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Combination of *Gymnema sylvestre* and *Combretum micranthum Methanol* Leaf Extracts Produced Synergistic Hypoglycaemic Activity in Alloxan Diabetic Mice

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Original Research Article

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Abstract: Herbal combinations when used together may sometimes produce enhanced, same or diminished effect. As a result, herb combinations used in the management of diabetes mellitus need to be thoroughly investigated to provide the best effect in reducing the major underlying cause (hyperglycemia) of the disease. Aqueous extracts of *Gymnema sylvestre* (GS) and *Combretum micranthum* (CM) leaves were subjected to solvent-solvent partitioning along different polarities. The best fractions (methanol) in terms of *in vivo* fasting blood glucose (FBG) in alloxan-diabetic mice were subjected to column chromatography and preparative-TLC to obtain sub-fractions and sub-sub-fractions respectively. Sub-fractions D and G for GS and CM respectively gave the best reduction in FBG *in vivo* while sub-sub-fractions D4 and G4 yielded highest percentage reduction in FBG in alloxan-diabetic mice. The probable mode of action of the phenolic compounds identified by UHPLC-QTOF-MS/MS in both plants could be by inhibiting activities of α-amylase and α-glucosidase. At a dose of 10 mg/kg body weight, a 1:1 combination of GS-D4 and CM-G4 sub-sub-fractions, containing mainly phenolic compounds, significantly ($P < 0.05$) reduced FBG to 58.25±7.54% in 2 hours and 69.11±6.78% in 4 hours in a synergistic manner.

Keywords: Herbal combination; synergy; diabetes mellitus

INTRODUCTION

Diabetes mellitus (DM) is defined as a syndrome characterized by hyperglycaemia and disturbances of carbohydrate, fat and protein metabolisms as a result of absolute or relative deficiencies in insulin action and/or secretion [1 - 6].

Total recovery from diabetes has yet to be reported and this explains the intensive studies done on it [7]. The International Diabetes Federation estimates that there are over 300 million people around the world with diabetes. This total is expected to reach close to 500 million within 20 years [8]. Drugs with plants as raw materials are increasingly favoured by people all over the world [9–11] for their unique advantages in preventing and curing diseases, rehabilitation and health care, especially in Europe, the United States, Africa and many Asian countries. In sub-saharan Africa, the use of multiple herbs as decoctions or in different combinations to treat chronic non-communicable diseases like diabetes mellitus and hypertension is common [12, 13]. However, some of these plants, when used together, can have either positive or negative effects on the individual. Moreover, scientific study of herbal remedies and their potential to cause interactions when used in combination need to be thoroughly understood and systematically studied so as to understand whether there is synergy, antagonism and/or additivity. The Plants *Gymnema sylvestre* R.Br. ("periploca of the woods" in English; "Kafi suga" in Hausa) (Asclepiadaceae) and *Combretum micranthum*, Fam. (Géézà in Hausa) (Combretaceae)) are used for the management of Diabetes Mellitus in the North-Western part of Nigeria [14, 15]. Researches have shown that phytochemicals when combined together, interact synergistically with each other in *in vivo* and *in vitro* conditions [16-20]. According to an ethnobotanical literature on traditional phytotherapy, species like *Asparagus racemosus, Butea monosperma, Cathanranthus roseus, Coccinia indica, Gymnema sylvestre, Syzygium cumini and Momordica charantia* are used, singly or in combinations, by communities for the treatment of diabetes [21]. Moreover, methanolic extract (75%) of *Terminalia chebula, Terminalia belerica, Emblica officinalis* and their combination named 'Triphala'(equal proportion of above three plant extracts) were found to inhibit lipid peroxide formation, scavenge hydroxyl and superoxide radicals *in vitro* and an oral administration of the extracts (100 mg/kg body weight) reduced blood sugar level in normal and in alloxan (120 mg/kg) diabetic rats significantly within 4 hours [22]. A combination of *Abroma augusta* and *Curcuma Ionga* have shown antidiabetic activity and

also reduced oxidative stress in diabetes - it also restored other general parameters, like TBARS, GSH, SOD and CAT activities in diabetic animals. The results were statistically analyzed and indicated that combination of herbal extracts showed better efficacy as compared to individual herbal plant extracts used [23].

Due to this, the current study looks at leaves of the plants *Gymnema sylvestre* (GS) and *Combretum micranthum* (CM) used concomitantly for the management of diabetes mellitus in the Northern part of Nigeria, so as to find the effect of their combination which shall be beneficial to the patients in reducing the fasting blood glucose.

MATERIALS AND METHODS

Materials

Plant material collection and identification

Fresh samples of the plants *Gymnema sylvestre* (GS) and *Combretum micranthum* (CM) were obtained from Shira Local Government Area (N 11^{O} 27' 29" and E 10^{O} 2' 48" or Latitude: 11.46 and Longitude: 10.05) of Bauchi State in Nigeria. The plants were identified at the herbarium unit of department of Biological Sciences, Bayero University, Kano (B.U.K.) and voucher specimens, with number BUKHAN 0349 (for *Gymnema sylvestre*) and BUKHAN 0272 (for *Combretum micranthum*) were then deposited in the same herbarium.

