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In vitro antibacterial activity of betulinic acid from *Psidium guajava* L

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Abstract
Utilizing chromatography and spectroscopic methods, 3-hydroxy-lup-20(29)-ene-28-oic acid or betulinic acid was isolated from *Psidium guajava* leaf extract and its structure was identified by spectroscopic methods. The minimum inhibitory concentration (MIC), was determined *in vitro* against *Klebsiella pneumoniae*, *Bacillus pumilus*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* by a microdilution method. Betulinic acid showed MIC of 0.025 and 0.056 mg/ml against *Staphylococcus aureus* and *P. aeruginosa* and this was comparable to the standard drug neomycin with an MIC of 0.072 mg/ml.

Keywords: Betulinic acid, *Psidium guajava*, isolation, spectroscopy, antimicrobial activity

Introduction
The rise of disease resistance to existing antimicrobial drugs pose a threat to human health. The World Health Organization (WHO) predicts that in a span of thirty years or fewer, antimicrobial resistance may overtake all other causes of mortality. Natural product-derived substances may help to curb this resistance (WHO, 2021) [1]. *P. guajava* L. is a small tree that thrives in tropical locations and is often known as guava. It has been widely used as a source of medicine throughout history to treat a variety of diseases (Kumar, *et al* 2021) [2]. In several cultures, the leaves, fruits, roots, and stem bark of the plant are used to relieve stomach-aches and diarrhea. In addition to being utilized as an anti-inflammatory, antispasmodic and cough suppressant. In addition to being used for the treatment of hypertension, cancer, obesity, and diabetes, the leaves, and pulp, can be utilized to treat gastrointestinal and respiratory conditions. (Ryu, 2012) [3]. The primary biochemical constituents of guava include triterpene acids, sesquiterpene alcohols, flavonoids, and essential oils (Shivani and Anjan, 2021) [4]. With more than 14,000 known structures, triterpenoids are a diverse subclass of terpenoids with structural variety. Several triterpenes are primarily pentacyclic, are found in large quantities in plant seeds, stem bark, roots, leaves, and waxy coatings. (Cardenas *et al*, 2019) [5]. Betulinic acid is a pentacyclic triterpene of the lupane class. It is reported to have good anti-tumor, antiinflammatory, anti-HIV, and anti-diabetic activities. Previously, it has been isolated from a variety of plants, including *Quisqualis fructus*, *Vitex negundo*, and *Chaenomeles lagenaria* (Ríos and Máñez, 2018) [6]. Several natural compounds have been shown to be effective against isolated strains of bacteria (Olga, 2019; Adeniran *et al.*, 2020; Masota *et al.*, 2023) [9, 7, 10] The guava plant is available worldwide and is used topically in the formulation of herbal drugs. It is therefore necessary to isolate and characterize its pure compounds and document the biological properties of these compounds against common infectious diseases and serve as baseline data for drug development.

Results
Isolation and structural elucidation of the compound: Guava leaves (500 g) were macerated with ethanol and the extract (15 g) was fractionated using a combination of silica gel column and preparative thin layer chromatography to give the compound as a white solid. The NMR (^{1}H, ^{13}C, HMQC, COSY and HMBC) spectra of the isolated compound were observed to be identical for what was described for betulinic acid (3β-hydroxy-lup-20(29)-ene-(28)-oic acid) (Oladosu *et al*, 2017) [8].

It was therefore identified as betulinic acid with the assignment of its ^{1}H and Carbon-13 chemical shift signals as in Table 1. The ^{1}H NMR (400 MHz, CD_3OD) showed six methyl proton singlet signals for H-23, H-24, H-25, H-26, H-27 and H-30 with their chemical shift values at δH (ppm) 0.98, 0.77, 0.84, 0.95, 0.72 and 1.71. Two downfield olefinic methylene proton signals of an isopropenyl unit were observed for H-29a (4.69 ppm) and H-29b (4.76 ppm). These in addition to the methyl proton signal at 1.71 ppm confirmed that the isolated compound is a lupane derivative. The ^{13}C NMR spectra (100 MHz, CD_3OD) of the isolated compound showed a chemical shift signal for the presence of a downfield oxymethine carbon (79.19 ppm) for the C-3 position (Table 1). The carbon signal at 177.6 ppm indicates a carboxylic acid carbon at the C-28. Proton-carbon single-bond correlations in the HSQC spectrum and proton-carbon long range correlations (HMBC) enabled the assignment of the proton and carbon signals for the compound (Table 1).

