

Methyl Gallate from the Anti-Hyperglycaemic Fraction of the Root Bark Extract of *Terminalia Superba*

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Abstract

Terminalia superba root bark is traditionally used for the treatment of diverse diseases including diabetes in the South Western part of Nigeria and part of Africa. This study evaluates the anti-diabetic activity of the extract and partitioned fractions of the root bark of *T. superba* for scientific validation of the ethno-medicinal claim.

The methanolic extract (**A**) of *T. superba* was partitioned with n-hexane, dichloromethane and ethyl acetate to give four fractions (**B₁ – B₄**). **A** was screened for its glucose lowering activity using oral glucose tolerance test (OGTT) and alloxan-induced diabetic rat models. **B₁ – B₄** were screened for their glucose lowering activity using oral glucose tolerance test (OGTT) model. In the OGTT model, three doses (100, 200 and 400 mg/kg) of the extract were administered to 24 h fasted induced hyperglycaemic rats. The most active dose was used in the alloxan-induced diabetic rat model for **A** and in the OGTT model for the **B₁ – B₄** to monitor the antihyperglycaemic activity. The mother liquor (**B₄**) being the most active fraction was purified and was further subjected to various chromatographic separations which afforded methyl 3,4,5-trihydroxybenzoate (methyl gallate).

The result showed that 200 mg/kg of the extract was the most active dose in the OGTT model with 33 % blood glucose level reduction that was comparable ($p > 0.05$) to the 39 % given by glibenclamide (5 mg/kg) at 4 h. In the alloxan-induced diabetic rat model, the extract at 200 mg/kg gave a significantly higher blood glucose reduction than the glibenclamide (5 mg/kg) throughout the 14-day period of treatment. The 31 and 40 % blood glucose level reduction given by ethyl acetate fraction (**B₃**) and the mother liquor (**B₄**) respectively at 4 h were comparable to the 39% activity elicited by glibenclamide (5 mg/kg) at the same hour. The structural elucidation of the methyl gallate isolated from the mother liquor (**B₄**) was carried out using 1D and 2D NMR spectra and the NMR data compared well with literature.

The study confirmed the ethno-medicinal use of the root bark of the plant in the treatment of diabetes. The methyl gallate isolated from the mother liquor (**B₄**) could be one of the constituents responsible for the activity observed in this fraction.

Keywords: *Terminalia superba*; root bark; anti-diabetes; chromatography; methyl gallate;

Introduction

Diabetes mellitus is a multifactorial metabolic disorder characterized by prolonged hyperglycaemic state, bringing about impairment in carbohydrate, protein and lipid metabolism, resulting from increased hepatic glucose production and diminished insulin secretion or impaired insulin action or both [1]. Globally, an estimated 422 million adults are living with diabetes as the prevalence is increasing rapidly and the number is projected to double by 2030 especially in urban populations of the developing countries due to trend of urbanization and lifestyle changes, such as increased sedentary life styles and perhaps most importantly, a “Western-lifestyle” [2].

Terminalia superba Engl. & Diels. (Combretaceae), commonly known as “Afara” among the Yorubas in Nigeria is a deciduous tree widely spread in West and Central Africa and also grown in the East and South Western part of Nigeria [3,4]. It is used ethno-medically as an anti-diuretic, anti-malarial, in the treatment of conjunctivitis, bronchitis and in ovarian troubles [3]. In Cameroon it is used in the treatment of various ailments, including diabetes mellitus [5]. Its antimicrobial, anti-diarrheal, antifungal, anti-ulcer activities have been reported [6, 7, 8]. The anti-diabetic activity of the leaves and stem bark has been reported [9, 10, 11]. Ellagic acid derivatives; 3, 4-di-*O*-methyllellagic acid 3-*O*-*b*-*D*-xylopyranoside, 4-*O*-galloyl-3,3-di-*O*-methyllellagic acid 4-*O*-*b*-*D*-xylopyranoside, 3,3-di-*O*-methyllellagic acid and 3,3-di-*O*-

methylellagic acid 4-O-β-D-xylopyranoside have been isolated from the plant [12]. The present study was designed to investigate the possible glucose lowering effect of the root bark extract of the plant and isolate the principle(s) responsible for the effect.

