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Research Paper

H-Proton NMR Spectra of Antihyperglycemic Triterpenoid Isolated from *Cussonia arborea*

P.E Aba[1]*, I.U Asuzu[1]

[1]Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, Enugu State, Nigeria

*Corresponding Author

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ABSTRACT

The aim of this study was to isolate and characterize the active principle responsible for hypoglycemic activity of *Cussonia arborea* using alloxan-induced diabetic rat. Thirty five (35) rats assigned into 7 groups of 5 rats per group were used for acute toxicity study. Groups 1-6 received 500, 1000, 2000, 3000, 4000 and 5000mg/kg extract while rats in group 7 received distilled water. Phytochemical composition of the extract was carried out. The extract was bioassay-guidedly fractionated using column and thin layer chromatography. Hyperglycemia was induced in rats by single intraperitoneal injection of alloxan monohydrate at the dose of 160mg/kg. Rats with Fasting Blood Sugar (FBS) levels higher than 126mg/dl were considered diabetic (hyperglycemic). Hypoglycemic fraction (subfraction 2:1) was subjected to ^{1}H proton Nuclear Magnetic Resonance (NMR). The acute toxicity study results showed that the methanol root bark extract of *C. arborea* did not produce any sign of toxicity even at the highest dose of 5000mg/kg body weight (bw) 48h post administration. Phytochemical analyses revealed the presence of terpenes, tannins, saponins, alkaloids, flavonoids and glycosides. Fractionation of the crude extract using column chromatography produced 7 fractions of which fraction 2 was most hypoglycemic in action (reduced FBS from 307.00±18.77mg/dl to 77.33±3.17mg/dl). Further purification of fraction 2 yielded 3 subfractions with subfraction 2:1 producing most profound antihyperglycemic activity (reduced FBS from 310.00±5.77mg/dl to 74.00±0.57mg/dl). Spectroscopic examination of subfraction 2:1 produced spectra typical of triterpenoids. It was concluded that the antihyperglycemic activity of *Cussonia arborea* root bark is attributed to its triterpenoid content.

Key words: *Cussonia arborea;* Antihyperglycemia; Triterpenoid; NMR.

INTRODUCTION

Diabetes is a complex and multifarious group of disorders that disturbs the metabolism of carbohydrates, fats and protein (kahn and Shecter, 1991). It is a metabolic disorder resulting from defects in insulin secretion or reduced sensitivity of

the tissues to insulin action or both (Lanza et al., 2001). Different types (insulin dependent *Diabetes mellitus* or type 1, non-insulin dependent *Diabetes mellitus* or type 2 and gestational *Diabetes mellitus*) of diabetes have been described with basic clinical signs of polydipsia, polyuria, polyphagia and asthenia (Kahn, 2005). Globally, as of 2010, an estimated 285 million people had diabetes with type 2 making up about 90% of the cases. In 2013, according to international diabetes federation, an estimated 381 million people had diabetes with its prevalence increasing rapidly and by 2030; this number is estimated to double (Wild et al., 2004). Middle-aged to older dogs and cats are equally affected by *Diabetes mellitus* (Kahn, 2005).

In the last two decades, research into antidiabetic medicinal plants has not been rewarded with marketable novel drugs (Dewanjee et al., 2008). Medicinal plants have been used traditionally in the management of the ailment. However, a lot of secrecy and exaggeration of effects have been associated with the use of medicinal plants. There is the need for standardization, hence this study. Literature search revealed that *Cussonia arborea* is used folklorically in the management of *Diabetes mellitus* (Amodou et al., 2008).

So, the aim of this study was to isolate and characterize using NMR, the hypoglycemic principle present in the root bark of *Cussonia arborea* for the first time.

MATERIALS AND METHODS

Chemicals and Reagents*:* Chloroform, Methanol, Ethylacetate, Hexane, Silica Gel,Glibenclamide, Alloxan monohydrate (Sigma Aldrich, UK).

