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Cytotoxicity and Isolation of Betullinic Acid from *Anthonotha noldeae* Stem Bark

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Abstract

The cytotoxicity and isolation of betullinic acid from *Anthonotha noldeae* stem bark were examined in this study. The stem bark was extracted using methanol (MeOH) via the cold maceration technique. The MeOH produced a crude extract of 125.18 g (6.78%), which was further divided into *n*-hexane, dichloromethane (DCM), and ethyl acetate (EtOAc) fractions with yields of 10.23%, 11.74%, and 12.22%, respectively. Cytotoxicity assays using brine shrimp lethality tests revealed that the DCM and EtOAc fractions exhibited substantial concentration-dependent toxicity, achieving 100% mortality at 500–1000 µg/mL. The LC_{50} values for the DCM and EtOAc fractions were 16.33 µg/mL and 28.64 µg/mL, respectively. Column chromatography of the fractions led to the isolation of the compound M1 (5 mg), characterized using FTIR, ^{1}H NMR, and ^{13}C NMR spectroscopy. FTIR analysis identified functional groups consistent with carboxylic acids and alkenes, while NMR spectroscopy confirmed the presence of six methyl groups, vinyl protons, hydroxyl groups, and carboxyl functionalities. The compound was identified as the bioactive triterpene known as betulinic acid. The findings provide novel insights into the phytochemistry of *Anthonotha noldeae*, highlighting its potential as a source of bioactive compounds and application in traditional medicine.

Keywords: *Anthonotha noldeae*; Stem bark; Betulinic acid; Cytotoxicity; Bioactive compounds.

INTRODUCTION

Traditional African medicine encompasses diverse practices beyond herbal remedies, including dietary modifications, therapeutic fasting, and physical therapies. These practices often reflect deep cultural understanding and are integrated into social contexts (Aftab & Rehman, 2021; Tella, 1979; Sofowora,1984). Traditional medicine heavily relies on plant-derived secondary metabolites (PSM), which are structurally diverse and possess diverse pharmacological activities. Historically, PSM has been pivotal in the creation of numerous modern drugs, including pain relievers (e.g., codeine and morphine), and antimalarials (e.g., quinine) (Mendoza & Silva, 2018; Wilson & Deering, 2019). Plants are a valuable source of potential antimicrobial agents, but their efficacy and safety are essential factors to consider (Prestinaci *et al.,* 2015). Cytotoxicity assays, especially the brine shrimp lethality assay (BSLA), play a crucial role in assessing the safety of plant-derived antimicrobial compounds. These assays assess potential toxicity to living cells, identifying promising candidates with minimal side effects and paving the way for the development of effective antimicrobial therapies (Celik, 2018; Davey, 2021).

Anthonotha is an understudied African genus with 17 species predominantly found in the Guineo-Congolian region. Traditional uses of these plants include treating various ailments such as cancer, aphrodisiac issues, and skin infections (Oyedemi *et al.,* 2018). While limited research exists, phytochemical analyses of some species, particularly *A. macrophylla* and *A. cladantha,* have revealed the presence of alkaloids, tannins, flavonoids, and terpenoids (Yakubu & Olutoye, 2016; Bongmo *et al.,*2023). *A. macrophylla* extract has promising anti-cancer and anti-estrogenic properties (Yakubu & Olutoye, 2016). However, most *Anthonotha species* remain poorly understood, with limited scientific investigation into their chemical composition and pharmacological potential. There is no such report on *Anthonotha noldeae* and other *Anthonotha species*. Additionally, *A. noldeae* was reportedly used in traditional medicine to treat pneumonia in cattle (Nodza *et al.,* 2022).

EXPERIMENTAL METHODOLOGY

Collection, Preparation of Plant, Extraction, and Fractionation

The stem bark of the plant was collected from Taraba State in Nigeria and identified by a Botanist in the Department of Botany, Gombe State University. The stem bark was air-dried under shade at room temperature and then milled to powder (Kwaji *et al.,* 2023). The powdered plant material was kept under dry and cool conditions until required for extraction. The stem bark (about 1.85 kg) was extracted by exhaustive cold maceration in methanol for 7 days with regular shaking at intervals to ensure thorough extraction. The extract was filtered using Whatman No. 1 filter paper into a clean and dry container. The crude extract was concentrated on a rotary evaporator at 45 °C under reduced pressure. The concentrate was allowed to dry at room temperature under shaking to obtain a crude extract which was covered with aluminium foil and stored in a refrigerator until required. The methanol crude extract of *Anthonotha noldeae* (100 g) was partitioned with n-hexane, DCM, and EtOAc successively by suspending 20 g of the extract in 10% aqueous methanol in a 500 ml separating funnel, and 250 ml of the solvent. The mixture was swirled and allowed to stand until two distinct layers were formed. The lower layer was drained through the tap of the separatory funnel and the upper layer was poured into a separate container. This procedure was repeated 3 times, for each solvent type to ensure increased yield (Al-Matani *et al.,* 2015; Yohanna *et al.,* 2021).

