*Chemistry Research Journal*, 2018, 3(2):49-55

*Available online www.chemrj.org*

**Research Article**

**ISSN: 2455-8990**
**CODEN(USA): CRJHA5**

# Isolation and Characterization of Betunilic Acid from the Stem Bark of *Zizyphus Spina-Christi*

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**Abstract** *Zizyphus spina-christi* belongs to the family of *Rhamnaceae*. The present work revealed the presence of a pentacyclic triterpenoid "Betunilic acid" from the ethanol stem bark of the plant material which was subjected to liquid column chromatography (LCC) and purified using PTLC to obtain ZS-P1. The structure was established by various spectroscopic studies and comparison with with available data. The bioactivity of this compound was carried out using some clinical pathogens and the activity compared with a standard drug.

**Keywords** *Zizyphus spina-christ (Rhamnaceae),* stem bark, Betunilic acid, Chemical and Spectral studies.

## 1. Introduction

Research is often performed to purify and isolate biologically active compounds from their natural sources for characterization and identification. Purified compounds that are identified can be used for further therapeutic research on a chronic condition or illness. [1,2,3] If the target compound is known, it is very easy to compare preliminary spectroscopic data with literature data or to make direct comparison with the standard sample. However, if the target compound is unknown and very complex, a comprehensive systematic approach involving a variety of physical, chemical and spectroscopic techniques is required. Information on the chemistry of genus or the family of plant under investigation could sometimes provide additional hints regarding the possible chemical class of the unknown compound [4]. The plant *Zizyphus spina-christi* from the family *Rhamnaceae* is readily distributed in the Sahara and Sahel, from Senegal to the Sudan and Arabia [5]. Different parts of the plant are being used for various medicinal purposes among the local populace of Borno State of Nigeria. These include its use as an abortificient by the local women and for the treatment of venereal diseases, intestinal infections and as a psychotropic agent [6]. Recently, much attention has been focused on verifying the effectiveness of Zizyphus against cancer. In this regard, it has been shown that Zizypus extract alone or in combination with other botanical formulations reveal anticancer activities on several tumor cell lines [7,8]. In the literature, quite a number of chemical constituents have been reported from different part of the family *Zizphus*. Ghannadi *et al.* [9] reported the presence of volatile oils form the leaves of Z. Spina-christi (L) wild. Weinges and Schick [10] reported the isolation of a Dodecaacetyl prodelphinidin B3 from the dried leaves of ***Ziziphus spina-christi***. Lee *et al*. [7] reported the presence of cytotoxic triterpenoids from the fruit of *Z. Jujube.* Hong *et al.* [11] reported the isolation of betulinic acid a 'pentacyclic triterpenoid' from the fruit of Z. Jujube. Fernanda, *et al*. [12] reported the extraction of Betulinic Acid from *Zizyphus Joazeiro using focused micro-wave assisted extraction*. Dubey and Goel [13] reported the evaluation of a downstream technique for

extracting Betulinic acid from the bark of Z. jujuba while Guo *et al*. [14] reported the isolation of triterpenic acid from the fruit of Zizyphus species. The isolation of betulinic acid from the stem bark extract is being reported for the first time in this study.

## Materials and Methods

*Plant Collection and Identification:*

The stem bark of *Z. Spina-christi* was cleaned, air-dried in a laboratory, for a number of days and pulverised using mortar and pestle. The coarse powder was weighed and stored at room temperature in a plastic container**.** 900 grams of the dried plant materials was placed in a thimble. The thimble and its contents were introduced into a soxhlet extractor connected to a condenser and extracted for eight (8) hours with 4 litres of 95% ethanol. The crude extracts obtained after drying the concentrate was defatted with petroleum ether and concentrated to dryness in vacuo at $40^0$C. The dried plant extract was weighed, labelled and stored at room temperature for further analysis.

### Isolation of ZP-P1 from Ethanol fraction

35g of the ethanol extract of the stem bark of *Zizyhus spina-christi* was pre-absorbed in sufficient celite. This was packed with 150g silica gel and subjected to Liquid Column Chromatography (LCC) using an isocratic solvent of hexane: chloroform at different ratios and by collecting 20ml fractions to obtain over 120 fractions. However, only three fractions were sufficient for phyto screening and antimicrobial assay and were labelled C, D&E. D&E were microbially active, hence were combined and re-subjected to column chromatography. Based on thin layer chromatographic (TLC) analysis, the resulting portion was combined into five (5) fractions D1-D5. Fraction D5 showed a single spot after crystallization using acetone. It was obtained as an amorphous white powder which weighed 30mg.