Animals

Adult male mice (15 – 25 g body weight) were purchased from the department of Pharmacology and Therapeutics of Ahmadu Bello University, Zaria. They were kept in standard metal animal cages at room temperature in the animal house of Biological Sciences Department, B.U.K., were allowed free access to food and water and allowed to acclimatize for a week prior to use. The protocol of the study was according to international guidelines i.e. the Organization for Economic Cooperation and Development (OECD) Test Guidelines (TG407) [24].

Chemicals, Reagents and Equipment

All the chemicals and reagents used for this work were of analytical grade and purchased from reputable chemical manufacturers, e.g. SIGMA-ALDRICH-FLUKA. The laboratory equipment were also of standard quality.

Methods

Preparation of Plant Material (Aqueous Extraction)

Two hundred grams of the air-dried plant samples of each plant were cold extracted with 2000ml of distilled water – 3X. The extracts were then filtered through Whatman No. 1 paper. The filtrate of the aqueous extract was then evaporated initially, in a hot-air oven at 40^{o}C and then lyophilised under vacuum at -80^{o}C and a pressure of 0.06 mbar for 72 hours. The dried/powdered extracts were weighed, per cent yield calculated and stored at -20^{o}C for further analyses [25].

Bioassay-guided studies

Liquid–liquid partition of GS and CM aqueous extract

The lyophilized extracts from leaves of GS and CM (100g each), were separately dissolved in 500 ml of 60% methanol. A successive partition of the extracts yielding hexane (4.59 g), ethyl acetate (4.2 g) and residual 60% methanol (33.10 g) GS fractions and of the CM extract affording hexane (13.52 g), ethyl acetate (7.44 g) and residual 60% methanol (26.60 g) fractions were subsequently subjected to tests of antidiabetic activity. The bioassay-guided fractionation was only carried out with the 60% methanol fractions of each plant extract (GS and CM) because they were the most active in reducing the FBG in the animals, gave the highest yield with varied phytochemical contents and good α-amylase and α-glucosidase inhibition (i.e. lowest IC_{50} values).

Column chromatography of the 60% methanol fractions of GS and CM

i) Fractionation

This was done according to the work of Ode *et. al.* [26] with slight modifications. Twenty-five (25) g of the 60% methanol fraction of GS and CM found to have highest hypoglycaemic effect were subjected to column chromatography. Silica gel 60 - 120G was used as the stationary phase while varying solvent combinations of increasing polarity (hexane:ethyl acetate 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40: 60, 30:70, 20: 80, and 10:90; ethyl acetate : methanol 100:0, 70:30, 50:50, 30:70 and 0:100.) were used as the mobile phase in a glass column of dimensions 5.5 x 80cm. The eluted fractions were collected in aliquots of 50 ml in conical flasks.

Sixteen (16) sub-fractions of GS, monitored by TLC (mobile phase n-hexane/ethyl acetate 30:70), were collected and subjected to antidiabetic activity (reduction in FBG). Sub-fraction D (0.169 g, Rf = 0.66) showed the highest reduction trend in antidiabetic activity and, therefore, chosen for further fractionation and bioassay.

In the case of CM, 7 sub-fractions, monitored by TLC (mobile phase n-hexane/ethyl acetate 30:70), were collected and subjected to antidiabetic activity too. Sub-fraction G (2.8 g, Rf = 0.83) showed the highest reduction trend in antidiabetic activity and, therefore, chosen for further fractionation and bioassay. The 1:1 combinations (GSCM) of the above sub-fractions were also used for the *in vivo* bioassay screening for antidiabetic activity.

ii) Further bioassay-guided fractionation with Preparative TLC and screening of the fractions

The sub-fractions D and G above were subjected to preparative thin-layer chromatography on glass plates with silica gel 60-120G using n-hexane/ethyl acetate (3:7) as mobile phase. Commercially – prepared TLC plates (Merck, Germany) were used for this study. The bands were removed from the plates with silica and extracted with absolute methanol.

The eluent was separated from the adsorbent by filtering through a small No. 3 SINTAGLASS funnel, GALLENKAMP, England. This process was repeated until it was satisfactory that all the eluent has been collected as much as possible. The collected eluent in methanol was evaporated to dryness at room temperature (37^{O}C). The eluents were subjected to further bioassay test to confirm hypoglycaemic activity. Eluents that exhibited maximal *in vivo* hypoglycaemic ability (sub-sub-fractions D4 with Rf = 0.26 and G4 with Rf = 0.36) were then labelled as the active sub-fraction(s) and subjected to hypoglycaemic, LC-MS, and *in vitro* α-amylase/α-glucosidase inhibitory activities studies.

In vitro alpha-amylase and alpha-glucosidase inhibition assay

The α-amylase and α-glucosidase inhibitory activities of samples were determined according to the method described by Kim *et al.*[27]. For alpha glucosidase inhibition, yeast α-glucosidase was dissolved in 100 mM phosphate buffer, pH 7.0, containing bovine serum albumin (2 mg/ml) and sodium azide (0.2 mg/ml) which was used as enzyme source. Paranitrophenyl- α-d-glucopyranoside was used as substrate. Samples were weighed and serial dilutions of 50, 100, 150, 200, 250 µg/ml were made up with equal volumes of dimethylsulfoxide and distilled water. Then 10µl of extract dilutions was incubated for 5 min with 50µl enzyme source. After incubation, 50µl of substrate was added and further incubated for 5 min at room temp. The pre substrate and post substrate addition absorbance was measured at 405 nm. The increase in absorbance on substrate addition was obtained. Each test was performed three times and the mean absorbance was used to calculate percentage α-glucosidase inhibition.

