Int J Biochem Mol Biol 2026;17(2):10-24
www.ijbmb.org /ISSN:2152-4114/IJBMB0170682

Original Article

Isolation, purification and characterization of betulinic acid derivative from the root bark of *Afzelia africana* and its antiplasmodial and antimicrobial activities

Bright Yaw Vigbedor[1], Clement Osei Akoto[4], Ralph Kwakye[1], Jonathan Osei-Owusu[3], Francis Ofosu-Koranteng[5], Ebenezer Asante Donkor[6], Gaston Hunkpe[2], Pius Kwashie[1], David Neglo[1], Henyo Collins Mawutor[2]

[1]Department of Basic Sciences, School of Basic and Biomedical Sciences, University of Health and Allied Sciences, Ho, Ghana; [2]Department of Biomedical Sciences, School of Basic and Biomedical Sciences, University of Health and Allied Sciences, Ho, Ghana; [3]Department of Biological, Physical and Mathematical Sciences, University of Environment and Sustainable Development, Somanya, Ghana; [4]Department of Chemistry, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana; [5]Pesticides Residue Analysis Laboratory, Ghana Standards Authority, Accra, Ghana; [6]KNUST Central Laboratory, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

Received December 11, 2025; Accepted April 25, 2026; Epub April 25, 2026; Published April 30, 2026

Abstract: Background: *Afzelia africana* is a tropical plant species with numerous documented ethnobotanical and medicinal properties. Aim/objective: The study focused on the isolation, purification, characterization and biological activity of betulinic acid from the bark of *A. africana*. Methodology: The series of methods employed include column fractionation and purification, characterization (MS, ^{1}H, ^{13}C, DEPT-135, HSQC, COSY and HMBC), antimicrobial (Micro-broth dilution, and synergistic effects), and antiplasmodial activity (MSF assay). Results: Repeated column chromatographic fractionation of the methanol (extract eluent: *n*-hexane-ethyl acetate (75:25)) and TLC profiling afforded a 15 mg white crystal characterized as betulinic acid. The compound reported good antiplasmodial activity against 3D7 strain of *P. falciparum* (IC_{50}: 12.89 ± 0.52 µg/mL). The compound exhibited varying degrees of bactericidal activities (MBC/MIC ≤ 4) against *S. mutans*, *C. albicans* 2, *B. subtilis, K. pneumonia* and bacteriostatic activities (MBC/MIC ≥ 4) against *S. aureus*, *E. coli*, methicillin-resistant *S. aureus, P. aeruginosa*, *S. typhi* and C. albicans 1. The compound further reported synergistic effects (FIC ≤ 0.5) against *E. coli, K. pneumonia, S. aureus* and *S. mutans*; partial synergism (FIC > 0.5 and < 1) against *S. typhi*, methicillin-resistant *S. aureus, B. subtilis* and indifferent effects (FIC > 1 and < 4) against *P. aeruginosa* when combined with tetracycline. The compound with Ciprofloxacin showed synergistic effect against *Klebsiella pneumoniae, Streptococcus mutans,* and *Salmonella typhi*. Additionally, it demonstrated further synergism with tetracycline against *Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus.* In the antifungal synergistic studies, the combination of the compound with fluconazole and nystatin, ciprofloxacin exhibited partial synergism (FIC > 0.5 and < 1) against one strain of *Candida albicans* and full synergism (FIC ≤ 0.5) against a second strain of *Candida albicans*. When combined with fluconazole and nystatin. Conclusion: Betulinic acid has been isolated for the first time from the bark of *Afzelia africana*. It demonstrated good antiplasmodial activity against *P. falciparum*, as well as various bactericidal and bacteriostatic effects against multiple microbial strains. Additionally, betulinic acid demonstrated synergistic effects when combined with tetracycline, ciprofloxacin, fluconazole, and nystatin.

Keywords: Antiplasmodial, antimicrobial, betulinic acid

Introduction

Betulinic acid, with the nomenclature, 3-β-hydroxylup-20(29)-en-28-oic acid, is a lupane-type pentacyclic triterpenoid characterized by its rigid 30-carbon skeletal structure. The structure consists of 4-fused 6-membered rings (A, B, C, D) and one 5-membered ring (E) [1]. The structure is also identified by the presence of three major functional groups comprising of a hydroxyl group at C-3 of ring A, an exocyclic isopropenyl group at C-20 of ring E, and a carboxylic acid moiety at C-28 of Ring E. These structural features make the compound flexible for

https://doi.org/10.62347/INVA5312

modification and enhancement of interactions with biological targets such as proteins and receptors. The lipophilicity and poor water solubility are attributed to the rigid hydrocarbon skeleton. Recent literature has demonstrated that betulinic acid exhibits anticancer, antiviral, and anti-inflammatory, antimalarial, antioxidant, and metabolic modulatory effects [2, 3]. The compound has been shown to regulate several inflammatory pathways exacerbated by stimulating the expression of GSH, SOD, and IL-10 while simultaneously suppressing TNF, COX-2, and NO. These molecular pathways provide the biochemical basis for its antioxidant and immunomodulatory effects [4]. The reported poor aqueous solubility and limited pharmacokinetic bioavailability of the compound have necessitated the development of nanoformulations and semi-synthetic derivatives. These structural modifications aimed to enhance therapeutic efficacy and improve systemic distribution to target molecules and receptors [3, 5]. Literature data suggests that betulinic acid possesses antiplasmodial potency and efficacy against both chloroquine-sensitive and multidrug-resistant isolates of *Plasmodium falciparum* [6, 7]. Moreover, in several structural activity and relationship studies, semi-synthetic derivatives have yielded analogues with nanomolar efficacy as low as 220 nM [8]. The inhibitory mechanism of the compound appears to be multi-targeted. The compound acts by suppressing essential parasitic glycolytic enzymes, such as hexokinase (PfHk) and lactate dehydrogenase (PfLDH), by occupying their catalytic sites, thereby impairing energy metabolism [7]. Furthermore, experimental data suggest that the compound disrupts parasitic homeostasis by interfering with calcium-regulated signaling pathways [8]. The compound is reported to display broad spectrum of antimicrobial efficacy, with significant growth inhibition documented in gram-positive species such as Bacillus subtilis and *Staphylococcus aureus* [9, 10]. In silico molecular modeling suggests that, betulinic acid as a catalytic antagonist, targeting beta-lactamase and DNA gyrase involved in antibiotic resistance and genomic replication, respectively [10].

Afzelia africana is a tropical plant species, utilized for its anti-inflammatory and antimicrobial properties [11, 12]. In Ghana, specifically across the Northern and Ashanti belts, the plant is a traditional alternative for malaria therapy [13, 14]. This ethnobotanical knowledge is substantiated by laboratory findings where bark extracts displayed promising *in-vitro* antiplasmodial action against *P. falciparum* [10]. Such scientific corroboration reinforces the reliability of these ethnomedicinal applications [13, 15]. The plant is further documented to exhibit notable organoprotective properties, significantly attenuating creatinine, ALT, and AST levels in chemically induced rat models [16]. The plant's pharmacological properties include the anthelmintic and antitrypanocidal effects, with studies showing significant inhibition of *H. contortus* egg development - an efficacy comparable to standard albendazole [17-19]. Bioassay-guided fractionation has further revealed superior antidiabetic and antioxidant potential, likely mediated by compounds such as eriodictyol (SC50: 2.14 µg/mL) and 3,3-di-O-methyl ellagic acid isolated from the bark of the plant [20-24].

Despite these ethnopharmacological properties, specific molecules responsible for the biological diversity of *A. africana* remain largely uncharacterised. Betulinic acid, with its established pharmacological profile [25], emerges as a primary candidate that may substantiate the plant's traditional medicinal applications. Consequently, the present studies focus on the isolation, purification, and characterization of betulinic acid from *A. africana* root bark through chromatographic separation and spectroscopic methods. Furthermore, this research evaluates the antiplasmodial and antimicrobial activities of the isolated compound to validate its therapeutic potentials.

Materials and methods

Materials, reagents and test organisms

Plant materials: The Bark of *Afzelia africana* was collected from forest of Nsuta (7.0129° N, 1.3783° W), Sekyere Central District of the Ashanti region of Ghana in March 2020. The plant was identified by Mr. Clifford Osafo Asare, at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Ghana. Voucher specimen of the bark ((KNUST/HM1/2019/SB/008) and leaves (KNUST/HM1/2019/L/002)) were deposited at the herbarium unit of the Faculty of Pharmacy and Pharmaceutical Sciences of

Kwame Nkrumah University of Science and Technology (KNUST), Kumasi for future reference.