### General Experimental Conditions

The 1H-nuclear magnetic resonance (NMR) spectra were recorded in $CD_3OD$ on Bruker AMX-500NMR spectrometers with TMS as an internal standard using UNIX operating systems at 400– 500MHz, respectively. The $^{13}$C-NMR spectra were recorded in $CDCl_3$ and $CD_3OD$ at 500MHz on a Bruker AMX-500 NMR spectrometer. The Fourier Transform Infrared (FTIR) spectra were recorded on a FTIR- 8400S spectrophotometer. Two dimensional NMR studies (COSY,HMBC, HSQC and NOESY) were conducted using similar instrument.Mas Spectrometry was conducted using TOF-MS, Angilient series ESI (HREST-MS) spectrometer.

Silica gel columns (60-120 mesh) was used for column chromatography (CC), pre-coated silica gel TLC (GF-254, 20 x 20 cm, 0.25 mm thick, Merck) were used to check the purity of the compound and were observed under ultraviolet (UV) light (250 and 600 nm), while 5% sulphuric acid, was used as a spraying reagent and also Iodine vapour.

## Results

### Spectra result for compound SB-C2 (Betunilic Acid)

The Structure of the component was determined spectroscopically using Nuclear Magnetic Resonance Spectroscopy (NMR), 1D-NMR and 2D-NMR, Fourier Transform Infrared Spectroscopy (FTIR) and also by comparing the obtained data with already existing literature. The results obtained are as shown in the tables and figures below.

**IR (cm-1):** 3450, 3075, 2870, 1650, 1600, 1450, 1350, 1100

The IR spectrum of compound ZS-P1 showed prominent stretching vibratuional peaks at OHstr, C=O str, C=C str, C-H str (unsaturated), C-H str (saturated) and bending vibrational peaks at ($CH_3$, $CH_2$, C-C).

**$^1$H NMR(δ)** 4.7135, 4.6327, 4.5837, 1.7065, 1.0036, 0.9753, 0.9515, 0.8576, 0.7516

$^1$H NMR(500mHz) spectrum of compound ZS- P1 confirmed the presence of five singlet at 0.75(3H,s,H-23) 0.85(3H, s, H-24) 0.95 (3H, s, H-25) 0.97 (3H, s, H-26), 1.00 (3H, s, H-27). These represent five angular methyl groups on the lupane skeleton. Another 3H singlet downfield at δ 1.70 (3H, s, H-30) represents the vinyl methyl

group C-30. The olefinic protons (H-29) resonate at 4.6 and 4.7ppm. For protons narrow range of chemical shift often results in peak overlap which was the case for compound ZS-P1.