Acarbose was used as positive control with various concentrations 50, 100, 150, 200, 250 µg/ml. Percentage α glucosidase inhibition was calculated according to the following formula [28].

$$\text{Percentage of inhibition} = \frac{\text{Control A405} - \text{Extract A405}}{\text{Control A405}} \text{X100}$$

The alpha amylase inhibition activity of the plant was assayed by the same method used for alpha glucosidase inhibition activity. For alpha amylase inhibition assay the enzyme porcine pancreatic amylase and substrate Paranitro phenyl alpha D-maltopentoglycoside were used and the absorbance measured at 540nm [28]. Percentage inhibition was calculated as follows:

$$\text{Percentage of inhibition} = \frac{\text{Control A540} - \text{Extract A540}}{\text{Control A540}} \text{X100}$$

In vivo bioassay screening of fractions

Experimental design (liquid-liquid partition)

The fractions from the liquid-liquid partition stage were used for this phase of the study. Mice of both sexes were weighed and assigned to groups of 3 mice each. All the mice were fasted for 12h prior to commencement of the experiment. A total of 27 mice were used for this phase of the experiment and grouped as outlined below:

Group I (GShex): given GS hexane fraction (600 mg/kg b. w.) orally;
Group II (GSetac): given GS ethyl acetate fraction (600 mg/kg b. w.) orally;
Group III (GSmeoh): given GS 60% methanol fraction (600 mg/kg b. w.) orally;
Group IV (CMhex): given CM hexane fraction (600 mg/kg b. w.) orally;
Group V (CMetac): given CM ethyl acetate fraction (600 mg/kg b. w.) orally;
Group VI (CMmeoh): given CM 60% methanol fraction (600 mg/kg b. w.) orally;
Group VII (normal control – non diabetic): given only distilled water (10 ml/kg) orally;
Group VIII (negative control - diabetic): received only distilled water (10 ml/kg) orally;
Group IX (positive control - diabetic): received glibenclamide (6 mg/kg) orally;

All the blood glucose concentrations (for determining hypoglycaemic activity) in the mice were determined using ACCU-CHEK Active® Glucometer and strips.

Experimental design (bioassay screening)

The sub-fractions from the column chromatography stage were used for this phase of the study. Mice of both sexes were weighed and assigned to groups of 4 mice each. All the mice were fasted for 12h prior to commencement of the experiment. A total of 100 mice were used for this phase of the experiment and grouped as outlined below:

Normal control group (non-diabetic), given only distilled water (10 ml/kg) orally;
Negative control (diabetic) group, received only distilled water (10 ml/kg) orally;

Positive control (diabetic) group, received glibenclamide (6 mg/kg) orally;
Groups (A – O) were given oral treatments with 40 mg/kg each of the *G. sylvestre* fractions (A – O) of the extracts respectively eluted from the chromatographic/TLC experiments.
Groups (B' – G') were given oral treatments with 40 mg/kg each of the *C. micranthum* fractions (B – G) of the extracts respectively eluted from the chromatographic/TLC experiments.

Group Z were given oral treatments with 40 mg/kg of 1:1 combination of fractions D and G of *G. sylvestre* and *C. micranthum* respectively. All the blood glucose concentrations (for determining hypoglycaemic activity) in the mice were determined using ACCU-CHEK Active® Glucometer and strips.

Further bioassay-guided fractionation - preparative TLC

Experimental Design (Further Bioassay-Guided Screening)

Sub- fractions D and G from the fractionation above were selected for re-fractionation and further studies. Mice of both sexes were weighed and assigned to groups of 4 mice each. All the mice were fasted for 12h prior to commencement of the experiment. A total of 60 mice were used for this phase of the experiment and grouped as outlined below:
Group A^1 (normal control – non diabetic), given only distilled water (10 ml/kg) orally;
Group B^1 (negative control - diabetic), received only distilled water (10 ml/kg) orally;
Group C^1 (positive control - diabetic), received glibenclamide (6 mg/kg) orally;
Groups D^1, E^1, F^1, G^1, and H^1, were given oral treatments with 10 mg/kg body weight each of the sub-fractions of *G. sylvestre* (D1, D2, D3, D4, and D5) respectively eluted from the preparative TLC experiments.
Groups I^1, J^1, K^1, L^1, M^1 and N^1 were given oral treatments with 10 mg/kg each of the sub-fractions of *C. micranthum* (G0, G1, G2, G3, G4 and G5) respectively eluted from the preparative TLC experiments.

Group O^1 animals were given oral treatments with 10 mg/kg body weight of 1:1 combination of sub-sub-fractions D4 and G4 of *G. sylvestre* and *C. micranthum* respectively. All the blood glucose concentrations (for determining hypoglycaemic activity) in the mice were determined using ACCU-CHEK Active® Glucometer and strips.

Determination of Combination Effect

To determine synergy or additive effect, interaction of the two fractions and sub-fractions of GS and CM were calculated as combination index (CI), estimated as

$$CI = \left[\frac{C_a}{IC_a}\right] + \left[\frac{C_b}{IC_b}\right]$$

where Ca and C_b are the concentrations of compounds/fractions A and B used together to achieve a fixed effect (ED_{50}). ICa and IC_b are the concentrations of A and B, respectively required individually to achieve the same effect. A CI value of less than, equal to, and more than one, indicates synergy, additivity, and antagonism respectively between these two compounds/fractions [29, 30].

