Available online on 15.07.2024 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

Open Access Full Text Article

Research Article

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

King Akpofure Nelson Esievo[1,2,3+]; Emmanuel Oluwadare Balogun[4,5]; Kingsley Oghenerukevwe Esievo[6]; Lauretta Oghenekevwe Esievo[7,8]; Edith Monica Esievo[9,10,*]; Dahiru Sani[11]; John Wassagwa[4,#]; Edward Oniovosa Uyovbisere[12]; and Emmanuel Tamajong Mumah[13]

1. *Lecturer/Resource Person, College of Veterinary Surgeons, Nigeria, Pathology Faculty, Samaru Zaria Study Centre, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.*
2. *Department of Veterinary Pathology, College of Veterinary Medicine, Federal University of Agriculture, Zuru, Kebbi State, Nigeria.*
3. *Consultant; Research and Diagnosis Unit, Kanesco Global Services Limited (RC829505) Hayin Mallam, Samaru, Zaria, Nigeria.*
4. *Department of Biochemistry, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria.*
5. *African Centre of Excellence for Neglected Tropical Diseases and Forensic Biotechnology, (ACENTDFB), Ahmadu Bello University, Zaria, Nigeria.*
6. *Department of Agronomy, Faculty of Agriculture, Ahmadu Bello University, Zaria, Nigeria.*
7. *Department of Theriogenology, and Production, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.*
8. *Department of Theriogenology, College of Veterinary Medicine, Federal University of Agriculture, Zuru, Kebbi State, Nigeria.*
9. *Department of Veterinary Surgery, College of Veterinary Medicine, Federal University of Agriculture, Zuru, Kebbi State, Nigeria.*
10. *Royal Veterinary College, University of London, Royal College Street, London NW1 OTU, United Kingdom.*
11. *Department of Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.*
12. *Department of Soil Science, Faculty of Agriculture, Ahmadu Bello University, Zaria, Nigeria.*
13. *Tama Veterinary Hospital, 542 S. Main Street, Milpitas Ca; 95035, USA.*

* *London School of Hygiene and Tropical Medicine, University of London, Keppel Street, London WCIE 7HT, United Kingdom*

\# *UNESCO International Centre for Biotechnology, Nsukka, 410001 Enugu State, Nigeria.*

Article Info:

Article History:

Received 24 April 2024
Reviewed 03 June 2024
Accepted 27 June 2024
Published 15 July 2024

Cite this article as:

Esievo KAN, Balogun EO, Esievo KO, Esievo LO, Esievo EM, Sani D, Wassagwa J, Uyovbisere EO, Mumah ET, Identification of Betulinic Acid and Trimethoxyellagic Acid as the Antidiabetic Compounds in *Anogeissus leiocarpus* Stem Bark Purified Extract, Journal of Drug Delivery and Therapeutics. 2024; 14(7):30-42

DOI: http://dx.doi.org/10.22270/jddt.v14i7.6668

*Address for Correspondence:

King Akpofure Nelson Esievo, Department of Veterinary Pathology, College of Veterinary Medicine, Federal University of Agriculture, Zuru, Kebbi State, Nigeria.

Abstract

Objectives: 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 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-do, $CDCl_3$, 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 macrovascuar complications of cardiovascular disease, nephropathy and retinopathy and blindness[1] had implicated DM as a leading cause of death by WHO[2], come 2030 with a more threatening scenario projected for 2035[3] hence, a challenging unresolved health problem for the 21st century[4].

The risk factor induced by diabetes on the prognosis and mortality of COVID-19 [5,6,7] and the national budgets/global economic implications remain memorable.

Obesity, a predisposing factor of diabetes[8] and in particular, the delayed or non-healing wounds (diabetic ulcer)[9,10,11,12], induced by chronic hyperglycaemia, create additional unquantifiable worrisome social menace. In Africa, age distribution and undiagnosed DM were reported[13].

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[18].

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 the 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 inbalance and the organic damages in alloxan-induced diabetic dogs[15]; (vi) in addition, it accelerated wound healing in alloxan-induced diabetic dogs[31]; (viii) and finally, it significantly established elevated serum sialic acids as a potent biomarker of diabetes mellitus which was predictive and prognostic[32].

Final purification produced three fractions and an interface[33]; the interface exhibited the antidiabetic and haemopoietic activities[33], had no effects on pregnancy, gestation, reproductive performance and no teratogenic effect on pups of Wistar rats[33]. In addition it has a wide range of safety value[33]: Optimisation for therapeutic hypoglycaemic dose, showed 5 mg/kg.bd.wt was hypoglycaemic and the interface segment had not expired after seventeen (17) months at room temperature, post purification[34].

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[35].

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-do, $CDCl_3$, 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:

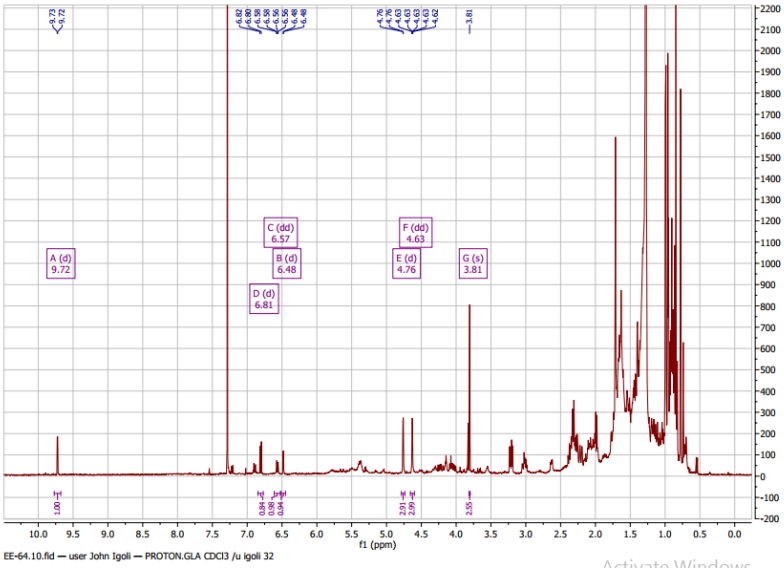


Figure 1: ^1H Nuclear Magnetic Resonance Spectra.

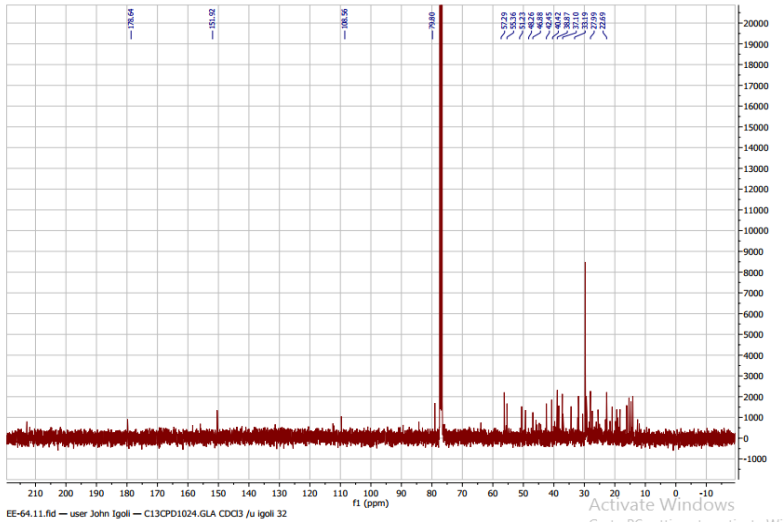


Figure 2: ^{13}C Nuclear Magnetic Resonance Spectra.