Isolation Procedure

The most active EtOAc extract fraction was dissolved in methanol, pre-adsorbed onto silica gel, and dried. Column packing was done using the wet slurry method. Gradient elution was carried out with *n*-hexane/DCM and DCM/EtOAc at a 5% increase in the volume of the eluting solvent to yield several fractions. TLC was used in monitoring the fractions. The compound was purified using a recrystallization procedure after washing impurities from fractions with a single spot (Kwaji *et al.,* 2018). 180 fractions were obtained and pulled together based on TLC value. An isolate labelled **M1** with R_f values 0.58 in hexane:Ethyl acetate (4:1) was obtained.

Cytotoxicity Assay

Incubation of Nauplii Eggs, Preparation of Stock, and Working Solution

Artemia salina eggs were hatched in a sterile brine solution by dissolving 20 grams of sodium chloride in 1 litre of distilled water, adjusting the pH to between 8.0 and 8.5 using a 1 molar solution of sodium hydroxide (NaOH), and aerating continuously for 36-48 hours. Actively motile *Nauplii* were then collected for the assay. Working solutions were prepared from the 2 mg/mL stock solutions by appropriate dilutions of each extract with approximately 4.5 mL of the brine solution to obtain 1,000 µg/mL and serially diluted to 1 µg/mL from stock solutions (Akano & Akinsomisoye, 2024).

Brine Shrimp In vitro Cytotoxicity Analysis

Ten *Artemia salina Nauplii* were transferred via Pasteur pipette into 6 test tubes each containing 4.5 mL of brine solution. Each tube contained 0.5 mL of a specific test solution. A positive control containing potassium dichromate ($K_2Cr_2O_4$) was included (Kwaji *et al.,* 2023). After a 24-hour incubation period, the number of surviving *Nauplii* in each tube was quantified, and the percentage mortality was calculated. The median lethal concentration (LC_{50}), the concentration of the test solution that resulted in the mortality of 50% of the *Nauplii* population after 24 hours of exposure, was determined graphically (Akano & Akinsomisoye, 2024).

RESULTS AND DISCUSSION

Cytotoxicity Studies

The cytotoxic properties of extracts were evaluated using BSLA due to the sensitivity of brine shrimp (*Artemia spp*) to toxic substances (Hassan *et al.,*2024). The results demonstrated an apparent concentration-dependent effect of the test substance on *Nauplii* mortality. This trend was most pronounced at the higher concentrations of 1000 and 500 µg/mL, where 100% mortality (10 dead *Nauplii* each) was observed for both EtOAc and DCM and vice versa (Suryawanshi *et al.,* 2020). A mortality of 80% (8 dead nauplii) at 250 µg/mL, 70% (7 dead nauplii) at 125 µg/mL, 60% (6 dead nauplii) at 62.5 µg/mL, and 20% (2 dead nauplii) at 1 µg/mL the EtOAc fraction. A similar trend was observed in the DCM fraction, 70% (7 dead nauplii) at 250 µg/mL, 60% (6 dead nauplii) at 125 µg/mL, and 62.5 µg/mL, and 10% (1 dead nauplii) at 1 µg/mL. A similar trend was reported by (Akano & Akinsomisoye, 2024). The cytotoxicity for both fractions is represented in **Figure 1a.**

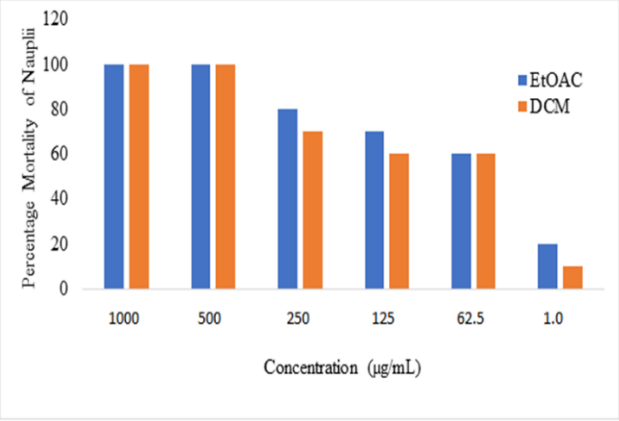


Figure 1a. %Mortality of Brine Shrimp Nauplii in DCM and EtOAc fractions.

