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Bioactivity-Directed Fractionation and Isolation of Bioactive Compounds from Acetone Extract of *Grewia mollis* Juss.

[1]Mshelia E. H., [2]Adamu H.M., [3]Watirahyel P. and [1]Agbogo U.V.

[1]Department of Chemistry, Nigerian Army University, Biu, Borno State, Nigeria

[2]Department of Chemistry, Abubakar Tafawa Balewa University, Bauchi, Nigeria

[3]Department of Integrated Science,

Federal College of Education (Tech.) Gombe, Gombe State, Nigeria.

Corresponding Author: vagbogo@gmail.com

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ABSTRACT

The bioactivity directed fractionation and isolation of bioactive compound from acetone extract of *Grewia mollis* was carried out. The acetone extracts of the root bark showed good bioactivities and were subjected to isolation using column and preparative thin layer chromatography (TLC) using different stationary phases and eluting solvent system mixtures. The isolated compound was then subjected to antimicrobial, antioxidant and cytotoxic assay using standard methods. The structural elucidation of the isolated compound was done by comparing the melting point and Rf-values of the isolate with the standards from literatures**,** IR, MS, and ^{1}H-NMR. The isolated compound from the acetone extract was lup-20(29)-ene-3,28-diol (betulin), a triterpenoid and showed some degree of bioactivities. Betulin showed moderate antioxidant activity by bioautography method while the cytotoxic activity revealed that the isolated compound had LC_{50} value of 81.92 µg/ml. The result for the antimicrobial activity of the isolate using bioautography also showed that the compound isolated inhibited all the test organisms moderately. The bioactivities of the isolated compound from the root bark of *Grewia mollis* used for the treatment of infections and inflammatory diseases were justified.

Keywords: Bioactivity, *Grewia mollis*, isolation, bioautography and betulin

INTRODUCTION

Medicinal plants are sources of crude traditional herbal medicines used in many indigenous communities to reduce disease risk and treat and manage some diseases [1]. This is attributed to their potent antimicrobial, antioxidant, and anti-inflammatory effects. It is estimated that 80–90%

of the world's population depends on conventional drugs to meet primary health care needs, many of which involve the use of plant extracts [2, 3]. These statistics support the belief that traditional herbal medicines are widely used by a significant proportion of the world's population [4]. Natural products, such as plant extracts, have opened new horizons for discovering therapeutic agents [5].

Research on plants with medicinal properties is ongoing to identify the chemical components responsible for their activities, to justify the ancient claims of traditional healers, and to prove the long-term healing potential of some of the medicinal plants [5]. Today, researchers are exploring plants broadly for their pharmacological activity as a source of major advances in drug discovery. Plants found in Nigeria are well known for their therapeutic properties, including anti-inflammatory and antimicrobial effects [6]. These plants contain a variety of bioactive compounds, such as alkaloids, flavonoids, tannins, saponins, and terpenoids, which significantly contribute to their medicinal value [6]

The phytochemicals found in medicinal plants have recently attracted significant attention because they may serve as a springboard for new chemical entities that can serve as lead ingredients in the selection process for developing a new drug [7]. It is well-established that most phytochemicals found in herbal plants, including phenolic, flavonoid, steroid, saponins, terpenoid, and coumarins, have biological and pharmacological effects on diseases and illnesses [7]. Research into medicinal plants and the industrial production of phytomedicine are enhanced by the current emphasis on the use of natural products in the identification, creation, and formulation of novel compounds to supplement pharmaceuticals and synthetic products

The choice of extraction technique is very important in quantitative and qualitative studies of bioactive compounds derived from plant sources [8]. The study of bioactive compounds has fueled a growing understanding of the various bioactive compounds derived from nature. Some areas, such as the pharmaceutical, food additive, and natural pesticide industries, have developed interest in the study of bioactive compounds derived from natural sources due to their less negative effect.