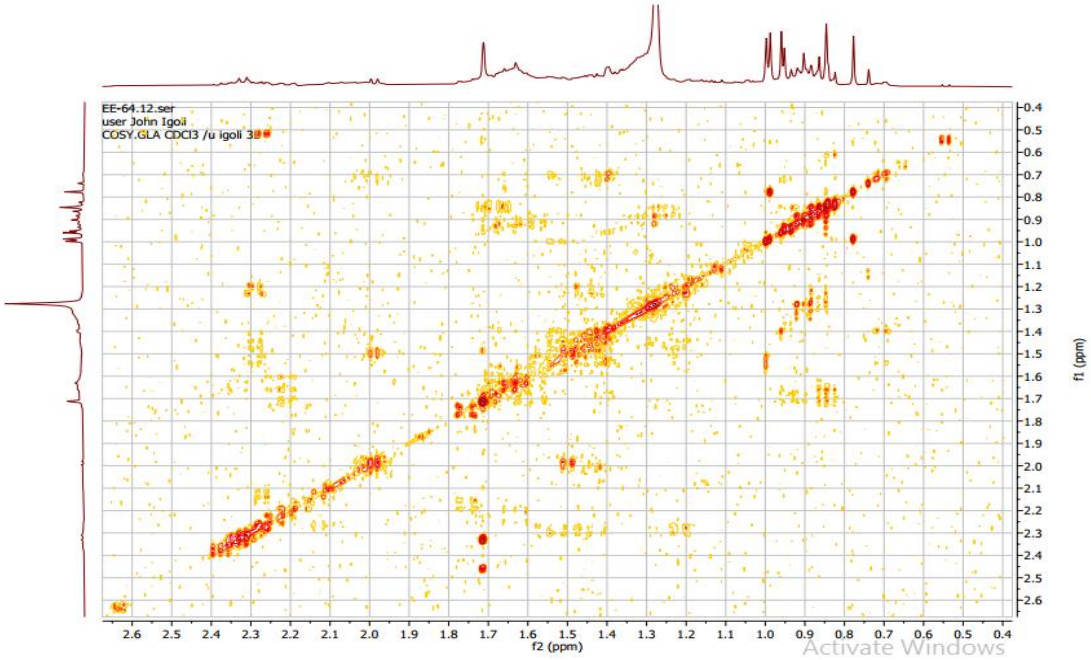


Figure 3: ^{1}H, ^{1}H-COSY (Correlated Spectroscopy) Spectra.

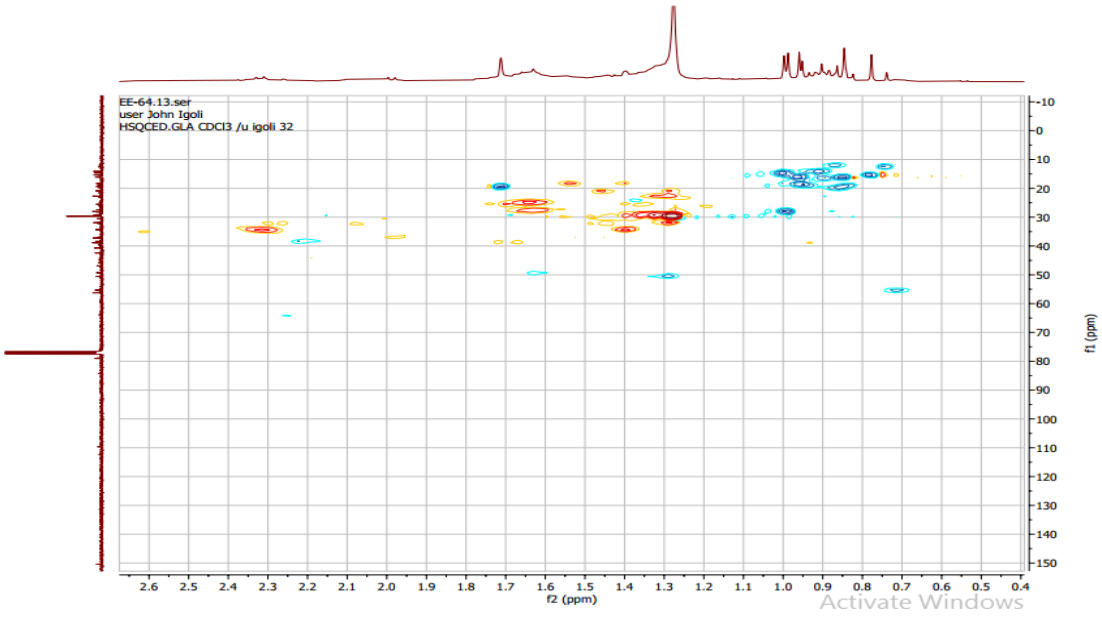


Figure 4: HSQC (Heteronuclear Single Quantum Correlation) Spectra.

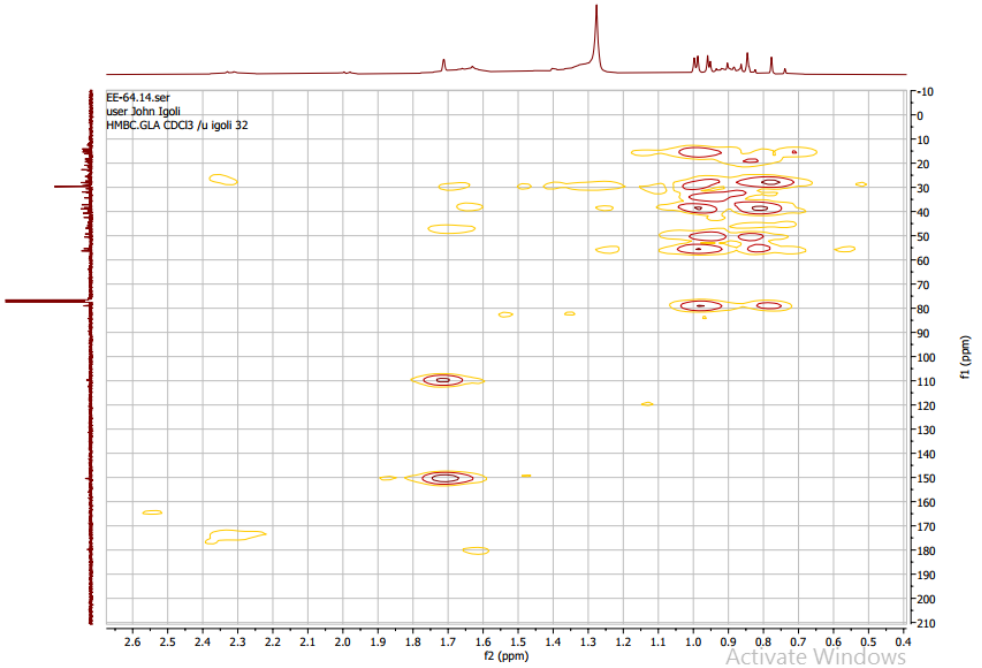


Figure 5: HMBC (Heteronulclear Multiple Quantum Correlation) Spectra.

The structure:

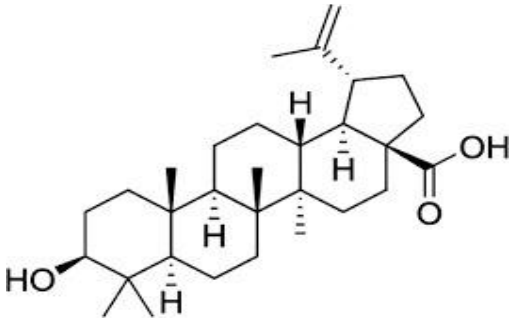


Figure 6: Chemical Structure of Betulinic Acid.