Table 1:1H-(400 MHz) and 13C-(100 MHz) NMR chemical shift data for Betulinic acid in CD_3OD

S/No	δ_C ppm	*Published	HSQC (δ_H ppm)	H-multi-plicity	H-H-COSY	HMBC
1	39.0	38.5	0.90,1.68	m	H_{-2}	
2	27.6	28.2	1.60	m	H_{-3},H_{1a}	
3	79.2	78.1	3.13 J(15.96, 11.24, 4.46)	ddd	H_{-2}	C_{-1}, C_{-23}, C_{-24}
4	38.2	39.4	-	-		
5	55.5	55.9	0.69	m	H_{-6a}	
6	18.5	18.7	1.40, 1.54	m	H_{-5}	
7	34.5	34.7	1.40	m		
8	41.0	41.0	-	-		
9	50.6	50.5	1.29	m		
10	37.3	37.5	-	-		
11	21.1	21.1	1.27, 1.41	m		
12	25.3	26.0	1.05, 1.75	m		
13	38.9	39.2	2.19	m		
14	43.0	42.8	-	-		
15	30.0	30.22	1.43, 2.25	m	H_{-15b},H_{-15a}	
16	32.1	32.8	1.42, 1.98	m	H_{-16a}, H_{-16b}	C_{-20},C_{-28}
17		56.6	-	-		
18	48.5	49.7	1.63	m	H_{-19}	C_{-28},C_{-30}
19	48.2	47.7	3.00	m	H_{-18}, H_{-21b}	C_{-20},C_{29},C_{18}, C_{-16}, C_{-30}
20	151.1	150.4	-	-		
21	29.9	31.1	1.27	m		
22	37.3	37.4	1.50,1.98	m		
23	28.2	28.5	0.98	s		
24	15.5	16.2	0.77	s		
25	16.3	16.3	0.84	s		
26	16.1	16.2	0.95	s		
27	14.7	14.8	0.99	s		
28	177.7	178	-	-		
29	109.5	109.5	4.63(J=4.6) 4.76(J=4.6)	sl, sl	H_{29a}, H_{29b}	C_{-19}, C_{-30}
30	19.5	19.4	1.71	s		C_{-20}, C_{-29}

m = overlapping proton multiplet, t = proton triplet, d = proton doublet, s = proton singlet.
*Oladosu *et al*, 2017 [8]

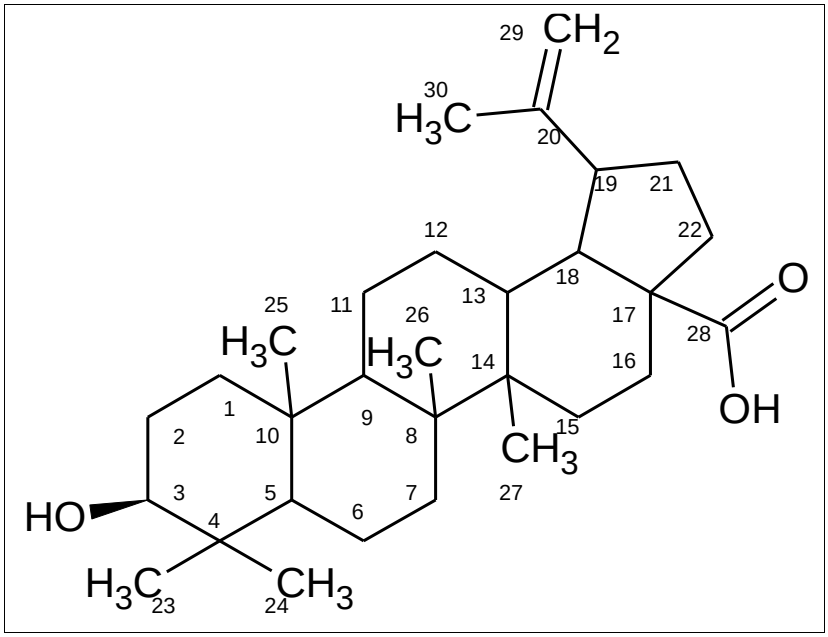

Fig 1: The structure of Betulinic acid (3β-hydroxy-lup-20(29)-en-(28)-oic acid).

