Identification of Betulinic Acid and Trimethoxyellagic Acid as the Antidiabetic Compounds in Anogeissus leiocarpus Stem Bark Purified Extract

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Abstract

Objective: The study aimed to identify the antidiabetic compounds purified from stem bark of Anogeissus leiocarpus and propose the mechanisms of action.

Design: Anogeissus leiocarpus stem bark was purified through ethyl acetate and n-hexane with minor exceptions. For very clear separation, cold acetone was added to trigger the precipitation. The precipitate was dissolved with a mixture of DCM-methanol (9:1), adsorbed it to silica gel (5 g), evaporated to free flowing powder and fractionated over silica gel (50 g) to realize 40 fractions. The gummy fractions were ignored. The light brown powder which possessed antidiabetic effect was selected for Nuclear Magnetic Resonance for structural elucidation.

Nuclear Magnetic Resonance (NMR) and Determination of Structure of Purified Compound: After column chromatography and TLC processes, along with the cold acetone, to the point of crystallization, the purified compounds, the light brown powder, were presented to NMR (Bruker Avance III, Spectrometer frequency 400 MHz; solvents DMSO-d6, CDCl3, Acetone-d6. Institute of Chemistry, Strathclyde University Glasgow UK.) for determination of compound structures and analyses.

Results: The NMR spectra and analyses revealed the existence of Betulinic acid and Trimethoxyellagic acid.

Conclusion: We show that Betulinic acid and Trimethoxyellagic acid are potent antidiabetic compounds in the stem bark extract of A. leiocarpus.

Keywords: Anogeissus leiocarpus, Betulinic Acid, Trimethoxyellagic Acid, Antidiabetic agent

INTRODUCTION

The global threat emanating from the worldwide increases in type 1 (T1DM) and type 2 diabetes mellitus (T2DM), without and with microvascular and macrovascular complications of cardiovascular disease, nephropathy and retinopathy and blindness had implicated DM as a leading cause of death by WHO2, come 2030 with a more threatening scenario projected for 2035 hence, a challenging unresolved health problem for the 21st century. The risk factor induced by diabetes on the prognosis and mortality of COVID-195,6,7 and the national budgets/global economic implications remain memorable.

Obesity, a predisposing factor of diabetes7 and in particular, the delayed or non-healing wounds (diabetic ulcer)8,10,11,12, induced by chronic hyperglycaemia, create additional unquantifiable worrisome social menace. In Africa, age distribution and undiagnosed DM were reported13.
The diabetes-induced global threat to human populations and their animals\(^{14,15,16,17,18}\) led to a very strong clamour for more research into other non-conventional drugs for diabetes mellitus and its life threatening complications.

Even, with the peculiarity of cattle, with comparatively low normal blood glucose, due to their reliance on the utilization of volatile fatty acids from rumen for energy metabolism\(^{19}\), the presentation of diabetes mellitus was reported\(^{16}\).

This strong clamour for the development of a non-conventional drug against DM had been directed towards medicinal plants and their phytoconstituents/phytochemicals\(^{4,20,21,22,23,24,25}\) due to the traditional use of medicinal plants to manage diabetes mellitus. One of these medicinal plants of the genus *Anogeissus* (axlewood, ghatti, button and chewing stick trees) of the Combretaceae, had eight species and of wide distribution in Asia and Africa\(^{26}\) had received attention. Under the context of traditional management of diabetes mellitus, more attention had been focused on one of its species, *Anogeissus leiocarpus*, for the underlisted endowed medicinal benefits it exhibited; (i). Extracts of *A. leiocarpus* of guill and perr leaf controlled hyperglycaemia and dyslipidaemia in alloxan-induced diabetic rats\(^{27}\); (ii). Antihyperglycaemia occurred in alloxan-induced diabetes in mice\(^{28}\), Similarly, crude ethanolic extracts of *A. leiocarpus* stem bark (iii) contained antioxidant activity in alloxan-induced diabetic rats\(^{29}\); (iv) attenuated dyslipidaemia in alloxan-induced diabetic dogs\(^{30}\); (v) ameliorated the deranged electrolytes, acid-base imbalance and the organic damages in alloxan-induced diabetic dogs\(^{31}\); (vi) in addition, it accelerated wound healing in alloxan-induced diabetic dogs\(^{32}\); (vii) and finally, it significantly established elevated serum sialic acids as a potent biomarker of diabetes mellitus which was predictive and prognostic\(^{33}\).