Reagents: Methanol (Sigma Aldrich, analytical grade), ethyl acetate (Sigma Aldrich, analytical grade), petroleum ether (Sigma Aldrich, analytical grade), chloroform (Sigma Aldrich, analytical grade), hexane (Sigma Aldrich, analytical grade), DPPH (Sigma Aldrich, analytical grade), ABTS (Sigma Aldrich, analytical grade), DMSO (Sigma Aldrich, analytical grade), Artesunate (Sigma Aldrich, analytical grade), silica gel 60 (230-400 mesh) (Sigma Aldrich, analytical grade).

Test organisms: The test organisms (bacteria and fungi) were obtained from the Microbiology unit of School of Basic and Biomedical Sciences, University of Health and Allied Science. These microbial organisms include: Methicillin resistant *Staphylococcus aureus* (NCTC 12493), *Staphylococcus aureus* (NCTC 12973), *E. coli* (NCTC 12241), *S. mutants* (ATCC 700610), *P. aeroginosa* (ATCC 4853), *S. typhii* (ATCC 14028), *K. pneumonia* (NCTC13440) and *Candida albicans* (ATCC 90028), fluconazole resistant *Candida albicans* (Clinical), *B. subtilis* (ATCC 10004). The 3D7 strain of *Plasmodium falciparum* was sourced from the Department of Immunology, Noguchi Memorial Institute for Medical Research, Legon, Ghana.

Extraction of plant material

The plant material was air-dried under shade for three weeks. One kilogram (1 kg) of the powdered plant material was macerated in a 5 L MeOH for 72 hours with occasional shaking. The extract was evaporated to dryness on water bath. The percentage yield was 12% w/w.

Column chromatographic fractionation and purification

A 70 g portion of the methanol extract was adsorbed onto 500 g of silica gel 60 (230-400 mesh) using 100 mL of methanol as solvent, which was subsequently dried under reduced pressure. The resulting brown powder was subjected to gradient column chromatography (CC) (open column, 15 × 15 cm), using a mobile phase gradient of n-hexane, n-hexane-ethyl acetate, and ethyl acetate. This process yielded several fractions, which were pooled into sub-fractions by combining eluates with similar Rf values as determined by thin-layer chromatography (TLC). This led to the recovery of fraction F2 (a 342 mg brown mixture), eluted with n-hexane-ethyl acetate (75:25). This mixture was further fractionated using CC (160.0 g silica gel) with an n-hexane-ethyl acetate (75:25) gradient to yield 15 mg of white crystals.

Structural elucidation of isolate

Mass Spectrometry (MS) analysis: The mass spectrum of the compound was determined using an Agilent Infinity 1290 LC coupled with a 6420 Triple Quadrupole MS (Perkin Elmer, Australia) equipped with an electrospray ionization (ESI) interface. The isolate was injected onto a Merck RP C-18 column (40-63 µm). A mobile phase of methanol: water (60:40) was employed.

The mass spectrum was generated under the following optimized conditions: (1) Fragmenter Voltage: 100 V; (2) Capillary Voltage: 2500 V; (3) Nebulizer Pressure: 30 psi; (4) Drying Gas Temperature: 350°C; (5) Mass Range: 100-600 Da. The resulting ions were sorted and separated according to their mass-to-charge ratio (m/z).

NMR spectroscopy analysis: A 5 mg sample of the compound was dissolved in 500 µL of acetone-d6. The 1-H NMR, 13-C NMR, DEPT-135, COSY, HSQC, and HMBC spectra were acquired using the Bruker 500 MHz Avance III HD NMR spectrometer (Germany). The 1-H NMR spectra were measured at 500 MHz, while the 13-C NMR spectra were recorded at 125 MHz.

Antiplasmodial activity of isolate

The anti-plasmodial effects of the isolated compound was determined using the Malaria SYBR Green I Fluorescence (MSF) assay, following previous protocol established by Vigbedor et al. [26], with slight procedural modification.

Cultivation and synchronization of parasite strains: Cryopreserved isolates of the chloroquine-sensitive 3D7 strain of *P. falciparum* were retrieved from liquid nitrogen and rapidly reconstituted in a 37°C water bath. A 0.2 mL of 12% NaCl was added, followed by 10 mL of 1.6% NaCl. A 10 mL of 0.9% NaCl was mixed

with 0.2% glucose. The resultant mixture was centrifuged at 1500 rpm for 5 minutes. The supernatant was decanted and erythrocyte pellet were mixed and equilibrated with a complete culture medium.

The culture medium was maintained under aseptic conditions in a NuAire class II laminar flow cabinet. All glassware were sterilized through autoclaving at 120°C and 15 atm for 20 minutes. The parasites were supported in an O^+ human erythrocytes suspended in RPMI 1640. The mixture was fortified with 10% human O^+ serum, 25 mM HEPES, 25 mM sodium bicarbonate, and 60 µg/mL gentamicin sulfate (pH 7.2). The incubation was conducted at 37°C in a gas-controlled medium comprising of 90% N_2, 5% O_2, and 5% CO_2. The culture was homogenized by synchronizing to the ring stage using a 5% (w/v) D-sorbitol treatment.

Anti-plasmodial assay protocol: The *in vitro* assessment was initiated by distributing 50 uL of the parasite inoculum (standardized to 0.5% parasitemia and 2% hematocrit) into 96-well microtiter plates. The test compound was solubilized in DMSO and subsequently diluted in RPMI 1640 to a 1% concentration. The test compound was tested by administering serially diluted concentrations of (100, 25, 6.25, 1.56, 0.39, and 0.098 µg/mL). Serially diluted Artesunate (ART) concentrations of (100, 25, 6.25, 1.56, 0.39, and 0.098 µg/mL), were applied as the positive control. Treated microplates were then incubated at 37°C over 48 hours duration.

Detection and spectrofluorometric analysis: The parasite viability was quantified by adding 100 µL of SYBR Green I lysis buffer (comprising 20 mM Tris-Cl, 5 mM EDTA, 0.008% Saponin, and 0.08% Triton-X 100) to each well. The plates were covered with aluminum foil to protect it from light interaction and then incubated at ambient temperature for one hour to facilitate DNA intercalation. The Fluorescence intensity was measured using a Tecan Infinite M200 microplate reader, with the wavelength of excitation and emission set at 490 nm, 530 nm respectively. The entire assay was performed in triplicate and the mean, standard deviation and test of statistical significance computed. The level of parasitemia was determined by inputting the absorbance data into the required relationship, and the percentage inhibition for each concentration was calculated by comparing the parasitemia of the control against the prepared compound concentrations.

The experiment was repeated 2 more times to achieve triplicate measurements. The parasitaemia was computed by inputing the absorbance data into the relation:

$$\%\ Parasitaemia = \left[\frac{\text{Fluorescence absorbance of Infected RBC after treatment}}{\text{Fluorescence absorbance of untreated RBCs}}\right] \times 100\%$$

The percentage inhibitions for the prepared concentrations were evaluated by inputting the experimental data of the parasitemia of control and prepared compound concentration.

$$\%\ Inhibition = \left[\frac{\text{Parasitaemia of control - Parasitaemia of compound}}{\text{Parasitaemia of control}}\right] \times 100$$

The percentage inhibition was plotted against the concentrations, and the antiplasmodial activity was evaluated by computing the IC_{50} (µg/mL). This value was determined using GraphPad Prism software (version 6.01), employing a nonlinear regression (curve fit) with Log (inhibitor) vs. response (three parameters) model.

Antimicrobial activity of isolate

Inoculum preparation: The microbes were grown on the nutrient agar (Oxoid, United Kingdom) plates within 18-24 hours at 37°C in an incubator. The suspensions of colonies were transferred from the plates with sterile inoculating loop into 15 mL of sterile saline in test tubes. Their turbidities were adjusted and compared to the 0.5 McFarland standards through eye reading. The inoculums were further diluted to 10^3 (Corliform forming Unit) CFU [27, 28].

