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Research Article – Phytochemistry

Isolation, characterization and biological properties of betulin from *Entada africana* Guill. and Perr. (Mimosaceae)

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Abstract

The present study is aimed at the isolation, characterization and evaluation of some biological properties of betulin from *Entada africana* stem bark extract. A dichloromethane soluble portion of the stem bark methanol/acetone (1:1 v/v) extract was subjected to gradient elution using ethyl acetate in hexane (5 – 30 %) on an open column. A pure compound was obtained with R_f = 0.61 in hexane/ethyl acetate (8:2 v/v) after repeated washing and recrystallization from methanol and coded Enac1. The pure compound was analyzed using IR, ^{1}H & ^{13}C NMR and GC-MS. Clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Staphylococcus aureus* were used to assess the antibacterial activity of the pure compound while its preliminary Cytotoxicity was evaluated using brine shrimp nauplii. Based on the spectroscopic data obtained and in comparison with literature, the isolated compound was identified as betulin. The minimum inhibitory concentration (MIC) of betulin ranged between 62.50 - 250.00 µg/mL for all the four bacterial isolates in the study while its fifty percent lethal concentration (LC_{50}) was 10.00 µg/mL. Significant Cytotoxicity with moderate antibacterial activity was observed. The study therefore justifies the existence of bioactive compounds in the stem bark of *Entada africana* and its use in traditional medicine.

Keywords: *Entada africana*, isolation, betulin, characterization, cytotoxicity

Introduction

Entada africana is a West African medicinal plant that is quite useful in the treatment of many ailments and diseases such as fever, diabetes, diarrhea, hypertension, arrow poison and as tonic (Bako *et al.*, 2005; Tibiri *et al.*, 2010). The leaves, stem bark, roots, seeds and its gum resins are used in the management of several ailments in traditional medicine in different regions of West Africa such as Mali, Niger, Cameroon and Nigeria. Due to widespread use, the plant is now considered to be an endangered species (Banzouzi *et al.*, 2007). Despite the existence of several reports on biological studies on the crude extracts of *E. africana* such as antileishmanial, antibacterial, anti-inflammatory, antioxidant and wound healing properties (Tibiri *et al.*, 2010; Mbatchou *et al.*, 2011; Njayou *et al.*, 2013; Marthe *et al.*, 2014; Kwaji *et al.*, 2017), only few reports exists on isolation of bioactive compounds such as the acidic wound healing polysaccharides (Diallo *et al.*, 2001) and the antiproliferative triterpene saponins (Cioffi *et al.*, 2006) from the root extracts of *Entada africana.* Therefore, this calls for further investigation of the bioactive constituents of *Entada africana* in order to understand more about its immense healing potential. Consequently the study is aimed at the isolation, characterization and evaluation of some biological properties of betulin from *E. africana* stem bark extract.

Materials and Methods

Isolation of Betulin from Entada africana Guill. and Perrott.

The isolation of compound was carried out in accordance with the method described by Teke *et al.* (2011) with slight modification. Briefly, a dichloromethane soluble fraction (9.35 gram) of acetone/methanol (1:1 v/v) extract was loaded onto a silica gel 60 (60-200) column. Gradient elution was carried out on an open column with solvent combination of hexane/ethyl acetate (5-30%) to yield several fractions of 100 mL each. The different fractions were combined on their thin layer chromatography (TLC) profiles after concentration on a rotary evaporator at 45 ^{o}C. Fractions 27-32 was rechromatographed and eluted isocratically using silica gel 60 (70-230 mesh) with a solvent combination of petroleum ether/diethyl ether (7:3) and recrystallized from methanol to yield a pure compound, Enac1 (200 mg). The retention factor, Rf, value was found to be 0.61 in hexane/ethyl acetate (8:2). The pure compound was kept for spectroscopic analysis.