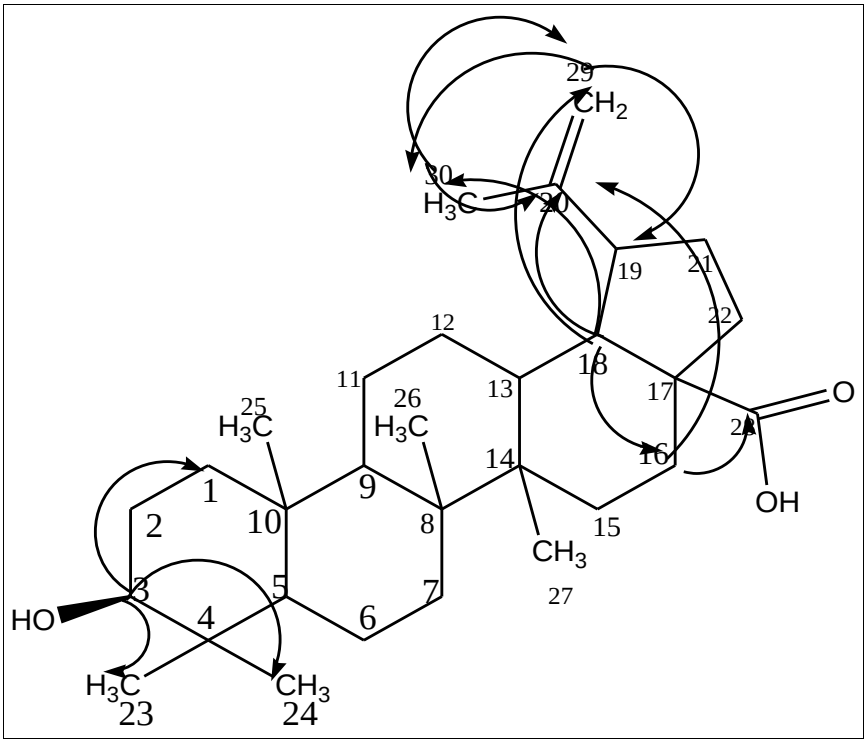

Fig 2: HMBC correlations for Betulinic acid

Antibacterial activity: Table 2 shows the MIC values for betulinic acid against the test microorganisms. The best activity was observed against *S. aureus* and *P. aeruginosa* at 0.25 and 0.056 mg/mL, respectively, which is comparable with the standard drug Neomycin which in this assay had an antibacterial activity of 0.072 mg/mL.

Table 2: Antibacterial activity of betulinic acid against some bacterial strains

Compound	Bacterial strains				
	K.P	B.P	E.C	S.A	P.A
Betulinic acid	7.39	1.75	6.25	0.025	0.056
Distilled H_2O	8.96	8.96	8.96	8.96	8.96
Neomycin	0.072	0.072	0.072	0.072	0.072

KP, Klebsiella pneumonia, BP, Bacillus pumilus, EC, Escherichia coli, SA, Staphylococcus aureus, PA, Pseudomonas aeruginosa.

Discussion

Betulinic acid, (3-hydroxy-lup-20(29)-ene-28-oic acid), is a lupane type pentacyclic triterpene found in plants especially in trees where it occurs up to 2.5% in their outer barks (Kumar *et al.*, 2021. Hordyjewska *et al.*, 2019) [2, 15]. It has been reported to show various biological activities. The *in vitro* antibacterial effect of betulinic acid on some selected clinical bacterial strains shows that betulinic has significant antibacterial effects against the tested organisms. Previous studies have reported the antimicrobial and anticancer activity of betulinic acid from other plants (Lou, *et al.*, 2021; Rodrigues, *et al.*, 2023) [13, 14]. This is an initial report of the antibacterial effects of betulinic acid from the leaves of *Psidium guajava* L.

Materials and Methods

Collection, identification, and preparation of plant material: Fresh leaves of guava were collected from around the University of Abuja, Gwagwalada Abuja, Nigeria. It was identified and authenticated at the Department of Botany, University of Abuja, Nigeria.