Identification of compounds

UHPLC-ESI- Q-TOF-MS/MS

The UHPLC system (Agilent infinity 1290, USA) was incorporated with a binary pump (G4220A), an auto sampler (HiP - ALS, G4226A), degasser (G1322A), Thermostatted Column Compartment (G1316C) and a DAD detector (G4212A) with a light-pipe flow cell (recording at 280 and 320 nm). This was coupled to Ultra-High-resolution-Quadrupole-Time-of-Flight Mass Spectrometer (G6550A) (USA) equipped with an ESI source (DUAL AJS ESI) operating on Auto-MS/MS mode. The analysis was achieved in the negative ion mode in a mass range from m/z 100-2000. The ESI source parameters were: capillary voltage 3.5 KV; nebulizing gas pressure 2.4 bar; drying gas temperature 200.0°C, drying gas flow 14.0 l/min; iFunnel 1RF 750.0 V; transfer time 50.0 µs; and pre-pulse storage 2.0 µs. The MS data were analyzed through Agilent Mass Hunter Qualitative Analysis software (B.06.00).

The UHPLC separation was achieved on a ZORBAX Eclipse Plus C18, Rapid Solution, 4.6x100mm, particle size 3.5micron column (Agilent Technologies, USA). Solvent A was water:methanol (60:40 v/v) and solvent B was acetonitrile (100%). Solvents were delivered at a total flow rate of 0.5 ml/min. The gradient profile was from 0% B (100%A) to 100% B (0%A) linearly in 50 min (Table 3.1). The injection volume was 2.00 µl [31, 32].

Statistical Analysis

IC_{50} calculations were performed with Microsoft Excel. Results were presented as mean ± standard error of mean (SEM). Statistical analyses were performed using two tailed Student's t-test at 95% confidence limit (i.e. $P < 0.05$ being significant); Pearson's Correlations were also run to make comparisons between parameters. SPSS version 17.0 computer software/programme was used to perform the statistical calculations.

RESULTS

Phytochemical Analyses

Quantitatively, the methanol fraction of GS and CM aqueous extracts showed presence of all phytochemicals assayed for (i.e. tannins, saponins flavonoids and alkaloids) with significantly ($P < 0.05$) high levels of flavonoids in both plants – 25.89 ± 0.06 and 14.74 ± 0.21g/100g dry weight respectively (Table 1).

Table-1: Quantitative Phytochemical Contents of Extracts of *G. sylvestre* and *C. micranthum* Leaves

Extract type	Phytochemical Composition (g/100g dry weight)			
	Tannins	Saponins	Flavonoids	Alkaloids
GSAq	1.48±0.02	ND	10.69±0.01	ND
GSHex	0.47±0.01	ND	6.07±0.07	34.00±0.01
GSEtac	0.27±0.02	ND	8.52±0.04	ND
GSMeoh	3.85±0.08	18.53±0.19	25.89±0.06	6.10±0.10
CMAq	1.06±0.07	ND	3.14±0.03	10.03±0.03
CMHex	0.04±0.01	ND	9.63±0.20	ND
CMEtac	0.27±0.01	ND	11.42±0.05	ND
CMMeoh	1.81±0.02	12.10±0.10	14.74±0.21	6.10±0.06

Values expressed as Mean ± SEM of triplicate measures. GSHex= *G. sylvestre* n-hexane; CMHex = *C. micranthum* n-hexane; GSEtac= *G. sylvestre* ethyl acetate; CMEtac = *C. micranthum* ethyl acetate; GSMeoh= *G. sylvestre* 60% methanol; CMMeoh = *C. micranthum* 60% methano. ND = Not Detected.

Bioassay – Guided Studies

4.8.1 Fractionation by column chromatography and pooling of fractions guided by TLC. Solvent partitioning was used to obtain hexane, ethyl acetate and aqueous-methanol (60%) fractions of both GS and CM aqueous extracts. The methanol partition/fractions of both GS and CM showed appreciable quantities of all the phytochemicals assayed for (Table 1) as well as gave the best reductions in FBG compared to the other solvents' partitions (Table 2). Hence they were chosen for the column chromatography studies.

The total recovery (i.e. eluted amount) of the GS extract was 12.04g (i.e. 48.17%) and 6.28g (25.12%) for CM. When the sub-fractions were administered to the diabetic mice, fraction D (n-hexane : ethyl acetate – 20:80) for GS and fraction G (ethyl acetate : methanol – 30:70) for CM gave the best percentage reduction in FBG between 2 and 4 hours of administration (Tables 3 and 4); they were chosen for further fractionation and isolation/characterization of the compounds. Table5 compared the 1:1 combination of the fractions (D and G) and an additive effect was established since the combination index for both fractions was approximately 1.