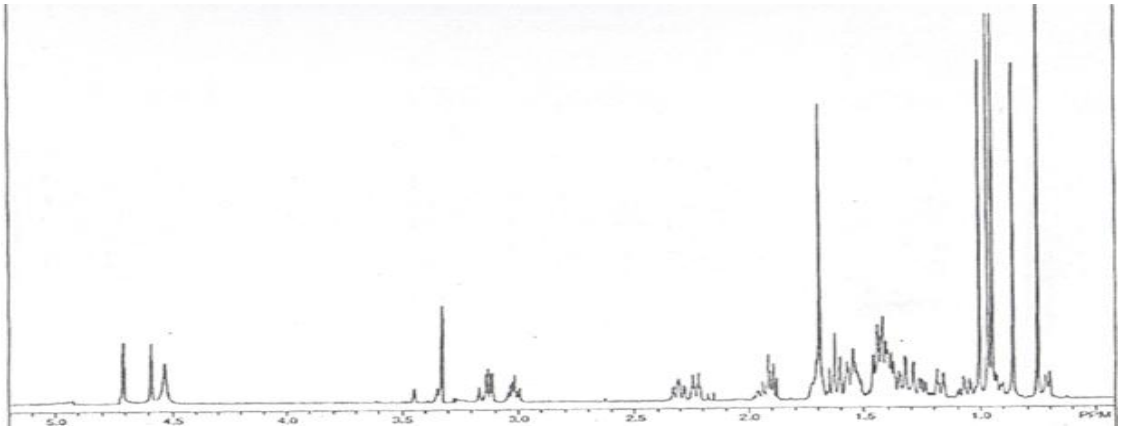


*Figure 1: $^{1}H$ NMR spectrum of compound ZS-P1 in MeOD at 500MHz*

**$^{13}$C NMR(δ):**180. 3142**,** 152.4123, 110.7041, 80.0180, 57.5366, 52.5593, 51.1426, 49.2678, 48.2730, 43.4524, 42.7093, 40.3719, 39.3636, 38.2161, 33.3428, 32.1504, 31.5442, 28.5216, 28.0223, 27.7039, 22.6964, 19.5653, 19.0729, 18.0996, 17.1359, 15.3508.

$^{13}$C NMR (500MHz) spectrum of compound ZS-P1 (Figure 2) showed 30 carbon atoms with signal at δ 180 (C-28) indicating the carbonyl atom of carboxylic group. Two signals at δ110 (C-29) and δ152 (C-20) were due to olefinic carbon atoms indicating that a double bond is present. Also there were signals at δ80 (C-3) and δ180 (C-28) showing that the hydroxyl and carbonyl groups were attached at these carbons, which were responsible for the high δ value. The signal at δ52.5 (C-9) and δ57.5 (C-17) were due to methine and carboxylic group attachment. While signals at δ51 (C-18) and δ49 (C-18) were due to another methine group and carbon double bond. With methylene attachment respectively.

In the APT/DEPT spectrum of the C-NMR (Figure 2), peaks were observed for six methyl groups ($CH_3$) at δ39.5(C-23), δ17(C-24), δ19.5(C-25) δ18(C-26) δ15(C-27) and δ28.5(C-30). Eleven methylene ($CH_2$) peaks at δ40(C-1) δ28 (C-2) δ19(C-6) δ36 (C-7) δ22(C-11) δ27(C-12) δ31.5(C-15) δ33(C-16) δ32.5 (C-21) δ38 (C-22) and δ110(C-29). Six CH (methine) peaks at δ80 (C-3) δ57(C-5) δ1152(C-9) δ48(C-13) δ51(C-18) and δ49(C-19). Finally, the quarternary carbon atom peaks with signal at δ40(C-4) δ42(C-8) δ38(C-10) δ43.5(C-14) δ57.5(C-17) δ152(C-20 and δ180(C-28). The high value of C-17 was due to the presence of carboxylic group attached to it at C-28 and also a cyclopentane ring. Similarly, C-20 is attached to adouble bond and methyl group while C-28 is part of a carboxylic group. The chemical shifts at δ 180, δ 152 and δ 110 were characteristic peaks for betulinic type of skeleton assigned to C-28, C-20 and C-29respectively.