Instruments and glassware*:* Glucometer (Accu Chek-Active), electronic weighing balance (Mettler, England), UV Lamp (YLN, China), Refrigerator (Haier Thermocool), Thin Layer chromatography plate and container (De Saga), Rotary evaporator (Buchii, Switzerland), NMR Machine (Bruker 450 Hz, UK), Glass wool, Gastic garvage, mortar and pestle, crocodile clip (Lagos, Nigeria).

Animals*:* Male albino Wistar rats weighing between 100g and 105g were obtained from the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka. The rats were acclimatized for two weeks. The environmental temperature where the animals were housed varied between 28-32°C. The animals were kept in stainless wire mesh cages and provided with good clean water *ad libitum*. They were fed with standard commercial feed (Guinea^{R} growers, Benin).

Plant Material*:* The root bark of the plant material (*Cussonia arborea*) used in this study was collected from Orukpa Local Government Area of Benue state, Nigeria and identified by a plant taxonomist, of Plant Science Department, University of Nigeria, Nsukka, Nigeria.

Preparation of the Plant Extract: Cold maceration method of extraction was employed. The root bark of *C. arborea* was air dried at a very low intensity of sunlight to avoid denaturation of the active ingredient. It was pulverized and stored in an air tight container pending its usage. About 2kg of the powdered stem bark was soaked in 10 liters of 80% methanol with intermittent shaking every 2h for 48h. The mixture was filtered using Whatmann No 1 filter paper. The filtrate was concentrated using rotary evaporator and the extract (CAE) stored at 4°C.

Acute Toxicity Test: Acute toxicity test according to the method of Aba et al., (2014) was used. Rats were assigned into 7 groups consisting of 5 rats per group. Groups 1-6 were given (orally) graded doses (500mg/kg, 1000mg/kg, 2000mg/kg 3000mg/kg, 4000mg/kg, and 5000mg/kg) of the extract respectively. The rats in group 7 received

distilled water (10ml/kg) to represent the negative control. The rats were observed closely for 48h for signs of toxicity such as sedation, dullness, anxiety, wretching, anaesthesia and death.

Phytochemical Test: The method of Trease and Evans (1989) was used.

Test for Presence of Tannins: 3ml of the extract was placed into 3 test tubes. A few drops of lead subacetate were added into the first test tube while 5ml of dilute sulphuric acid was added to the second tube. Into the third tube was added few drops of ferric chloride. Distilled water, yet in another test tube was used as a control. Brownish colour in lead acetate and yellow colour appearance in dilute sulphuric acid indicated the presence of tannins when the control showed no colour change. Bluish-black precipitate with ferric chloride shows the presence of tannins while the control is yellow.

Test for the Presence of Alkaloid*:* 1ml of the extract each in three test tubes was added few drops of Wagner`s reagent in test tube 1, Meyer`s reagent in test tube 2 and Draggendorff`s reagent in test tube 3 and distilled water in another test tube served as the control. Intense yellow colour in Wagners reagent when the control is light yellow indicates positivity. Slight yellow in Meyer`s reagent as the control remains colourless also indicates presence of alkaloid. Dirty yellow precipitate in Draggendorf`s reagent shows positivity as the control has brick-red precipitate.

Test for the Presence of Flavonoids*:* 1ml of extract was diluted to 5ml with distilled water in a test tube. 1ml of 20% sodium hydroxide was added and water was used as control. Intense yellow colour indicated the presence of flavonoids.

Test for the Presence of Glycosides*:* Into 5ml of the extract in a test tube was added 2ml of dilute sulphuric acid, heated, cooled and neutralized with equal volume of sodium hydroxide solution. This was boiled. About 5ml of Fehling`s`solutions I and II mixture was added and heated for 5 to 10mins and cooled. Water was used as negative control. Brick-red precipitate at the bottom of the test tube showed a positive test for glycoside as there was no such precipitate in the control tube (tube containing H_2SO_4, NaOH, Fehling`s I and II).