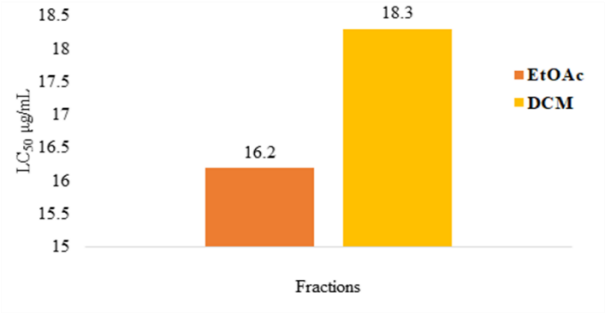


Figure 1b. LC50 of DCM and EtOAc fractions.

The toxicity of the extracts was also evaluated using LC_{50} values and compared to Meyer's and Clarkson's toxicity indices. The Meyer's index classified extracts with $LC_{50} < 1000$ µg/mL as toxic and those with $LC_{50} \geq 1000$ µg/mL as non-toxic (Meyer *et al.,* 1982). Clarkson's index classified extracts as non-toxic ($LC_{50} > 1000$ µg/mL), low toxic (500 µg/mL $\leq LC_{50} \leq 1000$ µg/mL), medium toxic (100 µg/mL $\leq$ LC50 $<$ 500 µg/mL), and highly toxic for $LC_{50} < 100$ µg/mL (Clarkson *et al.,* 2004). The LC_{50} of the fractions was analyzed by extrapolation from the graph equation. The LC_{50} for EtOAc and the DCM fractions were 16.33 µg/mL and 28.64 µg/mL, respectively. The EtOAc fraction is more toxic than the DCM fraction. This is evident in that it required a lower concentration of 16.33 µg/mL than the DCM fraction which required 28.64 µg/mL to cause the death of 50% of the nauplii. Although the EtOAc fraction was more toxic than the DCM fraction, both could be classified as strongly toxic based on Meyer's and Clarkson's toxicity indices. The LC_{50} of both fractions is represented in **Figure 1b.**

Spectroscopic Analysis of Isolated Compounds

The isolated compound was characterized via Fourier Transform Infrared (FT-IR), and Nuclear magnetic resonance (^{1}HNMR and ^{13}CNMR) spectroscopic methods.

FT-IR Analysis of M1

The compound was obtained as a white powder with an underlying pale yellow colour (5mg) with a melting point of 297 - 299 °C. The FT-IR spectra of **M1** in **Figure 2** were identified by correlating with the standard wavenumber (cm^{-1}) given in the literature (Nandiyanto *et al.,* 2019). The **M1** spectra revealed a broad peak between 3500 and 3200 cm^{-1} associated with the O-H stretch, and the absorption peak at 1387.16 corresponds to C=O stretching vibrations found in carboxylic acids. The isolated compound also showed peaks at 2914.80 cm^{-1} and 2847.43 cm^{-1} aligned with C-H (sp^3) stretching vibrations for methyl and methylene groups, respectively. The bending vibrations at 1172.93 cm^{-1} could also serve as a supporting guide for the methylene absorption observed at 2847.43 cm^{-1}. Another prominent peak was observed at 1734.05 cm^{-1} corresponding to C=O in carboxylic acid. An olefinic stretching (C=C bond vibration) was observed at 1620.46 cm^{-1} as a weak peak.

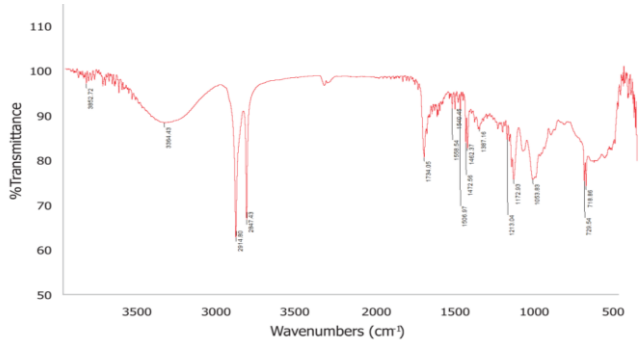


Figure 2. FT-IR spectrum of M1.