Table-2: Mean Fasting Blood Glucose and their Percentage (%) Changes for Alloxan-induced Diabetic Mice Administered Different Solvent Partitioned Fractions of *G. sylvestre* and *C. micranthum* Aqueous Leaf Extracts for Four Hours

Fraction	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hr	4hr
GShex	554.51±121.67	600.50±104.79	435.00±9.82	8.29±25.39	-21.55±21.26
GSetac	354.00±9.54	315.00±7.00	295.50±13.00	-11.02±1.00	-16.52±4.54
GSmeoh	287.25±87.50	241.50±76.00	141.75±25.50	-15.93±1.60	-50.65±11.66[a]
CMhex	483.00±74.14	587.00±64.68	453.00±74.07	21.53±11.58	-6.21±6.11
CMetac	334.01±17.07	331.50±6.00	304.99±4.33	-0.75±14.74	-8.68±13.34
CMmeoh	298.50±19.50	267.50±35.37	255.00±32.91	-10.38±10.88	-14.57±7.35
Normal	131.00±9.54	139.67±6.49	100.33±5.61	6.62±5.06	-23.41±6.67[b]
Diabetic control	536.50±35.50	579.00±33.00	470.00±58.00	7.92±1.37	-12.39±20.58[b]
Glibenclamide	301.50±112.50	258.00±79.00	92.50±10.50	-14.42±6.65	-69.32±9.25[c]

Values expressed as Mean ± SEM with those bearing different superscripts under the same column significantly different ($P < 0.05$); N = 3. (+) preceding a value means % increase in Fasting Blood Glucose; (-) preceding a value means % decrease in Fasting Blood Glucose; GSHex= *G. sylvestre* n-hexane fraction; CMHex = *C. micranthum* n-hexane fraction; GSEtac= *G. sylvestre* ethyl acetate fraction; CMEtac = *C. micranthum* ethyl acetate fraction; GSMeoh= *G. sylvestre* 60% methanolic fraction; CMMeoh = *C. micranthum* 60% methanolic fraction.

Table-3: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Different Fractions (40mg/kg body weight) of Aqueous/Methanolic Leaf Extracts of *G. sylvestre* for Four Hours

Fraction	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hrs	4hrs
A	271.33±74.05	169.00±27.18	190.33±15.86	-27.80±21.27	-09.91±38.00
B	259.00±133.00	312.00±182.00	325.50±19.50	14.60±11.42	65.43±77.42
C	224.00±95.69	122.67±15.84	121.00±11.93	-31.92±15.64	-29.54±21.47
D	213.33±67.66	132.33±25.10	129.33±19.55	-32.75±08.31[a]	-32.18±12.44[a]
E	232.67±107.74	112.00±31.32	84.67±18.41	-22.84±33.63	-41.23±24.37[a]
F	417.67±116.75	340.00±113.32	315.67±80.35	-22.49±10.10	-21.98±07.12
G	500.67±4.10	424.00±82.89	370.33±70.94	-15.03±17.28	-25.80±14.73
H	416.33±139.45	321.00±216.00	342.00±208.00	-10.12±16.46	01.31±07.60
I	256.33±63.85	242.00±112.07	195.00±55.43	-14.35±18.83	-24.98±04.69[a]
J	445.33±155.67	335.33±112.09	374.33±132.53	-21.01±07.40	-15.10±05.42
K	326.67±139.15	297.00±151.97	257.33±112.29	-15.70±07.78	-18.31±15.54
L	145.50±15.50	169.00±20.00	149.00±23.00	16.00±01.39	01.88±04.94
M	458.33±142.67	448.33±161.67	448.33±161.67	-08.25±09.75	01.00±0.01
N	596.33±4.67	512.67±68.86	453.00±32.62	-14.20±10.97	-24.03±05.45[a]
O	305.00±148.36	298.67±155.77	143.00±1.00	-04.14±10.23	-07.78±09.94
Normal	87.33±2.91	95.33±10.33	89.67±12.68	08.96±09.89	02.80±14.40
Diabetic control	210.33±62.59	196.00±78.23	205.33±77.65	-11.78±08.79[b]	-06.46±07.36[b]
Glibenclamide	260.00±103.68	170.67±28.67	130.00±20.11	-22.93±14.65	-40.90±11.46[a]

Values are expressed as mean + standard error of mean with those bearing different superscripts being significantly (P< 0.05) different. N = 4.

Table-4: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Different Fractions (40mg/kg body weight) of Aqueous/Methanolic Leaf Extracts of *C. micranthum* For Four Hours

FRACTION	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hrs	4hrs
B'	408.50±171.50	368.50±231.50	332.50±210.50	-19.37±22.82	-27.45±21.07
C'	319.00±159.00	206.00±80.00	154.40±44.50	-30.71±09.46	-44.81±13.56[a,b]
D'	305.50±116.50	229.00±90.00	210.50±88.50	-25.43±01.02[a]	-32.30±03.15[a]
E'	453.00±31.00	259.00±18.00	238.00±15.00	-42.28±07.92[b]	-47.44±00.29[b]
F'	290.00±62.00	185.50±27.50	186.00±16.00	-35.10±04.39[b]	-34.03±08.59[b]
G'	433.50±167.50	148.00±6.00	120.00± 11.00	-60.50±13.88[c]	-66.31± 15.56[b]
Normal	87.33±2.91	95.33±10.33	89.67±12.68	08.96±09.89	02.80±14.40
Diabetic control	210.33±62.59	196.00±78.23	205.33±77.65	-11.78±08.79[d]	-06.46±07.36[c]
Glibenclamide	260.00±103.68	170.67±28.67	130.00±20.11	-22.93±14.65	-40.90±11.46

Values are expressed as mean + standard error of mean with those bearing different superscripts being significantly (P< 0.05) different. N = 4.