***Test for the presence of saponins*:**

a. Frothing Test*:* Exactly 3ml of extract was diluted to 10ml with distilled water. The solution was shaken vigorously for 30 seconds and allowed to stand. Much foaming indicated the presence of saponin.

b. Emulsifying Test: One drop of olive oil was added into the test solution and shaken vigorously. Saponin was used as positive control while distilled water served as negative control. Emulsification indicated the presence of saponin.

Test for Sterols/Terpenes: 10ml extract was evaporated to dryness in a beaker. The residue was dissolved in 1ml acetic acid anhydride and 1ml chloroform. This was transferred to a dry test tube into which 2ml of concentrated sulphuric acid was added. Formation of a brownish or violet ring at the zone of contact with supernatant indicated the presence of sterols/terpenes.

Bioassay-Guided Fractionation/Isolation of Active Compound

Column Chromatography: was used to separate the different phytochemical components of the plant extract. The method of Ergon (1967) was used.

Table- 1: The mixture proportions used during fractionation are summarized as:

	Hexane	Chloroform	Ethylacetate	Methanol
1	100	-	-	-
2	80	20	-	-
3	60	40	-	-
4	40	60	-	-
5	20	80	-	-
6	-	100	-	-
7	-	80	20	-
8	-	60	40	-
9	-	40	60	-
10	-	20	80	-
11	-	-	100	-
12	-	-	80	20
13	-	-	60	40
14	-	-	40	60
15	-	-	20	80
16	-	-	-	100

Bioassay-guided Screening of the Fractions: The fractions (7) gotten after chromatography were subjected to screening to ascertain the particular fraction that has the antidiabetic properties. Diabetes was induced in groups 1-9 rats as described before by the method of Venugopal et al., (1998). The rats were injected with alloxan monohydrate dissolved in distilled water at dose of 160mg/kg body weight intraperitoneally, after overnight fasting (18h). Meanwhile before the injection with alloxan monohydrate, the blood glucose levels of the rats were taken using Accu-Check glucometer. This was done by tail snip of the rats and allowing blood to drop on the glucometer strip. The value was digitally read off on the screen of the glucometer. After induction, the rats were kept in clean stainless cages and fed with commercial feed and were also given clean water for about 2 days before they came down with diabetes. On the 2nd day, diabetes was confirmed. The rats were fasted overnight before the assessment of their glucose blood status on the 2nd day. The FBS values above 7mMol/L (126mg/dl) were considered diabetic.

The pre-extract administration blood glucose level was determined. The rats were assigned to 10 groups consisting of 3 rats each. Groups 1-7 received the same dose (12.5mg/kg) of different fractions of CAE; group 8 rats received 2mg/kg glibenclamide (Standard control) while rats in groups 9 and 10 received the vehicle (10ml/kg distilled water) to serve as negative and normal controls respectively.

Following the administration of the fractions, blood was collected from the rats by tip tail cut after 1h, 3h and 6h post extract administration for blood glucose analysis. The fraction which was able to reduce significantly, the blood-glucose level was taken as the fraction that has the anti-hyperglycemic property.

Bioassay-guided Purification of Active Fraction: The active fraction obtained from the experiment above was subjected to preparative thin layer chromatography for further purification. This was done using the method of Ergon (1969).

Purification of Active Fraction 2: Eighteen (18) rats were assigned into 6 groups of 3 rats per group. Diabetes again was induced in 15 rats (Groups 1-5) by the method of Venugopal et al., (1998). The rats in groups 1-3 were treated with 1.25mg/kg of the subfraction 2 and the rats in groups 4 and 5 were treated with 2mg/kg glibenclamide and 10ml/kg distilled water respectively while rats in group 6 were not induced but

treated with the vehicle (10ml/kg distilled water). Thereafter, blood samples were taken 1h, 3h and 6h post treatment for glucose analysis.

Characterization and Structural Elucidation of the Active Compound: This was done using NMR spectroscopy.

***Statistical Analysis*:** Statistical package for social sciences version 20 was employed. One-way Analysis of Variance (ANOVA) was used to compare the means of the FBS levels and their differences separated using Duncans Multiple Range post hoc test.