Preparation of extract: The plant material was dried and powdered, and 500 g of the powder was extracted by maceration using 500 mL of 70% ethanol and 30% distilled water. The extract was filtered to obtain a red-brown gummy solid extract after removal of the solvent on a water bath at 40 °C. The extract was kept in a clean, sterile, airtight glass container and stored at -4°C.

Chromatography of the extract.

About 30 g of the extract was fractionated on a column chromatography grade silica gel column (particle size 60-120 mesh) (Sayed, 2021) [11], the column was then eluted with dichloromethane. About 160 fractions were collected in the first run of the column. The fractions were analysed by TLC and the spots were observed under UV light at 256 and 366 mm and thereafter the plates were sprayed with 10% sulfuric acid reagent and heated to determine the UV active compounds as well as any UV-inactive compounds. Fractions 11, 13, 15, 17, 19, 21 and 23 appeared semi-pure under UV light. Therefore, further 13 and 12 fractions purification was performed using a smaller column and preparative thin-layer chromatography. Fractions 11 to 13 were combined and purified using DCM and n-hexane (9:1) to give a single compound. Spectroscopic analysis of the isolated compound via NMR spectra was carried out on a Brucker Avance III (400 MHz) spectrometer in CD_3OD.

Analytical data: Betulinic acid: White solid (15.2 mg), Melting point: 316-318°C. IR (film): ν_{max} 3400, 3000, 2923, 1465, 1100 and 757 cm^{-1}. ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; Antibacterial activity *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 1958), *Staphylococcus aureus* (ATTC 6538), *Klebsiella pneumoniae* (ATTC 13047) and *Bacillus pumilus* (ATCC 14884) were obtained from the University of Abuja (Mueller-H) and maintained on agar. Minimum inhibitory concentration (MIC) were determined (Anja *et al.*, 2010). The compound was dissolved in extraction solvent to a concentration of 50 mg/mL. Betulinic acid was assayed at 12.5 mg/mL on a 96-well microtitre plate and serially diluted two-folds to 0.098 mg/mL before loading each well with 100 L bacterial cultures. As a positive control, each bacterium was treated with 0.1 6 mg/mL of the antibiotic Neomycin. Negative controls were the solvent used to dissolve the compound and wells without bacteria. The tests were performed thrice. The microplates were incubated for 24 hours at 37°C. 40 L of p-iodonitrotetrazolium violet (INT) dissolved in water at a concentration of 0.2 mg/mL as an indicator of bacterial growth was added to the wells and incubated at 37°C for 30 minutes. The lowest concentration of betulinic acid that completely inhibited bacterial growth was recorded as the MIC.

Statistical analysis: The average of three duplicate MICs was used to represent the data. It was statistically analyzed (using GraphPad Prism 5 for Windows 7).

