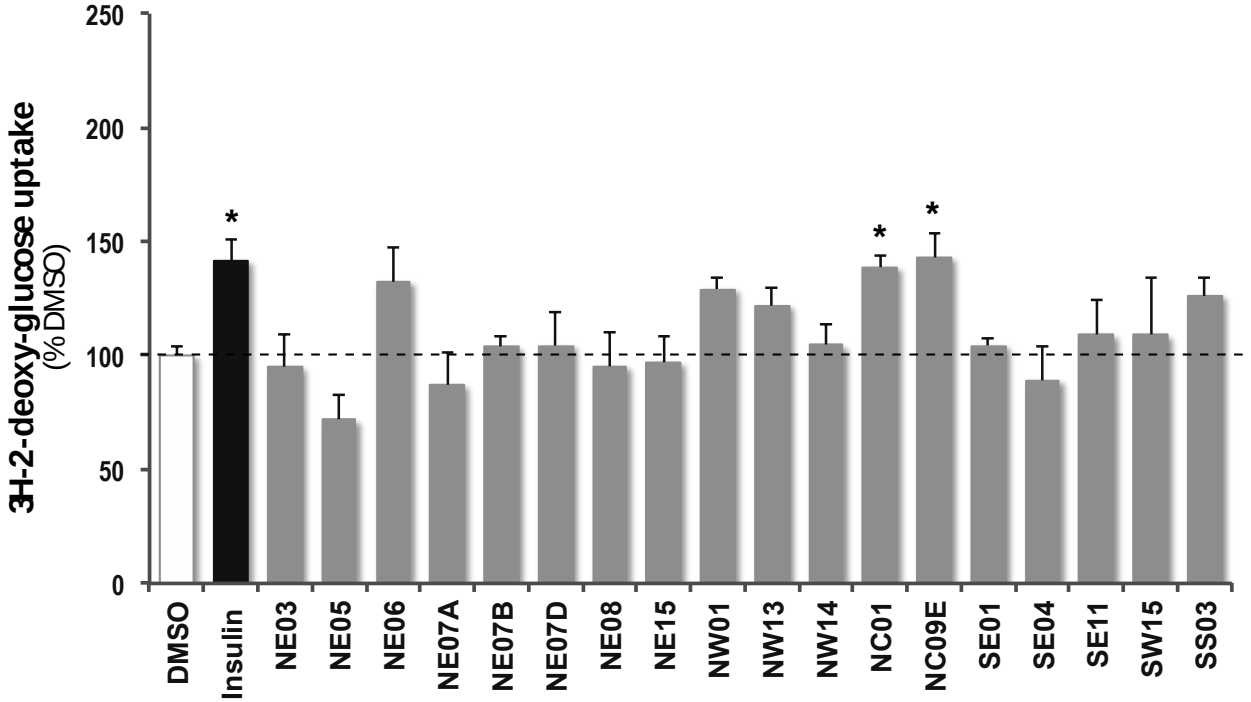

Figure 2: Effect of extracts on ^{3}H-deoxy-glucose uptake in C2C12 muscle cells.

Cells were treated with vehicle, recipe extract concentrations for 18 h or insulin for 30 min. Results are presented as % relative to vehicle-treated basal condition for 3 separate experiments each with 2 replicates.*, significantly increased uptake at $p<0.05$ compared with vehicle (one-way ANOVA and Dunnet's post hoc). Insulin concentration used was 100 nM while the non-toxic concentrations (µg/ml) of extracts used were 12.5 (SE04), 25 (NE03, NE07A, NE07B, SW15), 50 (NE07D, NE08, NW14, NC01) and 100 (NE05, NE06, NE15, NW01, NW13, NC09E, SE01, SE11, SS03) respectively.

Table 1 :Methods of extraction, yields and phytochemical constituents of selected Nigerian antidiabetic plant preparations collected from TMPs.