RESULTS AND DISCUSSION

The acute toxicity study results showed that the methanol root bark extract of *C. arborea* did not produce any sign of toxicity even at the highest dose of 5000mg/kg body weight (bw) 48h post administration.

Table-2: Acute toxicity test at varying doses of the methanol root bark extract against rats.

Group	Dose (mg/kg)	Signs of Toxicity/Death
1	5 00	None
2	1000	None
3	2000	None
4	3000	None
5	4000	None
6	5000	None
7	Distilled water	None

The result of acute toxicity test of the extract is presented in table-2. Treatment of rats orally with varying doses (500, 1000, 2000 3,000, 4000 and 5000mg/kg) of the methanol root bark extract of *Cussonia arborea* did not result in toxicity signs nor deaths of the treated animals (**Table-2**). This agrees with the studies on the methanol stem bark extract of *C. arborea* which was found to be safe when administered to rats even at the highest dose of 3, 200mg/kg (Aba, et al., 2014). This observation is also in agreement with the finding of Fabricant and Farnswort (2001) who reported that medicinal plants have been documented to have advantage in toxicity consideration based on their long term use by human. This is the first time acute toxicity studies on the methanol root bark extract of *C. arborea* was carried out.

Phytochemical evaluation of *C. arborea* root bark extract revealed the presence of saponins, glycosides, alkaloids, tannins, flavonoids and terpenes (**Table-3**).

Table-3: Phytochemistry of the methanol root bark extract of *Cussonia arborea.*

Phytochemical constituents	Tests	Concentration
Saponins	Froathing Emulsifying	+++ +++
Glycosides	Sulphuric acid +Fehlings I and II	++
Tannins	Lead acetate Ferric chloride Sulphuric acid	+ + +
Alkaloids	Wagner Meyer Dragendorff`s	+ ++ ++
Flavonoids	Sodium hydroxide	++
Terpenes	Acetic acid anhydride+ chloroform + sulphuric acid	+++

The result of the phytochemical studies of the methanol root bark extract of *Cussonia arborea* revealed the presence of saponins and terpenes in very high quantity, glycosides, alkaloids, flavonoids, in relatively high amount, tannins, were found in moderate concentrations.

Researchers have reported different phytochemical compounds in medicinal plants (Tapondjou et al., 2003). The finding of saponins, glycosides and tannins agrees with the results of our earlier studies (Aba et al., 2014) on the methanol stem bark extract of *C.arborea*. However, flavonoids were absent in the methanol stem bark extract of *C.arborea*. There is dearth of information on the phytochemistry of *C.arborea* root bark extract. We report for the first time, the phytochemical components of *C.arborea* root bark extract. In their earlier studies, Tapondjou et al., (2003) and Dovgii et al., (2005) reported the presence of saponins from *Cussonia bancoensis* and glycoside from *Cussonia paniculata* respectively.

Chromatographic analysis of *C. arborea* extract yielded seven different fractions (**Table-4**).

Table-4: Effect of different fractions of *C. arborea* on Fasting Blood Sugar (FBS) levels of alloxan induced diabetic rats.