Table-5: Mean Fasting Blood Glucose and Their Percentage (%) Changes of Alloxan-Induced Diabetic Mice Administered Fractions D and G (and their 1:1 Combinations) at 40mg/kg body weight of Aqueous/Methanolic Leaf Extracts of *G. sylvestre* and *C. micranthum* Respectively for Four Hours

Fraction	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hrs	4hrs
D	213.33±67.66	132.33±25.10	129.33±19.55	-32.75±08.31	-32.18±12.44 **(0.95)**
G'	433.50±167.50	148.00±6.00	120.00± 11.00	-60.50±13.88[a]	-66.31± 15.56[a] **(1.18)**
D:G' (1:1)	227.33±9.82	211.00±77.16	153.00±15.13	-09.04±29.74	-32.81±05.08[b]
Normal	87.33±2.91	95.33±10.33	89.67±12.68	08.96±09.89[b]	02.80±14.40[c]
Diabetic control	210.33±62.59	196.00±78.23	205.33±77.65	-11.78±08.79[c]	-06.46±07.36[c]
Glibenclamide	260.00±103.68	170.67±28.67	130.00±20.11	-22.93±14.65	-40.90±11.46

Values are expressed as mean + standard error of mean with those bearing different superscripts being significantly (P< 0.05) different. N = 4. Values in parentheses represent Combination Index (CI). 4.8.2 Further fractionation (preparative TLC)

Preparative TLC of GS – D was used to isolate 5 compounds whereas CM – G revealed 6 compounds. Fasting Blood Glucose analyses showed sub-fractions D4 for GS and G4 for CM to have the highest percentage reductions (Table 6). When 1:1 combination of D4:G4 was also administered, a synergistic effect was observed with combination index of 0.23 for GS - D4 and 0.29 for CM – G4 (Table 6). Thin Layer chromatograms of GS – D4 revealed more co-eluting compounds which are mostly phenolics whilst that of CM – G4 showed a single spot of a trimethyl ether.

Alpha-amylase and Alpha-glucosidase Inhibition

Moreover, the subfractions D4 (for GS) and G4 (for CM) gave significantly better α-amylase and α-glucosidase inhibitory activities when compared to standard acarbose (Figure 1). However, acarbose was found to have better IC_{50} (137.13µg/ml for α-amylase and 83.47µg/ml for α-glucosidase) than the 1:1 combination of D4:G4 with IC_{50} of 258.75µg/ml (for α-amylase) and 636.28µg/ml (for α-glucosidase) (Figure 2). Significantly ($p < 0.01$) negative correlations were established between % decrease in FBG and inhibitory activities of both alpha-amylase/alpha-glucosidase for sub-fraction D4 of GS. In case of sub-fraction G4 of CM and the 1:1 combination of both plants, a positive correlation was observed between the % decrease in FBG and the inhibitory activities of both carbohydrate-hydrolyzing enzymes under study.

Table-6: Mean Fasting Blood Glucose and their Percentage (%) Changes of Alloxan-induced Diabetic Mice Administered Subfractions (10mg/kg body weight) of *G. sylvestre* (Fractions D1-D5), *C. micranthum* (Fractions G0-G5) and 1:1 Combinations of Most Active Subfractions (D4:G4) Leaf Extracts for Four Hours

SubFraction	Mean Fasting Blood Glucose (mg/dl)			% Change in Fasting Blood Glucose	
	0hrs	2hrs	4hrs	2hr	4hr
D1	438.25±44.51	301.75±51.68	525.00±35.71	-31.56±7.44	22.80±13.44
D2	569.50±10.46	412.25±82.58	550.00±0.01	-27.97±13.82	-3.33±1.72
D3	331.25±23.44	262.75±16.47	271.00±23.28	-20.48±2.56	-18.07±5.47
D4	311.50±6.46	254.75±19.73	239.25±14.15	-18.48±4.92^{a}	-23.34±3.38^{a} **(0.23)**
D5	316.00±18.49	261.00±18.07	265.75±18.29	-17.06±5.78	-15.53±5.93
G0	321.67±32.30	161.33±65.26	266.33±34.81	-48.68±22.39	-17.33±7.28
G1	381.33±46.74	330.33±129.19	361.67±109.33	-19.17±24.45	-8.90±17.77
G2	373.67±86.73	325.00±99.59	404.33±145.89	-16.13±7.90	-1.09±21.37
G3	283.00±63.69	227.67±93.85	148.67±34.57	-25.50±16.72	-39.17±20.51
G4	453.67±71.49	273.33±77.82	154.00±68.13	-32.52±22.88	-64.65±13.71^{b} **(0.29)**
G5	406.33±30.02	395.33±73.84	404.00±58.56	-4.17±10.74	-1.25±8.45
D4:G4 (1:1)	350.50±73.14	130.25±5.96	94.50±6.59	-58.25±7.54^{b}	-69.11±6.78^{b}
Normal	87.33±2.91	95.33±10.33	89.67±12.68	8.96±9.89^{c}	2.80±14.40^{c}
Diabetic control	210.33±62.59	196.00±78.23	205.33±77.65	-11.78±8.79^{d}	-6.46±7.36^{c}
Glibenclamide	260.00±103.68	170.67±28.67	130.00±20.11	-22.93±14.65	-40.90±11.46^{d}

Values expressed as Mean ± SEM with those bearing different superscripts under the same column significantly different ($P < 0.05$); N = 4. (-) preceding a value means % decrease in Fasting Blood Glucose. Values in parentheses represent Combination Index (CI).