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 positon 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

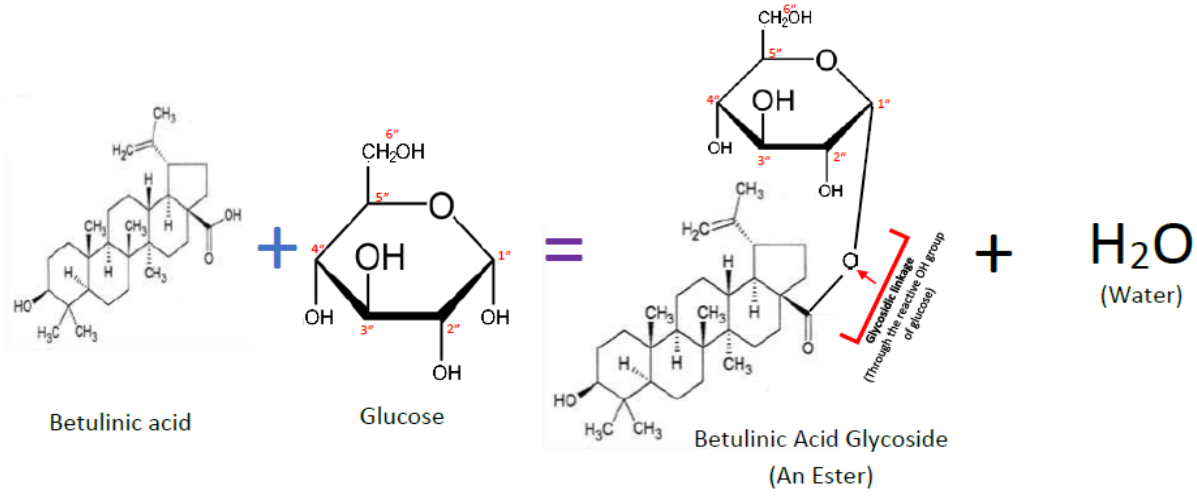


Figure 7: Reaction 1

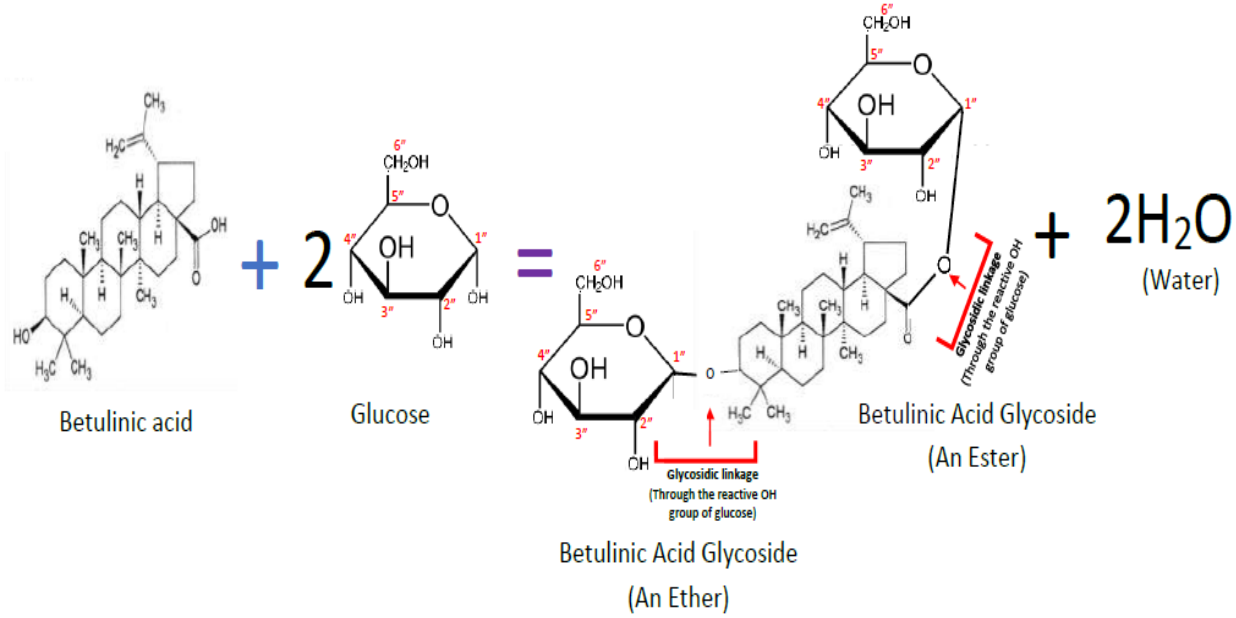


Figure 8: Reaction 2

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:

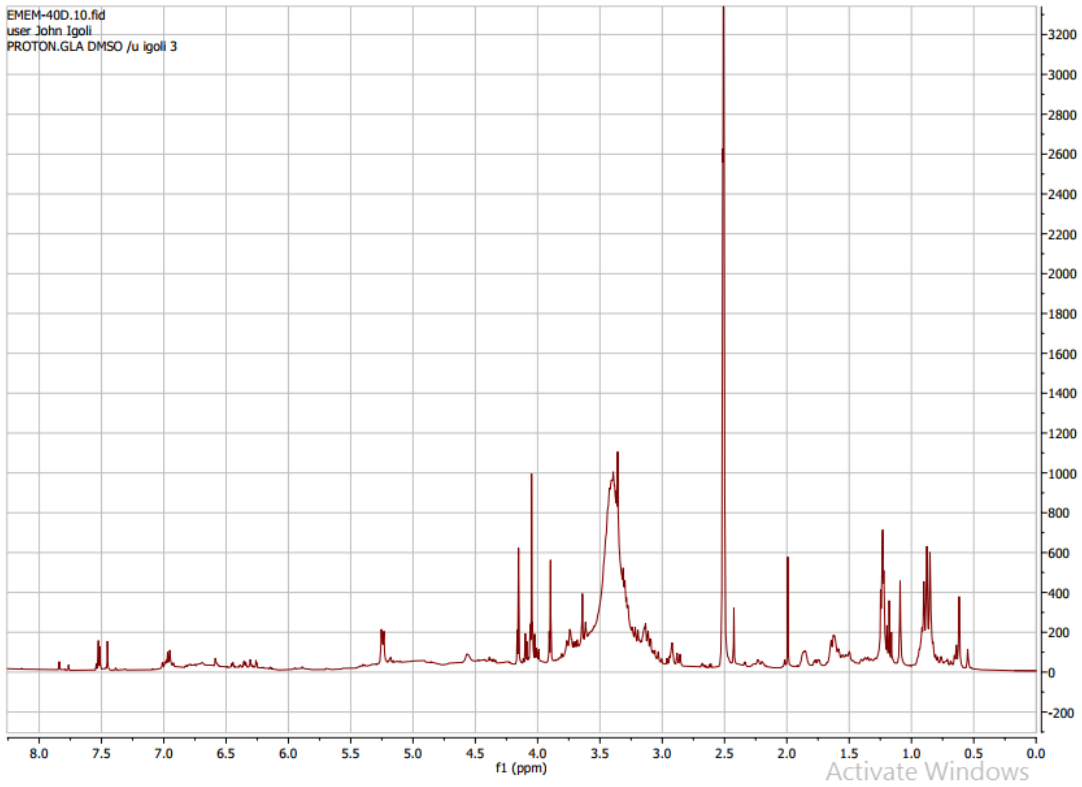


Figure 9: ^{1}H Nuclear Magnetic Resonance Spectra.

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

EMEN-20D.10.fid; EMEN-52-53D.10fid; EMEN-60D.10.fid; EMEN-66D.10.fid; EMEN-67D.10.fid; EMEN-80D.10.fid; EMEN-104D.10.fid are very similar to Fig. 9, hence are not included.