Analysis of Sample (Enac1)

Infrared spectrum of the sample was obtained and recorded on Perkin Elmer Universal ATR spectrum 100 FT-IR spectrometer, while the ^{1}H NMR and ^{13}C NMR spectra were recorded on 400 MHz Bruker Avance II Ultrashield with the sample initially dissolved in deuterated chloroform ($CDCl_3$). The GC-MS analysis for molecular mass determination was carried out on Shimadzu GC-MS-QP2010SE. The complete analysis was done at the Department of Chemistry, University of Kwazulu-Natal, Durban South Africa.

Minimum Inhibitory Concentration

Modified macroboth resazurin assay was used to determine the minimum inhibitory concentration (MIC) as described by Satyajit *et al.* (2007) and Karuppusamy &

Rajasekaran, (2009). Briefly, two fold serial dilutions of plant extract (1.80 ml, 100 mg/mL) and gentamicin (1.80 ml, 1 mg/mL) in a set of 9 test tubes was carried out with Mueller Hinton broth. To each test tube was added 0.20 mL of standardized bacterial inoculums (10^6cfu/mL). This was incubated for 12 hrs at 37°C for bacteria. Then 50 µL of 0.1% resazurin solution was added to each test tube and incubated for further 5 hrs. The MIC corresponds to the lowest concentration which prevented colour change from purple to pink in the test tubes.

Cytotoxicity Test

The brine shrimp lethality test was used to evaluate the preliminary cytotoxicity of Betulin. Brine shrimps nauplii were hatched from brine shrimp eggs in sterile artificial sea water (38 g/L NaCl and adjusted to pH 8.5 with 1M NaOH) in a plastic container with a porous middle partition through which hatched nauplii can move to an electrically illuminated zone of the container. A pinch of the brine shrimp eggs was added to one of the less illuminated compartment of the container and allowed to hatch overnight in the brine solution. Ten hatched nauplii per test tube were taken in 4.5 mL of brine solution. To each of the test tubes, 0.5 mL of either extract or standard at different concentrations (100 µg/mL, 1,000 µg/mL, 10,000 µg/mL) was added. Each test tube will now have a final concentration of 10 µg/mL, 100 µg/mL and 1000 µg/mL respectively. These were then incubated for 24 hrs under light. For each concentration, tests were carried out in triplicate. Percentage mortality rate for each concentration was calculated. The 50% lethality concentration was obtained using Probit analysis on software SPSS 16.0. Potassium dichromate was used as standard (Krishnaraju *et al*., 2005; Chaitali *et al*., 2010).

Results and Discussions

From 9.35 gram dichloromethane soluble fraction, the percentage recovery of Enac1 was found to be 2.10% as presented in Table 1 below. Enac1 was a powdery white substance that is soluble in hexane and chloroform.

Table 1 *Entada africana* Isolated Compound

Sl. No.	Compound	Rf value	Mass of isolate	Percentage recovery
1	Enac1	0.61	200 mg	2.10 %

EnacI

The IR spectrum of Enac1(Table 2) showed a broad absorption band at 3331 cm^{-1} and a sharp peak at 1042 corresponding to O-H and C-O stretching bands respectively. Other absorptions bands observed at 2925.43-2872.11 cm^{-1} are typical of C-H bond stretching bands. A weak band at 1638.41 cm^{-1} indicates the presence of a C=C absorption band (Elvira *et al*., 2009; Correa *et al.*, 2014; Barakat & Saleh., 2016).

In the proton NMR (Table 3), a doublet of doublets was seen at δ 3.19 ppm which shows the presence of α-oriented hydrogen at C-3. Furthermore, doublets can also be seen at δ 4.67, 4.55 and 3.79 ppm together with a methyl group at δ 1.66 ppm clearly suggests the presence of a lupane-triterpene nucleus. This is further substantiated by the C-13 NMR spectrum (Table 3). The characteristic C=C double bond of a lupane type nucleus was observed as shifts at δc 150.98 and 109.32 ppm. Also observed are the typical chemical shifts at δc 79.0 and 60.5 ppm of C-3 and C-28 to which hydroxyl (OH) groups are attached.