Final purification produced three fractions and an interface\(^{34}\), the interface exhibited the antidiabetic and haemopoietic activities\(^{35}\), had no effects on pregnancy, gestation, reproductive performance and no teratogenic effect on pups of Wistar rats\(^{36}\). In addition it has a wide range of safety value\(^{37}\), Optimisation for therapeutic hypoglycaemic dose, showed 5 mg/kgbd.wt was hypoglycaemic and the interface segment had not expired after seventeen (17) months at room temperature, post purification\(^{38}\). Preliminary structural investigation for an elucidation and insight into the mechanism of action using nuclear magnetic resonance (NMR) showed fraction A to be Lupeol, a pentacyclic triterpene, an anti-inflammatory compound\(^{39}\).

This study was designed for further structural investigation on the interface, which revealed the existence of Betulinic acid and Trimethoxyellagic acid and discussed along the mechanism of antidiabetic activity of *A. leiocarpus*.

**MATERIALS AND METHODS**

**Sample Collections, Processing and Purification.**

The fertility assessment of the tree grown soils, the harvesting of the *Anogeissus leiocarpus* stem bark, the processing and the purification protocols of fractionation, column chromatography, with ethyl acetate and n-hexane and thin layer chromatography followed standard procedures as detailed earlier\(^{15,29,30,31,32,33,34,35,36,37,38}\).

There were minor exceptions: much earlier the interface precipitations occurred because the separation funnel was shaken too vigorously. For very clear separation, cold acetone was added to trigger the precipitation. The precipitate was dissolved with a mixture of DCM:methanol (9:1), adsorbed it to silica gel (5 g), evaporated to free flowing powder and fractionated it over silica gel (50 g) to realize 40 fractions. The gummy fractions were ignored. The light brown powder which possessed antidiabetic effect was selected, for Nuclear Magnetic Resonance for structural elucidation.

**Nuclear Magnetic Resonance (NMR) and Determination of Structure of Purified Compound.**

After column chromatography and TLC processes, along with the cold acetone to the point of crystallization, the purified compounds, the light brown powder were presented to NMR (Bruker Avance III, Spectrometer frequency 400 MHz; solvents DMSO-d6, CDCl3, Acetone-d6). Institute of Chemistry, Strathclyde University, Glasgow UK) for determination of compound structures and analyses.

**RESULTS**

The purified compound was approximately 100 mg.

The 1D and 2D NMR spectra under different solvents and their structural characterization, are presented below.

**Nuclear Magnetic Resonance Spectra of Betulinic Acid:**

![1H Nuclear Magnetic Resonance Spectra.](image-url)
Figure 2: $\text{\textsuperscript{13}C}$ Nuclear Magnetic Resonance Spectra.

Figure 3: $\text{\textsuperscript{1}H}$-$\text{\textsuperscript{1}H}$ COSY (Correlated Spectroscopy) Spectra.

Figure 4: HSQC (Heteronuclear Single Quantum Correlation) Spectra.
The potent chemical reaction between betulinic acid and glucose produced the end-products of betulinic acid glycoside, an ester and water. The reactions could occur through glycosidic linkages with the reactive hydroxyl (OH) group at C position 1 of two molecules of glucose, linked with the carboxylic (COOH) and the hydroxyl (OH) groups of the betulinic acid.

**Potent Chemical Reaction of Betulinic Acid and Glucose**
Figure 8: Reaction 2

i. Formation of R-O-R (ether) linkage with elimination of water molecule when simple monosaccharide (glucose) couples or polymerizes.

ii. Cross-OH condensation between monosaccharide and other –OH of other biological molecules.

iii. The –COOH and –OH condensation (to form ester glycoside) occurs far more readily than the –OH and –OH condensation (to form ether glycosides).

Nuclear Magnetic Resonance Spectra of Trimethoxyellagic acid:

Figure 9: $^1$H Nuclear Magnetic Resonance Spectra.

Additional $^1$H Nuclear Magnetic Resonance Spectra (14 in number) for:


are very similar to Fig. 9, hence are not included.
Figure 10: $^{13}$C Nuclear Magnetic Resonance Spectra.