^{1}H NMR and ^{13}C-NMR Analysis of M1

The ^{1}H NMR (600 MHz, CHLOROFORM-*D*) spectrum of **M1** showed peaks at δ 4.74 (d, *J* = 1.3 Hz, 1H), 4.61 (s, 1H), 3.19 (dd, *J* = 10.2, 3.5 Hz, 1H), 2.94 (td, *J* = 10.8, 4.9 Hz, 1H), 2.35 (t, *J* = 7.5 Hz, 1H), 2.22 - 2.12 (m, 2H), 2.03 -1.92 (m, 2H), 1.69 (s, 3H), 1.06 (d, *J* = 7.4 Hz, 6H), 0.97 (d, *J* = 5.0 Hz, 7H), 0.94 (s, 3H), 0.82 (s, 3H), 0.75 (s, 3H), 0.69 (d, *J* = 9.4 Hz, 1H). The ^{1}H-NMR spectrum showed peaks typical of a triterpene nucleus. The peaks correspond to 6 methyl groups in ppm at δ 0.75 (3H, s), 0.82 (3H, s), 0.94 (3H, s), 0.97(6H, d), and 1.69 (3H, s). Similarly, a pair of singlets at δ 4.61 and 4.74 containing one H each due to the vinyl protons at carbon 29 were observed. The presence of a doublet with one proton intensity at δ 3.19 (dd, *J* = 10.2, 3.5 Hz) could be attributed to the proton attached to the carbon bearing the hydroxyl group at C-3. The chemical shifts indicate that compound **M1** is a lupane-type triterpene typical of betulinic acid (Ogunmoye *et al.,* 2017). This submission is supported by comparing the ^{1}H NMR data with several works of literature. Some studies have focused on the chemical shifts of the methyl groups at δ 0.75, 0.82, 0.94, 0.9, and 1.69 along with protons at δ 4.74 and 4.61. Furthermore, the chemical shift reported for betulinic acid from *A. cladantha* supports the identification of **M1** as betulinic acid (Bongmo *et al.,*2023; Ogunmoye *et al.,* 2017; Dais *et al.*, 2017). The ^{1}H NMR of M1 is given in Figure 3.

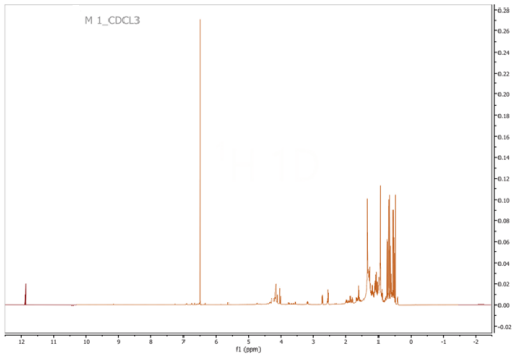


Figure 3. 1H NMR Spectrum of M1

^{13}C-NMR Analysis of M1

The ^{13}C-NMR (600 MHz, CHLOROFORM-*D*) spectrum of **M1** showed peaks at δ 14.6 (1C, s), 15.4 (1C, s), 16.0-16.2 (2C, 16.0 (s), 16.2 (s)), 18.3 (1C, s), 19.5 (1C, s), 21.2 (1C, s), 25.2 (1C, s), 27.6-27.9 (2C, 27.6 (s), 27.9 (s)), 30.1 (1C, s), 31.0 (1C, s), 34.2-34.3 (2C, 34.2 (s), 34.3 (s)), 36.9 (1C, s), 37.8 (1C, s), 38.0 (1C, s), 38.5-38.9 (2C, 38.5 (s), 38.9 (s)), 41.1 (1C, s), 42.1 (1C, s), 46.6 (1C, s), 48.6 (1C, s), 50.4 (1C, s), 55.3 (1C, s), 55.9 (1C, s), 78.8 (1C, s), 110.7 (1C, s), 148.8 (1C, s), 178.9 (1C, s). The observed signals correspond to six methyl groups (CH_3) at C-23, C-24, C-25, C-26, C-27, and C-30. Eleven methylene groups (CH_2) were observed at C-1, C-2, C-6, C-7, C-11, C-12, C-15, C-16, C-20, C-21, and C-29 while 6 methine groups (CH) were observed at C-3, C-5, C-9, C-13, C-18, C-19. Additionally, 1 carbonyl carbon (C=O) at C-28, and 6 quaternary carbons at C-4, C-8, C-10, C-14, C-17, and C-22 were also observed. The characteristic signals of C-OH and C=O (for COOH) at 78.8 and 178.9 for carbons C-3 and C-28 respectively are typical of betulinic acid (Bongmo *et al.,*2023; Ogunmoye *et al.,* 2017; Dais *et al.*, 2017). Based on the literature available, this is the first phytochemical investigation of *A. noldeae* leading to the isolation and cytotoxicology of betulinic acid. Betulinic acid is a lupane-type triterpene with a molecular formula of $C_{30}H_{50}O_3$ and a molecular weight of 456.7 g/mol. Betulinic acid is a promising alternative treatment for viral infections like HIV and selected cancers (e.g., melanoma, leukaemia, and prostate). It has anti-inflammatory properties that reduce inflammation in conditions like arthritis. It acts as an antioxidant to safeguard cells from oxidative stress damage potentially reducing ageing. Betulinic acid has been extensively researched for its antibacterial, antimalarial, and antiparasitic properties (Ghaffari *et al.,* 2012; Jiang, *et al.,* 2021). The ^{13}C NMR of M1 is given in **Figure 4**.