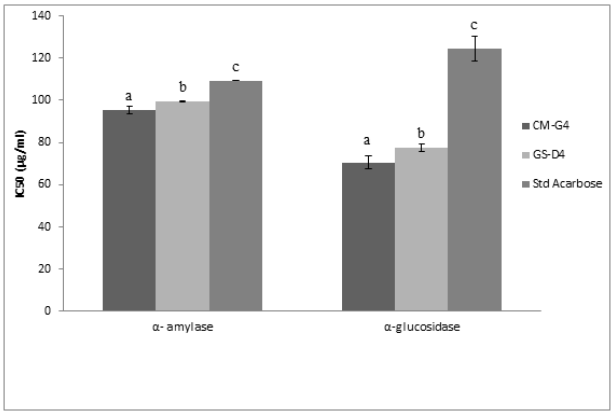

Fig-1: In vitro α- amylase and α-glucosidase Inhibition Activities of G.sylvestre (Subfraction D4) and C. micranthum (Subfraction G4) methanolic Leaf Extracts

Values are expressed as mean ± standard error of mean with those bearing different superscripts on same cluster being significantly (P< 0.05) different.

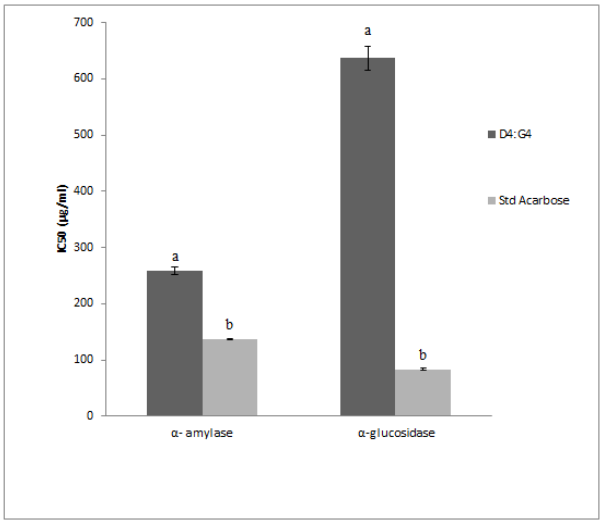

Fig-2: In vitro α- amylase and α-glucosidase inhibitory activity of 1:1 combination (Subfractions D4:G4) of G. sylvestre and C. micranthum Methanolic Leaf Extracts

Values expressed as Mean ± SEM of triplicate measurements, with those bearing different superscripts on the same cluster significantly different (P < 0.05).

Isolation and Characterization of Compounds

Ultra-high-performance liquid chromatography – mass spectroscopy (UHPLC-MS) analyses: The eluted fractions (from preparative TLC) which gave the highest reduction in FBG were analyzed by LC – MS (UHPLC-ESI-QTOF/MS – MS); GS – D4 gave four major separate peaks and about five minor ones. The mass spectra of the peaks identified some phenolic compounds and a few other ones using the ESI – Molecular Formula Extractor (MFE) Algorithm. The compounds were identified base on their retention times and mass-to-charge ratio (m/z) and listed in Table 7. Chromatogram of CM – G4 by LC – MS gave a single major peak at a retention time of 2.003 and a mass – to – charge ratio (m/z) of 329.1392 from the mass spectrum. The compound was identified as a trimethyl ether (Table 8).

Table-7: Compound Composition of Subfraction 4 of Fraction D [N-Hexane:Ethylacetate (20:80)] for *G. sylvestre* Aqueous/Methanolic Leaf Extract Using LC/MS in the Negative Ion Mode

S/N	RT	Mass	Name	Formula	DBFormula	DBDiff(ppm)	Hits(DB)
1	1.993	164.0475	4-Hydroxycinnamic acid	$C_9 H_8 O_3$	$C_9 H_8 O_3$	-0.75	10
2	2.514	186.125	(3S)-6-Hydroxy-3-isopropenyl-heptanoate	$C_{10} H_{18} O_3$	$C_{10} H_{18} O_3$	3.31	10
3	2.558	228.1362	(-)-11-hydroxy-9,10-dihydrojasmonic acid	$C_{12} H_{20} O_4$	$C_{12} H_{20} O_4$	-0.16	10
4	19.962	200.1777	4-Methyl-undecanoic acid	$C_{12} H_{24} O_2$	$C_{12} H_{24} O_2$	-0.39	10
5	21.063	380.162	2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate	$C_{23} H_{24} O_5$	$C_{23} H_{24} O_5$	0.87	6
6	21.704	292.2036	Colnelenic acid	$C_{18} H_{28} O_3$	$C_{18} H_{28} O_3$	0.94	10
7	23.724	586.349	6,8a-Seco-6,8a-deoxy-5-oxoavermectin "2a" aglycone	$C_{34} H_{50} O_8$	$C_{34} H_{50} O_8$	2.73	2
8	24.049	300.2083	9,13-di-cis-retinoic acid	$C_{20} H_{28} O_2$	$C_{20} H_{28} O_2$	2.01	10
9	24.83	408.1934	Glyinflanin A	$C_{25} H_{28} O_5$	$C_{25} H_{28} O_5$	0.79	10