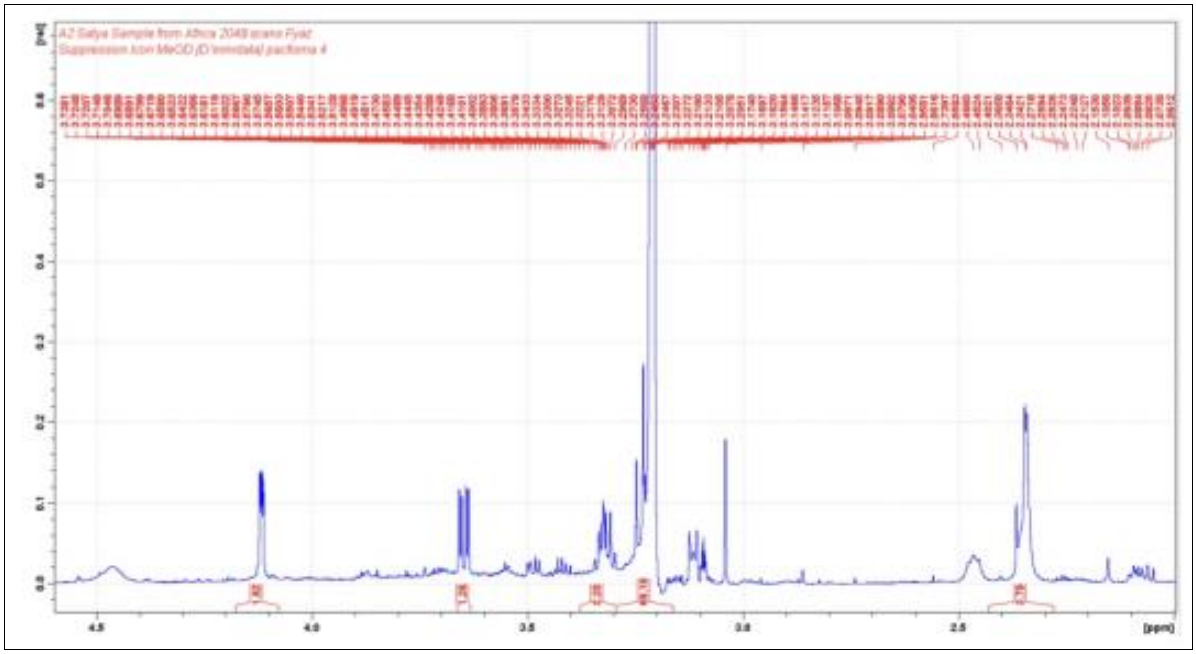

Fig 3: H1 proton spectrum

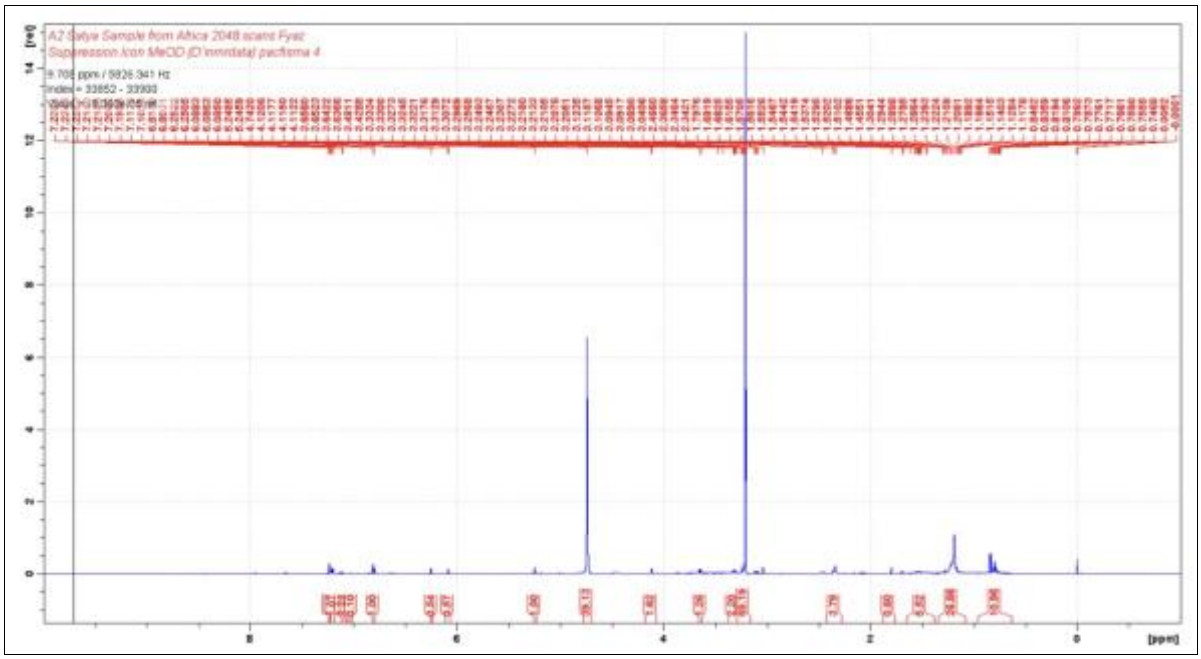

Fig 4: Showing the ^{1}H proton spectrum expansion between…..?