Minimum inhibitory concentrations of the compound and standard antibiotics: The Minimum Inhibitory Concentrations (MICs) of the compound and the reference drugs (Tetracycline, fluconazole) were determined according to the method described by Eloff (1998) [29] and in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. Each well of the 96-well microtiter plates (Bio-Tek Instruments GmbH, Germany) was filled with 150 µL of Mueller-Hinton broth (Oxoid Limited, United Kingdom). This was followed by the preparation

of two-fold serial dilutions of the compound and the reference drugs. The concentrations ranged from 0.3 to 200 mg/mL for the compound (specifically: 200.00, 100.00, 50.00, 25.00, 12.50, 6.25, 3.125, 1.5625, and 0.7813 mg/mL) and 0.063 to 128 µg/mL for the antibiotics. One well served as the positive control (inoculated with the organism and broth), while the broth stock in a test tube served as the negative control (broth only, without the organism). After the serial dilution preparations, each well was inoculated with 150 µL of the standardized microbial suspension, and subsequently adjusted to 10^6 CFU/mL. The microtiter plates were incubated at 37°C for a 24-hour period. A volume of 40 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.1% w/v) was introduced into each well, and further incubated for half an hour at a temperature of 37°C. The MICs of the betulinic acid isolate were evaluated as the lowest concentration of the compound or reference drug that showed no change in color after visual inspection. This parameter served as the primary metric for evaluating the inhibitory capacity of the isolated compound.

Assessment of Minimum Bactericidal (MBC) and Fungicidal (MFC) concentrations: The lethal thresholds (MBC and MFC) were established in accordance with the Clinical and Laboratory Standards Institute (CLSI) protocols, with modifications as suggested by Mogana et al. (2020). After completing the MIC determination, representative samples were collected from all wells that showed no visible growth.

The bacterial isolates were aliquoted and transferred aseptically onto Nutrient Agar (NA) plates, while fungal samples were inoculated onto Sabouraud Dextrose Agar (SDA). The resulting subcultures were incubated at 37°C for 48 hours. The Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were determined as the lowest concentration that resulted in no colony growth, indicating that 99.9% of the original bacterial or fungal inoculum had been killed.

Determination of synergistic effect of isolate with selected antimicrobial agents: The *In-vitro* analysis of the interaction between the isolate and the antibiotics (tetracycline and ciprofloxacin) against the bacterial strains and the antifungals (fluconazole, ketoconazole and nystatin) against *C. albicans* strains, were evaluated by adopting the checkerboard microdilution assay as described previously by Khodavandi *et al*. (2010) and Dickson *et al*. (2006) [31, 32] with minor modifications. The tested concentrations for each antibiotic and each test plant samples ranged from (1/32) × MIC to 2 × MIC. The mode of the interactions were measured by calculating the Fractional Inhibitory Concentration Index (FICI) by inputting the MIC values into the relation:

$$\text{FIC index} = \frac{[\text{MIC of antibiotic in combination}]}{[\text{MIC of antibiotic alone}]} + \frac{[\text{MIC of fraction in combination}]}{[\text{MIC of fraction alone}]}$$

The interaction was considered synergistic if the FICI was ≤ 0.5, partial synergistic if FICI was > 0.5 and < 1, additive if FICI was =1, no difference if the FICI was > 1 and ≤ 4, and antagonistic if the FICI was > 4.0.

Statistical analysis

The antiplasmdial procedure was done in triplicate (n=3), and data are expressed as mean ± Standard Deviation (SD). Statistical comparisons between groups (e.g., test extract vs. positive control) were performed using the Student's t-test. The one-way Analysis of Variance (ANOVA) was utilized, followed by Tukey's post-hoc test for inter-group comparisons. Differences were considered statistically significant when the *p*-value was less than 0.01 ($P < 0.01$). Data processing, graph plotting, and statistical analyses were conducted using SPSS, GraphPad Prism 5.00 (San Diego, California, USA), and Microsoft Excel 2013.

Results and discussion

Structural elucidation of isolate

The structural identity of betulinic acid (MF: $C_{30}H_{34}O_3$) is characterized by the presence of the lupane carbon skeleton comprising of five fused rings of four hexacyclic (A, B, C and D) and one pentacyclic (ring E) system. The structure is further marked by the attachment of a hydroxy (OH) group at C3, carboxyl (COOH) group at C17, an olefin (H_2C=CH-) moiety at C19, two methyl (CH_3) groups at C4 and four other methyl groups at C8, C10, C14 and C20. The structure of betulinic acid was success-

Table 1. Summary of NMR spectra data of isolate

Carbon number	^{13}C-NMR	DEPT-135	Proton number	^{1}H-NMR	HSQC
	^{13}C Chemical shift assignment	Type of carbon		^{1}H-Chemical shift assignment	Carbon-Hydrogen connectivity
C1	38.7	CH_2	H1	0.94	$C_{38.67}$-$H_{0.94}$
			H2	1.64	$C_{38.67}$-$H_{1.64}$
C2	27.4	CH_2	H3	1.55	$C_{27.37}$-$H_{1.55}$
			H4	1.60	$C_{27.37}$-$H_{1.60}$
C3	77.7	O-CH	H5	3.13	$C_{77.67}$-$H_{3.13}$
C4	38.7	C	-	-	-
C5	55.4	CH	H6	0.74	$C_{55.43}$-$H_{0.74}$
C6	18.2	CH_2	H7	1.40	$C_{18.2}$-$H_{1.40}$
			H8	1.50	$C_{18.2}$-$H_{1.50}$
C7	34.3	CH_2	H9	1.38	$C_{34.32}$-$H_{1.38}$
			H10	1.44	$C_{34.32}$-$H_{1.44}$
C8	40.6	C	-	-	-
C9	50.6	CH	H11	1.38	$C_{50.55}$-$H_{1.38}$
C10	37.1	C	-	-	-
C11	20.8	CH_2	H12	1.36	$C_{20.80}$-$H_{1.36}$
			H13	1.24	$C_{20.80}$-$H_{1.24}$
C12	25.5	CH_2	H14	1.05	$C_{25.51}$-$H_{1.05}$
			H15	1.70	$C_{25.51}$-$H_{1.70}$
C13	38.1	CH	H16	2.36	$C_{38.13}$-$H_{2.36}$
C14	42.3	C	-	-	-
C15	30.4	CH_2	H17	1.46	$C_{30.44}$-$H_{1.46}$
			H18	1.46	$C_{30.44}$-$H_{1.46}$
C16	31.9	CH_2	H19	1.45	$C_{31.92}$-$H_{1.45}$
			H20	1.54	$C_{31.92}$-$H_{1.54}$
C17	55.9	C	-	-	-
C18	49.0	CH	H21	1.63	$C_{49.04}$-$H_{1.55}$
C19	47.1	CH	H22	3.05	$C_{47.06}$-$H_{3.05}$
C20	150.7	C=	-	-	-
C21	29.6	CH	H23	2.10	$C_{29.57}$-$H_{2.10}$
			H24	1.46	$C_{29.57}$-$H_{1.44}$
C22	36.6	C	H25	1.94	$C_{36.64}$-$H_{1.95}$
			H26	1.56	$C_{36.64}$-$H_{1.51}$
C23	27.7	CH_3	H27	0.96,	$C_{27.67}$-$H_{1.00}$
C24	15.2	CH_3	H28	0.74	$C_{18.58}$-$H_{0.90}$
C25	15.7	CH_3	H29	0.84	$C_{15.74}$-$H_{0.90}$
C26	15.6	O=C-	-		-
C27	14.1	CH_3	H30	0.99	$C_{14.12}$-$H_{1.00}$
C28	176.7	CH_3	H31	1.03	$C_{15.74}$-$H_{0.99}$
C29	109.1	$=CH_2$	H32	4.60	$C_{109.12}$-$H_{4.60}$
			H33	4.73	$C_{109.12}$-$H_{4.73}$
C30	18.6	CH_3	H34	1.71	$C_{18.58}$-$H_{1.75}$

fully characterized by the application of spectroscopic techniques such as NMR (1H, 13C, DEPT-135, HSQC, COSY and HMBC) and MS (-ve ESI-MS).