Material and Methods

General

NMR spectra were measured on Bruker DPX Avance 500 instrument using methanol-d₄ as solvent and internal standard. For TLC, pre-coated silica gel 60F254 plates were used and compounds were detected under ultra-violet (UV) lamp (254 nm) and further visualized by spraying with vanillin-sulphuric acid solution. Accu-Chek Glucometer with Accu-Chek test strips. alloxan monohydrate (SIGMA chemicals, USA).

Plant material

The root bark of *T. superba* was collected from the wild on Obafemi Awolowo University Campus, Ile-Ife, Osun State, Nigeria. It was authenticated by Mr. Ademoriyo of the Department of Botany, OAU, Ile-Ife and its voucher specimen, IFE-17575 was prepared and deposited at the herbarium.

Extraction and partitioning

The root bark of *T. superba* was air-dried and powdered with 1.5 kg of the powdered root bark extracted exhaustively with methanol. The methanolic solution was concentrated *in-vacuo* to obtain the crude extract (306.26 g; 20.4 %). The crude extract was re-taken in aqueous methanol (1:1, 1 L) and successively partitioned with n-hexane (5×400 mL), dichloromethane (4×400 mL) and ethyl acetate (16×400 mL) and the solutions were concentrated *in vacuo* to obtain their corresponding n-hexane (7.22 g), dichloromethane (4.23 g), ethyl acetate (81.82 g) and mother liquor (212.36 g) fractions respectively. The mother liquor was retaken in 1 L of distilled water and further partitioned with butanol (10×400 mL) to obtain butanolic fraction (152.54 g).

Isolation of the compound and its spectroscopic data

The butanolic fraction (50.0 g) was adsorbed on 50 g of silica gel and was left to dry overnight to avoid solvent interference. The adsorbed fraction was loaded on glass column dry and gradiently eluted using n-hexane, ethyl acetate and methanol. The eluent (100 mL) was collected from the column into each conical flask and 84 column fractions were obtained. These were bulked into ten column fractions according to their TLC profile. A brown solid (18.21 g) obtained at EtOAc/MeOH (92:8), was further subjected to repeated column chromatography on silica gel and Sephadex LH20 to yield a white crystalline solid (172.00 mg).

Methyl 3,4,5-trihydroxybenzoate (Methyl gallate)

White crystalline solid; IR (KBr): ν^{\max} (cm⁻¹) 3570 (OH), 1682 (C=O), 1601 and 1528 (C=C of the benzene ring), 1210 (C-O-C of ester). ¹H and ¹³C NMR, Table 3.

Bioassay

Animals

Healthy Wistar rats of either sex (150 g, average weight) that were used for the experiments, were bred under standard conditions (temp. 27±3°C, relative humidity 65%, natural 12 h day-night) and housed in different cages in the animal house, Department of Pharmacology, Faculty of Pharmacy, O.A.U., Ile-Ife, Nigeria. The rats were fed on a standard pellet diet (Bendel Feeds, Benin, Nigeria), with water given *ad libitum*. They were acclimatized for at least 5 days before commencement of the experiments. Five groups of rats with five rats in each group were fasted for 24 h before administration of either glucose, extract, fractions, drugs or vehicle [13]. All animal experiments conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Academies Press [14].

Glucose lowering activity of extract and fractions

Glucose (10 g/kg, *p.o.*) was administered to 24-hour fasted normal rats and those that were hyperglycaemic [blood glucose level ≥ 7.0 mmol/L (126 mg/dL)] after 0.5 hour (T₀) were selected and randomized into groups of five with five rats in each group. They were administered (*p.o.*) with extract (100, 200, 400 mg/kg), or 1 % Tween 80 in normal saline (negative control), or glibenclamide (5 mg/kg, positive control). At 0.0, 0.5, 1.0, 2.0 and 4.0 hours after administration of the test agents, a drop of blood that was taken from the tip of the tail of each rat was dropped onto a glucometer strip and the blood glucose level was directly read off the glucometer. The blood glucose levels at 0.0 h (T₀) were taken as 100 %, while those at other times were expressed as percentages of these values. Also, glucose lowering activity of the partition fractions were similarly assayed at 200 mg/kg, the highest active dose of the extract [15].