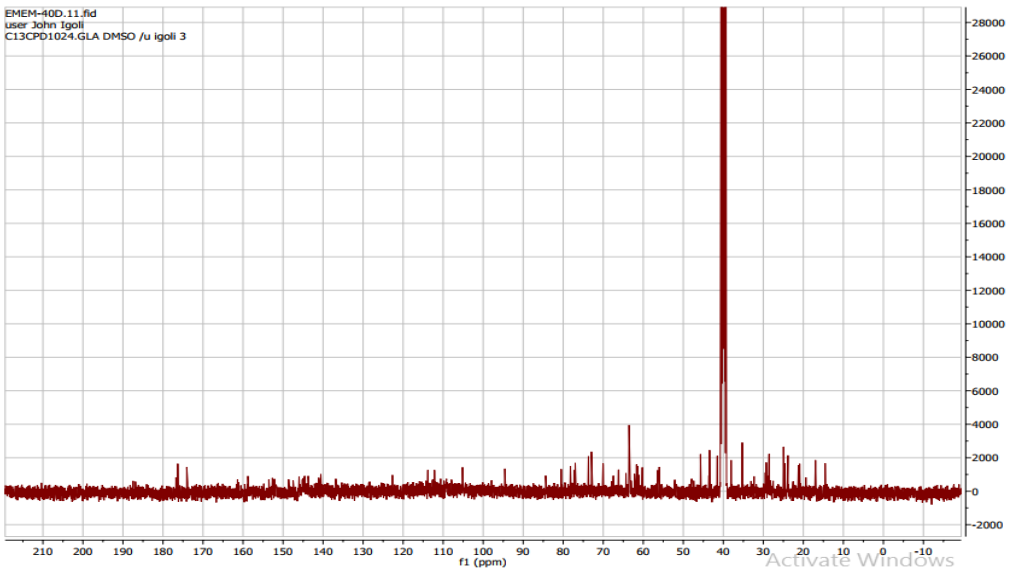


Figure 10: ^{13}C Nuclear Magnetic Resonance Spectra.

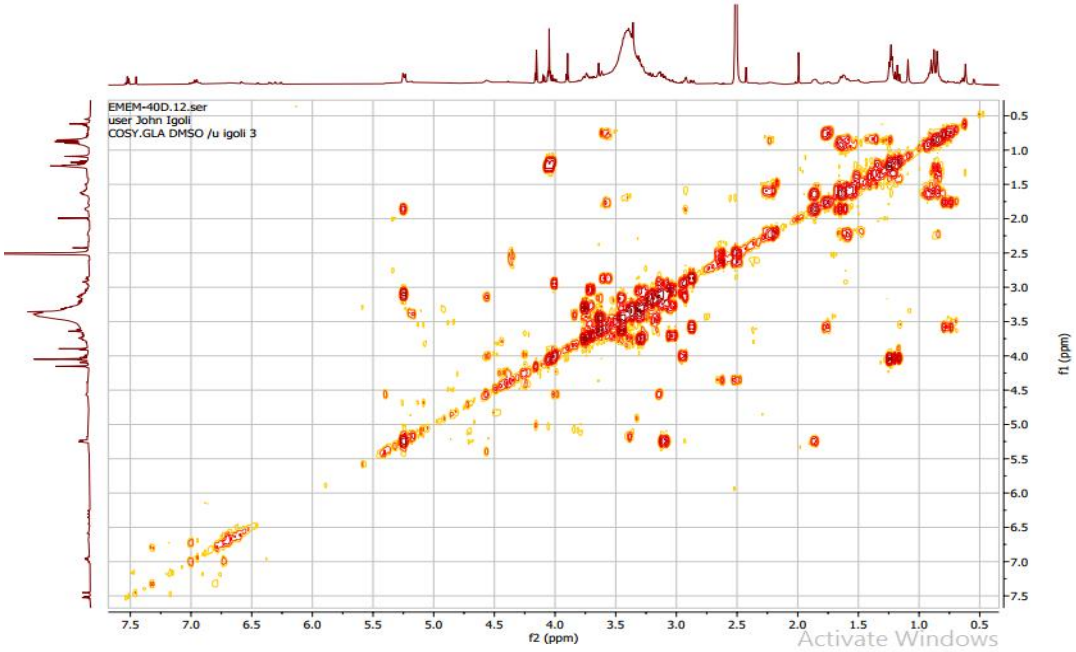


Figure 11: ^{1}H, ^{1}H-COSY (Correlated Spectroscopy) Spectra.

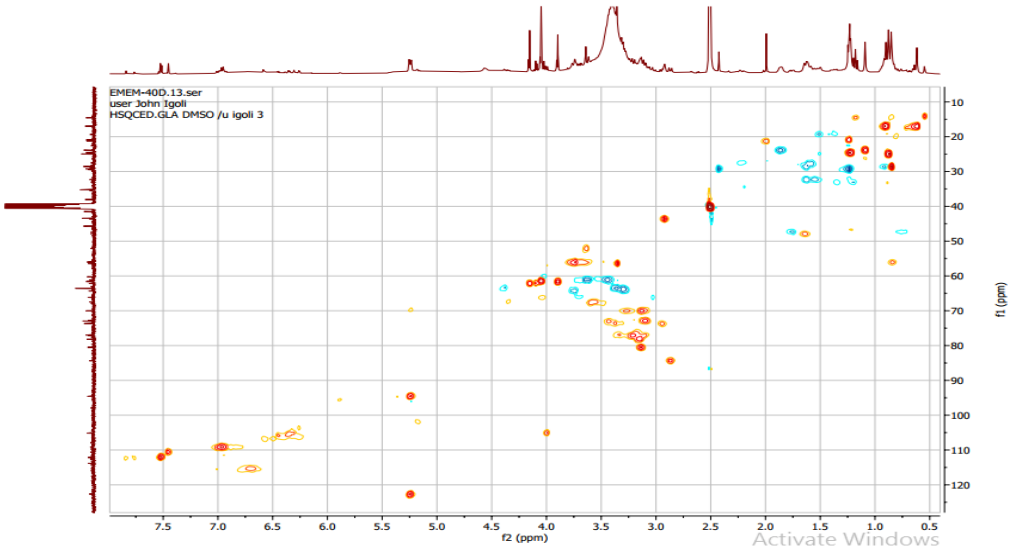


Figure 12: HSQC (Heteronuclear Single Quantum Correlation) Spectra.

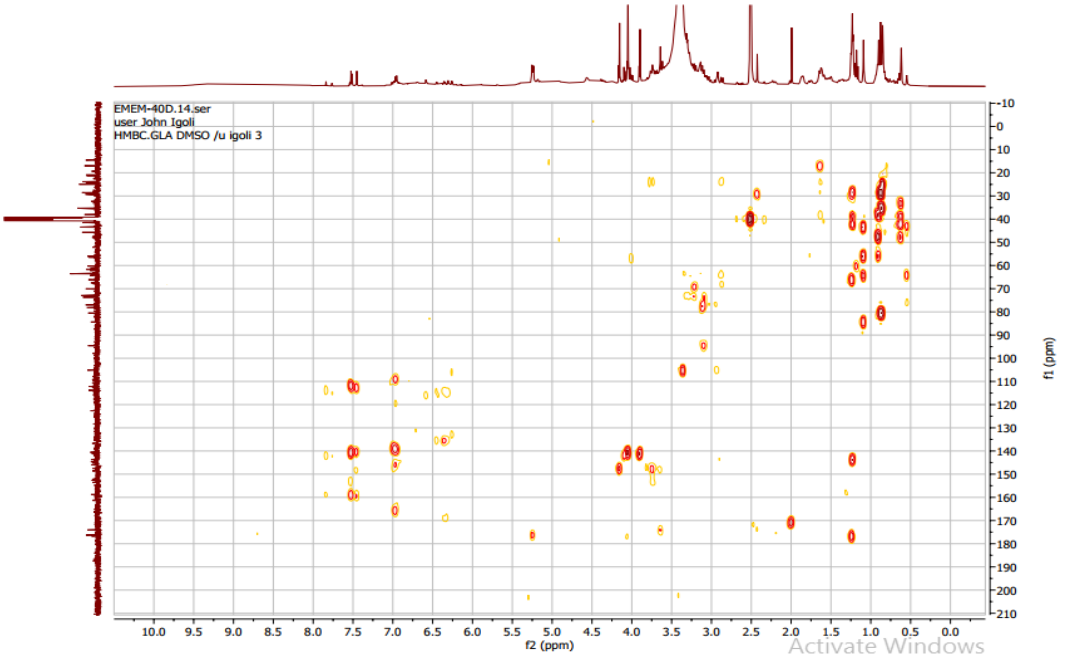


Figure 13: HMBC (Heteronuclear Multiple Quantum Correlation) Spectra.