NMR analysis: A detailed spectra analysis and summary of all NMR data are presented in the Supplementary Data 1, 2, 3, 4, 5, 6, **Table 1** and **Figure 1**. The illustration of the various

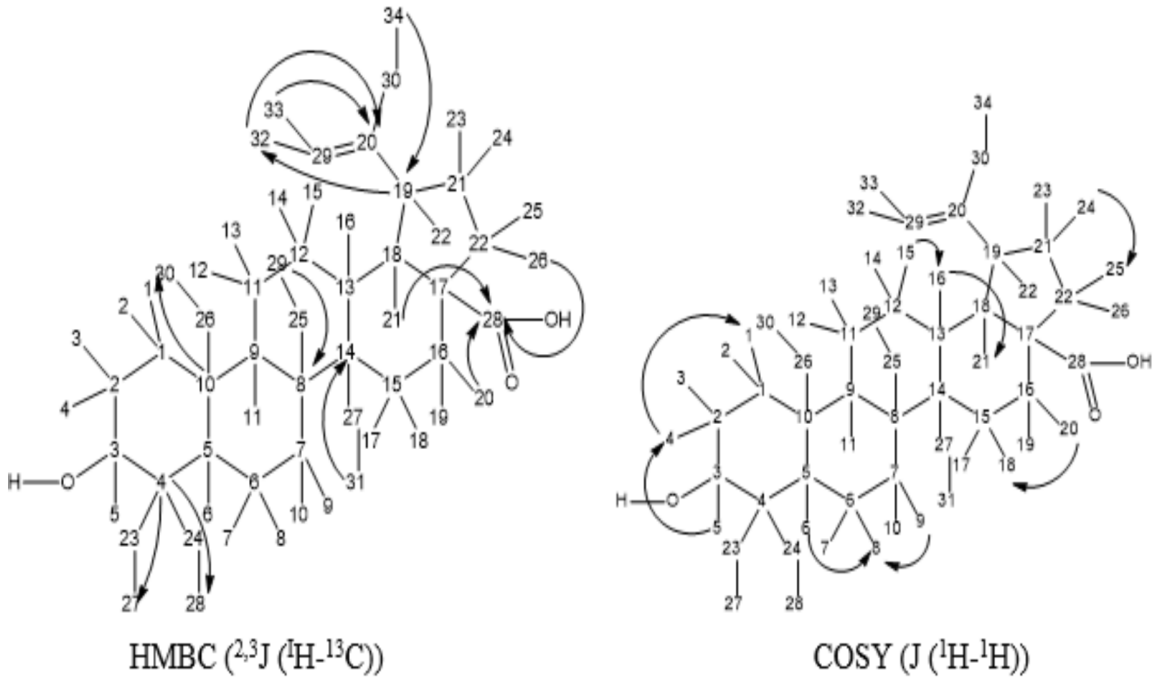

Figure 1. Illustration of HMBC (^{2,3}J (^{1}H-^{13}C)) and COSY (^{2,3}J (^{1}H-^{1}H)) of isolate.

^{2,3}J-(^{1}H-^{13}C)-(HMBC) and the (^{1}H-^{1}H)-(COSY) in the compound are indicated in **Figure 1**. Based on the analysis, the isolate was identified as betulinic acid (MF: $C_{30}H_{46}O_3$, M: 456.5). The comparison of the ^{1}H and ^{13}C-NMR spectra of the isolate with betulinic acid (literature) [33, 34] are indicated in **Table 2**.

LC-MS analysis: Retention time (Rt): 0.58-0.82 minutes (methanol/water (60:40) on a C-18 column). The mass spectrum analysis showed a prominent molecular ion peak (m/z: 455.5). Further confirmatory information regarding the compound was obtained via ESI-MS (negative ion mode). The fragmentation pattern (**Figures 2** and **3**) was consistent with lupane-type triterpenes, displaying characteristic peaks for betulinic acid at m/z 455.5 [M-H$^+$], 437 [M-H-H_2O], 203 [M-$C_{16}H_{28}$O-OH], 191 [M-$C_{16}H_{24}O_2$-OH], 177 [M-H-$C_{16}H_{24}O_2$-2CH_3], 137 [M-$C_{20}H_{30}O_2$-OH] and 123 [M-$C_{21}H_{32}O_2$-OH] [35].

Antiplasmodial activity of isolate

The contribution of natural products to the interventions in malaria treatment is enormous and can never be underemphasized. Plant materials have served as a natural factory for the biosynthesis of numerous secondary metabolites with wide structural varieties [36, 37]. The quest to discover potent antiplasmodial agents from plants has been successfully carried out by experimental procedures that seeks to screen plants with ethno-antimalarial properties. Several screening experiments have proceeded through *in-vitro* or *in-vivo* methods [38]. In the current study, the *in-vitro* antiplasmodial activity of betulinic acid, an isolate from the root bark extract of *A. africana* is reported. The test involved the culturing of the asexual stage of 3D7 strain of *P. falciparum* and subsequent treatment with betulinic acid. The evaluation of the antiplasmodial activity was done by computing the % inhibition of serially diluted concentrations of the compound ranging from 100-0.098 µg/mL. Subsequently, the plot (**Figure 4**) of the % Inhibition versus concentration. The IC_{50} (µg/mL) was evaluated by employing the GraphPad Prism software package 5.00 (San Diego, California, USA) with non-regression (curve fit) with the Log (Inhibitor) versus response (three parameters).

The results indicate that betulinic acid exhibits moderate antiplasmodial activity (IC_{50} = 12.89 ± 0.52 µg/mL, *P* value: < 0.01), which is weaker than that of the standard drug artesunate (IC_{50} = 0.03 ± 0.01 µg/mL, *P* value: < 0.01). Despite its comparatively lower potency, the compound demonstrates promising activity

Table 2. Comparison of ^{1}H and ^{13}C-NMR of isolate with literature (Betulinic acid)

Carbon number	^{13}C-NMR		Proton number	^{1}H-NMR	
	^{13}C-NMR (Isolate)	^{13}C-NMR (Betulinic acid)		^{1}H-NMR (Isolate)	^{1}H-NMR (Betulinic acid)
C1	38.7	39.0	H1	0.94	0.86
			H2	1.64	1.64
C2	27.4	27.6	H3	1.55	1.52
			H4	1.60	1.61
C3	77.7	78.2	H5	3.13	3.17
C4	38.7	39.1	-	-	-
C5	55.4	55.5	H6	0.74	0.66
C6	18.2	18.4	H7	1.40	1.50
			H8	1.50	-
C7	34.3	34.5	H9	1.38	1.35
			H10	1.44	1.40
C8	40.6	40.8	-	-	-
C9	50.6	50.7	H11	1.38	1.25
C10	37.1	37.3	-	-	-
C11	20.8	21.0	H12	1.36	1.37
			H13	1.24	-
C12	25.5	25.7	H14	1.05	1.04
			H15	1.70	1.68
C13	38.1	38.1	H16	2.36	2.20
C14	42.3	42.5	-	-	-
C15	30.4	30.2	H17	1.46	1.14
			H18	1.46	1.49
C16	31.9	32.9	H19	1.45	1.27
			H20	1.54	-
C17	55.9	56.3	-	-	-
C18	49.0	49.2	H21	1.63	1.61
C19	47.1	48.1	H22	3.05	3.00
C20	150.7	150.3	-	-	-
C21	29.6	30.6	H23	2.10	1.99
			H24	1.46	1.44
C22	36.6	37.0	H25	1.94	1.96
			H26	1.56	1.55
C23	27.7	27.9	H27	0.96	0.95
C24	15.2	15.5	H28	0.74	0.91
C25	15.7	16.4	H29	0.84	0.96
C26	15.6	16.7	H30	0.99	0.91
C27	14.1	15.0	H31	1.03	0.96
C28	176.7	180.3			-
C29	109.1	108.9	H32	4.60	4.58
			H33	4.73	4.71
C30	18.6	19.6	H34	1.71	1.67

and remains a valuable candidate for antimalarial lead optimization and structure - activity relationship (SAR) studies. The IC_{50} value observed in this study is consistent with previous

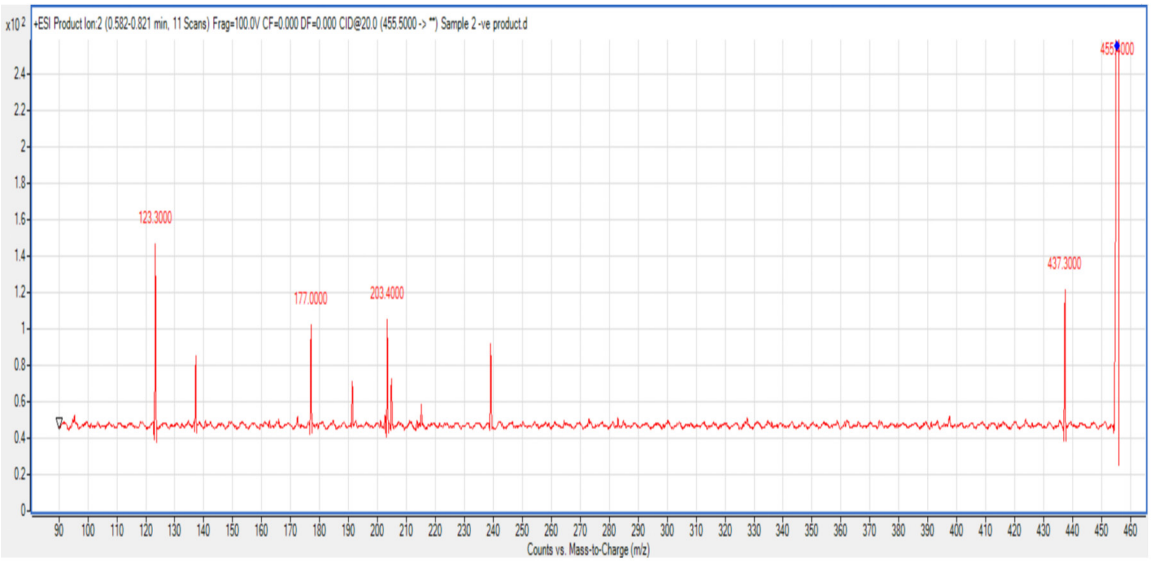

Figure 2. Mass spectrum of betulinic acid.

