

Antidiabetic Activity, Molecular Identification and In silico Docking of Compounds in Chromatographic Fractions of *Tephrosia bracteolata* Guill. & Perr. (Fabaceae) Leaves

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Abstract

Background

Recent efforts for the complementary treatment of diabetes have focused on medicinal plants and their bioactive compounds. *Tephrosia bracteolata* is one of such plants used in the management of diabetes but its anti-diabetic principles are yet to be identified. This study was aimed at identifying the compounds responsible for the antidiabetic activity of the ethylacetate fraction of *Tephrosia bracteolata* leaves and subsequently, carryout an *in silico* molecular docking of these compounds against key targets in the pathophysiology of diabetes.

Methods

The ethylacetate fraction (EAF) of *T. bracteolata* leaves was fractionated using Silica gel column chromatography to yield 100 fractions. Pooling together of fractions with similar thin layer chromatographic (TLC) mobility profile afforded seven major fractions (SF1- SF7). Preliminary phytochemical studies were carried out on the fractions using standard methods. The antidiabetic activity of the fractions was subsequently evaluated (at a dose of 200 mg/kg) against alloxan- induced diabetes in adult mice. GC-MS analysis was carried out on the fraction with the highest activity. Subsequently, some of the identified active compounds were docked against key targets in the pathology of diabetes using Auto Dock tool.

Results

Preliminary phytochemical analysis revealed the presence of terpenoids, saponins, steroids, glycosides, flavonoids, tannins and alkaloids in varying proportions in the fractions. The sub-fractions produced varying degrees of significant ($p < 0.05$) decrease in FBS at 12h and 24h- post-treatment. GC-MS analysis of the most active fraction (SF5) revealed the presence of thirty- six compounds among which are some that have been reported to possess direct or indirect antidiabetic properties. These are Mome-inositol, 2-methoxy-4-vinylphenol, 1-D-thio-glucitol, 4-Piperidinone, Hexadecanoic acid, 9- octadecanoic acid, n-hexadecanoic acid and D- allose. Molecular docking studies (Auto Dock tool) between Mome inositol, 1-D-thio-glucitol and alpha-glucosidase showed that Mome inositol ($- 6.7$ kcal/mol) had a stronger affinity to the enzyme. Similarly, for sodium glucose co-transporter 2 (SGLT 2), Mome inositol ($- 6.5$ kcal/mol) had a stronger affinity than 1-D-thio-glucitol.

Conclusions

The identified compounds in the fraction could be responsible for the observed antidiabetic properties of the fraction of *T. bracteolata*.

Background

Diabetes mellitus (DM) is a cluster of metabolic conditions identified and characterized by the occurrence of elevated levels of glucose in the blood caused by defects in insulin secretion and insulin resistance [1]. The gradual progression of this disease affects other organs of the body, and serious complications appear after the onset of diabetes. It is the most prevalent and rapid-growing worldwide problem and arise as a huge health and socioeconomic burden [2]. In 2017, 425 million adults were diabetic globally and these figures are predicted to reach 629 million by 2045. The developing economies of Africa and Asia contribute a significant fraction of this figure. The prevalence of diabetes mellitus in Nigeria has increased from 2.2% as reported by in 1997 from a national survey to 3.9% in 2025 as estimated by W.H.O [3].

Alloxan, a toxic glucose analogue when administered to vertebrates leads to the destruction of pancreatic β -cells. Consequently, an insulin- dependent type of diabetes mellitus (Type 1) develops in the animals. Alloxan is selectively toxic to insulin producing pancreatic β -cells because it preferentially accumulates in β -cells through uptake via glucose transporter-2 (GLUT2) [4]. Alloxan, in the presence of intracellular thiols, generate reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid. The β -cell toxic action of alloxan is initiated by free radicals formed in this redox reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of β -cells [5]. Administration of alloxan elevates serum glucose, which signifies induction of diabetes mellitus.

Tephrosia bracteolata Guill. & Perr. (Fabaceae) is wide spread in Tropical Africa. *T. bracteolata* is an erect shrub, usually 2 to 3 feet high but may reach 8 feet [6, 7]. The plant has been used for various purposes in folklore medicine. The root is used as medicine for venereal diseases, e.g., treatment of pregnant women with syphilis, the aerial parts has been used for animal grazing [8]. It has also been reported to possess analgesic anti- inflammatory and anti- pyretic properties [9]. Information obtained through personal communications in herbal markets around Lokoja and Bassa Local governments of Kogi state, Nigeria confirmed that *T. bracteolata* leave- preparation is used for the treatment of whitlow, toothache, ear-ache, open wound and diabetes. A study by Egharevba *et al.* [10] revealed the presence of alkaloids, steroids, tannins, flavanoids and terpenoids in the hexane and ethylacetate extracts of the leaves of *T. bracteolata*.

Medicinal plants continue to play a key role in the healthcare system of a large number of many countries of the world. This is particularly true in the developing countries, where herbal medicine has a long and uninterrupted history of use. Recognition and development of medicinal and economic benefits of these plants are on the increase in both developing and industrialized nations [11]. These plants are specifically screened for the presence of bioactive compounds which are known to possess therapeutic properties [12, 13]. The process of utilization of the bioactive compounds involves identification, isolation and characterization for use in pharmaceutical and food industries [14]. This study therefore, was aimed at investigating the antidiabetic activity, molecular identification and in silico docking of compounds in the

the active fraction of *Tephrosia bracteolata* leaves using gas chromatography in tandem with mass spectrometry and Autodocking too.