Sample	Method of extraction	% Yield	Carbo hydrates	Tannins	Flavonoids	Phenols	Alkaloids	Balsams	Anthra-quinones	Cardiac-glycosides	Saponins	Terpenes	Sterols
NE03	D	3.3	+	+	-	-	+	-	+	-	+	+	+
NE05	CM	7.4	-	-	-	+	-	+	+	-	+	-	-
NE06	CM	6.1	+	+	-	+	+	-	+	-	+	+	+
NE07A	D	4.5	+	-	+	+	+	-	+	-	+	+	+
NE07B	D	7.5	+	-	-	+	-	+	+	-	-	+	+
NE07D	CM	6.0	-	-	+	+	+	-	-	+	-	-	-
NE08	HI	8.5	+	-	+	+	-	+	-	+	-	-	-
NE15	HI	11.0	+	+	-	-	+	-	+	-	+	+	+
NW01	HI	5.1	+	+	+	+	-	+	+	+	+	-	-
NW13	D	7.8	+	+	+	+	-	-	-	-	+	+	+
NW14	D	6.9	+	+	-	-	+	-	-	+	+	+	+
NC01	HI	1.8	+	-	+	+	+	+	-	+	+	-	-
NC09E*	D	1.0	+	-	+	+	+	+	-	+	+	-	-
SE01	HI	1.0	+	+	-	+	-	+	-	-	+	+	+
SE04	D	6.2	+	+	+	+	-	-	-	-	+	+	+
SE11	D	2.5	+	+	+	+	+	+	-	+	+	-	-
SW15	HI	5.0	+	+	+	+	-	+	-	+	-	+	+
SS03	HI	2.4	+	-	+	+	+	-	-	-	+	-	-

HI=Hot infusion. D=Decoction. CM=Cold maceration. *With 0.5 g red potash.
%Yield=weight of freeze dried extract/weight of dry plant material x 100%.+ =present. - = absent.
NE=North East Nigeria. SE=South East Nigeria. NW=North West Nigeria. SW= South West Nigeria. NC=North Central Nigeria. SS=South South Nigeria.

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Table 2: Blood glucose concentration in normal mice before and after oral administration of glucose and extracts of selected Nigerian antidiabetic plant preparations

	Blood glucose concentration (mg/dL)				
Time (min) post oral glucose load	0	15	30	60	90
Samples					
Vehicle	73±4 (0)	275±13 (277)	312±19 (327)	176±13(140)	119±7 (63)
Metformin	76±4 (0)	122±8*** (61)	128±11*** (69)	86±6 (14)	70±4 (-8)
NE03	91±5 (0)	339±23 (273)	256±18 (181)	165±18 (82)	125±11 (37)
NE05	64±4 (0)	275±27 (329)	307±16 (379)	172±17 (169)	97±5 (52)
NE06	72±1 (0)	303±18 (320)	274±40 (280)	157±22 (118)	101±6 (41)
NE07A	86±4 (0)	279±21 (224)	258±34 (200)	151±24 (76)	112±9 (30)
NE07B	94±5 (0)	335±33 (256)	280±33 (198)	185±19 (97)	146±8 (55)
NEO7D	87±5 (0)	243±24 (179)	280±22 (222)	183±13 (111)	131±8 (51)
NE08	90±6 (0)	263±18 (192)	281±10 (212)	146±10 (63)	108±9 (22)
NE15	85±9 (0)	259±21 (204)	219±31 (157)	114±25 (34)	85±15 (0)
NW01	63±4 (0)	215±27 (240)	214±28 (240)	93±9 (47)	80±8 (26)
NW13	80±9 (0)	297±30 (272)	266±16 (233)	142±9 (77)	107±8 (34)
NW14	65±2 (0)	225±25 (245)	226±18 (247)	166±20 (155)	126±7 (124)
NC01	81±10 (0)	138±24** (70)	128±20*** (22)	99±16 (22)	101±19 (25)
NC09E	62±6 (0)	158±34* (154)	168±38* (171)	133±25 (114)	107±9 (72)
SE01	80±9 (0)	229±32 (186)	252±35 (215)	135±12 (68)	94±9 (17)
SE04	92±5 (0)	172±29* (87)	301±24 (228)	274±29 (197)	120±13 (31)
SE11	65±7 (0)	200±54 (208)	179±45* (175)	98±18 (50)	80±13 (22)
SW15	65±4 (0)	305±76 (369)	236±36 (263)	155±23 (138)	94±6 (44)
SS03	63±5 (0)	250±36 (297)	247±27 (292)	129±23 (105)	87±16 (38)