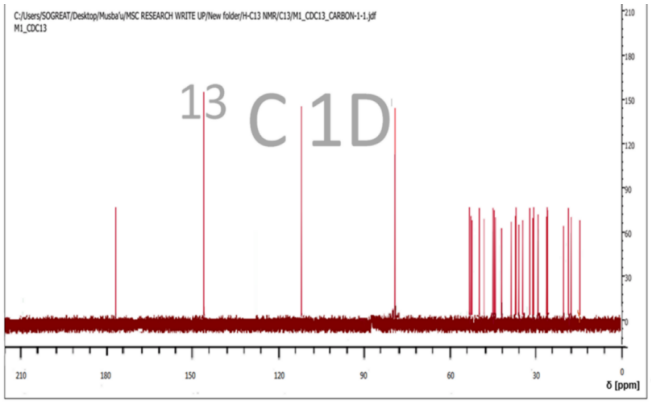
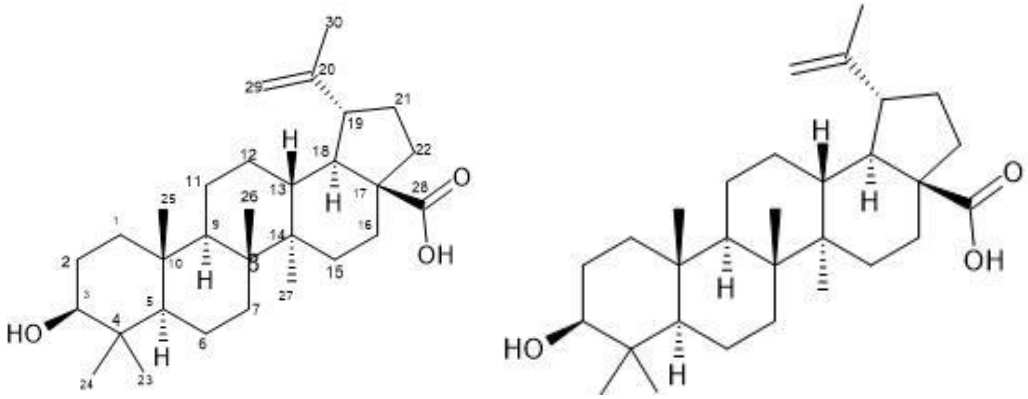


Figure 4. ^{13}C-NMR spectrum of M1

The proposed structure of compound M1 (Betulinic acid (Lup-20(29)-en-28-oic acid) is depicted in Figures 5a and 5b, showcasing the number and unnumbered carbons, respectively, after extensive analysis and literature comparisons.



(a) **(b)**

Figure 5. M1-Betulinic acid (Lup-20(29)-en-28-oic acid)

CONCLUSION

The study demonstrated that *A. noldeae* (Rossberg) Exell & Hillc is a potential source of therapeutic agents for anti-cancer drugs. The compound isolated, previously found in several plants, has widely documented anti-retroviral, anti-cancer, anti-inflammatory and antioxidant potentials. However, this is the first report of the phytochemical investigation and cytotoxic activity leading to the isolation of betulinic acid from the stem bark of *A. noldeae* (Rossberg) Exell & Hillc. Consequently, this study has made a significant contribution to the fields of natural product chemistry and pharmacology. The authors opine that the discovery of Betulinic acid *A. noldeae* could open novel therapeutic applications, targeting cancer and protecting against oxidative stress. Overall, this research underscores the importance of exploring diverse plant species for bioactive compounds.

Competing Interests: The authors declare that there are no competing interests.

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