Methods

Collection and identification of plant material

Fresh leaves of *T. bracteolata* Guill. & Perr. (Fabaceae) were collected from a natural habitat along the River Niger Area of Lokoja, Kogi State, Nigeria. The plant sample was identified and authenticated at the Herbarium Unit of the Department of Biological Sciences, Federal University, Lokoja, Kogi State, Nigeria by Mr. Gbenga, an ethnobotanist, and was assigned a voucher number (FULH/0765) which was deposited at the herbarium for future reference.

Chemicals, reagents and drugs

Drangendorff's reagent, Wagner's reagent, Mayer's reagents, 5% Iron (III) chloride, 1% and aluminium (III) chloride (BDH, England). Bromine water, Aqueous ammonia, concentrated tetraoxosulphate (VI) acid (BDH, England), concentrated Hydrogen chloride acid, -naphthol and lead acetate solution, Ethanol ($\geq 99.5\%$), hexane ($\geq 98.5\%$), chloroform ($\geq 98.5\%$), ethylacetate ($\geq 99.8\%$) and methanol ($\geq 99.8\%$) (Sigma-Aldrich, UK), Pre-coated silica gel 60F₂₅₄ on aluminium sheets, silica gel (60–120 mesh) for column chromatography (Sigma-Aldrich, UK), Alloxan monohydrate (Sigma-Aldrich, UK), metformin (Glucophage®- Bristol Myers Squibb Company) and distilled water.

Experimental animals

Adult Male Swiss mice weighing 25–30 g obtained from the Animal House Facility of Salem University, Lokoja, Kogi State, Nigeria, were used for the study. They were kept in well-ventilated stainless-steel cages under standard laboratory conditions guaranteed of a 12 h dark/light cycle, and were maintained on standard rodent feed and potable drinking water *ad libitum*. The animals were acclimatized to the laboratory environment for a period of 7 days prior to use, and accorded humane care in line with the institutional ethical committee, and the International Guidelines for Handling of Laboratory Animals [15].

Preparation and processing of plant material

The leaves of *T. bracteolata* were collected from a natural habitat along the River Niger Area of Lokoja, Kogi State, Nigeria. The plant was identified by Mr. Gbenga at the Herbarium Unit of the Department of Biological Sciences, Federal University, Lokoja, Kogi State, Nigeria. The plant was assigned a voucher number FULH/0765 and kept for future reference. The leaves of *T. bracteolata* were rinsed with distilled water to remove all debris, shade-dried for fifteen days (15) days and subsequently pulverized using an electric blender.

Crude extraction and solvent partitioning

A known weight, 2000 g of the pulverized leaves was macerated in ethanol and allowed to stand for 72h after which it was filtered with muslin sieved/ Whatman No. 1 filter paper. The filtrate was concentrated using rotary evaporator to obtain the ethanol extract (EETB). The EETB was fractionated according to the method of Uzor et al. [16]. A known weight, 200 g of the EETB was triturated with 400 g of silica gel, mesh size 60–120 to increase the surface area then partitioned successively with ethyl acetate to obtain the ethyl acetate fraction (EAF).

Fractionation of the EAF

Based on the results from our previous study, the EAF was subjected to further fractionation by using column chromatographic technique.

Chromatographic conditions

A glass column of dimensions (75 by 3.5 cm) was used. Silica gel, 60–120 mesh size was used as the stationary phase. The column was packed as a wet slurry, and elution was done using various solvent combinations of varying polarities.

Sample loading

The EAF (5g) was loaded by the dry loading method [17]; the sample was dissolved in minimum amount of suitable organic solvent, mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the previously packed column.

Solvent system and elution: Elution of the column was done using various solvent combination of varying polarity. The following solvents systems were used in the elution process; hexane: chloroform 100:0, 80:20, 60:40, 40:60, 20:80, 0:100; chloroform: ethyl acetate 100:0, 80:20, 60:40, 40:60, 20:80, 0:100. Ethyl acetate: methanol 100:0, 80:20, 60:40, 40:60, 20:80, 0:100 and methanol: water 100:0, 80:20, 60:40, 40:60, 20:80, 0:100. The various eluates were collected into seven sub-fractions based on their similarities in TLC profile (SF1-SF7).

Phytochemical analysis of sub-fractions

Phytochemical analysis of the EAF and the sub-fractions were determined by the methods of Trease and Evans [18] and Harborne [19].

Investigation of antidiabetic property of the sub-fractions in mice

Induction of diabetes in mice

Adult mice were fasted for 12h and subsequently injected (i.p) with alloxan solution at a dose of 150 mg/kg b.w Mice with blood glucose level of 200 mg/dl or more after 48 h were selected for the study.

Experimental design

A total of 102 adult male Swiss mice (25-30g) were used for the study. Following seven (7) days of acclimatization to the laboratory environment, they were randomly distributed into 10 groups of six mice each. The animal groupings and treatment administered is represented as follows:

Group 1: Normal control (1ml/kg b.w normal saline)