Table 2 FT-IR Spectrum of Enac1

Sl. No.	Frequency (cm^{-1})	Type of Vibration
1	3331.91	O-H stretching
2	2925.43	C-H stretching due to CH_3,
3	2872.11	CH_2 stretching of the first ring
4	1638.41	C=C stretching
5	1379.57	CH_3 and CH_2 bending vibrations
6	1042.68	C-O stretching due to CH_2OH
7	1014.60	C-O stretching of methine carbon
8	879.37	Ω (H-C-CH) of an umbrella alkene

The Mass spectrum data of Enac1 (Table 4) revealed the molecular ion mass to be 442 [M^+ $C_30H_{50}O_2$]. Subsequent fragment peaks of m/z 411, 234, 203, 189 and 81 confirm the presence of a lupane-type nucleus (Maryam *et al.*, 2013). Consequently based on comparison of spectra data with literature, Enac1 was found to be lup-20(29)-ene-3,28-diol with the molecular formula $C_{30}H_{50}O_2$ commonly known as betulin. The structural formula is as shown in Fig. 1 below. This is the first time that betulin is isolated from the stem bark extract of *Entada africana.*

Despite the discovery of this molecule about 200 years ago from other sources, there is still a growing interest in betulin due to its wide spectrum of biological activities such as antiviral, anticancer, antibacterial, anti-inflammatory (Maryam *et al.*, 2013) and hepatoprotective with potential for application in several therapeutic domains such as the anti-infectives, oncology and immunology (Sylwia *et al.*, 2015). A comparative study of the hepatoprotective effects of betulin, betulinic acid and oleanolic acid against ethanol-induced toxicity indicates that betulin exhibited the best hepatoprotective activity and even stimulated cell growth at 10-5 mM alcohol concentration. Lupeol, a structurally related triterpene to betulin reportedly offered hepatoprotective activity in cadmium-treated rats by improving tissue redox reaction and by inhibition of lipid peroxidation. The hepatoprotective mechanism of betulin is assumed to follow the same pattern (Agnieszka & Martyna, 2005). It is considered as the drug of the future partly due to its potency, availability and high selectivity index which are critical properties of a molecule with drug properties (Boryczka *et al.*, 2013).

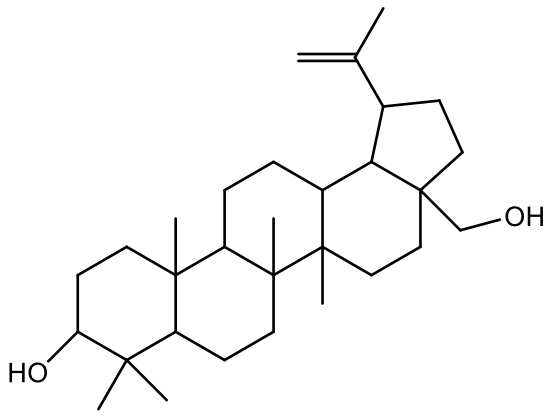

Fig. 1 Betulin Structure

Antibacterial Activity of Betulin

The lowest MIC for compound Enac1 was 62.50 µg/mL

Table 3 δ ^{13}C and ^{1}H NMR for Enac1and Literature Values

Carbon	Literature	Enac1		Literature	DEPT
C-atom	δC	δC	δH	δH	Enac1
1	38.8	38.3			CH_2
2	27.4	27.5		1.52	CH_2
3	79.0	79.0	3.19(1H, dd)	3.15	CH
4	38.3	38.1			C
5	55.4	55.3			CH
6	18.3	18.3			CH_2
7	34.3	34.3			CH_2
8	41.0	40.8			C
9	50.6	50.5			CH
10	37.4	37.2			C
11	20.9	20.9			CH_2
12	25.6	25.2			CH_2
13	37.0	37.2			CH
14	42.8	42.8			C
15	27.1	27.4			CH_2
16	29.3	29.7			CH_2
17	47.8	48.0			C
18	47.8	48.3			CH
19	48.8	48.3			CH
20	150.6	150.9			C
21	29.8	29.9			CH_2
22	34.0	34.3			CH_2
23	28.0	28.0	0.95(3H, s)	0.99	CH_3
24	15.3	15.4	0.77(3H, s)	0.80	CH_3
25	16.1	16.1	0.81(3H, s)	1.00	CH_3
26	16.1	16.1	0.95(3H, s)	1.02	CH_3
27	14.7	14.6	1.01(3H, s)	1.04	CH_3
28	60.8	60.5	3.70(1H, d)	3.56	CH_2
29	109.6	109.9	4.67(1H, s)	5.11	CH_2
30	19.4	19.3	1.66(3H, s)	1.82	CH_3

^{1}H- and ^{13}C-NMR Appendix I (a & b).