Group	FBS pre induction	FBS 0h	FBS 1h	FBS 3h	FBS 6h
Diabetic rat + 12.5mg/kg F1	79.00±1.52^{a}	293.00±1.73^{bc}	286.33±2.02^{c}	282.66± 4.80^{c}	273.00±11.67^{c}
Diabetic rat + 12.5mg/kg F2	77.66± 2.84^{a}	307.00±18.77^{c}	211.33±5.54^{b}	94.67±5.84^{ab}	77.33±3.17^{a}
Diabetic rat + 12.5mg/kg F3	75.33 ± 2.18^{a}	282.00±8.02b^{c}	237.00±9.07^{c}	119.00±7.23^{b}	99.33±6.06^{b}
Diabetic rat + 12.5mg/kg F4	75.33 ± 1.33^{a}	295.33±5.17^{bc}	278.00±4.04^{c}	247.33±23.66^{c}	244.33±21.69^{bc}
Diabetic rat + 12.5mg/kg F5	81.33± 2.33^{a}	290.33±2.60^{bc}	279.66±2.02^{c}	277.33±1.45^{c}	271.66±4.70^{c}
Diabetic rat + 12.5mg/kg F6	78.00± 5.03^{a}	294.33±6.17^{bc}	287.00±8.50^{c}	279.33±8.41^{c}	267.00±6.11^{c}
Diabetic rat + 12.5mg/kg F7	78.33± 4.17^{a}	284.00±9.07^{bc}	274.00±13.01^{c}	269.66±12.91^{c}	252.33±26.20^{bc}
Diabetic rat + 2mg/kg Glibenclamide	75.00±0.57^{a}	287.33±14.52^{bc}	208.66±3.52^{b}	85.66±2.60^{ab}	80.33±3.48^{a}
Diabetic rat + 10ml/kg Distilled water	73.00± 2.51^{a}	273.33±14.52^{b}	272.00±10.11^{c}	257.33±16.17^{c}	223.00±2.51^{b}
Non-diabetic rat +10ml/kg Distilled water	74.00±0.57^{a}	76.00±0.57^{a}	74.33±0.88^{a}	75.33±3.17^{a}	76.00±1.52^{a}

Different superscripts along the same column indicate significant difference at $P<0.05$

The FBS levels of the rats treated with fraction 2 were significantly ($P< 0.05$) lower than the FBS of the diabetic untreated rats (Group 5) 3h and 6h post treatment but was statistically comparable to that of the normal control rats and to that of the rats treated with glibenclamide (Group 4 rats). The FBS levels of the rats treated with fraction 2 were significantly ($P< 0.05$) lower than the FBS of the diabetic untreated rats (Group 5) 3h and 6h post treatment but was statistically comparable to that of the normal control rats and to that of the rats treated with glibenclamide.(**Table-4**).

Table-5: Effect of Fraction 2 subfractions of *C. arborea* root bark extract on the FBS levels of alloxan-induced diabetic rats.

Group	FBS pre induction	FBS 0h	FBS 1h	FBS 3h	FBS 6 h
Diabetic rat + 1.25mg/kg $subF_1$	75.00±0.57[a]	310.00±5.77[b]	196.33±3.17[b]	81.33±3.17[a]	80.00±0.57a
Diabetic rat + 1.25mg/kg subf1	76.33±0.88[a]	313.33±12.01[b]	310.33±9.66[c]	297.00±3.51[b]	298.66±6.35[b]
Diabetic rat + 1.25mg/kg subf1	75.42±0.71[a]	299.97±10.55[b]	305.24±78[c]	289.92±7.23[b]	291.58±5.45[b]
Diabetic rat + 2mg/kg Glibenclamide	76.00±1.52[a]	313.33±17.63[b]	197.003.51[b]	78.33±1.45[a]	77.00±0.57[a]
Diabetic rat + 10ml/kg Distilled water	76.33±0.88[a]	300.00±5.77[b]	295.66±6.69[c]	296.33±3.17[b]	294.00±4.00[b]
Non-diabetic rat + 10ml/kg DW	76.00±1.15[a]	74.66±1.45[a]	74.67±0.88[a]	77.33±3.17[a]	75.00±1.52[a]

Different superscripts along the same column indicate significant difference at $P<0.05$ The FBS levels of the rats treated with subfractions of the fraction 2 showed a significant ($P< 0.05$) reduction when compared with the diabetic untreated (Group 5) rats 3h post treatment but was statistically comparable to those of the rats treated with standard drug (Glibenclamide) and normal control**.** Upon further purification and separation by bio-assay guided technique of fraction 2, subfraction 1 of the 3 subfractions demonstrated hypoglycemic potential after reducing the FBS levels of the diabetic rats from 310.00 ± 5.77 to 80.00 ±0.57mg/dl 6h post administration (**Table-5**). The FBS levels of the rats treated with subfraction 1 of the fraction 2 showed a significant ($P< 0.05$) reduction when compared with the diabetic untreated rats 3h post treatment but was statistically comparable to those of the rats treated with standard drug and normal control rats. Aba, et al., (2014) reported significant reductions in the FBS levels of alloxan-induced diabetic rats treated with fractions of methanol stem bark extract of *C. arborea*. This is also in agreement with the findings of Abo and Lawal **(**2013**)** who reported antihyperglycemic activities of different fractions of *Physalis angulata* extract.