Group 2: Diabetic control + 1ml/kg b.w normal saline

Group 3: Diabetic + 150 mg/kg b.w Metformin hydrochloride

Group 4: Diabetic + 200 mg/kg b.w SF1

Group 5: Diabetic + 400 mg/kg b.w SF1

Group 6: Diabetic + 200 mg/kg b.w SF2

Group 7: Diabetic + 400 mg/kg b.w SF2

Group 8: Diabetic + 200 mg/kg b.w SF3

Group 9: Diabetic + 400 mg/kg b.w SF3

Group 10: Diabetic + 200 mg/kg b.w SF4

Group 11: Diabetic + 400 mg/kg b.w SF4

Group 12: Diabetic + 200 mg/kg b.w SF5

Group 13: Diabetic + 400 mg/kg b.w SF5

Group 14: Diabetic + 200 mg/kg b.w SF6

Group 15: Diabetic + 400 mg/kg b.w SF6

Group 16: Diabetic + 200 mg/kg b.w SF7

Group 17: Diabetic + 400 mg/kg b.w SF7

Induction of experimental diabetes

The mice were fasted overnight with free access to water before the induction of diabetes. Diabetes was induced by a single intra-peritoneal injection of Alloxan monohydrate (Sigma St. Louis, M.S., USA.) at a dose of 100 mg/kg body weight dissolved in 0.9% cold normal saline solution [20]. Since Alloxan is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, the rats were treated with 20% glucose solution orally after 6 h, and subsequently kept on 5% glucose solution bottles in their cages for the next 24 hours to prevent hypoglycaemia [21]. After 48 h of Alloxan treatment, blood

samples were collected from tail vein of the mice and blood glucose measured by the glucose oxidase method of Beach and Turner [22] using the glucometer (Fine Test glucometer and test strips, Fine Test® Milpitas, U.S.A). Mice having fasting blood glucose level greater than 200mg/dl were considered diabetic and were subsequently used for the study [23].

Determination of fasting blood glucose concentration

All blood samples were collected from the tail vein of the rats at intervals of 0 h, 3 h, 6 h, 12 h and 24 h post-treatment. Fasting blood glucose levels were determined by the glucose oxidase method of Beach and Turner, (1958) using a digital glucometer (Fine Test glucometer and test strips, Fine Test® Milpitas, U.S.A), and the results were expressed in the unit of mg/dl [24]

Principle

Glucose in the sample reacts with glucose oxidase in the glucose electrode strips, oxidizing glucose to gluconic acid and temporary transfer of two electrons from the glucose molecule to the enzyme. The reduced enzyme reacts with the mediator, transferring a single electron to each of the two mediator ions, thus returning to its original state. At the electrode surface, the reduced mediator is re-oxidized providing amperometric signal whose magnitude is proportional to the glucose concentration in the sample.

Procedure

The data chip from the strip pack was inserted into the chip holder beside the device. The glucometer test strip was inserted into the strip holder of the device (just in front of the device). The tail of the mice was punctured using sterile lancet and blood samples from the animal was allowed to drop (one drop) on the sensor part of the glucometer strip in front of the device. The results were displayed on the device screen and recorded accordingly.

Identification of active principles

The fraction with highest antidiabetic action (SF5) was selected and subjected to GC-MS for identification of active principles.

GC-MS analysis of the SF5

Chemical composition of the SF-5 was determined by GC/MS-QP-2010 plus Ultra (Shimadzu, Kyoto Japan) using a DB-5 MS fused silica capillary column (30 × 0.25 mm internal diameter, film thickness 0.25 µm). For GC-MS detection an electron ionization system with ionization energy of 70 eV was used. Carrier gas was Helium gas, flow rate was 1.2 ml/ min. Injector and MS transfer line temperature were set at 260°C and 270°C respectively. Oven temperature was initially maintained for 2 min, and then increased to 210°C at a rate of 8°C/min to 280°C; hold time was 10 min. Samples were completely dissolved in absolute ethanol and 0.3 µL was injected through auto-sampler in the split mode, split ratio was 1:100. Relative percentage of each constituent was expressed as percentages by peak area normalization. Each

component was identified based on its column retention time relative to computer-based matching of mass spectra with those of standards (NIST and Wiley libraries for GC-MS system).

In silico studies

Alpa-glucosidase (PDB ID: 2JKP) and SGLT2 (PDB ID: 7VSI) were obtained from the Protein Data Bank. Preparation of the proteins prior to molecular docking was performed using AutoDock tools which involved, addition of charges, addition of polar hydrogen and grid set-up. The 3D structure of 1-Thio-D-Glucitol and Mome inositol in sdf format were downloaded from Pubchem. These sdf files were then converted to PDB files using Pymol software [25]. A site specific molecular docking was performed targeting Glutamate (GLU) 532 in Alpha-glucosidase and Serine (Ser) 287 in SGLT2 due to their role in the catalytic mechanism of the target proteins using AutoDock Vina Software [26] and their binding affinities/energy reported in Kcal/mol. The renderings for the receptor-surface view (3D view) were computed using Pymol software [25] while the 2D diagrams of the interactions were computed using Bovia-Discovery studio Visualizer.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation, and statistical differences between means were determined by one- way ANOVA followed by Duncan's post-hoc test for multiple comparison tests using SPSS version 20. Values were considered significant at $P \leq 0.05$.

Results

Phytochemical composition of the EAF

Phytochemical analysis of EAF revealed the presence of phenols, terpenes, saponins, steroids, glycosides, flavonoids, tannins and alkaloids in varying proportions.

Table 1
Phytochemical composition of the EAF

Phytochemicals	Ethylacetate fraction (Bioavailability)
Phenols	++
Terpenes	++
Saponins	+
Steroids	+
Glycosides	+
Flavonoids	++
Tannins	+
Alkaloids	+

Key: + (slightly present), ++ (moderately present).

Fractionation of the EAF

Based on the previous study by Idakwoji *et al.*, [17], the ethylacetate fraction (EAF) of the extract of *T. bracteolata* showed a more promising antidiabetic activity amongst the nhexane, chloroform, methanol and aqueous fractions. Based on this, the EAF was subjected to further fractionation using column chromatographic technique. The fractionation afforded one hundred (100) fractions which were pooled into seven (7) sub-fractions (SF1- SF7) based on similarities in TLC mobility profiles. The TLC profiles of the sub-fractions are presented in Table 2 below.