Figure 11: $^1$H, $^1$H-COSY (Correlated Spectroscopy) Spectra.

Figure 12: HSQC (Heteronuclear Single Quantum Correlation) Spectra.
Figure 13: HMBC (Heteronuclear Multiple Quantum Correlation) Spectra.

The structure:

Figure 14: Chemical Structure of Trimethoxyellagic Acid.

The potent chemical reaction between Trimethoxyellagic acid and glucose produced the end-products of trimethoxyellagic acid glycoside and water. Similarly, the reaction is through the glycosidic linkage with the reactive hydroxyl (OH) group at C position 1 of glucose and the hydroxyl (OH) group of the trimethoxyellagic acid.

Figure 15: The Potent Chemical Reaction of Trimethoxyellagic Acid and Glucose.

i. Formation of R-O-R (ether) linkage with elimination of water molecule when simple monosaccharide (glucose) couples or polymerizes.

ii. Cross-OH condensation between monosaccharide and other –OH of other biological molecules.
Chemical Shift Data of Proton and Carbon NMR of the Betulinic Acid and Trimethoxyellagic Acid

Table 1: NMR Data Table of Betulinic Acid

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<th>$^1$H (δ ppm)</th>
<th>Position</th>
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<td>19</td>
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Table 2: NMR Data Table of Trimethoxyellagic Acid

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<td>-</td>
<td>111.8 (C)</td>
<td>-</td>
<td>-</td>
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</tr>
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<td>114.6 (C)</td>
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<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
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<tr>
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<td>140.4 (C)</td>
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<td>112.6 (CH)</td>
<td>7.25 (1H, s)</td>
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<td>7’</td>
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<td>61.3 (CH$_3$)</td>
<td>4.02 (3H, s)</td>
<td>56.5 (CH$_3$)</td>
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Betulinic acid was isolated as a white powder. The combined 1H and 13C NMR data indicated the presence of one carboxylic acid carbon, two tertiary methyl groups, one exo-methylene protons (each 1H), a carbonyl proton, and several methine signals. Correspondingly, the 13C NMR spectrum showed signals for thirty carbons, which were categorized as six methyls, eleven methylenes, six methines, and seven quaternary carbons.

Trimethoxyellagic acid was isolated as a pale-yellow amorphous powder in this study; the structure of the compound was determined through extensive one-dimensional and two-dimensional NMR analysis. The 1H NMR spectrum displayed five signals: two aromatic protons at δ 7.52 (s, 1H) and δ 7.44 (s, 1H), and three methyl groups at δ 3.90 (s, 3H), 4.04 (s, 3H), and 4.04 (s, 3H). C-NMR spectrum showed seven-teen carbon signals, among which six are assigned to one sugar unit, eleven to the aglycone moiety, which were attributed to two methyl signals, two methine signals, two carbonyl carbons and five quaternary carbons.

**DISCUSSION**

The identification of betulinic acid, a pentacyclic triterpendoid and trimethoxyellagic acid in the ethyl acetate and n-hexane purified extracts of *A. leiocarpus* stem bark in the current study, is remarkably significant in the elucidation of the mechanisms of action of *A. leiocarpus* anti hyperglycaemic, accelerated wound healing activities in types 1 and 2 diabetes mellitus and the accompanying life threatening complications.

This is remarkable because of the finding of the betulinic acid and trimethoxyellagic acid in the interface of purified extract which possessed antidiabetic effect.

Mechanisms advanced as modes of actions of phytochemicals in medicinal plants for the treatment of diabetes mellitus, included effects on activity on pancreatic β-cells (synthesis, release, cell regeneration/revitalization); insulin-like activity of the plant extract; increase in insulin sensitivity and increase in protective/inhibitory effect against insulinase activity.

In an adequately designed alloxan-induced type 1 diabetes mellitus in dogs, aberrations occurred in the electrolytes and base balance that produced hyperkalemia and hyperchloridaemia in the diabetic dogs. In addition, the crude ethanolic extracts of the *A. leiocarpus* stem bark, that comprehensively and scientifically contained the betulinic acid, as evidenced in the present study, adequately ameliorated the hyperkalemia and hyperchloridaemia produced in the diabetic dogs.