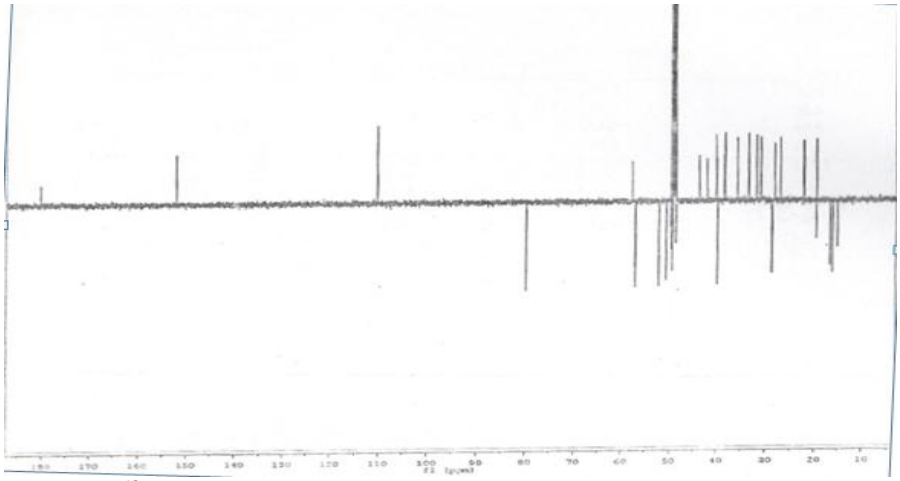


*Figure 2: $^{13}C$ NMR with APT spectrum of compound ZS-P1 in MeOD at 500MHz*

[2]DNMR analysis of Compound ZS-P1 (Table 1) showed HSQC spectrum which reveals connectivity's between the carbon atoms in the skeleton of the molecule and between C-H as well as position of unsaturation. The heteronuclear single quantum coherence (spectra) showed that there is an interaction between proton at 3.10ppm (H-3) and that of C-3 at 80.0 ppm. There is connectivity for proton H-5 at 0.80 ppm and C-5 at 57 ppm. . Connectivity exist for H-9 at 1.25 ppm and C-9 at 52.5 ppm. While proton H-18 at 1.70 ppm was connected to C-18 at 51.0 ppm. Similarly proton H-19 at 3.4 ppm was connected to C-19 at 49 ppm with proton H-29 at 4.7 ppm connected to C-29 at 110 ppm. The δ shift at 110 ppm for C-29 and δ shift at 180 ppm and 152 ppm for C-28 and C-20 were characteristic peaks for betunilic type skeleton (Figure 3).

**Table 1**: HSQC Correlation spectra analysis of Compounds ZS-P1

| $^{13}$C | C (ppm) | H(ppm) | $^{13}$C | C(ppm) | H (ppm) |
|---|---|---|---|---|---|
| **C-1** | **40** | **0.90** | **C-16** | **33** | **2.5** |
| **C-2** | **28** | **1.75** | **C-17** | **57.5** | |
| **C-3** | **80** | **3.1** | **C-18** | **51** | |
| **C-4** | **40** | | **C-19** | **49** | |
| **C-5** | **57** | **0.80** | **C-20** | **152** | |
| **C-6** | **19** | **1.45** | **C-21** | **32.5** | |
| **C-7** | **36** | **1.35** | **C-22** | **38** | |
| **C-8** | **42** | **1.80** | **C-23** | **39.5** | |
| **C-9** | **52.5** | | **C-24** | **17** | **0.85** |
| **C-10** | **38** | | **C-25** | **19.5** | **0.95** |
| **C-11** | **22** | **1.35** | **C-26** | **18** | **0.97** |
| **C-12** | **27** | **1.8** | **C-27** | **15** | **1.0** |
| **C-13** | **48** | | **C-28** | **180** | |
| **C-14** | **43.5** | | **C-29** | **110** | |
| **C-15** | **31.5** | | **C-30** | **28.5** | **1.7** |