Table 2
TLC profiles of sub-fractions of the EAF

Sub-fraction	Number of major spots	R _f values
1	4	0.19, 0.44, 0.63, 0.76
2	2	0.81, 0.89
3	3	0.22, 0.43, 0.88
4	3	0.49, 0.74, 0.82
5	2	0.39, 0.75
6	2	0.73, 0.88
7	2	0.69, 0.79

Qualitative phytochemical composition of the EAF sub-fractions

Results showed the presence of flavonoids in all the sub-fractions except SF1 and SF5. Terpenoids were also detected in all the fractions except SF6 and SF7. Saponins were detected only in SF3. Alkaloids were detected in all the sub- fractions except SF2. Steroids were detected in only SF1, SF2 and SF3 while tannins were detected only in SF4 and SF5 (Table 3).

Table 3
Qualitative phytochemical composition of the EAF sub-fractions

Phytochemicals	SF1	SF2	SF3	SF4	SF5	SF6	SF7
Phenols	-	-	+	+	++	+	-
Terpenoids	+	+	+	+	+	-	-
Saponins	-	-	+	-	-	-	-
Steroids	+	++	-	-	++	+	+
Glycosides	-	-	-	-	++	+	+
Flavonoids	-	+	+	+	+	+	-
Tannins	-	-	-	+	+	-	-
Alkaloids	+	-	+	+	+	+	+
Key: + (slightly present), ++ (moderately present), - (not detected), SF (sub-fraction)							

Effect of sub-fractions of the EAF on 24 h fasting blood sugar levels of diabetic mice

The effect of the sub- fractions of ethylacetate fraction on the FBS of diabetic Wistar rats is presented in Table 4. Following alloxan administration, there was a significant ($P < 0.05$) increase in FBS in all treatment groups compared to non-diabetic control. following the administration of the subfractions at 200 and 400 mg/kg, all except SF5, showed no statistically significant ($P > 0.05$) decrease in FBS at 3 and 6 h- post treatment compared to diabetic control. At 12 and 24 h post- treatment, all subfractions showed significant ($P < 0.05$) decrease in FBS compared diabetic control. However, the reduction in FBS produced by metformin and SF5 were significantly higher compared to other subfractions.

Table 4
Effect of sub-fractions of the EAF on 24 h fasting blood sugar levels of diabetic mice

Treatment	0h	3h	6h	12h	24h
Normal control	72.3 ± 5.01 ^a	72.7 ± 3.61 ^a	73.0 ± 2.28 ^a	71.2 ± 4.88 ^a	72.5 ± 4.89 ^a
Diabetic control	326.7 ± 114.55 ^b	367.5 ± 101.37 ^c	393.2 ± 91.24 ^c	456.7 ± 80.14 ^d	451.8 ± 87.81 ^d
150 mg/kg b.w met	323.2 ± 111.97 ^b	343.5 ± 110.05 ^c	327.2 ± 85.48 ^c	233.3 ± 68.27 ^b	207.0 ± 63.80 ^{ab}
200 mg/kg b.w SF1	323.0 ± 108.32 ^b	331.0 ± 113.73 ^c	329.8 ± 113.99 ^c	351.2 ± 105.13 ^c	332.7 ± 110.44 ^c
400 mg/kg b.w SF1	321.0 ± 103.11 ^b	335.0 ± 123.46 ^c	334.5 ± 102.13 ^c	345.2 ± 126.19 ^c	330.2 ± 111.28 ^c
200 mg/kg b.w SF2	318.2 ± 102.10	327.3 ± 100.92 ^c	323.8 ± 98.69 ^c	339.7 ± 83.61 ^{b,}	320.8 ± 95.83 ^c
400 mg/kg b.w SF2	320.4 ± 106.05 ^b	323.8 ± 109.12 ^c	323.1 ± 92.45 ^c	320.1 ± 44.18 ^c	320.8 ± 99.71 ^c
200 mg/kg b.w SF3	324.1 ± 111.41 ^b	327.5 ± 106.17 ^c	320.5 ± 102.98 ^c	343.8 ± 97.14 ^c	322.2 ± 107.79 ^c
400 mg/kg b.w SF3	321.2 ± 101.81 ^b	321.4 ± 98.81 ^c	320.2 ± 99.07 ^c	328.8 ± 17.22 ^c	320.5 ± 94.55 ^c
200 mg/kg b.w SF4	322.2 ± 98.02 ^b	307.8 ± 96.82 ^{bc}	302.7 ± 98.97 ^{bc}	310.7 ± 94.12 ^b	308.5 ± 96.27 ^{bc,}
400 mg/kg b.w SF4	324.5 ± 86.31 ^b	312.4 ± 75.28 ^{bc}	301.8 ± 94.26 ^{bc}	301.6 ± 84.46 ^b	298.2 ± 42.15 ^{b,}
200 mg/kg b.w SF5	320.0 ± 98.75 ^b	298.5 ± 97.51 ^b	293.8 ± 94.81 ^b	228.0 ± 98.32 ^{ab}	204.3 ± 15.03 ^{ab}
400 mg/kg b.w SF5	325.0 ± 68.43 ^b	291.4 ± 74.11 ^b	283.6 ± 42.44 ^b	210.3 ± 55.40 ^{ab}	200.3 ± 23.04 ^{ab}
200 mg/kg b.w SF6	320.0 ± 67.74 ^b	320.3 ± 71.27 ^c	317.7 ± 98.38 ^{bc}	339.5 ± 96.50 ^c	330.8 ± 94.37 ^c
400 mg/kg b.w SF6	320.0 ± 81.74 ^b	316.3 ± 60.21 ^{bc}	315.2 ± 49.56 ^{bc}	314.3 ± 56.41 ^{bc}	310.3 ± 71.23 ^c
200 mg/kg b.w SF7	324.2 ± 81.15 ^b	317.3 ± 92.25 ^{bc}	304.7 ± 85.34 ^{bc}	324.3 ± 99.66 ^b	328.5 ± 97.31 ^c

Treatment	0h	3h	6h	12h	24h
400 mg/kg b.w SF7	324.2 ± 27.34 ^b	315.3 ± 78.56 ^{bc}	310.4 ± 79.64 ^{bc}	300.4 ± 99.66 ^{bc}	297.6 ± 44.64 ^c

Data are presented as mean ± SD. (n = 6). Mean values having different lowercase alphabets as superscripts down the columns are considered significant (p < 0.05).