The structure:

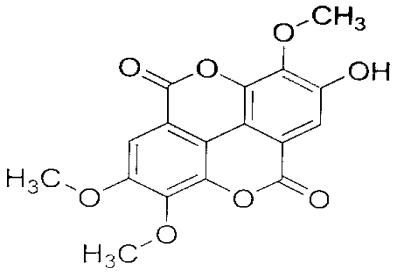


Figure 14: Chemical Structure of Trimethoxyellagic Acid.

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.

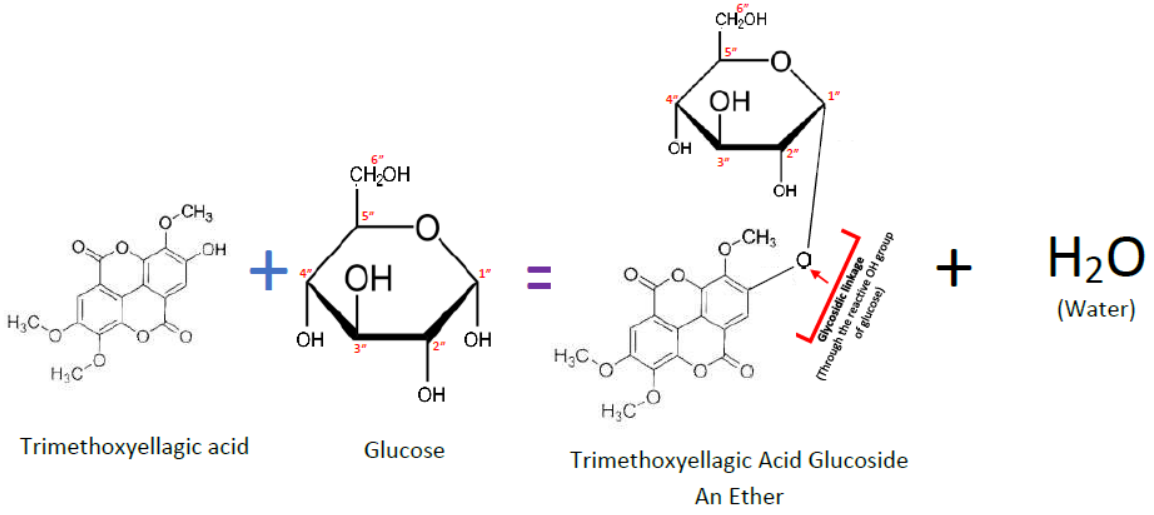


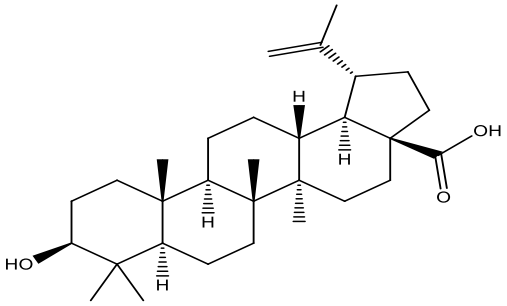
Figure 15: The Potent Chemical Reaction of Trimethoxyellagic Acid and Glucose.

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.

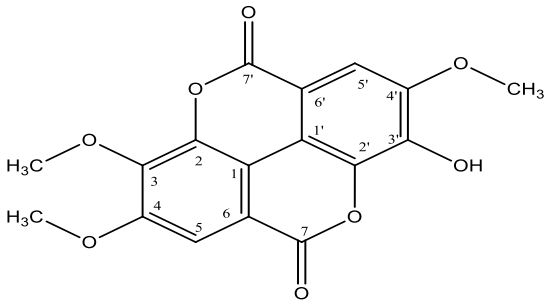
Chemical Shift Data of Proton and Carbon NMR of the Betulinic Acid and Trimethoxyellagic Acid

Table 1: NMR Data Table of Betulinic Acid



Betulinic acid			Betulinic acid Continued		
Position	^{13}C (δ ppm)	^{1}H (δ ppm)	Position	^{13}C (δ ppm)	^{1}H (δ ppm)
1	38.8 (t)	1.64, 0.89	16	32.2 (t)	2.30, 1.21
2	27.4 (t)	1.59	17	57.2 (s)	-
3	79.1 (d)	3.20	18	46.9 (d)	1.57
4	38.9 (s)	-	19	49.3 (d)	3.00
5	55.4 (d)	0.68	20	150.4 (s)	-
6	18.3 (t)	1.53, 1.38	21	29.7 (t)	2.00, 1.44
7	34.4 (t)	1.41	22	37.1 (t)	2.34, 1.14
8	40.8 (s)	-	23	28.0 (q)	0.99
9	50.6 (d)	1.29	24	15.4 (q)	0.76
10	37.3 (s)	-	25	16.1 (q)	0.85
11	20.9 (t)	1.48, 1.18	26	16.2 (q)	1.26
12	25.6 (t)	1.63, 1.06	27	14.7 (q)	0.97
13	38.5 (d)	1.64	28	180.4 (s)	-
14	42.5 (s)	-	29	109.7 (t)	4.74, 4.61
15	30.6 (t)	1.66, 1.06	30	19.4 (q)	1.69

Table 2: NMR Data Table of Trimethoxyellagic Acid



	Experimental Values		Literature Values	
Position	^{1}H δ ppm (mult.)	^{13}C (δ ppm)	^{1}H δ ppm (mult.)	^{13}C (δ ppm)
1	-	114.6 (C)	-	
2	-	139.7 (C)	-	
3	-	141.3 (C)	-	
4	-	140.9 (C)	-	
5	7.52 (1H, s)	112.0 (CH)	7.53 (1H, s)	
6	-	111.8 (C)	-	
7	-	158.2 (C)	-	
1′	-	114.6 (C)	-	
2′	-	139.7 (C)	-	
3′	-	140.4 (C)	-	
4′	-	140.9 (C)	-	
5′	7.44 (1H, s)	112.6 (CH)	7.25 (1H, s)	
6′	-	111.8 (C)	-	
7′	-	158.2 (C)	-	
3- OCH_3	3.90 (3H, s)	61.7 (CH_3)	3.96 (3H, s)	61.6 (CH_3)
4- OCH_3	4.04 (3H, s)	61.0 (CH_3)	4.02 (3H, s)	57.0 (CH_3)
4′- OCH_3	4.04 (3H, s)	61.3 (CH_3)	4.02 (3H, s)	56.5 (CH_3)

Betulinic acid was isolated as a white powder. The combined ^{1}H and ^{13}C NMR data indicated the presence of one carboxylic acid carbonyl group and one C=C double bond, which together accounted for two out of the seven degrees of unsaturation. This left the remaining five degrees of unsaturation to be attributed to four hexacyclic rings and one pentacyclic core within the compound. The ^{1}H NMR spectrum revealed six tertiary methyl groups, two exo-methylene protons (each 1H), a carbonylic proton, and several methine signals. Correspondingly, the ^{13}C 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 ^{1}H 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 triterpenoid [39,40,41,42,43,44] 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* antihyperglycaemic, 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 [33,34].