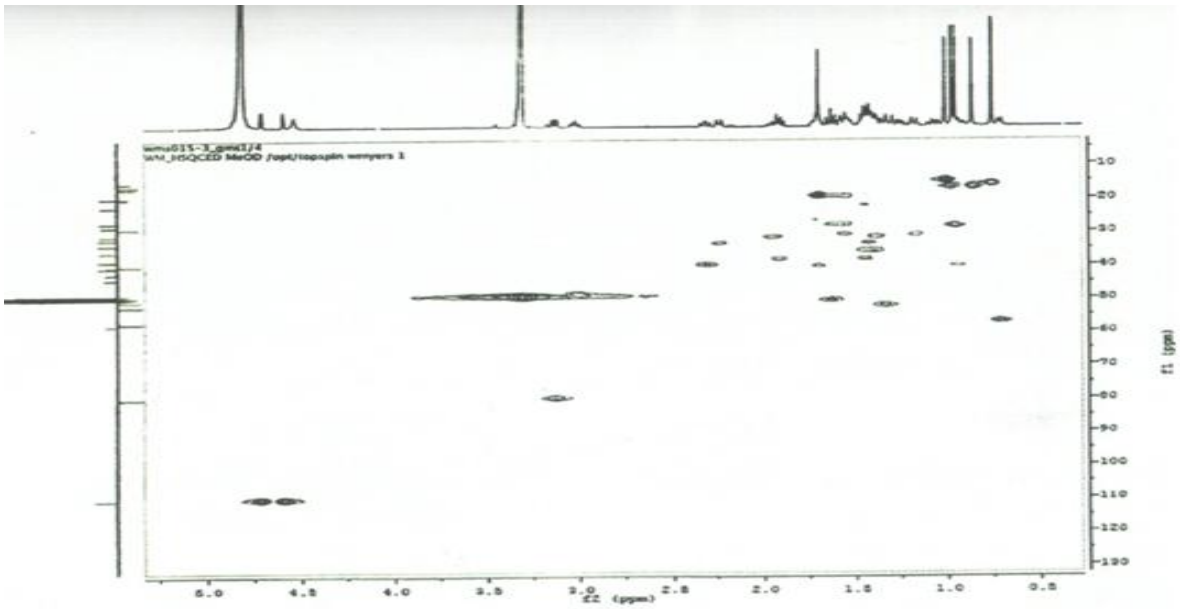


*Figure 3: HSQC spectrum of compound ZS-P1 in MeOD at 500MHz*

$^{2}$DNMR (HMBC) spectrum of Compound ZS-P1(Table 2) revealed strong long range correlation s between proton H-23, H-24 and C-3 at 80 ppm. Also, interaction was observed between proton (H-30) and carbon (C-20) at 152ppm. Proton at H-29 showed interaction with carbon at 110 ppm (C-29).Interactions between proton at H-30 and C-30 was also recorded at 28.5 ppm (Figure 4).

**Table 2**: HMBC (long range correlation) spectra analysis of Compounds ZS-P1

| $^{13}$C | C (ppm) | H(ppm) |
|---|---|---|
| **C-3** | **80 .0** | **H-23, H- 24** |
| **C-20** | **152** | **H-30** |
| **C-29** | **110** | **H-29** |
| **C-30** | **28.5** | **H-30** |

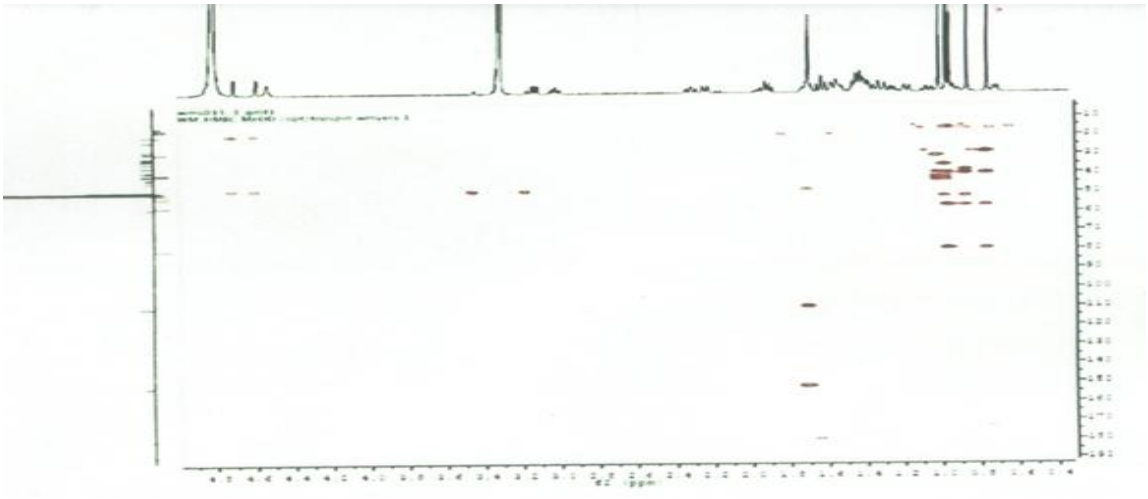


*Figure 4: HMBC spectrum of compound ZS-P1 in MeOD at 500MHz*

The mass spectrum of Compound ZS-P1 showed that the molecular ion peak appeared at m/z 456 in electron impact (EI) spectrum. This represents the molecular weight of the compound which was analysed as $C_{30}H_{24}O_3$. The spectrum recorded a base peak of 189 and other fragmented ions at 207, 327 and 395 which indicated a loss of water molecule as part of the fragmentation in hydroxylupenoic skeleton.