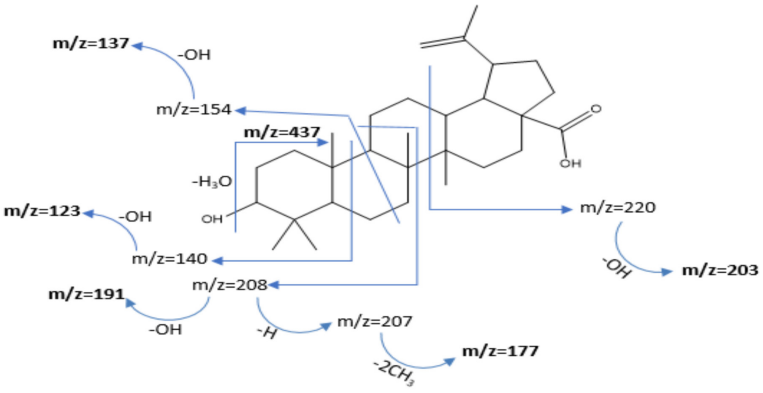

Figure 3. Illustration of the fragmentation pattern of betulinic acid.

reports, including those of Egbubine et al. (12.60 µg/mL) and Bringmann et al. (10.46 µg/mL against the chloroquine-sensitive NF54 strain) for *P. falciparum* asexual blood stages. Bringmann et al. also reported IC_{50} values of 19.6 and 25.9 µg/mL against the chloroquine-sensitive T9-69 and chloroquine-resistant K1 strains, respectively [39, 40]. Even though, the exact antiplasmodial mechanism of action of betulinic acid is not fully understood, studies have proposed potential molecular targets and pathways. Betulinic acid and its metabolite classes have been documented with *P. falciparum* enzymes through hydrogen bonding and hydrophobic interactions. The resultant effect includes the formation of a ligand - topoisomerase - DNA complex that hinders DNA replication in *P. falciparum* [40]. Further investigations suggest that betulinic acid and its derivatives could disrupt the erythrocyte lipid bilayer. This could result in the formation of echinocyte or stomatocyte and thereby impairing merozoite invasion and parasite growth [38].

The inhibition of β-hematin and calcium-dependent pathways has been extensively studied as a key mechanism for many pentacyclic secondary metabolites that possess structural features linked to substituents such as hydroxyl (-OH), carboxylic acid (-COOH), and olefins (C=C). The incorporation of these molecular entities into the host erythrocyte membrane may destabilize intracellular pH, ultimately inhibiting the growth and development of *P. falciparum*. This observation is supported by studies conducted by Rocha et al. [40] and Olanlokun et al. [41]. Moreover, the hydroxyl (-OH) and carboxylic acid (-COOH) groups in betulinic acid play crucial roles as sites for hydrogen bonding and ionic interactions, which enhance its binding affinity to proteins, enzymes, or receptors. The -COOH moiety, particularly at C-28, has been shown to be essential for the cytotoxic properties of betulinic acid and

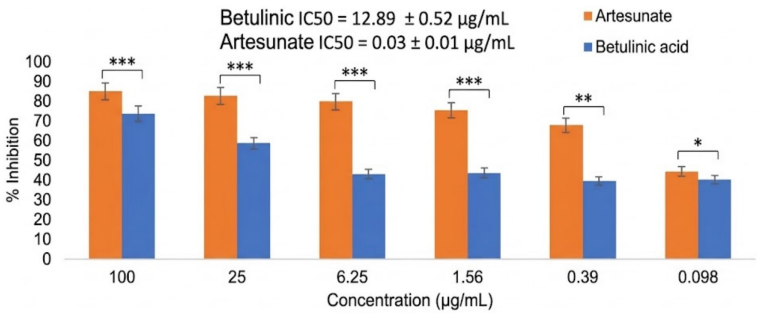

Figure 4. *In-vitro* antiplasmodial activity of Artesunate and Betulinic acid against *P. falciparum* (3D7 strain) at varying concentrations. *Statistical significance for comparisons between groups at each concentration is indicated as: P < 0.05*, **P < 0.01, P < 0.001. All measurements are from triplicate assays (n=3).

related triterpenes [42, 43]. Additionally, the olefinic bond (C=C) and the rigid pentacyclic backbone help maintain a stable three-dimensional structure, allowing for hydrophobic interactions and integration into membranes or the hydrophobic pockets of target proteins [44].

These functional groups could contribute to the compound's ability to interact with multiple biological targets, which may account for its broad pharmacological properties [45]. These structural features and the mechanistic observations reinforce the value of betulinic acid as a versatile scaffold with significant promise for the design and development of novel antimalarial therapeutics.

Antimicrobial activity of isolate

The search for new therapeutic agents for infectious diseases continues to draw heavily from both synthetic and natural sources [46]. The antimicrobial potentials of these compounds have been accessed through standardized microbial assay methods such as the MIC, MBC and their ratios (MBC/MIC) [47]. These parameters determine the minimum inhibitory and bactericidal concentrations. They measure the lowest concentration of an antimicrobial agent that prevents visible microbial growth [48], and the 99.9% lethal concentrations respectively. The assessment of these parameters provides valuable insights into whether a compound exhibits bacteriostatic or bactericidal activity [49]. Several literature data have classified antimicrobial agents with MIC values up to 8 mg/mL as potential candidates for drug development against microbial strains [30]. Moreover, antimicrobial agents with MBC/MIC ratio ≥ 4 typically indicates a bacteriostatic effect, whiles ratios ≤ 4 suggests bactericidal activity [30].

In the present study, the antimicrobial properties - including both the bacteriostatic and bactericidal effects of betulinic acid isolated from the stem bark of *A. africana* were evaluated by determining its MIC, MBC, and MBC/MIC ratios against ten different microbial strains (**Table 3**). The MIC results revealed that, the compound exhibited broad spectrum of inhibitory (bacteriostatic) activity (MIC ≤ 8 mg/mL) against nearly all tested microbial strains. The reported activity of the compound against *S. aureus, E. coli, B. subtilis*, and *P. aeruginosa* is corroborated by similar findings reported by Ibrahim et al. [50] and Fontanay et al. [51]. The MBC values further demonstrated the compound's broad-spectrum of bactericidal activity across all tested strains. The most potent effects were observed at relatively low concentrations (MBC < 4 mg/mL) against *B. subtilis, S. mutans*, and *K. pneumoniae*. Based on the MBC/MIC ratios, the compound exhibited clear bactericidal activity (MBC/MIC ≤ 4) against *B. subtilis, S. mutans, C. albicans* (strain 2), and *K. pneumoniae*, while demonstrating bacteriostatic effects (MBC/MIC ≥ 4) against *E. coli, S. aureus*, MRSA, *S. typhi, P. aeruginosa*, and *C. albicans* (strain 1). Interestingly, the most pronounced activity was observed against *S. mutans, B. subtilis*, and *K. pneumoniae*, where it acted at lower and potentially safer concentrations. The findings of these antimicrobial assays could contribute to the well-documented antimicrobial activities reported for the crude extracts of *A. Africana*. In fact, previous studies [16, 17] reported high susceptibility of *S. aureus* and *B. subtilis* to crude extracts of the plant, therefore, supports its traditional uses as antimicrobial agents. Moreover, the antimicrobial activity of betulinic acid is previously observed against *Streptococcus pyogenes*, *E. coli, S. aureus, Enterococcus faecalis*, and related pathogens [52, 53]. These findings validate the findings of this study and

Table 3. MIC, MBC and MBC/MIC ratios for isolate and antibacterial agents

Bacterial strain	Antibacterial activity of isolate MIC, MBC/mg/mL			MICs of standard antibacterial agent/µg/mL	
	MIC	MBC	MBC/MIC ratio	CIPRO	TET
SM	0.78	1.56	2.0^{bc}	125.00	7.81
SA	1.56	25.00	16.0^{bs}	7.81	7.81
EC	1.56	12.50	8.0^{bs}	125.00	7.81
CA2	12.5	50.00	4.0^{bc}	125.00	7.81
MRSA	3.125	25.00	8.0^{bs}	250.00	15.63
BS	1.56	1.56	1.0^{bc}	125.00	7.81
KP	3.125	3.125	1.0^{bc}	125.00	7.81
PA	3.125	25.00	8.0^{bs}	125.00	7.81
ST	1.56	50.00	32.1^{bs}	3.90	15.63
CA1	6.25	50.00	8.0^{bs}	-	-

bc: bactericidal; bs: bacteriostatic.