Identification of active components

Table 5
Identification of bioactive compounds in SF-5

Peak No.	R. Time	Area	Area%	Name of compound
1	5.479	53822793	1.35	2-cyclopenten-1-one, 2-hydroxy-
2	8.811	441042	0.11	4-piperidinone, 2,2,6,6-tetramethyl-
3	11.908	841673	0.21	2-methoxy-4-vinylphenol
4	12.585	761111	0.19	2-propanone, 1-(3,5,5-trimethyl-2-cyclohexane-1-ylidene)-
5	12.988	3888243	0.98	1-(3,6,6-trimethyl-1,6,7,7 a-tetrahydrocyclo
6	13.329	1251207	0.31	Ethanone, 1-(2,3-dihydro-1,1-dimethyl-1h-inden-4-yl)-
7	13.539	2621869	0.66	1-(2-[2n-2-methyl-furan-2-yl]-propyl)-cyclopropyl
8	13.635	693935	0.17	3,3-dimethyl-4-phenyl-4-penten-2-one
9	14.575	1779787	0.45	D-allose
10	15.152	1241100	0.31	3-(tert-butyl)-4-methoxyphenyl acetate
11	15.763	6421829	1.62	.alpha.-d-glucopyranoside, methyl
12	15.907	614813	0.15	Megastigmatrienone 2
13	15.993	2413208	0.61	1-s-hexyl-1-thio-d-glucitol
14	16.214	18677393	4.70	1,3,4,5-tetrahydroxy-cyclohexanecarboxy
15	16.694	4071548	1.02	Hexanoic acid, tridec-2-ynyl ester
16	18.088	252100743	63.43	Mome inositol
17	19.035	6544023	1.65	Hexadecanoic acid, methyl ester
18	19.516	15934297	4.01	N-hexadecanoic acid
19	20.669	1013280	0.25	9,12-octadecadienoic acid (z,z)-, methyl ester
20	20.731	6397519	1.61	9-octadecenoic acid (z)-, methyl ester
21	20.964	1204762	0.30	Methyl stearate
22	21.195	2015839	0.51	9-octadecenoic acid (z)-
23	21.567	1083136	0.27	Octadecanoic acid, ethyl ester
24	22.080	602604	0.15	Oxacyclohexadecan-2-one, 16-methyl-13-ni'
25	22.491	6540877	1.65	Glycidyl palmitate
26	23.977	9357771	2.35	Glycidylolate

Peak No.	R. Time	Area	Area%	Name of compound
27	24.171	3980523	1.00	Glycidyl palmitate
28	24.436	10885630	2.74	Bis(2-ethylhexyl) phthalate
29	25.530	1911035	0.48	9-octadecenoic acid (z)-, 2,3-dihydroxypropyl ester
30	25.778	3309520	0.83	Oleoyl chloride
31	26.604	2826684	0.71	Decanedioic acid, bis(2-ethylhexyl) ester
32	31.123	1564235	0.39	Spirostan-6-ol, 3-amino-, (3.beta.,5.alpha.,6.alpha.,25r)
33	34.449	1680824	0.42	16-hentriacontanone
34	40.133	7523042	1.89	Cyclodocosane, ethyl-
35	44.203	5493173	1.38	No hit compound
36	48.501	4351466	1.09	Neronine, 4.beta,5-dihydro-

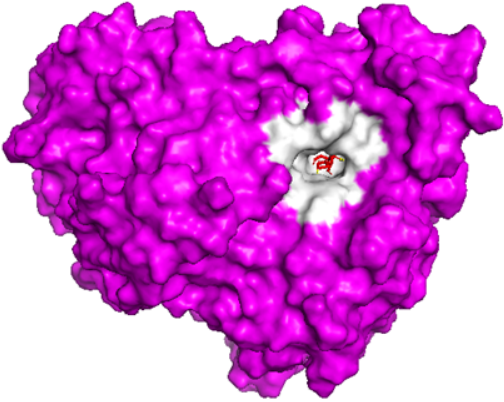
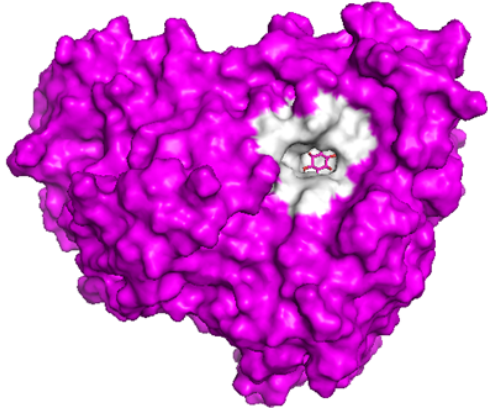
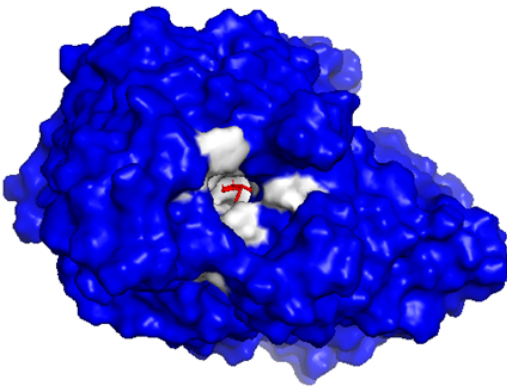
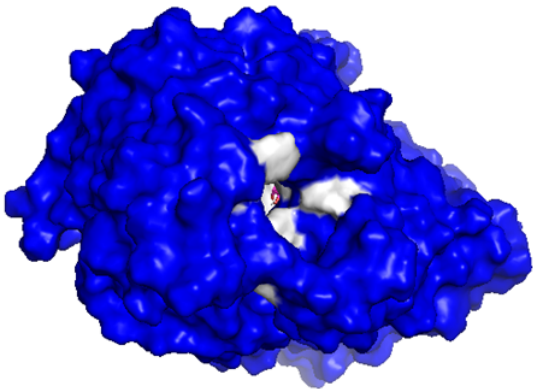
Table 6
Binding Affinity of Ligands against target proteins