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[22,25].

In an adequately designed alloxan-induced type 1 diabetes mellitus in dogs, aberrations occurred in the electrolytes and acid-base balance that produced hyperkalemia and hyperchloridaemia in the diabetic dogs[15]. 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[15].

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 extrracts[22,25]. 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 [40]; 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 betulinc acid to stimulate insulin secretion [40].

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[32]. 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[32]. Indeed, similar findings, of elevated sialic acids occurred in type 2 diabetes mellitus in human patients [45,46]. 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, ST3Gal1 and ST6Gal1 promoted stabilization of erythrocyte mass that led to recovery of anaemia in *Trypanosoma-brucei* infected pigs [47].

In addition, N-acetylneurarinic acids (sialic acids) attenuated high-fat diet-induced inflammation and oxidative stress in rats [48] and was linked to the dynamics of elevated serum sialic acids as a manifestation of diabetes [32]. Additionally, the crude ethanolic extracts of *A. leiocarpus* stem bark attenuated the dyslipidaemia of alloxan-induced type 1 diabetic dogs [30]. An overwhelming support comes from the study that, this betulinic acid, contained in the crude ethanolic extracts [29, 30, 31, 32] as evidenced in the present study, was reported to inhibit high-fat diet-induced obesity [41].

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 [39] as further observed [42,43].

Betulinic acid was referred to as a lupane type triterpene [49]. 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 [35]. It is therefore, reasonable to surmise that these betulinic acid, in the present study, Lupeol [35] 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 [44]. 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 [50,51,52,53].

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 [33,34,35] 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 [33,34,35]. This discovery of Trimethoxyellagic acid in the interface of the purified *A. leiocarpus* stem bark is emphatically,

comprehensibly and remarkably a pharmacological antidiabetic compound.

The anti-inflammatory activity of the extract from *Euphobia acaulis* rhizomes [54] was linked to ellagic-acid glycoside 3,3|-di-O-methyl ellagic acid 4-rutinoside, 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 *Euphobia 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 [45,46] 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 [32,47]. These latter observations are supported by the potent glycosidation chemical reactions of betulinic acid and trimethoxyellagic acid (below) with glucose and the potent insulin secretagogue effect of betulinic acid [40] and the hyperkalemia and hyperchloridaemia in diabetic dogs [15].

In a novel and articulately 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 admistration alleviated the postprandial blood glucose fluctuations in mice, for its control of postprandial hyperglycaemia [61]. 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 antidiabetic effects of betulinic acid was mediated by the activation of AMP activated protein kinase pathway [62]. 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 trimethoxyellagic 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 trimethoxyelllagic and betulinic acids as the antidiabetic 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 macrocylic 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 trimethroxyellagic acid in the circulation and indirect evidence of intact entries of betulinic and trimethoxyellagic 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 trimethoxyellagic 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. leiocarpus* 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. leiocarpus* stem bark directly with hypoglycaemia and hence antidiabetic property.

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

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 24hrs only.

Acknowledgements:

The Authors acknowledge the technical supports of Adamu Mohammed, Ibrahim Kabiru of the Department of Pharmacognosy and Drug Development; Dennis Otie of the Department of Veterinary Pharmacology and Toxicology A.B.U, Zaria. The NMR structural analysis was performed by Professor J. Igoli, with assistance of Dr J. Anyam, of the Department of Chemistry, Federal University of Agriculture, Makurdi, Nigeria and is highly acknowledged. The assistance of Professor A.O. Oyewale of the Department of Chemistry, Ahmadu Bello University, Zaria is highly acknowledged.

Authors Contributions

Esievo, KAN: Conceptualisation; Supervision; Investigation; Writing, Editing,

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

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

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

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

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

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

Uyovbisere, EO: Supervision, Investigation; Soil composition; Weather conditions; Writing; Editing.

Mumah, ET: Investigation; Writing, Editing.

Funding Source: The study was partly funded by the Research and Diagnosis Unit of Kanesco Global Services Limited (RC 829505) with additional funds from supervising Authors.

Conflict of Interests: Authors declare no conflict of interests

Ethical Approval: No experimental animal invasive involvement

REFERENCES

1. Quresh M, Gammoh J, Shakil J, Robbins R, Update on management of Type 2 diabetes for cardiologists. Methodist DeBakey Cardiovascular Journal. 2018; 14(4): 273-280. https://doi.org/10.14797/mdcj-14-4-273 PMid:30788013 PMCid:PMC6369620

2. World Health Organization (WHO), GLOBAL report on diabetes 1. Diabetes mellitus-epidemiology. 2. Diabetes mellitus-prevention and control. 3. Diabetes, Gestational 4. Chronic Disease. 5. Public Health. 1. World Health Organization. France 2016

3. Guariguata L, Whiting DR, hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Research Clinical Practice. 2014; 103(2), 137-149. https://doi.org/10.1016/j.diabres.2013.11.002 PMid:24630390

4. Amjad S, Jafri, Sharma AK, Serajuddin M. A novel strategy of nanotized herbal drugs and their delivery in the treatment of diabetes: Present status and future prospects. Journal of Herbal Medicine, 2019; 17:100279. https://doi.org/10.1016/j.hermed.2019.100279

5. Guo W, et al., Diabetes is a risk factor for the progression and prognosis of COVID-19. Diabetes/Metabolic Research Review. 2020; 36(7): 33319. https://doi.org/10.1002/dmrr.3319 PMid:32233013 PMCid:PMC7228407

6. Hussain A, Bhowmik B, Moreira NC. COVID-19 and diabetes: Knowledge in progress. Diabetes Research and Clinical Practice. 2020; 162: 1-9. https://doi.org/10.1016/j.diabres.2020.108142 PMid:32278764 PMCid:PMC7144611

7. Zhu L., et al., Association of blood glucose control and outcomes in patients with COVID-19 and pre-existing type 2 diabetes. Cell Metabolism. 2020; 31(16): 1068-1077. https://doi.org/10.1016/j.cmet.2020.04.021 PMid:32369736 PMCid:PMC7252168

8. Kumar V, et al. Anti-diabetic, anti-oxidant and anti-hyperlipidemic activities of Melastoma malabathricum Linn. Leaves in streptozotocin induced diabetic rats. BMC complement. Alternative Medicine. 2013; 13(1): 1-19. https://doi.org/10.1186/1472-6882-13-222 PMid:24010894 PMCid:PMC3847142

9. Armstrong DG, Boulton AJ, and Bus SA. Diabetic foot ulcers and their recurrence. New England Journal of Medicine, 2017; 376(24), 2367-2375. https://doi.org/10.1056/NEJMra1615439 PMid:28614678

10. Dave P. The Challenges of Chronic Wound Care and Management. Asian Journal of Dental and Health Sciences, 2024;4(1):45-50. https://doi.org/10.22270/ajdhs.v4i1.70

11. Ponnanikajamideen M, Rajeshkumar S, Vanaja M, and Annadurai G. Anti-diabetic and wound healing effect of antioxidant gold nanoparticles synthesized using insulin plant (Chamaecostus Cuspidatus). Canadian Journal of Diabetes 2018; https://doi.org/10.1016/j.jcjd.2018.05.006 PMid:30413371

12. Blakytny R, and Jude E. The molecular biology of chronic wounds and delayed healing in diabetes. Diabetic Medicine. 2006; DOI: 10.1111/j.1464-5491.2006.01773.x, 594-608. https://doi.org/10.1111/j.1464-5491.2006.01773.x PMid:16759300