Table 4. Synergistic effects of isolate with standard antibacterial agents against bacterial strains

Strain	MIC (CIP)/µg/mL	FIC (CIP + BA)	INT.	MIC (TET)/µg/mL	FIC (TET + BA)	INT.
EC	125.00	0.904	PS	7.81	0.110	S
KP	125.00	0.094	S	7.81	0.110	S
SA	7.81	0.990	PS	7.81	0.062	S
SM	125.00	0.381	S	7.81	1.125	I
ST	3.90	0.065	S	15.63	0.531	PS
MRSA	250.00	0.990	PS	7.81	1.062	I
BS	125.00	1.500	I	7.81	0.563	PS
PA	125.00	3.000	I	7.81	1.125	I

CIP: Ciprofloxacin; TET: Tetracycline; INT: Interpretation; BA: Betulinic acid; FICI: Fractional Inhibitory Concentration Index, [synergistic (S) if the FICI ≤ 0.5; partial synergistic (PS) if FICI > 0.5 and < 1; additive (AD) if FICI=1; no synergism or indifferent (I) if the FICI > 1 and ≤ 4; antagonistic (A) if the FICI > 4.0].

Table 5. Synergistic activity of isolate with standard antifungal agents against fungal strains

Agent	CA1	CA2	Combination	CA1-1		CA-2	
	MIC	MIC		F1CI	INT.	FICI	INT.
FLC	64	> 64	BA + FLC	1.400	I	0.250	S
KET	512	512	BA + KET	0.525	PS	0.560	PS
NYX	128	128	BA + NYS	0.531	PS	0.281	S

FLC: Fluconazole; KT: Ketoconazole; NYX: nystatin; INT: Interpretation; FICI: Fractional Inhibitory Concentration Index.

reinforces the therapeutic effects of *A. africana*.

Antibacterial synergistic effects of isolate: Given the strong antimicrobial activity demonstrated by the compound, its synergistic interactions with standard antibacterial agents, including ciprofloxacin and tetracycline were evaluated. The results (**Table 4**) revealed varying degrees of synergism across the bacterial isolates. The compound exhibited synergistic effects against *K. pneumoniae, S. mutans*, and *S. typhi*; partial synergism against methicillin-resistant *E. coli, S. aureus*, and MRSA; and indifferent effects against *B. subtilis* and *P. aeruginosa* when combined synergistically with ciplofloxcine. Moreover, the compound demonstrated synergistic activity against *E. coli*, *K. pneumoniae*, and *S. aureus*; partial synergism against *S. typhi* and *B. subtilis*; and indifferent effects against *S. mutans*, MRSA, and *P. aeruginosa* when combined with tetracycline.

Antifungal synergistic activity of isolate: The synergistic effects of the isolate with fluconazole, ketoconazole, and nystatin were further evaluated using the checkerboard assay (**Table 5**). The interpretation of the interactions fol-

lowed the established criteria: synergistic (S) when FICI ≤ 0.5; partial synergistic (PS) when FICI > 0.5 and < 1; additive (AD) when FICI=1; indifferent (I) when FICI > 1 and ≤ 4; and antagonistic (A) when FICI > 4.0 [54, 55]. The results demonstrated varying degrees of synergism against *Candida albicans* strains 1 and 2. Specifically, the compound showed indifferent interaction with *C. albicans* 1 but full synergism with *C. albicans* 2 when combined with fluconazole. The synergistic interaction with ketoconazole, the compound demonstrated partial synergism against both strains. Finally, nystatin with betulinic acid showed partial synergism against *C. albicans* 1 and complete synergism against *C. albicans* 2. These findings suggest that the compound may serve as a promising candidate for single-agent or combination therapy against *C. albicans* infections.

The isolate is a lupane-type triterpenoid, a class recognized for its diverse pharmacological profile, most notably its anticancer, anti-inflammatory, antimicrobial, antiplasmodial, and antioxidant properties [56, 57]. Earlier work showing that crude extracts of *Afzelia africana* synergize with several antibiotics against drug-resistant bacteria [58], The strong synergistic interactions observed in this study further validate the plant's broad antimicrobial potential. Betulinic acid is known to exert its pharmacological activity by modulating multiple signaling pathways. This compound, or its derivatives induces apoptosis through the intrinsic mitochondrial pathway. This mechanism promotes the generation of reactive oxygen species (ROS) and activates pro-apoptotic p38 MAPK and SAP/JNK kinases. The resulting cascade effect regulates cell proliferation, gene expression, differentiation, and survival [59-61]. The compound is also known to suppress cell proliferation through down-regulation of ERα expression and inhibition of NF-κB signaling, including blockade of IκBα phosphorylation, NF-κB nuclear binding, and NF-κB-dependent gene transcription [57]. Owing to these mechanisms, lupane triterpenoids have been explored for therapeutic applications in cardiovascular diseases, cancer, inflammation, microbial infections, protozoal diseases, viral infections, and hepatotoxicity [57, 62, 63]. Additional mechanisms of action attributed to betulinic acid include the activation of the Nrf2 pathway and upregulation of its downstream antioxidant enzymes HO-1 and NQO1, along with suppression of NF-κB activity [64]. It has also been shown to inhibit LPS-induced IL-17 and IF-γ production in encephalitogenic T cells [65]. Other experimentally validated properties include anti-inflammatory, anticancer, hepatoprotective, antiviral, anti-ulcer, and antimicrobial activities. Mechanistically, the compound can inhibit lipid peroxidation, normalize ADP and AMP levels [66], and disrupt bacterial growth by interfering with cell wall synthesis, increasing membrane permeability, and suppressing protein synthesis at the 30S ribosomal subunit [67, 68]. The observed antiplasmodial and antimalarial activities coupled with the already rich pharmacological profile of betulinic acid and other isolated compounds such as eriodictyol, 3,3-di-O-methyellagic acid further validates the ethnomedicinal properties of *Afzelia africana*.

Conclusion

The study reports for the first time, the isolation and characterization of betulinic acid from the root bark of *Afzelia africana*. The compound demonstrated promising antiplasmodial activity against *Plasmodium falciparum* (3D7) with an IC_{50} of 12.89 µg/mL. The compound further demonstrated broad-spectrum of bactericidal activity, with the highest potency (MBC < 4 mg/mL) against *B. subtilis, S. mutans*, and *K. pneumoniae*. Based on MBC/MIC ratios, it exhibited bactericidal activity (ratio ≤ 4) against *B. subtilis, S. mutans, K. pneumoniae*, and *C. albicans* (strain 2), while showing bacteriostatic effects (ratio ≥ 4) against *E. coli, S. aureus,* MRSA, *S. typhi*, *P. aeruginosa*, and *C. albicans* (strain 1). When compound was combined with ciprofloxacin, it exhibited full synergism against *K. pneumoniae*, *S. mutans*, and *S. typhi*; partial synergism against *E. coli, S. aureus*, and MRSA; and indifferent effects against *B. subtilis* and *P. aeruginosa*. In combination with tetracycline, it showed full synergism against *E. coli, K. pneumoniae*, and *S. aureus*; partial synergism against *S. typhi* and *B. subtilis*; and indifferent effects against *S. mutans*, MRSA, and *P. aeruginosa*. The interaction revealed that the compound showed full synergism for fluconazole and nystatin *C. albicans* 2. Partial synergism was noted for ketoconazole against *C. albicans* 1 and 2 and nystatin against *C. albicans* 1. An indifferent

interaction was recorded for fluconazole against *C. albicans* 1. These results validate the traditional use of *A. africana* as an antimicrobial agent and align with previous reports on the efficacy of crude extracts and betulinic acid against similar pathogens.