The subsequent amelioration of these derangements of electrolytes by the crude ethanolic extracts of *A. leiocarpus* stem bark is being speculated to be linked, in part, to the insulin-like activity of medicinal plant extracts. This speculation derives enormous support from the effect of betulinic acid on the potent insulin secretagogue and antidiabetic activity mediated by potassium and chloride channels; this novel and articulately designed in vitro study showed that betulinic acid triggered calcium influx, partly, through ATP-dependent potassium channels and partly, a calcium-dependent chloride channels, an intricately interwoven process that allowed betulinic acid to stimulate insulin secretion.

Beta-cell regeneration and/or revitalization, as a mechanism of antidiabetic activity of *A. leiocarpus* stem bark is being given attention due to the chronic hyperglycaemia-induced injuries and subsequent desialylation of the cells of the capillaries' endothelium, internal organs and plasma glycoproteins that led to elevated serum sialic acids in alloxan-induced diabetic dogs. In addition, these processes confirmed elevated serum sialic acids as a potent biomarker of alloxan-induced diabetes mellitus in dogs and it was predictive and *A. leiocarpus* stem bark showed its prognostic value following successful treatment. Indeed, similar findings, of elevated sialic acids occurred in type 2 diabetes mellitus in human patients.

Under the context of revealing mechanism of action of the antidiabetic activity of *A. leiocarpus*, the study of role of the sialyltransferase, in the enzymatic “mopping up” and replacement of free plasma sialic acids onto the desialyted red blood and endothelial cells, as a revitalization process, is almost completed; this latter, is a follow-up from a novel and articulately designed study that showed that upregulation of sialyltransferases, ST3Ga11 and ST6Ga11 promoted stabilization of erythrocyte mass that led to recovery of anaemia in *Trypanosoma-brucei* infected pigs.

In addition, N-acetylneuraminic acids (sialic acids) attenuated high-fat diet-induced inflammation and oxidative stress in rats and was linked to the dynamics of elevated serum sialic acids as a manifestation of diabetes. Additionally, the crude ethanolic extracts of *A. leiocarpus* stem bark attenuated the dyslipidaemia of alloxan-induced type 1 diabetic dogs; the overwhelming support comes from the study that, this betulinic acid, contained in the crude ethanolic extracts, promoted stabilization of erythrocyte mass that led to recovery of anaemia in *Trypanosoma-brucei* infected pigs.

Under the context of the objectives of this study, additional supports are derived from a review that covered between 1990 and 2003, which showed that betulinic acid and its derivatives exhibited antibacterial, anti-inflammatory and antioxidant activities, amongst others as further observed.

Betulinic acid was referred to as a lupane type triterpene. Indeed, from an earlier investigative study, anti-inflammatory Lupeol, a pharmacologically active pentacyclic compound was identified, with its structure in ethyl acetate and n-hexane purified extracts of *A. leiocarpus* stem bark. It is therefore, reasonable to surmise that these betulinic acid, in the present study, Lupeol and trimethoxyellagic acid discussed below, with their enormous medicinal properties and benefits, in the treatments of type 1 and type 2 diabetes mellitus are therapeutically useful, synergistically, directly or as industrial synthetic products for a remarkable enhancement of their activities. This hypothesis derives enormous support from the synthesis, through a stepwise structure optimization, of betulinic acid derivatives that exhibited enhanced anti-alpha-glycosidase activity, as a management tool for type 2 diabetes mellitus. This latter glucose management process involved inhibiting alpha-glycosidase and hence, prevention of hydrolysis of carbohydrates and regulation of blood glucose of postprandial hyperglycaemia and the risk of complications of diabetes.

The discovery of Trimethoxyellagic acid in the ethyl acetate and n-hexane purified extracts of *A. leiocarpus* stem bark is comprehensively very remarkable, in particular, from the fact that, in the present study, it in addition, existed in the interface precipitates of the purification processes. This interface precipitation is reproducible, evidenced from its production in an earlier study in an enormous quantity that led to its usage for bioassay study and an emphatic identification of the erythropoietic, thrombopoietic and hypoglycaemic, hence antidiabetic activities resident in the interface. This discovery of Trimethoxyellagic acid in the interface of the purified *A. leiocarpus* stem bark is emphatically,
comprehensively and remarkably a pharmaceutical anti-diabetic compound.