13. International Diabetes Federation, IDF Diabetes Atlas, 8th ed. 2017.

14. Ihedioha JI, and Enahoro G. Prevalence of diabetes mellitus and reference values for the fasting blood glucose levels of locally available breeds of dogs in Warri, Nigeria. Comparative Clinical Pathology. 2019; 28, 1107-1112. https://doi.org/10.1007/s00580-019-02938-7

15. Num-Adom SM, Adamu S, ALuwong T, Ogbuagu NE, Umar IA and Esievo KAN. Ethanolic extract of Anogeissus leiocarpus ameliorates hyperglycaemia, hepato-renal damage, deranged electrolytes and acid-base balance in alloxan-induced diabetes in dogs. Scientific African. 2022; 16:e01183. https://doi.org/10.1016/j.sciaf.2022.e01183

16. Meier H. Diabetes mellitus in animals: A review. Diabetes. 1960; 9(6): 485-489. https://doi.org/10.2337/diab.9.6.485

17. Jayalakshmi K, Selvaraj P, Veeraselvam M, Yogeshpriya S, Venkasan M. Diabetic Ketoacidosis in a buck: a case report. Iranian Journal of Veterinary Research. 2019; 20(3) 213-217.

18. Sharma GU. Diabetes mellitus in a buffalo-cow. Indian Veterinary Journal.1941; 17: 370-372.

19. Esievo KAN. Veterinary clinical pathology. 1st Edit Spectrum Books, Ibadan; Safari Books (Export) Limited, Jersey JE 2 3NP United Kingdom, 2017; 37:118-122; 157-158.

20. Rao MU, Sreenivasulu M, Chengaiah B, Reddy KJ, Chetty CM. Herbal medicines for diabetes mellitus: A review. International Journal of Pharmaceutical Technology Research. 2010; 2(3) 1883-1892.

21. Gaikwad SB, Mohan GK and Rani MS. Phytochemicals for diabetes management. Crops. 2014; 5(Suppl. 1: M2). 11-28. https://doi.org/10.2174/2210290601405010011

22. Govindappa M, A review on role of plant(s) extracts and it's phytochemicals for the management of diabetes. Journal of Diabetes and Metabolism. 2015; 6: 565.

23. Mursiti S, Matsjeh S, Mustofa J, The hypoglycaemia effect of alkaloid compounds from oil free Mahagony seeds (Swietenia macrophylla,King). European Journal of Medicinal Plants 2016; 16(4) 1-5. https://doi.org/10.9734/EJMP/2016/18574

24. Raj R and Tripathi SS. Medications of diabetes mellitus and antidiabetic medicinal plants: A review. International Journal of Indian Herbs Drugs .2016; 1:19-28.

25. Bacanli M, Aydin S, Basaran N and Basaran A. Effects of phytochemicals against diabetes. Advances in Food and Nutrition Research. 2019; 128. https://doi.org/10.1016/bs.afnr.2019.02.006 PMid:31351526

26. Singh D et al., The genus Anogeissus: A review on ethanopharmacology, phytochemistry and pharmacology. Journal of Ethnopharmacology. 2016; 194: 30-56. https://doi.org/10.1016/j.jep.2016.08.025 PMid:27566202

27. Onoja US et al., Effect of Anogeissus leiocarpus guill and perr leaf on hyperglycaemia and associated dyslipdaemia in alloxan-induced diabetic rats. Dhaka University Journal of Pharmaceutical Science. 2018; 17(1), 65-72. https://doi.org/10.3329/dujps.v17i1.37120

28. Motto EA, et al., Anthihyperglycemic activity of total extract and fractions of Anogeissus leiocarpus Journal of Drug Delivery & Therapeutics. 2020; 10(3), 107-113. https://doi.org/10.22270/jddt.v10i3.4078

29. Num-Adom SM et al., Antidiabetic and antioxidant activities of ethanolic extract of Anogeissus leiocarpus in alloxan-induced type 1 diabetes mellitus in Wistar rats. EC Diabetes Metabilic Research. 2022b; 6(1).

30. Num-Adom SM et al. Dyslipidaemia of alloxan-induced type 1 diabetes in dogs is attenuated by ethanolic extracts of Anogeissus leiocarpus stem bark. Diabetes & Obesity International Journal.2022c; 7(3). https://doi.org/10.23880/doij-16000260

31. Anefu EO, Ogbuagu NE, Adamu S and Esievo KAN. Healing activities of ethanolic extract of Anogeissus leiocarpus on surgically-induced skin wounds in alloxan-induced diabetic dogs. EC Diabetes Metabolic Research. 2022; 6(3).

32. Esievo KAN et al. Elevated serum sialic acids, a potent biomarker of alloxan-induced type 1 diabetes in dogs by ethanolic extract of Anogeissus leiocarpus. Journal of Diabetes and Metabolic Disorders. 2021; 20: 179-186. https://doi.org/10.1007/s40200-021-00726-1 PMid:34178829 PMCid:PMC8212296

33. Esievo KAN et al., Acute and delayed oral toxicity studies and observations on pregnancy, gestation and reproductive performance of Wistar rats administered limit dose of purified extract from stem bark of antidiabetic Anogeissus leiocarpus (African Birch Tree). Journal of Drug Delivery and Therapeutics. 2023; 13(10): 11-27. https://doi.org/10.22270/jddt.v13i10.5965

34. Esievo LO et al., Effect of extract (interface) from stem bark of antidiabetic Anogeissus leiocarpus (African Birch Tree) on random blood glucose levels of adult female Wistar rats: Optimisation for therapeutic hypoglycaemic dose. Journal of Drug Delivery & Therapeutics. 2023; 13(12): 125-132. https://doi.org/10.22270/jddt.v13i12.6108

35. Esievo KAN et al., Antiinflammatory lupeol and antidiabetic compound coexist in ethyl acetate and n-hexane extracts from stem bark of Anogeissus leiocarpus (African Birch Tree): The therapeutic advantages. Journal of Drug Delivery & Therapeutics. 2024; 14(1): 121-126. https://doi.org/10.22270/jddt.v14i1.6271

36. Ebada SS, Edrada RA, Lin W, Proksch P. Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates Nature Protocol. 2008; 3(12): 1820 - 1830. https://doi.org/10.1038/nprot.2008.182 PMid:18989260

37. Harvey D. Modern analytical chemistry (1st Ed.), McGraw-Hill. 2000; Pp 547 - 589.

38. Mbaveng AT et al. Antimicrobial activity of the crude extracts and five flavonoids from the twigs of Dorstenia barteri (Moraceae). Journal of Ethnopharmacology. 2008; 116(3): 483 - 489. https://doi.org/10.1016/j.jep.2007.12.017 PMid:18280679

39. Yugeeswari P and Sriram D. Betulinic acid and its derivatives: A review on their biological properties. Current Medicinal Chemistry. 2005; 12 (6) 657-666. https://doi.org/10.2174/0929867053202214 PMid:15790304

40. Castro AJG et al., The potent insulin secretagogue effect of betulinic acid is mediated by potassium and chloride channels. Archive Biochemistry and Biophysics. 2018; 648: 20-26. https://doi.org/10.1016/j.abb.2018.04.015 PMid:29704483

41. Kim K-D et al., Betulinic acid inhibits high-fat diet-induced obesity and improves energy balance by activating AMPK. Nutrition, Metabolism & Cardiovascular Diseases. 2019; 29: 409-420. https://doi.org/10.1016/j.numecd.2018.12.001 PMid:30799179

42. Hordyjewska A, Ostapiuk A, Horecka A, Kurzepa J. Betulin and betulinic acid: triterpenoids derivatives with a powerful biological potential. Phytochemical Review. 2019; 18: 929-951. https://doi.org/10.1007/s11101-019-09623-1