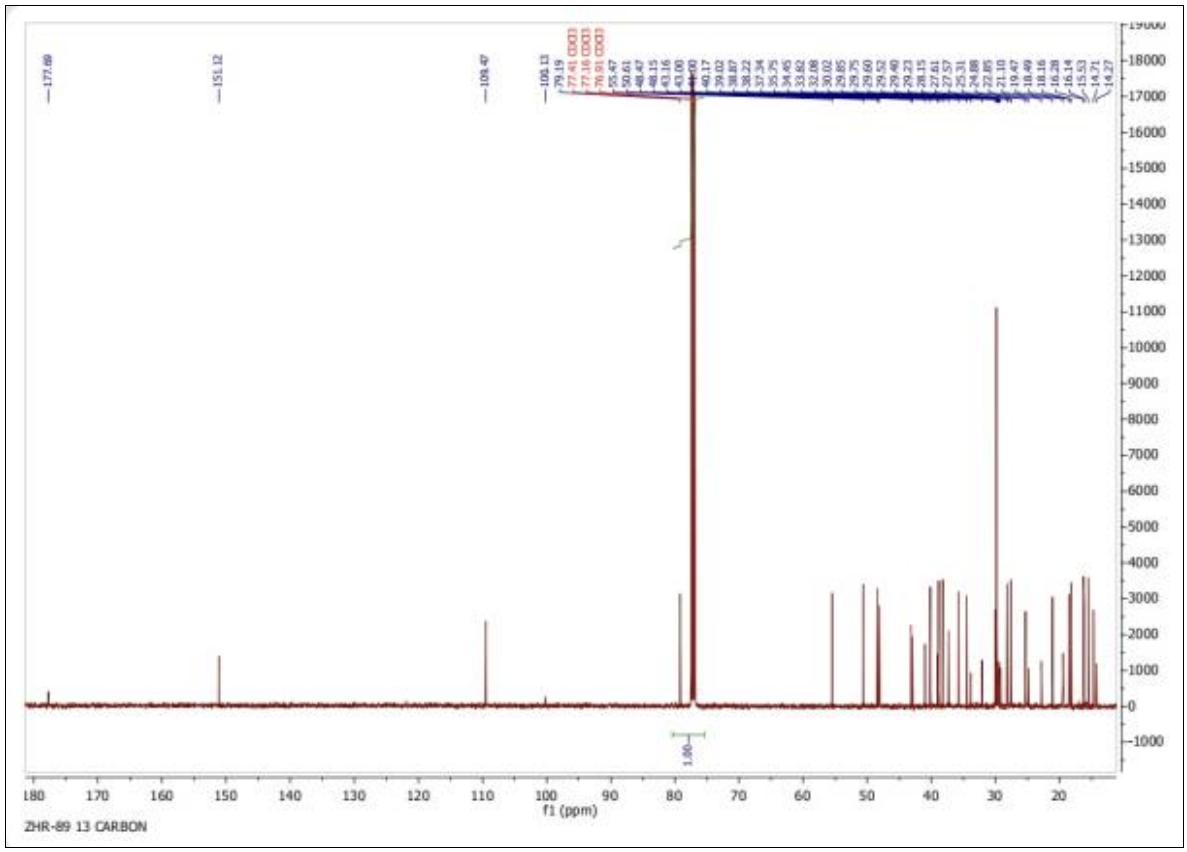

Fig 5: Showing the Carbon-13 spectrum

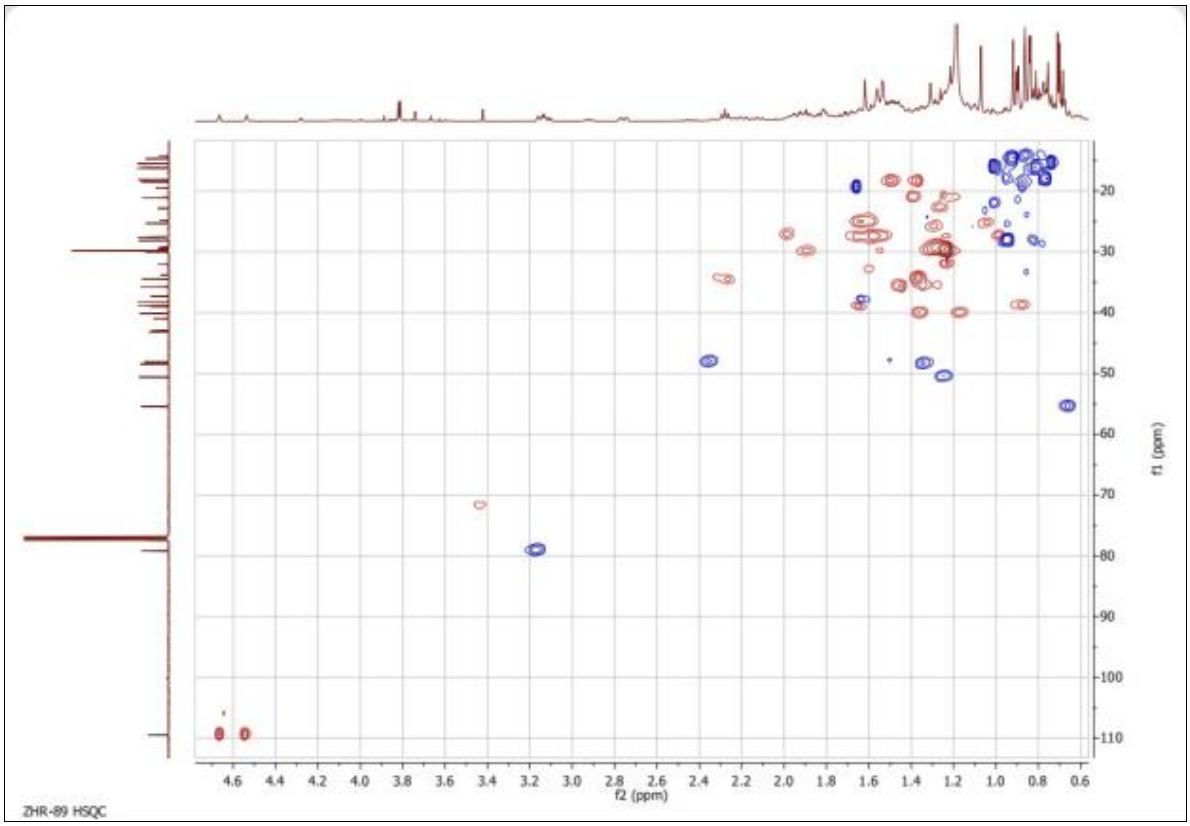

Fig 6: Showing the HSQC spectrum