Nuclear magnetic resonance spectroscopy is a modern tool used in determining the molecular structure, dynamics and characterization of materials at molecular level (Wuthrich, 1990). The basis for the use of NMR is the distinct difference in resonance frequency of every nuclei in the same magnetic field referred to as chemical shift and the nuclei of neighboring NMR active substances known as spin-spin or J-coupling. J-coupling allows the identification from peak intensities. This information from NMR is used to determine accurately the unique molecular structure of compounds (Molodtsov et al., 2004). In spectroscopic analysis of our active compound, the NMR spectra indicated that the active compound was a triterpene (**Figure-1**).

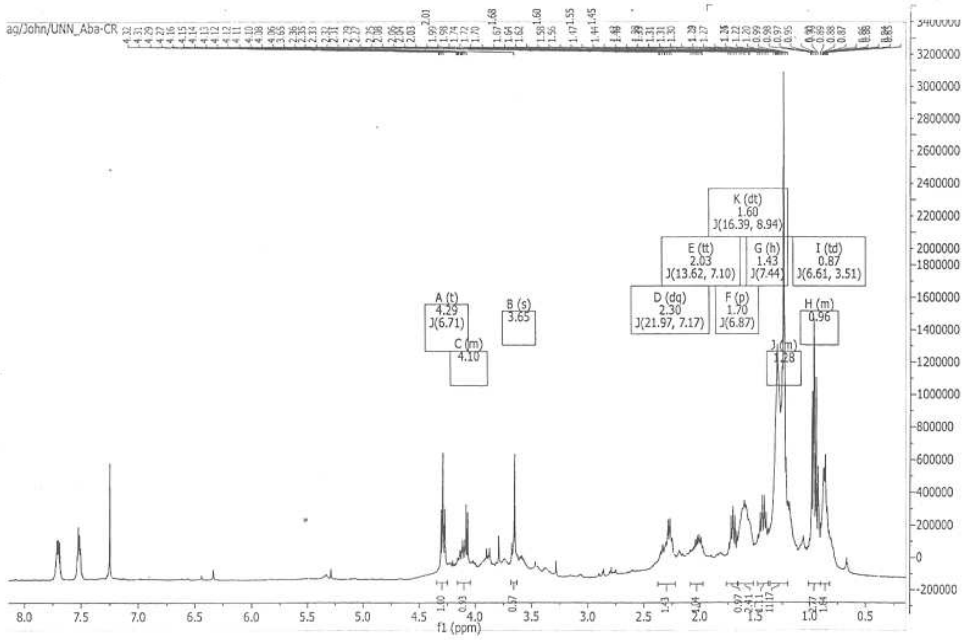

Figure-1: ^{1}H Proton NMR Spectra of the Active Compound-Triterpene

Triterpenes constitute a large structurally diverse group of natural compounds biogenetically derived from active isoprene. Triterpenes have been reported to possess antidiabetic activities. They inhibit enzymes involved in glucose metabolism, prevent the development of insulin resistance and normalize plasma glucose and insulin levels (Nazaruk and Borzy-kluczyk, 2014). This is in agreement with the findings of Min-Jia et al.,(2008) who reported antidiabetic activities of triterpenoids isolated from bitter melon. Similarly, Santos et al., (2012) reported antihyperglycemic activities of alpha and beta amyrins which are pentacyclic triterpenes. Oleanolic acid is a triterpene found in a variety of plants and has been demonstrated to have a variety of biological effects including antidiabetic effect (Castellano et al., 2013; Zhang et. al., 2004). Here we report antidiabetic effect of triterpene isolated from methanol root bark extract of *C. arborea* for the first time.

Conflict of interest: Authors confirm no conflict of interest.

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