Table-8: Compound Composition of Sub-fraction 4 of Fraction G [Ethylacetate:Methanol (30:70)] For *C. micranthum* Aqueous/Methanolic Leaf Extract Using LC/MS In The Negative Ion Mode

S/N	RT	Mass	Name	Formula	DB Formula	DB Diff (ppm)	Hits (DB)
1	2.242	330.1464	3-Deshydroxysappanol Trimethyl Ether	$C_{19}H_{22}O_5$	$C_{19}H_{22}O_5$	1.01	10

DISCUSSION

Phytochemical compounds have been shown to possess significant antidiabetic properties as obtained in the current study. Compounds like gymnemagenin and gymnemic acids in *G. sylvestre* extract [33] were found to be antihyperglycemic. Polyphenolic compounds have been implicated in the management/treatment of diabetes mellitus and other diseases [31, 34, 35, 36]. Resveratrol, anthocyanins and other condensed tannins have all demonstrated antihyperglycemic activity, whether by reducing obesity or other proposed mechanisms. Three theaflavins, from black tea or fermented *Camellia sinensis*, as well as two more flavonoids from *Artemisia dranunculus* L., exhibit glucose-lowering activity via down regulation of hepatic gluconeogenesis. In another study, ethyl acetate and n-butanol fractions of crude leaf extract of *C. micranthum* revealed a number of polyphenolic compounds including catechins, glycosylflavones, flavans, galloylated C-glycosylflavone derivatives and a flavan-piperidine alkaloid. The catechins and flavans were the active compounds by antioxidant capacity and epicatechin was identified as a glucose-lowering compound by phosphoenolpyruvate carboxykinase (PEPCK) inhibition [37].

Pancreatic and intestinal glucosidases are the key enzymes in dietary carbohydrate digestion and inhibitors of these enzymes are found to be effective in retarding glucose absorption to suppress hyperglycaemia. Inhibition of these enzymes reportedly decreased blood glucose levels in diabetic patients. The result of this study indicates that the extracts possess significant inhibitors of α–amylase and α-glucosidase and may thus be effective in reducing postprandial hyperglyceamia which may not be unconnected with the polyphenolic contents of the plants under study [38 – 40]. Findings of this work correlates well with those of earlier studies conducted using *G. sylvestre*, which reported reduced blood glucose and increased plasma insulin levels in both type 1 and type 2 diabetic rats [7, 41, 42].

Drug/herbal combinations when used together may sometimes produce enhanced, same or diminished effect. In this interaction there might be three different types of behaviours namely synergy, antagonistic and additive/indifferent. In the current study, GS and CM, in a 1:1 combination synergistically caused a significant reduction in FBG (Table 6), which might be as a result of combined effect of the phytochemicals (phenolics) present in them, (Table 7). This could be attributed to the restoration of integrity and functioning of β-cells, insulin mimetic potential of the compounds which modulate membrane receptors to enhance peripheral glucose entry into tissues or insulin sensitivity, and antioxidant properties [20, 42, 43, 44, 45, 46].

In the current study, the compounds were automatically profiled by a combination of in-house database as well as UHPLC-Q-TOF-MS analysis and further identified by their retention times and MS/MS spectra including characteristic MS/MS fragmentation ions. Thus, nine (9) main compounds were identified, for GS, majority of which are phenolics (Table 7), e.g. 2,2-Dimethyl-3-[4-(acetyloxy)phenyl]-4-ethyl-2H-1-benzopyran-7-ol acetate, Glyinflanin A and (-)-11-hydroxy-9,10-dihydrojasmonic acid in the GS subfraction, whereas 3-Deshydroxysappanol Trimethyl Ether was identified for the plant, CM. The similarity in structure of the conventional α-amylase/α-glucosidase's substrate (glucose) and that of the 3-Deshydroxysappanol Trimethyl Ether in the CM plant and other polyphenols in the GS could be attributed to the inhibition of the hydrolysing enzymes, thereby leading to reduction in postprandial glucose levels in the mice. Thus, the synergism observed upon combination of the plants could be associated with combined antioxidants property of the phenolics in GS [44, 47, 48, 49, 50] (which reverses the oxidative stress due to STZ) and the inhibition of the carbohydrate-hydrolyzing enzymes by the trimethyl-ether present in CM [33, 42, 51, 52, 53, 54].

CONCLUSION

At a dose of 40 mg/kg body weight, a 1:1 combination of GS-D and CM-G fractions significantly ($P < 0.05$) reduced FBG from 9.04 – 32.81±5.08% in an additive manner, while at a dose of 10 mg/kg body weight, a 1:1 combination of GS-D4 and CM-G4 sub-fractions, containing mainly polyphenolic compounds, significantly ($P < 0.05$) reduced FBG to 58.25±7.54% in 2 hours and 69.11±6.78% in 4 hours in a synergistic manner.

Authors' Contributions

1. I.A. (PhD): Conceptualized and designed the research, acquired the data (conducted the experiment), analyzed the data generated from the study, wrote and revised the manuscript and approved final manuscript.

2. O. E. (PhD, Professor): Assisted in supervision of the research and approved final manuscript.
3. N. A. J. (PhD, Professor): Assisted in supervision of the research and approved final manuscript.
4. U. I. A. (PhD, Professor): Supervised the research, participated in the experimental analysis, data analysis and approved final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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