Target Proteins	1-thio-d-Glucitol	Mome inositol
Alpa-glucosidase (PDB ID: 2JKP)	-6.1 Kcal/mol	-6.7 Kcal/mol
SGLT2 (PDB ID: 7VSI)	-5.7 Kcal/mol	-6.5 Kcal/mol

Table 7
2-D diagram of protein-Ligand Interactions

Target Proteins	1-thio-d-Glucitol	Mome inositol
Alpa-glucosidase (PDB ID: 2JKP)		
SGLT2 (PDB ID: 7VSI)		

Table 8
3-D diagram of protein-Ligand Interactions

Target Proteins	1-thio-d-Glucitol	Mome inositol
Alpa-glucosidase (PDB ID: 2JKP)		
SGLT2 (PDB ID: 7VSI)		

Discussion

Medicinal plants that possess anti-hyperglycaemic properties usually contain certain phytochemicals such as terpenoids, saponins, alkaloids, glycosides, phenols and flavonoids. These phytochemicals were found in higher concentrations in the ethylacetate fraction which could be responsible for the higher anti-hyperglycaemic activity. Phenolic compounds such as flavonoids found in the extract and fractions (Table 1) possess antioxidant properties which might have played a role in inhibiting and scavenging the free radicals generated by alloxan consequently, the regeneration of the beta-cells which led to the release of insulin (pancreatotropic action). It could also be that, the extract and fraction might have facilitated the uptake of glucose by the peripheral cells [28].

In our previous study, we reported the antidiabetic potentials of the ethanol extract of *Tephrosia bracteolata* leaves (Fig. 1) [27]. Based on the result of the penultimate studies, the ethyl acetate fraction (EAF) was subjected to further fractionation by using column chromatographic technique. The fractionation afforded one hundred (100) fractions which were pooled into seven (7) sub-fractions (SF1-SF7) based on similarities in TLC mobility profiles. The TLC details of the sub-fractions are presented in Table 6. SF 1 had 4 TLC spots, SF3 and SF4 had 3 spots each while SF5, SF6 and SF7 had 2 spots each.

The sub-fractions were investigated for anti- hyperglycaemic activity using alloxan model of diabetes in mice.

The results of the antidiabetic studies carried out in mice with the EAF sub-fractions (SF1-SF7) are summarized in Table 4. The fractions at dose levels of 200 and 400 mg/kg and the standard drug- metformin at a dose of 150 mg/kg significantly ($p < 0.05$) reduced FBS of rats at 12 and 24 h post-treatment compared to the diabetic control. However, the results clearly show that the antidiabetic activity resides mostly with SF5. SF5 at the dose of 200 mg/kg afforded the best FBS reduction of 26.45 and 34.10% after 12 and 24 h, an effect which was significantly ($P < 0.05$) different from the control and comparable to 150 mg/kg metformin which produced a FBS reduction of 27.82 and 35.95% respectively. SF4 also produced a significant reduction (1.19%) in FBS of the mice at 200 mg/kg after 24 h. However, other sub-fractions did not seem to produce remarkable blood sugar reduction.

In the various sub-fractions (SF1-SF7), alkaloids, saponins, glycosides, flavonoids are present in medium to high concentrations. SF5, which showed the best reduction in blood glucose of the animals among the fractions, appears to have the highest concentration of the phytochemicals among the sub-fractions (Table 3). Several reports have shown that flavonoids, steroids, terpenoids or phenolic acids are known to be bioactive antidiabetic principles [29, 30, 31]. Flavonoids have been noted as one of the most numerous and widespread groups of phenolic compounds in higher plants [32]. Some of them, due to their phenolic structure, are known to be involved in the healing process of free-radical mediated diseases including diabetes [33]. Flavonoids are known to regenerate the damaged β -cells in the alloxan-induced diabetic mice and acts as insulin secretagogues [34]. Thus, the antidiabetic activity of the ethanol leaf extract of *T. bracteolata* may reside with any of the constituents of SF5 or a combination of these constituents. It was on this note; SF5 was selected and subjected to GCMS analysis for identification of the antidiabetic principles of ethanol leaf extract of *T. bracteolata* (Fig. 2 and Table 5).

The GC-MS analysis of SF5 revealed a number of bioactive compounds with reported direct or indirect antidiabetic properties hence could be responsible for the anti- diabetic activity of the ethanol leaf extract of *T. bracteolata*. Some of the compounds are highlighted below.