Anti-diabetic activity of the extract on alloxan-induced diabetic rats

Groups of rats were injected (i.p.) with 150 mg/kg alloxan monohydrate dissolved in normal saline [16]. For six days, they were fed and water was given *ad libitum*. The rats with blood glucose (BG) levels ≥ 11.0 mmol/L (200 mg/dL) were considered diabetic. The diabetic rats were randomized into groups of three with five rats in each group. They were administered daily for fourteen days with 1 % Tween 80 in normal saline (negative control) or extract (200 mg/kg) or glibenclamide (5 mg/kg) (positive control) dissolved in the vehicle. Their BG levels were determined and recorded on 1, 4, 7, 10 and 14 days after administration of test agents, as reported by Ojezele and Abatan, 2011.

Statistical analysis

Data obtained from this study were expressed as the mean ± SEM for the number (N) of animals in the group. One way analysis of variance (ANOVA) was first used followed by Student-Neuman-Keuls' test to determine the source of significant differences for all determinations and p < 0.05 was considered to be statistically significant.

Results and Discussion

Glucose lowering activity of the extract

There was significant time dependent reductions in the blood glucose levels up to the fourth hour in glucose-induced hyperglycaemic rats administered with 1 % tween 80 in normal saline (negative control) (Table 1) that was caused by homeostatic regulatory mechanism in the normal animals. This observation confirmed that the pancreases of the rats were functioning well [17]. The *T. superba* extract at 100 mg/kg lacked activity at 0.5-2 h but gave 20 % activity at 4 h. However, at 200 and 400 mg/kg, its activity was time dependent up to the fourth hour with 200 mg/kg showing the highest activity at 4 h. The anti-hyperglycaemic activity of the extract at 200 mg/kg was comparable ($p > 0.05$) to glibenclamide (5 mg/kg) at all-time points (Table 1) suggesting similar minor extrapancreatic and major insulin stimulation mechanism of action of glibenclamide [18]. Furthermore, based on similar results, extracts of *Uvaria afzelii*, *Chrystophyllum albidum*, *Xylopia aethiopica* and *Parquetina nigrescens* have been reported to have insulin stimulation as their mechanism of action [15, 19, 20].

Anti-diabetic activity of the extract on alloxan-induced diabetic rats

The alloxan-induced diabetic rats that were given 1 % tween 80 in normal saline were consistently hyperglycaemic throughout the 14 days of treatment indicating that they were permanently diabetic. The anti-hyperglycaemic activity of the extract at 200 mg/kg, the most effective anti-hyperglycaemic dose in glucose-loaded rats (Table 1), daily administered for 14 days to the diabetic rats was significantly higher than that of glibenclamide (5 mg/kg) at all times (Figure 1). Also, the time-dependent effect shown by the extract, similar to glibenclamide, may suggest insulin release as its major mechanism of action, which was earlier suggested from its anti-hyperglycaemic action using the glucose-loaded model (Table 1). This suggested the usefulness of the extract of *T. superba* in the management of prolonged hyperglycaemic conditions typified by the alloxan-induced rats. Similar to glibenclamide, significant anti-hyperglycaemic activity in glucose-loaded and drug induced diabetic rats have been reported for the methanolic extracts of *Uvaria afzelii*, *S. cayennensis*, *J. tanjorensis* and *B. monandra* [21, 22, 23].