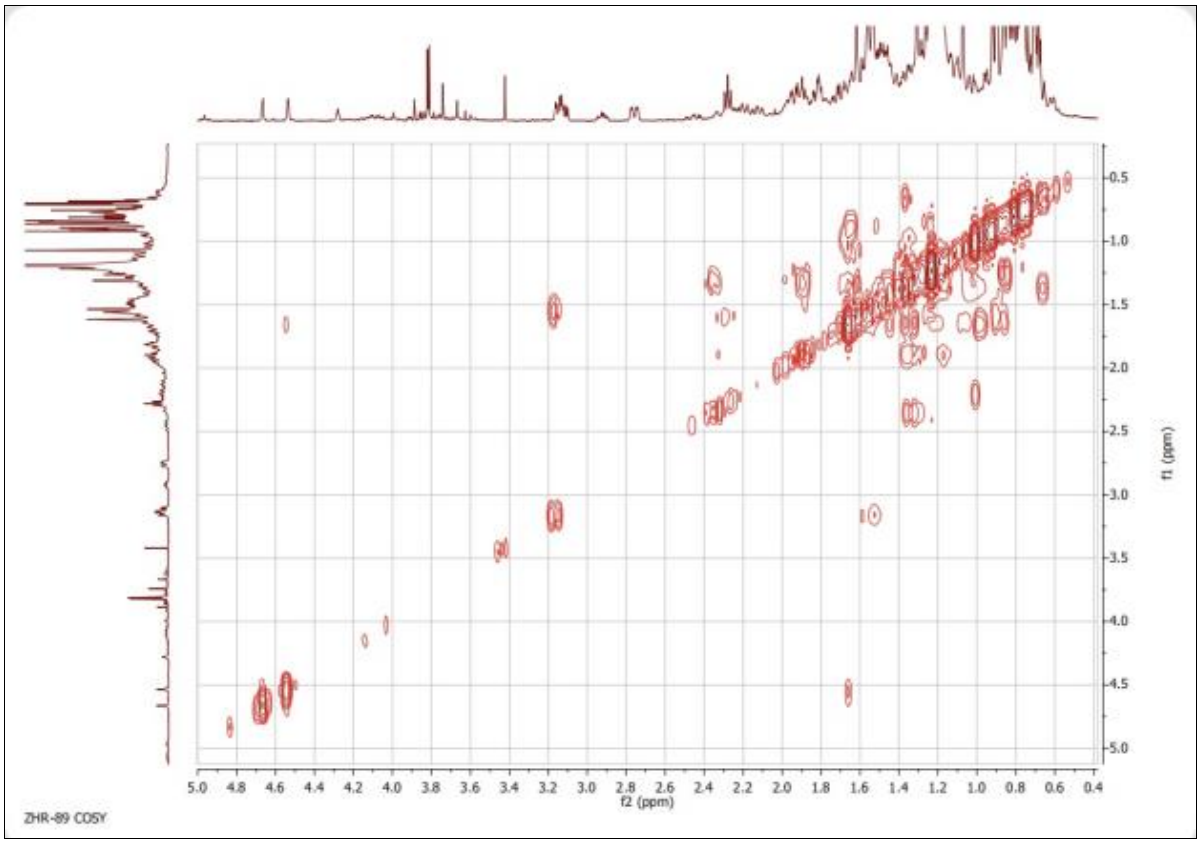

Fig 7: Showing the COSY spectrum

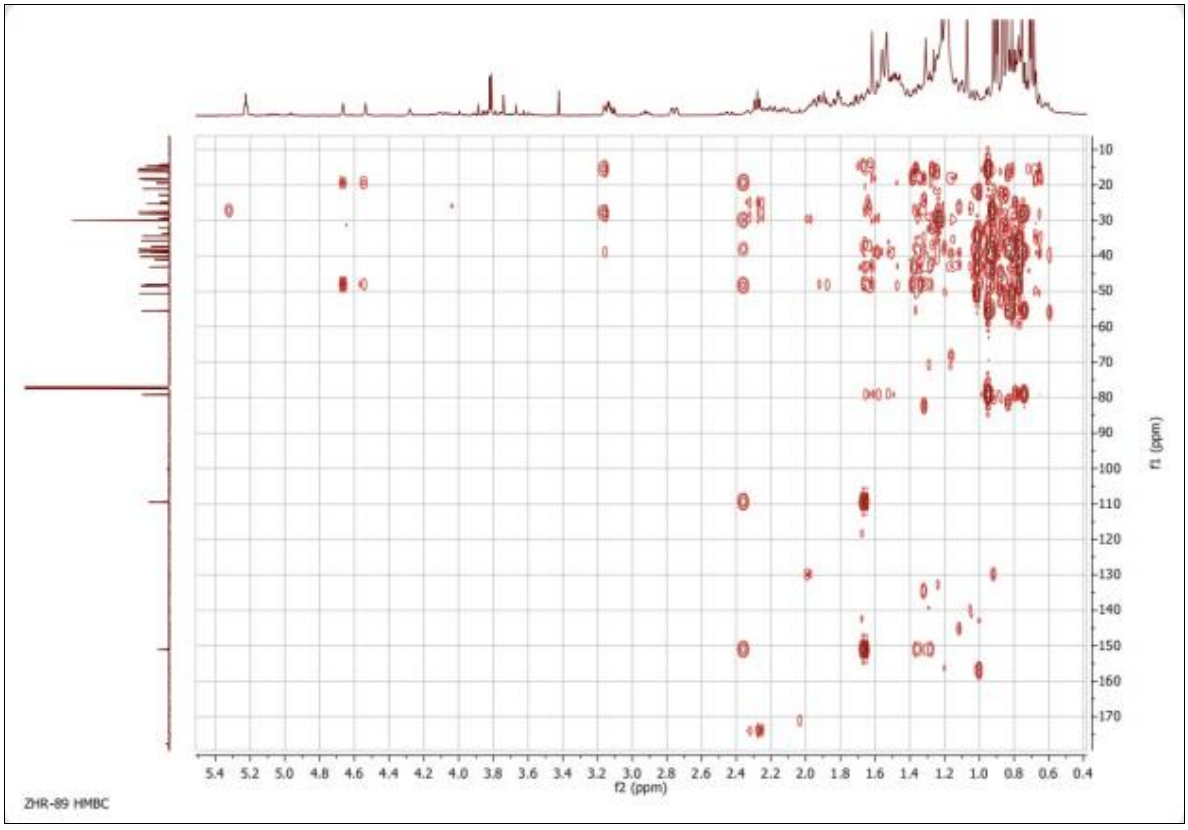

Fig 8: Showing the HMBC spectrum

Conclusion

Betulinic acid, was obtained from the hydroethanolic extract of *Psidium guajava* leaves. Antibacterial activity assay show comparable results to Neomycin.

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