Mome inositol: Inositol is a cyclitol present in animal and plant cells. It can be present in nine distinct stereoisomers, myo-inositol being the most represented. D-chiro-inositol is an inositol isoform derived from myo-inositol through an epimerization process and this reaction is insulin dependent [35]. Both myo- and d-chiro-inositol showed insulin mimetic effects in animal models of insulin resistance [36, 37]. Inositol has been mainly used as a supplement in treating several pathologies such as gestational diabetes (GDM) [38]. Inositol plays a preventive role in GDM onset [39]. In addition, inositol has been studied as a therapeutic option for the treatment of GDM [40, 41]. The main effect of inositol when used in pregnancy is decreasing the level of insulin resistance. Consequently, a potential role of inositol as a treatment option could be hypothesized for type-2 diabetes which is characterized by insulin resistance and deficiency of insulin secretion from the pancreas [42]. It showed for the first time a direct beneficial effect of the supplementation with the association of myo- and d-chiro-inositol on glycemic parameters

of subjects with T2DM. Particularly, a significant reduction in blood glucose and HbA1c levels was registered with inositol supplementation [43].

The compound, 2-methoxy-4-vinylphenol is a natural occurring phenolic compound. Different groups of phenolic compounds have different biological characteristics. However, they are usually known for their antioxidant properties. Many studies have shown that diabetes mellitus is associated with increased formation of free radicals and decreased antioxidant potential, leading to oxidative damage of cell components [44]. Hence, phytochemicals with antioxidant properties could be useful in managing diabetes and its complication. Studies have reported that 2-methoxy-4-vinylphenol is used as anti-inflammatory, antioxidant, antimicrobial, flavouring and odour agent [45, 46, 47]. The reported antioxidant property of 2-methoxy-4-vinylphenol might have contributed to the anti-diabetic activity of the extract though there is no clear evidence supporting this supposition.

1-thio-D-glucitol: Luseogliflozin, a novel and orally bioavailable drug is a 1-thio-D-glucitol derivative. It is a highly selective inhibitor of SGLT2 [48, 48]. The sodium glucose cotransporter 2 (SGLT2) inhibitors are gaining attention as a new drug class for treatment of diabetes mellitus. Inhibition of SGLT2 promotes urinary glucose excretion by preventing the reuptake of filtered glucose in the proximal tubules of the kidney, consequently lowering plasma glucose levels [48, 49]. Because SGLT2 inhibitors have an insulin-independent mechanism of action, these are expected to improve glycemic control with a low risk of major hypoglycemic events [50]. Luseogliflozin lowered glucose levels by promoting urinary glucose excretion in animal models [51]. Results from previous clinical studies have shown that once daily administration of luseogliflozin leads to significant improvements in HbA1c as well as other glycemic parameters. In addition, the drug was found to be well tolerated, with a favorable safety profile [52, 53].

Hexadecanoic acid, methyl ester, 9- octadecanoic acid, methyl ester, 9,12-octadecadienoic acid (*Z, Z*)-, methyl ester and n-hexadecanoic acid: fatty acids such as hexadecanoic acid, methyl ester and 9-octadecanoic acid, methyl ester was reported to be potential antidiabetic agents. Balogun *et al.* [54] also reported that some fatty acid compounds were thought to be able to inhibit the α -glucosidase. 9,12-octadecadienoic acid (*Z, Z*), methyl ester has the property of anti-inflammatory, anti-arthritis, hepatoprotective, hypocholesterolemic, 5-alpha reductase inhibitor, insectifuge and antiarthritic activity [55, 56]. Aparma *et al.* [57] also reported the anti-inflammatory and antioxidant activity of n-hexadecanoic acid.

4- Piperidinone: 4-piperidinone is a derivative of piperidine. Piperidines are organic compounds consisting of a six membered ring with a molecular formula (CH₂)₅NH. This heterocyclic amine consists of a six-membered ring containing five methylene bridges (-CH₂-) and one amine bridge (-NH-). The modified side chains are responsible for the antibiotic, antifungal, anti-oxidant, anti-inflammatory and anticancer properties [58]. Piperidine and its derivatives have a major impact in the medical field due to their wide range of pharmacological activities. The piperidine moiety is an essential pharmacophore which is found in numerous alkaloids, pharmaceuticals, agrochemicals and as synthetic and biological intermediates

[59]. Piperidine and its derivatives are ubiquitous building blocks in the synthesis of pharmaceuticals and fine chemicals.

D-allose: D- allose is a C-3 epimer of D-glucose with 80% of the sweetness of sucrose. D- allose is one of many rare sugars that exist in very small quantities in nature [60]. Its anti-inflammatory [61], anti-oxidative [62] and inhibitory effects on osteoclast differentiation [63] have been reported. It is believed that when D- allose is a part of a meal, the normal rise in blood sugar is suppressed hence effective in preventing and treating diabetes. It is also believed that it possesses antioxidant properties.

Molecular docking of the most abundant compounds in SF5- Mome inositol and 1-thio-d-glucitol against two key targets in the pathophysiology of diabetes- alpha-glucosidase and SGLT 2 revealed strong affinity between the compounds and the protein targets (Tables 7 and 8). The docking between Mome inositol, 1-D-thio-glucitol and alpha-glucosidase showed that Mome inositol (– 6.7 kcal/mol) had a stronger affinity to the enzyme. Similarly, for sodium glucose co-transporter 2 (SGLT 2), Mome inositol (– 6.5 kcal/mol) had a stronger affinity than 1-D-thio-glucitol. This observation provides an insight into the possible mechanism of action of SF5. The possible inhibition of alpha-glucosidase by the plant could be useful in blunting post-prandial hyperglycaemia. Inhibition of SGLT2 as discussed above is a useful way of reducing hyperglycaemia by preventing the re-uptake of filtered glucose in the proximal tubules of the kidney back into circulation. The inhibition of these two key targets by the most abundant compounds in SF5 is a pointer to the therapeutic potentials of SF5. These two compounds therefore can serve as lead for the discovery of new drugs.