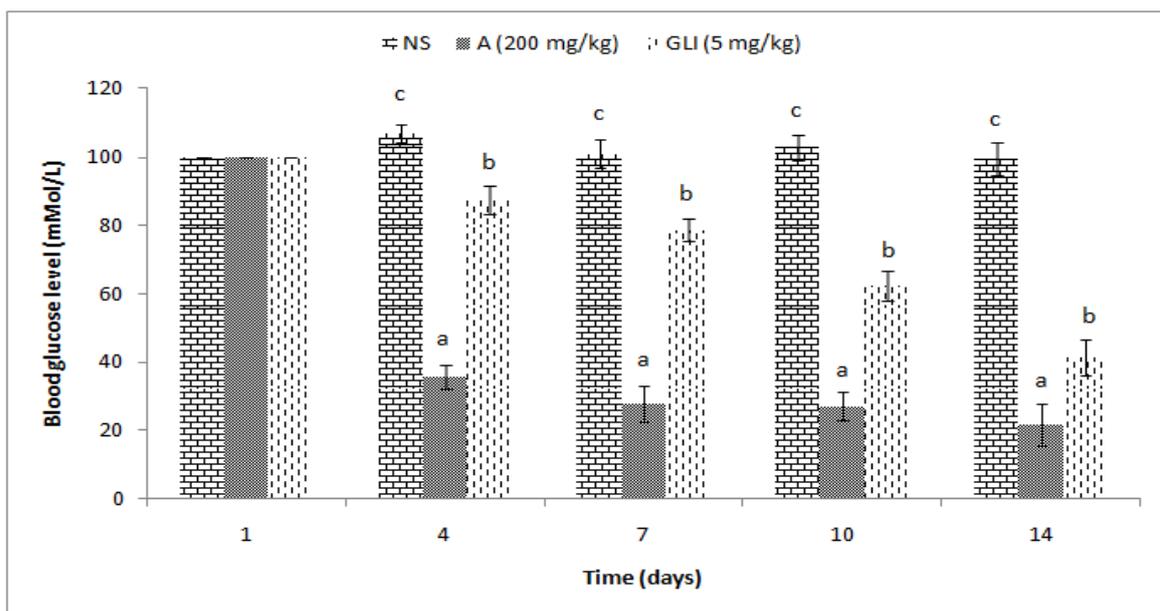


Figure 1: Anti-hyperglycaemic activity of *T. superba* using alloxan-induced diabetic rats

Data show the mean \pm SEM blood glucose levels at different time points expressed as percentages of levels at day 1 (T_0), $n = 5$. Values with different superscripts within each time points are significantly different ($p < 0.05$, one-way analysis of variance followed by the Student-Neuman-Keuls' test). NS: < 1 % of Tween 80 in normal saline (negative control); A: *Terminalia superba* methanolic root extract at 200 mg/kg; Glib (5): Glibenclamide at 5 mg/kg (positive control).

Table 1: Dose related hyperglycemia lowering effect of *T. superba* crude extract

Extract/Drug Doses (mg/kg)	Blood glucose level as percentage of T0 (reduction in blood glucose relative to negative control at Tt)				
	0 h	0.5 h	1 h	2 h	4 h
GLU (10)	100	83.8±3.8 ^a	85.9±0.5 ^b	76.5±1.7 ^b	74.2±2.0 ^c
A (100)	100	76.8±3.8 ^a -8.40%	86.8±6.9 ^b (-1.07%)	77.0±3.6 ^b (-0.7)	58.8±5.6 ^{ab} (20.8%)
A (200)	100	75.3±2.9 ^a (10.1%)	68.2±2.7 ^a -20.60%	54.0±1.9 ^a (29.4%)	50.1±1.3 ^{ab} (32.5%)
A (400)	100	75.7±6.0 ^a -9.60%	73.0±4.5 ^{ab} (15.0%)	63.5±7.4 ^{ab} (16.9%)	58.7±6.0 ^{ab} (20.9%)
GLI (5)	100	75.6±6.7 ^a -9.70%	70.7±6.7 ^{ab} (17.7%)	58.3±6.4 ^a (23.7%)	45.3±6.9 ^a (38.9%)

Data show the mean ± SEM blood glucose levels at different time points expressed as percentages of levels at 0 h (T₀), n = 5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (p < 0.05, one-way analysis of variance followed by the Student–Neuman–Keuls’ test). GLU: Glucose in 1% of Tween 80 in normal saline (negative control); A: Root bark extract of *T. superba*; GLI: Glibenclamide.

Glucose lowering activity of the partitioned fractions

Apart from the dichloromethane partition fraction (B₂) of *T. Superba*, other fractions gave similar profile of activities to glibenclamide indicating that they contained the various concentrations of the insulintropic constituents of the extract. The extract, its ethyl acetate fraction (B₃) and glibenclamide (5 mg/kg) elicited comparable blood glucose reduction at all time points

in the glucose-loaded rats. However, the aqueous fraction (B₄) showed significantly higher (p < 0.05) activity than glibenclamide at 0.5-1 h and comparable activity (p > 0.05) at 2-4 h indicating an additional extra-pancreatic activity to its insulin stimulating effect (Table 2). The overall order of anti-hyperglycaemic effect of the extract and its partition fractions at 4 h therefore is, B₄ > GLI > A > B₃ > B₁ > B₂. This showed B₄ as the most active fraction and thus its choice for further purification (Table 2).

Table 2: Anti-hyperglycaemic effect of the partition fractions of *T. superba* root extract in glucose loaded rats.