Acknowledgements

Due acknowledgement goes to all who contributed to the formulation of this research piece with sole dedication to the memory of the late Dr. Samuel Osafo Acquaah.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bright Yaw Vigbedor, School of Basic and Biomedical Sciences, University of Health and Allied Sciences, PMB 31, Ho 00233, Volta Region, Ghana. Tel: 233-245052908, 233-506421166; E-mail: ybvigbedor@uhas.edu.gh

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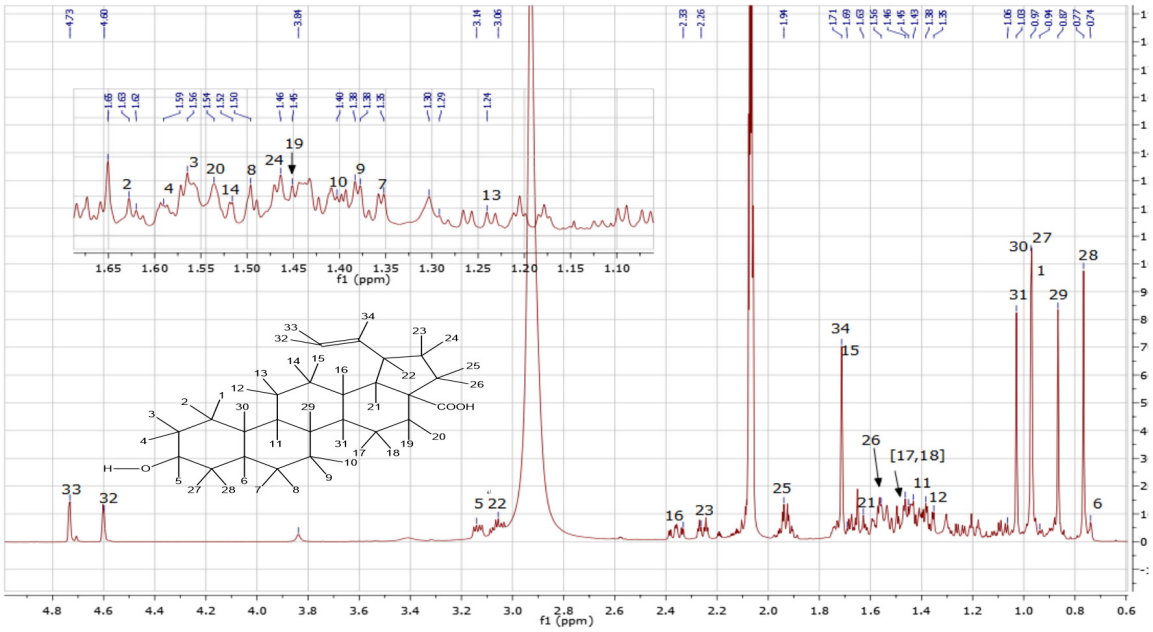
Supplementary Data 1

Proton NMR spectrum (^{1}H-NMR)

The ^{1}H-NMR (Supplementary Figure 1) revealed the presence of 34 different signals consistent with betulinic acid. The signals comprises of 32 aliphatic and 2 olefinic protons such as H1 (0.94), H2 (1.64), H3 (1.55), H4 (1.60), H5 (3.13), H6 (0.74), H7 (1.40), H8 (1.50), H9 (1.38), H10 (1.44), H11 (1.38), H12 (1.36), H13 (1.24), H14 (1.05), H15 (1.70), H16 (2.36), H17 (1.46), H18 (1.46), H19 (1.45), H20 (1.54), H21 (1.63), H22 (3.05), H23 (2.10), H24 (1.46), H25 (1.94), H26 (1.56), H27 (0.96), H28 (0.74), H29 (0.84), H30 (0.99), H31 (1.03), H32 (4.60), H33 (4.73), H34 (1.71).

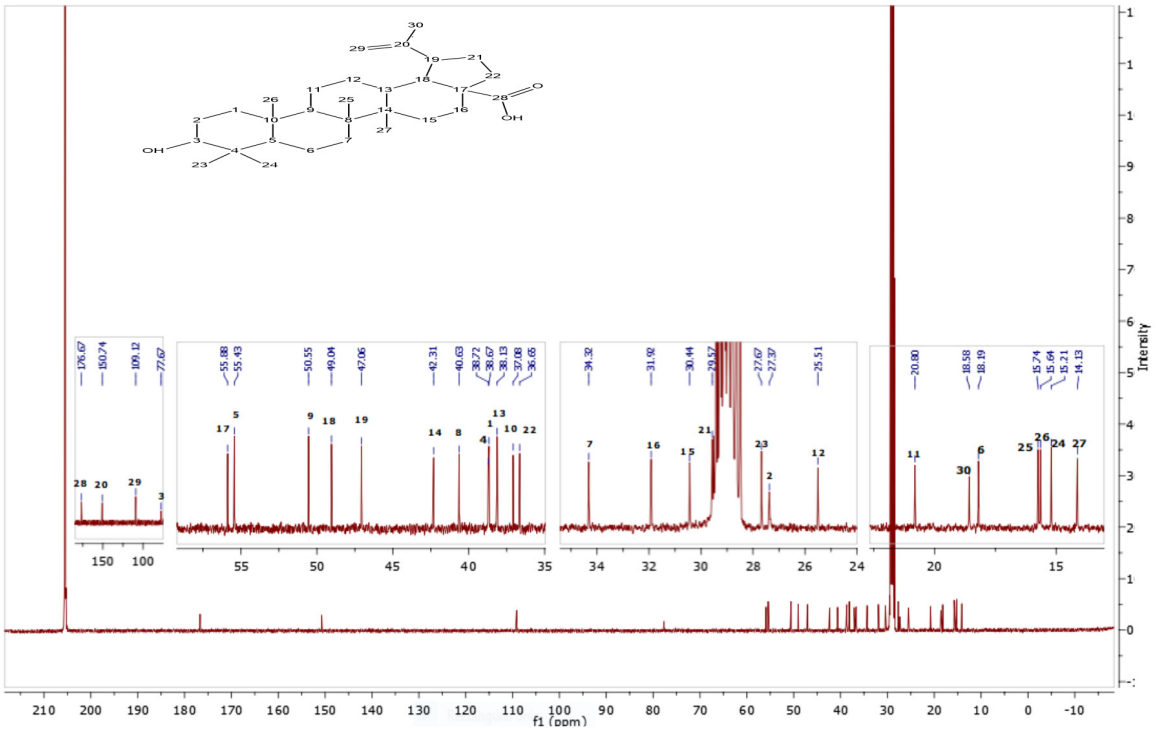


Supplementary Figure1. ^{1}H-NMR spectrum of betulinic acid.

Supplementary Data 2

Carbon-13 NMR spectrum of isolate

^{13}C-NMR: The ^{13}C-NMR (Supplementary Figure 2) revealed the presence of 30 different signals consistent with betulinic acid comprising of 27 aliphatic 2 olefinic and 1 carboxylic carbon. The signals include C1 (38.7), C2 (27.4), C3 (77.7), C4 (38.7), C5 (55.4), C6 (18.2), C7 (34.3), C8 (40.6), C9 (50.6), C10 (37.1), C11 (20.8), C12 (25.5), C13 (38.1), C14 (42.3), C15 (30.4), C16 (31.9), C17 (55.9), C18 (49.0), C19 (47.1), C20 (150.7), C21 (29.6), C22 (36.6), C23 (27.7), C24 (15.2), C25 (15.7), C26 (15.6), C27 (14.1), C28 (176.7), C29 (109.1) and C30 (18.6).