The anti-inflammatory activity of the extract from *Euphoria acaulis* rhizomes 54 was linked to ellagic-acid glycoside 3,3’-di-O-methyl ellagic acid 4-rutinose, along with 3, 4, 3’-tri-O-methyl ellagic acid 4-rutinoside and the 3,4,3’-tri-O-methyl ellagic acid isolated from the rhizomes of *Euphoria acaulis* structurally identified and their anti-inflammatory activity was established 55. Any additional anti-inflammatory activity of the trimethoxyellagic acid in the interface of the purified extract of *A. leiocarpus* stem bark, while reinforcing the anti-inflammatory activities of coexisting Lupeol 35 and betulinic acid, discovered in the present study, may not effectively control diabetic pains 56,57,58. This opinion has arisen from the backdrop of the observations from the treatment of naturally occurring type 2 diabetes mellitus in a female Wistar rat using interface only, of the purified extract, (part of another manuscript).

Of the eight species of the genus *Anogeissus*, two *Anogeissus latifolia* and *A. leiocarpus* had been widely investigated pharmacologically 26 but *A. leiocarpus* received more attention with regards to treatment of experimentally induced diabetes mellitus. Potent chemical reaction of betulinic acid through carboxylic group, with glucose at the latter’s reactive hydroxyl group, at position carbon one, a glycosidic linkage, leads to betulinic acid glycoside, an ester, to mop-up or scavenge glucose molecules in the circulation and ameliorate the hyperglycaemia of diabetic patients and animals. Additionally, the hydroxyl group on the betulinic acid is exposed to a glycosidic linkage with another molecule of glucose to form betulinic acid glycoside, an ether, this time around. This latter glycosidation reaction with the hydroxyl group of the betulinic acid may readily be achieved after a chemically saturated reaction environment with the betulinic acid carboxylic group. These two chemical reactions, namely, ester and ether productions may jointly mop up or scavenge more glucose molecules from the circulation and further ameliorate the hyperglycaemia of diabetic patients and animals. It is being suggested that the glycosidation processes of betulinic and gives further support for its contribution, partly, for the amelioration of diabetes mellitus. This presupposes that the betulinic acid administered orally, enters the circulation intact, to some extent for reason provided below.

Elevated plasma/serum sialic acids concentrations that signaled ongoing diabetes mellitus in humans 45, 66 and dogs 32 were restored to normal concentrations in treated human diabetic-patients 59 and diabetic dogs treated with ethanolic extracts of *A. leiocarpus* stem bark 32. This may be linked to insulin dependent diabetes mellitus (IDDM) in humans 60 and dogs 32 with sialic acid glucose receptors in addition to a role credited to sialyltransferase activity 32, 67. These latter observations are supported by the potent glycosidation chemical reactions of betulinic acid and trimethoxylglycidic acid (below) with glucose and the potent insulin secretagogue effect of betulinic acid 40 and the hyperkalemia and hyperchloridaemia in diabetic dogs 35.

In a novel and articularly designed study, betulinic acid was verified to exhibit a very strong non-competitive inhibitory effect against intestinal α-glucosidase by binding to the active site of this enzyme, which changed the microenvironment and secondary structure of the α-glucosidase 61. The study concluded that, oral betulinic acid administration alleviated the postprandial blood glucose fluctuations in mice, for its control of postprandial hyperglycaemia 62. In addition, the significance and usefulness of betulinic acid in the treatment of diabetes mellitus as observed in the current study, receives more support from the report that anti-diabetic effects of betulinic acid was mediated by the activation of AMP activated protein kinase pathway 63. Indeed, in addition, betulinic acid inhibited high-fat diet-induced obesity and improved energy balance by activating AMKP 41. Hence, the syntheses and anti-α-glucosidase activity evaluation of betulinic acid derivatives were undertaken 44.

Sugars are common in polar extracts and ellagic acids are quite polar; they easily come together during separation, fractionation or purification of polar extracts. During the current study, thorough examinations of the spectra of the trimethoxyellagagic acid indicated its purification as an entity. The potent chemical reaction of trimethoxyellagic acid through its hydroxyl group with glucose, at the latter’s reactive hydroxyl group at position carbon one, a glycosidic linkage, leads to trimethoxyellagic acid glycoside, an ether, to mop up or scavenge glucose molecules in the circulation to ameliorate the hyperglycaemia of diabetic patients and animals.