Extract/Drug dose (mg/kg)	Blood glucose level as percentage of T0 (reduction in blood glucose relative to negative control at Tt)				
	0 h	0.5 h	1 h	2 h	4 h
GLU (10)	100	83.8±3.8 ^c	85.9±0.5 ^c	76.5±1.7 ^c	74.2±2.0 ^c
A (200)	100	75.3±2.9 ^b (10.1%)	68.2±2.7 ^b (20.6%)	54.0±6.2 ^{ab} (29.4%)	50.1±1.3 ^a (32.5%)
B1	100	82.2±4.4 ^c -1.90%	76.8±6.1 ^{bc} (10.6%)	62.5±3.1 ^b (18.2%)	53.4±2.4 ^b (28.0%)
B2	100	82.5±6.7 ^c -1.60%	77.1±4.9 ^{bc} (10.2%)	73.7±1.5 ^{bc} (3.7%)	65.9±4.6 ^{bc} (11.1%)
B3	100	72.9±4.0 ^{ab} (13.0%)	70.2±2.2 ^b (18.3%)	57.3±5.3 ^{ab} (25.0%)	50.9±5.5 ^{ab} (31.4%)
B4	100	64.1±3.4 ^a -23.50%	53.3±2.7 ^a (37.9%)	48.04±2.5 ^a (37.2%)	44.6±1.6 ^{9a} (39.9%)
GLI(5)	100	75.6±6.7 ^{bc} (9.8%)	70.7±6.9 ^b (17.7%)	58.3±6.4 ^b (23.8%)	45.3±6.9 ^a (38.9%)

Data show the mean ± SEM blood glucose levels at the different time points expressed as percentages of levels at h (T₀), n = 5. Values in parentheses represent the percentage reductions in blood glucose levels relative to negative control for each time point. Values with different superscripts within columns are significantly different (p < 0.05, one-way analysis of variance followed by the Student–Neuman–Keuls’ test). Tween 80: < 1 % of Tween 80 in normal saline (negative control). A: Extract of *T. superba*; B₁ – n-Hexane fraction, B₂ – DCM fraction, B₃ – EtOAc fraction, B₄ – Aq-MeOH fraction, GLI – Glibenclamide (positive control).

Structural elucidation of the isolated compound

The $^1\text{H-NMR}$ (MeOD, 500 MHz) spectrum showed two signals for aromatic protons at $\delta 7.00$ (s, 2H) and non-aromatic protons at $\delta 3.82$ (s, 3H). The $^{13}\text{C-NMR}$ (125 MHz) showed signals at 169.8, 146.7, 139.8, 121.5, 109.9 and 52.2. The analysis of the carbon-13 and the DEPT-135 gave molecular formula of $\text{C}_8\text{H}_8\text{O}_2$. In the HMQC, the protons at 3.82 showed correlations with carbon-13 at 52.2 and protons at 7.00 showed correlations with carbon-13 at 109.9. In the HMBC, proton at 3.82 showed correlations with carbon-13 at 169.8 and protons at 7.00 showed correlations with carbon-13 at 169.8, 146.7, 139.8 and 121.5 as shown in (Table 3). The NMR data compared well with published data [24]. Thus the compound was identified as methyl 3,4,5-trihydroxybenzoate, (Figure 2).

Table 3: ^1H - and ^{13}C -NMR data and HMBC correlations of the isolated compound

Carbon atom	^1H (multiplicity)	^{13}C	HMBC ^1H to ^{13}C
1	-	139.8	-
2 & 6	7.05 (s)	109.9	169.8, 139.8, 146.7, 121.5
3 & 5	-	146.7	-
4	-	121.5	-
7	-	169.8	-
OCH ₃	3.86 (s)	52.2	169.8

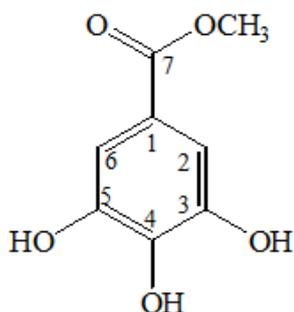


Figure 2: Structure of methyl 3,4,5-trihydroxybenzoate

Conclusion

The anti-hyperglycemic effect exhibited by the extract of root bark of *T. superba* both in glucose-loaded rats and alloxan-induced hyperglycaemic rats in this study justified its ethno-medicinal anti-diabetic usage and methyl gallate that was isolated from the aqueous methanol, most active fraction of the extract is being proposed as the constituent or one of the principles responsible for the activity observed.

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