Values are means ±SEM for 4-6 mice per sample. Metformin and extracts were administered orally at 400 and 800 mg/kg body weight respectively. Vehicle was administered at $p<0.05$, ** $p< 0.01$, *** $p<0.001$ versus blood glucose concentration of vehicle treated mice at the corresponding time points. % glycaemia is given in parenthesis.

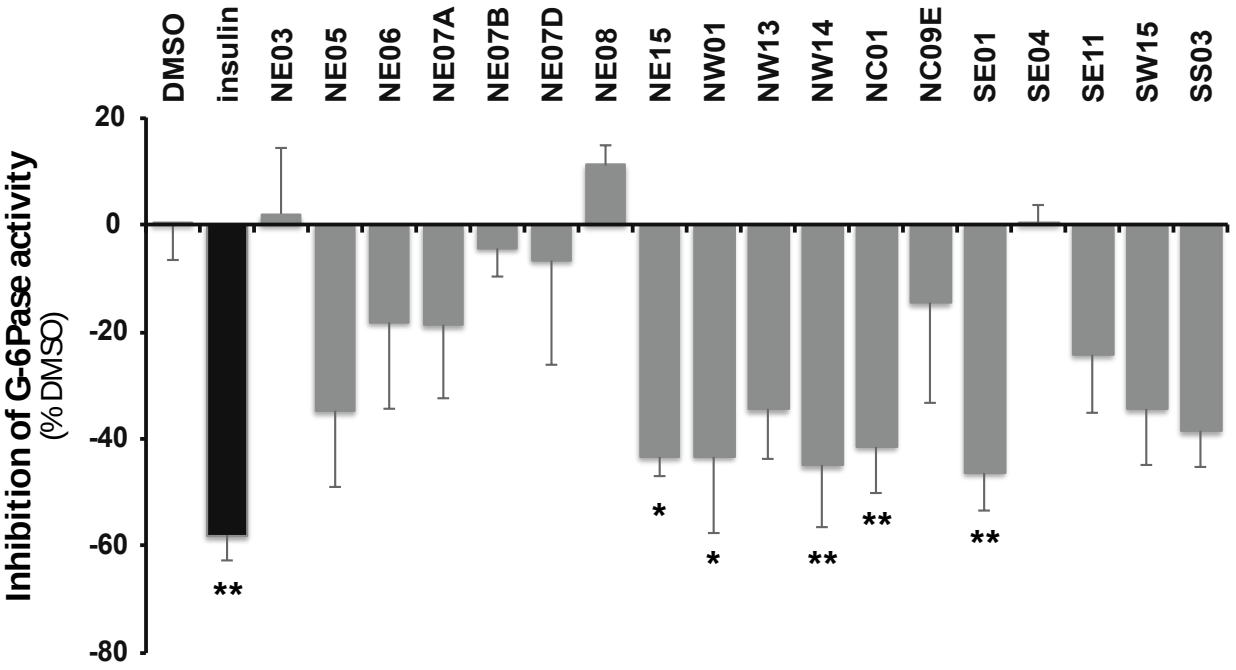

Figure 3: Effect of extracts on glucose production in H4IIE hepatoma cells.

Cells were treated with vehicle, extract concentrations or insulin for 18 h. Results are presented as % relative to vehicle-treated basal condition for 3 or 4 separate experiments each with 4 replicates.*, significantly increased uptake at $p<0.05$ compared with vehicle (one-way ANOVA and Dunnet's post hoc). Insulin concentration used was 100 nM while the non-toxic concentrations (µg/ml) of extracts used were 6.25 (SE04), 12.5 (NE03, NE07A), 25 (NE07B, NE07D NE08, SW15), 50 (NE05, NE06, NE15, NW01, NW14, NC01, NC09E, SE11, SS03) and 100 (NW13, SE01) respectively.

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