Table 4 Mass spectral data of Enac1

Enac1 GC-MS m/z	442[M^+ $C_{30}H_{50}O_2$], 411(5.8), 313(00), 288(04), 271(00), 245(06), 234(9.6), 220(7.7), 203(32.6), **189(100),** 175(38), 161(26), 147(42), 135(36.5), 121(0), 107(48), 95(42.2), 81(36.5), 69(28.8), 55(34.6), 41(30.7), 21(9.6)

on *Salmonella typhi* while the highest MIC value was observed for *Klebsiella pneumoniae* and *Staphylococcus aureus* at 250 µg/mL. This implies that Enac1 is very active on *S. typhi* and therefore could potentially be used in the management of infections or diseases associated with *S. typhi*. Additionally, it may also be used as a bacteriostatic agent for *K. pneumoni*ae and *Staph. aureus* related infections. The gentamicin (control) lowest MIC value of 3.96 µg/mL was observed for *Staphylococcus aureus*, a gram positive bacterium with a less complicated cell wall structure compared to the gram negative bacteria (*E. coli, K. pneumonia* and *S. typhi*). However, the highest MIC value of gentamicin was seen for *Salmonella typhi* at 31.25 µg/mL as expected (Table 5). According to Teke *et al*. (2011) classification of antibacterial activity, MIC values of 24.4 to 78.2 µg/mL is considered significant (i.e. MIC < 100 µg/mL) , while 100 < MIC = 625 µg/mL is moderate or weak (MIC > 625 µg/mL) against various resistant pathogens. On the basis of such a classification, betulin may be considered to possess a significant to moderate antibacterial activity. Several compounds isolated from *Entada abyssinica*, a member of the same genus as *Entada africana* exhibited moderate activity against several pathogenic organisms. The reported antibacterial activity is consistent with those obtained in this study. Two of the isolated compounds (methyl-3,4,5-trihydroxybenzoate, and benzene-1,2,3-triol) with MIC values ranging between 250 - 500 µg/mL demonstrated moderate antibacterial activity.

Table 5 Antibacterial of Activity of Betulin

Bacteria	Betulin	Gentamicin
	MIC (µg/mL)	MIC(µg/mL)
Escherichia coli	125	7.81
Kleb. pneumoniae	250	15.63
Salmonella typhi	62.50	31.25
Staph. aureus	250	3.96

Cytoxicity of Betulin

Probit analysis of the brine shrimp test data (Appendix Ic) of the isolated compound betulin gave an LC_{50} of 10.00 µg/mL while that of the covariate standard substance potassium dichromate gave LC_{50} of 8.33 µg/mL. According to Meyer *et al*. (1982) and McLaughlin *et al*. (1998) the isolate is cytotoxic and indicates the presence of a bioactive compound or a potential anticancer agent. Adoum (2009) reported that the crude chloroform fraction of *Entada sudanica* (syn. *Entada africana*) stem bark extract had quite significant cytotoxic effect with LC_{50} value of 10 µg/mL while the ethanol and aqueous fractions had LC_{50} values of 8 µg/mL and 6 µg/mL respectively. The observed higher Cytotoxicity values of the crude extracts could be indicative of the synergistic (additive) effects of the various phytocompounds. In herbal medicine the plant had been

used for the treatment of cancer, ulcers and malaria diseases. This high cytotoxic nature of the plant justifies its use in traditional medicine as an anticancer agent both within and outside Nigeria by some native tribes (Adoum, 2009).

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.21839/jaar.2018.v3i2.138

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