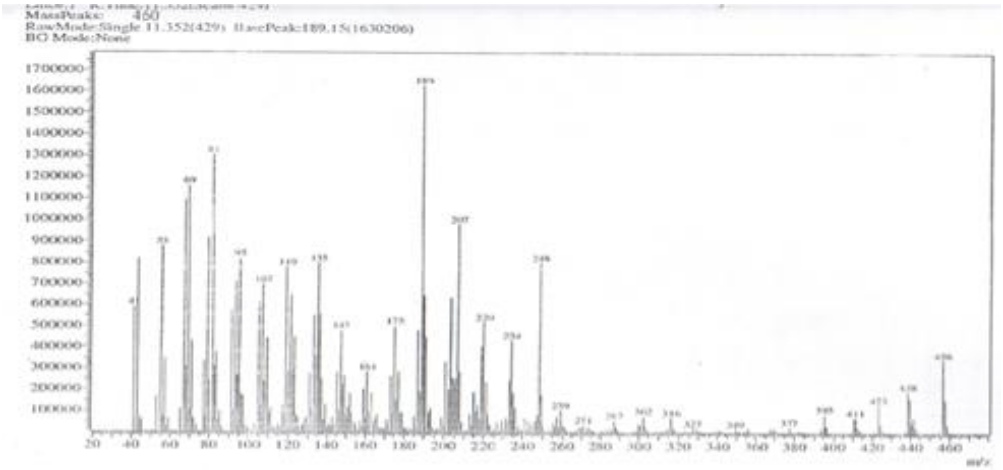


*Figure 5: Mass Spectrum of Compound ZS-P1*

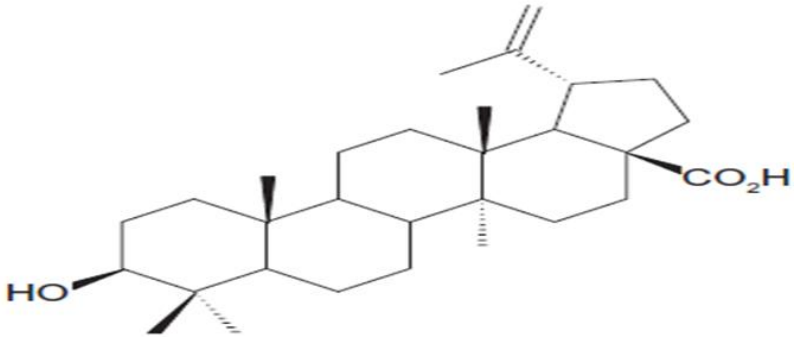
**Discussion**

In the elucidation of Compound ZS-P1, Chemical shift for $^{13}C$ NMR ranged between 15ppm-180 ppm while that of proton was between 1 ppm-5 ppm. The characteristic of the various carbon was deciphered in the APT spectra that is absorption signals or multiplicities for methyl carbons (primary carbon nuclei, quartet) were recorded within 15-40 ppm, methine carbons (tertiary carbons nuclei, doublet) were at 48-80 ppm and quaternary carbon (singlet) were at 37.5-57.5 (5 carbons) and then two others at 150 ppm and 180 ppm, were carbonyl (C=O) carbon. The remaining were methylene (secondary nuclei, triplet) carbons whose ranges were within 20-58 ppm including an alkenyl carbon at 110 ppm. The data from $^{13}C$ NMR, APT and HSQC spectrum indicated that there were seven carbons without protons, eleven (11) carbons with two (2) protons , six(6) carbons with one (1) proton and six (6) carbon with three (3) protons

Narrow range of chemical shift and peak overlap was evident in the proton NMR. However, a combination of IR signals and other spectroscopic tools alongside literature data enabled the interpretation. Forty six (46) protons were initially recorded. Two additional protons were later detected, one from ann aldehydic carbon with with IR signal at 1650 $cm^{-1}$ and δ 180 in the APT spectrum and an hydroxyl function with IR signal at 3400$cm^{-1}$ and δ80 which gave a total of forty eight (48) protons. An addition of three oxygens gave m/z 456 which represents the mass spectrum of compound ZS-P1. A spectral agreement was obtained on comparison with data obtained in earlier studies carried out by Peng et al. (1998) , Sharma et al. (2010) and Yunusa et al. (2012) on betunilic acid [15-17].

**Conclusion**

The $^{13}C$, $^{1}H$ NMR, HMQC, HMBC, APT and MS and IR for compound ZS-P1 were deciphered with multiplicities, peak integration and and molecular weight in order to establish the type of hydrogen, carbon and functional group and hence the molecular formula. Thus spectral studies have confirmed that the isolated compound ZS-P1 with a molecular formula $C_{30}H_{48}O_3$ to have hydroxylupenoic acid skeleton which conforms with 3-betahydroxy-lup-20-(29)-en-28-oic acid commonly known as betulinic acid.




**Acknowledgement**

The authors wish to appreciate the kind assistance of Prof Isa Marte Hussaini and Prof Ibrahim Iliya for helping in elucidating the structure and also to our University for the grants that aided the work.

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