43. Oliveira-Costa JF, Meira CS, das Neves MVG, Reis BP ZCD, Soares MBP, Anti-inflammatory activities of betulinic acid: A review. Frontiers in Pharmacology. 13: 883857 https://doi.org/10.3389/fphar.2022.883857 PMid:35677426 PMCid:PMC9168372

44. Wu X-Z et al., Synthesis and anti-α-glucosidase activity evaluation of betulinic acid derivatives. Arabian Journal of Chemistry. 2023; 16:104659. https://doi.org/10.1016/j.arabjc.2023.104659

45. Englyst NA, Crook MA, Lumb P, Stears AJ, Masding MG, Wootton SA, et al., Percentage of body fat and plasma glucose predict plasma sialic acid concentration in type 2 diabetes mellitus. Metabolism. 2006; 55: 1165-1170. https://doi.org/10.1016/j.metabol.2006.04.014 PMid:16919534

46. El-Sayed MS, El Badawy A, Abdelmoneim RO, Mansour AE, Khalil ME, Darwish K, Relationship between serum sialic acid concentration and diabetic retinopathy in Egyptian patients with type 2 diabetes mellitus. Benha Medical Journal. 2018; 35: 257-263. https://doi.org/10.4103/bmfj.bmfj_17_18

47. Atata JA, Enam SJ, Ogbuagu NE, Balogu EO, Adamu S, Esievo KAN, Upregulation of sialytransferases ST3Gal 1 and ST6Gal 1 promotes stabilization of erythrocyte mass and recovery of anemia in Trypanosoma brucei brucei-infected pigs. Research in Veterinary Science 2022; 145: 102-108. https://doi.org/10.1016/j.rvsc.2022.02.012 PMid:35180660 PMCid:PMC9148701

48. Yida Z, Imam MU, Ismail M, Ismail N, Ideris A, Abdullah MA. High fat diet-induced inflammation and oxidative stress are attenuated by N-acetyl neuraminic acid in rats. Journal of Biomedical Science. 2015; 22: 96 https://doi.org/10.1186/s12929-015-0211-6 PMid:26498218 PMCid:PMC4619312

49. Maurya SK, Devi S, Pandey VB, Fitother 1989; 60: 468.

50. Ali F et al., Hydrazinyl arylthiazole based pyridine scaffolds: Synthesis, structural characterization. In vitro alpha-glucosidase inhibitory activity and in silico studies. European Journal of Medicinal Chemistry. 2017; 138: 255-272. https://doi.org/10.1016/j.ejmech.2017.06.041 PMid:28672278

51. Hameed S et al., Synthesis of benzotriazoles derivatives and their dual potential as alpha-amylase and alpha-glucosidase inhibitors in vitro: Structure-activity relationship, molecular docking and kinetic studies. European Journal of Medicinal Chemistry. 2019; 183: 111677. https://doi.org/10.1016/j.ejmech.2019.111677 PMid:31514061

52. Rafique R et al., Synthesis of new indazole based dual inhibitors of alpha-glucosidase and alpha-amylase enzymes, their in vitro, in silico and kinetic studies. Bioorganic Chemistry. 2020; 94:103195. https://doi.org/10.1016/j.bioorg.2019.103195 PMid:31451297

53. Zheng PF, et al., in vitro and in silico studies of bis (indol-3-yl) methane derivative as potential alpha-glucosidase and alpha-amylase inhibitors. Journal of Enzyme Inhibition and Medicinal Chemistry. 2021; 36(1): 1938-1951. https://doi.org/10.1080/14756366.2021.1971976 PMid:34459690 PMCid:PMC8409970

54. Singh GB, Kaur S, Satti NK, Atal CK and Maheshewarri JK. Anti-inflammatory activity of Euphorbia acaulis Roxb. Journal of Ethnopharmmacology. 1984; 10:225-233. https://doi.org/10.1016/0378-8741(84)90004-7 PMid:6727400

55. Bindra RS, Satti NK and Suri OP. Isolation and structures of ellagic acid derivatives from Euphobia aculis. Phytochemistry. 1988; 27(7): 2313-2315. https://doi.org/10.1016/0031-9422(88)80150-X

56. Toth C, Lander J, Wiebe S. The prevalence and impact of chronic pain with neuropathic pain symptoms in the general population. Pain Medicine.2009; 10(5): 918-929. https://doi.org/10.1111/j.1526-4637.2009.00655.x PMid:19594844

57. Weng YC, Tsai SS, Lyu RK et al., Diabetic distal symmetrical polyneuropathy: correlation of clinical, Laboratory and electrophysiologic studies in patients with type 2 diabetes mellitus. Journal of Diabetes Research. 2020; article 6356459, 1-11. https://doi.org/10.1155/2020/6356459 PMid:32695829 PMCid:PMC7362296

58. Li S, Li X, Xie Z, Wei X, Yu C, Cheung CW, Xia Z, Tian G. N-acetylcysteine attenuates hyperalgesia in rats with diabetic neuropathic pain: Role of oxidative stress and inflammatory mediators and CXCR4. Journal of Diabetes Research. 2021; Article ID 8862910. https://doi.org/10.1155/2021/8862910

59. Rahman I, Malik AS, Bashir M, Khan R, Igbal M. Serum sialic changes in non-insulin dependent diabetes mellitus (NIDDM) patients following better melon (Momordica charantia) and rosiglitazone (Avanda) treatment. Phytomedicinal. 2009; 16 401-405. https://doi.org/10.1016/j.phymed.2009.01.001 PMid:19362455

60. Valhakar GS. and Haldankar VA. RBC membrane composition in insulin dependent diabetes mellitus in context of oxidative stress. Indian Journal of Clinical Biochemistry. 2008; 23 (3) 223-226. https://doi.org/10.1007/s12291-008-0050-2 PMid:23105758 PMCid:PMC3453436

61. Chen S et al. Binding interaction of betulinic acid to α-glucosidase and its alleviation on postprandial hyperglycaemia. Molecules. 2022; 27 (8) 2517. https://doi.org/10.3390/molecules27082517 PMid:35458714 PMCid:PMC9032457

62. Song T-J, et. al. Antidiabetic effects of betulinic acid mediated by the activation of the AMP-activated protein kinase pathway. Plos one. 2021; 16 (4) e 0249109 https://doi.org/10.1371/journal.pone.0249109 PMid:33819291 PMCid:PMC8021171

63. Zhang S, Liu X, Zhang Z, He L, Wang Z. and Wang G. Isolation and Identification of the Phenolic Compounds from the Roots of Sanguisorba officinalis L. and Their Antioxidant Activitie. Molecules, 2012; 17: 13917-13922. https://doi.org/10.3390/molecules171213917 PMid:23178307 PMCid:PMC6268844

64. Noviany and Osman, H. Structure Elucidation of Betulinic Acid from Sesbania grandiflora Root Journal of Physics: Conf. Ser. 2021; 1751 012090. https://doi.org/10.1088/1742-6596/1751/1/012090

65. Harsunen, MH et al., Reduced blood leukocyte and neutrophil numbers in the pathogenesis of type 1 diabetes. Hormones Metabolism and Research. 2013; 45 (6) 467-470. https://doi.org/10.1055/s-0032-1331226 PMid:23322517

66. Hillson R. Diabetes and the red blood cells. Practical Diabetes. 2015; 32: 124-126. https://doi.org/10.1002/pdi.1939

67. Num SM, Oladele SB, Esievo KAN and Useh NM. Some observations in wistar rats administered ethanol extracts of the stem barks of Anogeissus leiocarpus. Asian Journal of Pharmacology and Toxicology. 2014; 2(3) 4-10.