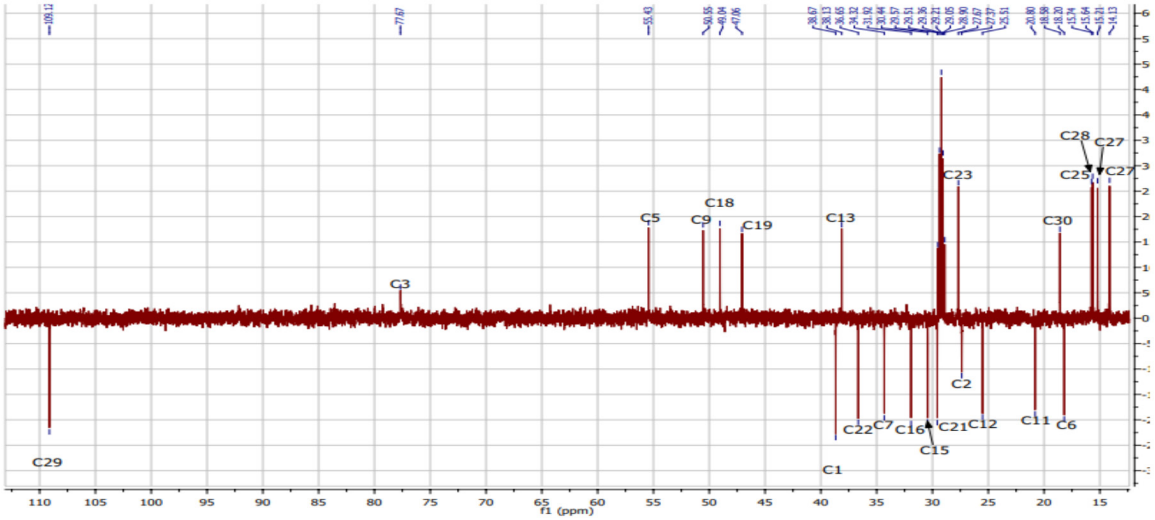


Supplementary Figure 2. ^{13}C-NMR spectrum of isolate.

Supplementary Data 3

DEPT-135 NMR spectrum

DEPT-135 NMR: The DEPT-135 NMR (Supplementary Figure 3) revealed the presence of 22 different signals comprising of X methine (CH: C3 (77.7), C5 (55.4), C9 (50.6), C13 (38.1), C18 (49.0)), methelene (CH2: C1 (38.7), C2 (27.4), C6 (18.2), C7 (34.3), C11 (20.8), C12 (25.5), C15 (30.4), C16 (31.9), C21 (29.6), C22 (36.6), C29 (109.1)) and methyl (CH3: C23 (27.7), C24 (15.2), C25 (15.7), C26 (15.6), C27 (14.1), and C30 (18.6)).

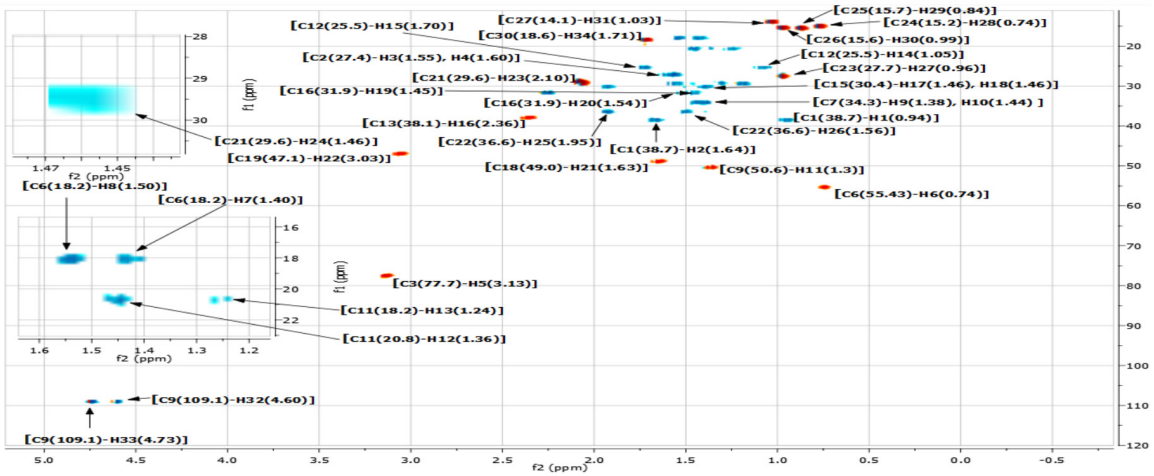


Supplementary Figure 3. DEPT-135 NMR spectrum of isolate (Betulinic acid).

Supplementary Data 4

HSQC NMR spectrum of isolate

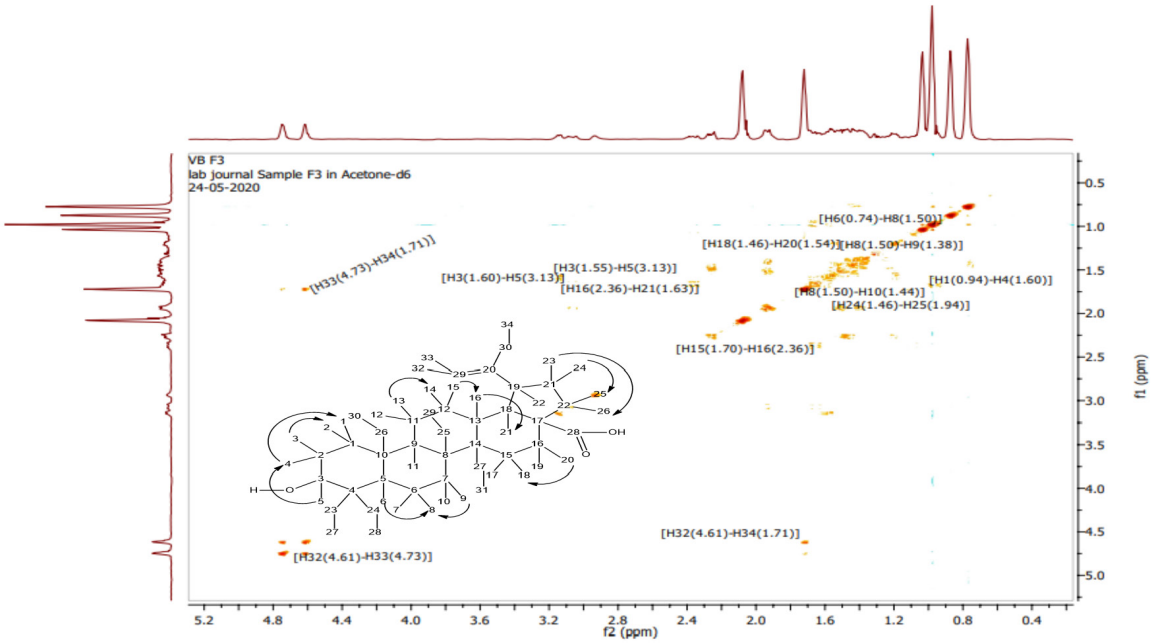
HSQC NMR (Supplementary Figure 4): The HSQC-NMR revealed the presence of 22 different direct [^{1}H-^{13}C] coupling signals comprising of [C1 (38.7)-H1 (0.94)], [C1 (38.7)-H2 (1.64)], [C2 (27.4)-H3 (1.55)], [C2 (27.4)-H4 (1.60)], [C3 (77.7)-H5 (3.13)], [C5 (55.4)-H6 (0.74)], [C6 (18.2)-H7 (1.40)], [C6 (18.2)-H8 (1.50)], [C7 (34.3)-H9 (1.38)], [C7 (34.3)-H10 (1.44)], [C9 (50.6)-H11 (1.38)], [C11 (20.8)-H12 (1.36)], [C11 (20.8)-H13 (1.24)], [C12 (25.5)-H14 (1.05)], [C12 (25.5)-H15 (1.70)], [C13 (38.1)-H16 (2.36)], [C15 (30.4)-H17 (1.46)], [C15 (30.4)-H18 (1.46)], [C16 (31.9)-H19 (1.45)], [C16 (31.9)-H20 (1.54)], [C18 (49.0)-H21 (1.63)], [C19 (47.1)-H22 (3.05)], [C21 (29.6)-H23 (2.10)], [C21(29.6)-H24 (1.46)], [C22 (36.6)-H25 (1.94)], [C22 (36.6)-H26 (1.56)], [C23 (27.7)-H27 (0.96)], [C24(15.2)-H28 (0.74)], [C25 (15.7)-H29 (0.84)], [C26 (15.6)-H30 (0.99)], [C27 (14.1)-H31 (1.03)], [C29(109.1)-H32 (4.60)], [C29 (109.1)-H33 (4.73)], [C30 (18.6)-H34 (1.71)].



Supplementary Figure 4. HSQC-NMR spectrum of isolate.

Supplementary Data 5

COSY NMR spectrum of isolate



Supplementary Data 6

HMBC NMR spectrum of isolate