It is being suggested that these glycosidation processes in part ascribe trimethoxyellagic and betulinic acids as the anti-diabetic compounds in the interface of ethyl acetate and n-hexane purified extracts of *A. leiocarpus* stem bark and that these compounds, scientifically and conceivably are acting synergistically, with the production and elimination of water into the circulation.

The chemical shift data of proton and carbon NMR of betulinic acid and trimethoxyellagic acid are adequately supportive 63, 64.

Anaemia, a consistent presentation in human diabetic patients 65, 66 and diabetic dogs 15, 32 was morphologically classified as macrocytic and regenerative 15, 32; the anaemia was restored to normal values by enhanced haematopoiesis by ethanolic extracts of *A. leiocarpus* 15, 32, 67. Paradoxically, during the studies of bioassay, acute and delayed toxicity and effects on reproductive performance, 2,000 mg/kg bd.wt of purified interface extracts *A. leiocarpus* stem bark produced normocytic normochromic anaemia in Wistar rats over a 24hr period 33. The normocytic normochromic anaemia was ascribed to hydraemia, due to an expanded plasma volume in the absence of an immediate erythropoiesis 33 although reticulocytosis / macrocytosis, an indicator of enhanced erythropoiesis and haematopoiesis occurred thereafter 33.

These observations give support and credence to the production and elimination of water molecules from the glycosidation processes of betulinic acid and trimethoxylglycidic acid in the circulation and indirect evidence of intact entries of betulinic and trimethoxylglycidic acids into the circulation from oral administration of the purified interface extracts. It is conceivable that 2000 mg/kg bd.wt will produce and eliminate high water molecules into the circulation.

The clinical implications of these observations are as follows: for human or animal patients presenting with advanced diabetes mellitus and anaemia that requires Physician’s or veterinary Clinician’s discretion for a higher therapeutic dose of these betulinic and trimethoxylglycidic acids, cognizance must be taken of a further deterioration or exacerbation of the anaemia within the first 24hrs which must be avoided. During a molecular biology study, 100 mg/kg, bd. wt of the purified interface extract of *A. leiocarpus* was effective in Wistar rats (not part of this manuscript). In addition from optimization study 5 mg/kg/bd.wt was hypoglycaemic in Wistar rats; the dose was recommended to maintain normoglycaemia for a while, after “crashing” down DM with a higher dose 34.

An enormous and advantageous detoxification effect was observed from the urea evaluation from the oral
administration of the purified interface to the Wistar rats 33,34 but no adverse effects from ester or ether 33.

Although, ellagic acid a bioactive compound was isolated from the genus Anogeissus 26, this appears to be the first report linking trimethoxyellagic acid in the ethyl acetate and n-hexane interface extract of A. leioecarpus stem bark with antidiabetic activity following a bioassay study with Wistar rats 33.

**CONCLUSION**

This appears to be the first report linking betulinic acid and trimethoxyellagic acid in the interface of ethyl acetate and n-hexane extracts of A. leioecarpus stem bark directly with hypoglycaemia and hence antidiabetic property.

The existence of these antidiabetic compounds, betulinic and tremethoxyellagic acids and the antiflammatory Lupeol 35 in the stem bark of A. leioecarpus is therapeutically and economically advantageous and requires a strategic sustainability and industrial plans for A. leioecarpus.

Presenting advanced cases of types 1 and 2 diabetes mellitus accompanied with the characteristic anaemia, requiring therapeutic discretion for a higher dose of these betulinic and trimethoxyellagic acids must avoid exacerbating the existing anaemia within the first 2 hrs only.

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**Authors Contributions**

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Balogun, EO: Supervision; Investigation; Writing; Editing.

Esievo, KO: Investigation; Soil composition; Ethanolic Extraction and Purification, Writing.

Esievo, LO: Investigation; Ethanollic Extraction and Purification; Writing.

Esievo, EM: Investigation; Ethanollic Extraction and Purification; Writing.

Sani, D: Supervision; Investigation; Bioassay; Writing; Editing.

Wassagwa, J: Investigation; Purification; Writing; Editing.

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