Conclusions

The present study demonstrated that sub- fraction 5 (SF5) obtained from the ethyl acetate fraction of the crude extract through column chromatography possess significant anti-diabetic activity against alloxan induced diabetes mellitus. This was evident in its ability to lower the FBS of diabetic mice at various time intervals. The active principles of SF5 as established by GC-MS analysis which might have contributed directly or indirectly to its antidiabetic activity include Mome inositol, 2-methoxy-4-vinylphenol, 1-thio-D-glucitol, Hexadecanoic acid, 4-Piperidinone and D-allose. The most abundant of these compounds showed high binding affinity against two molecular targets in the pathophysiology of diabetes. This study therefore, shows that there is a prospective future in the use of *T. bracteolata* leaves as a source of natural medicine for management of diabetes mellitus.

Abbreviations

GC-MS: Gas chromatography-mass spectrometer

EETB: Ethanol extract of *Tephrosia bracteolata*

EAF: Ethyl acetate fraction

HbA1c: Glycated hemoglobin

TLC: Thin layer chromatography

SGLT2: Sodium- glucose co- transporter- 2

SF: Sub-fraction

Declarations

Ethics approval and consent to participate

. Animals were handled in accordance to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NRC 2011), and the ethical approval was obtained from the Ethics and Biosafety Committee, Faculty of Biological Sciences, University of Nigeria, Nsukka (approval number: UNN/FBS/EC/1037).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

PAI; Conceived, designed and performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

JMO: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

SCO: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

AOO: Conceived and designed the experiments; Analyzed and interpreted the data.

WOO: Conceived and designed the experiments; Analyzed and interpreted the data

DAZ: Conceived and designed the experiments; Analyzed and interpreted the data.

TBM: Conceived and designed the experiments; Analyzed and interpreted the data

RSI: Conceived and designed the experiments; Analyzed and interpreted the data.

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Figures



Figure 1

Tephrosia bracteolata

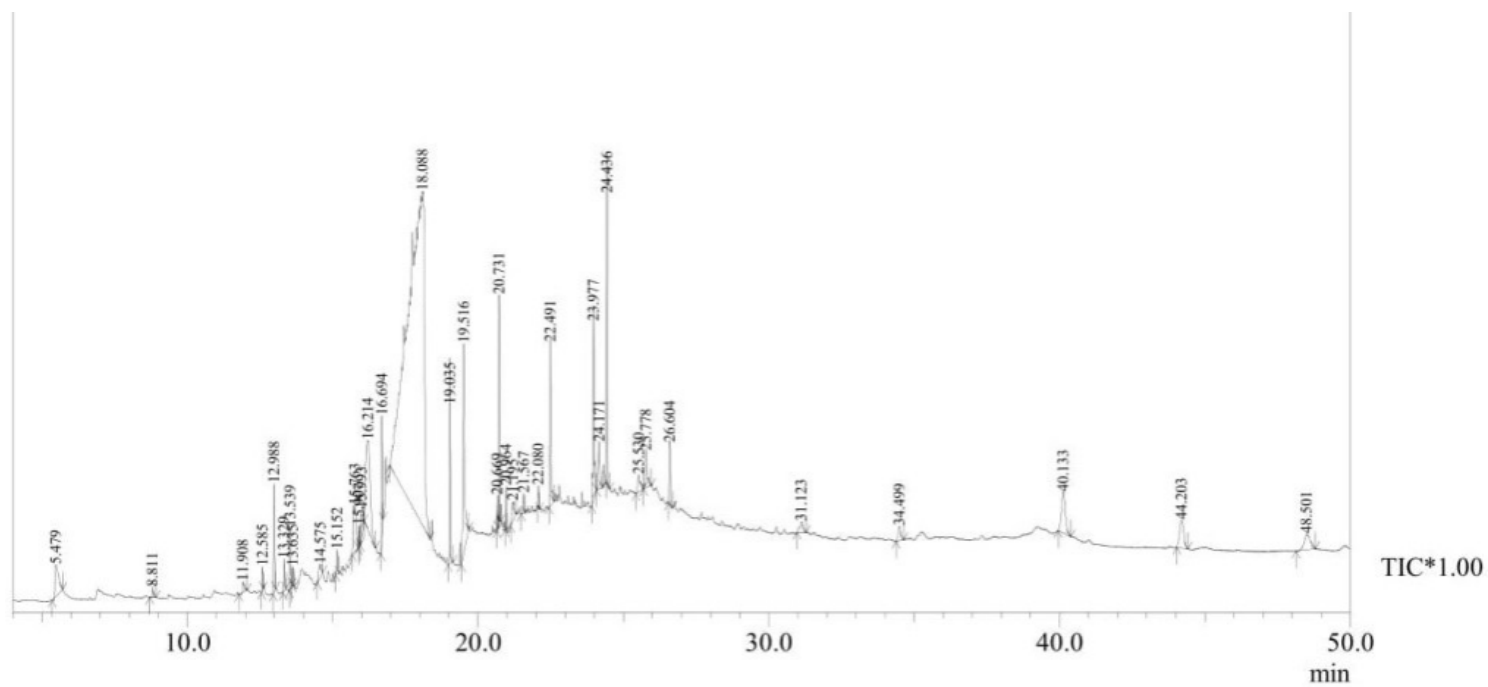


Figure 2

GC-MS profile of SF5