With advances in phytochemical analytical techniques, several active principles of many medicinal plants have been isolated and introduced as valuable drugs in modern systems of medicine. The most important of these bioactive compounds are alkaloids, flavonoids, tannins and phenolic compounds [9-11]. Methods used to extract medicinal plants include maceration,

infusion, decoction, percolation, digestion, Soxhlet extraction, superficial extraction, ultrasound-assisted extraction, and microwave-assisted extraction. The fractionation of the extracts into simpler fractions was usually done using a fractionating funnel, while the isolation and purification of the secondary metabolites utilize chromatography techniques, including thin-layer chromatography, column chromatography (CC), high-performance liquid chromatography (HPLC), paper chromatography (PC), and gas chromatography (GC) [12-14].

The choice of appropriate extraction, isolation, and purification methods depends on the nature of the plant material, solvent used, pH of the solvent, temperature, and solvent-to-sample ratio. It also depends on the intended use of the final products [2,12, 14, 15].

This study was carried out to isolate, purify, characterize, and determine the bioactivity of the isolated active compound from *Grewia mollis*, a plant used in medicinal preparation and food by the people of North Eastern Nigeria.

MATERIALS AND METHODS

Collection and preparation of plant material

The root bark of *Grewia mollis* was collected in Hawul Local Government Area of Borno State, Nigeria in April 2026. The soil was removed by gently washing the roots under running tap water, then the root bark was removed, air-dried in the shade at room temperature, and pulverized into powder.

Extraction of plant material

The extraction was carried out using a Soxhlet apparatus using solvents of increasing polarity serially with hexane, ethyl acetate, acetone, ethanol, and water as solvents for 8 hours [16].

Antibacterial activity (Bioautography)

Masoko and Eloff [17] and Begue and Kline [18] methods were used to determine the antibacterial activity.

Brine shrimps Cytotoxicity analysis

The cytotoxic activity of the isolated compound was carried out as described by Abdulrani *et al.* [19], Adoum [20], Clarkson and Thompson [21], Meyer et al. [22].

Qualitative analysis of antioxidants (Bioautography*)*

The qualitative antioxidant activity of the isolated compound was done using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described by Masoko and Eloff, [17]; Brand-William *et al.* [23].

Purification

The solvent-solvent separation was carried out as described by Gailliot [29]; Houghton and Ramon [30]; Suffness and Douros, [31] to purify the extract.

Isolation

Fractions F_3, F_4, F5, F6, and F8 from the chloroform fraction of the acetone extract of the root bark of *Grewia mollis* showed some degree of antimicrobial activity on the test organisms using the disc method. The highest antibacterial activity was shown by fraction F_5, but the fraction was very small to be subjected to another column since it showed about 4 spots on TLC when eluted with EMW and sprayed with P-anisaldehye/ sulphuric acid spray reagent. Fractions F_3 and F6 also showed good activity but were not used for further separations due to their small quantities, and they also showed several spots on TLC. Fraction F_4 showed moderate antibacterial activity with about 4 spots on the TLC when developed in EMW and sprayed with P-anisaldehye/sulphuric acid spray mixture. About 2.3201g of F_4 was soaked on glass wool and introduced into the column containing silica gel 60-120 mesh and eluted with the solvent system hexane: chloroform (9:1, 8:2,6:4), and five fractions were obtained after the recombination of the fractions based on their TLC profile.

All fractions showed some antibacterial and antioxidant activity by bioautography, with the largest zones of inhibition in fraction FAC4 (0.3211 g) and fraction FAC5 (1.2320 g); the remaining fractions were negligible in quantity and were not considered for further separation because they showed several spots. Fraction FAC_5 was re-subjected to several column chromatography using different columns and solvent systems, followed by re-separation on preparative TLC using TLC silica pre-coated (0.5- 1 mm) and eluted with chloroform: hexane: methanol (16:4:1) to obtain three fractions. Fraction one was washed with hexane and ethyl acetate to obtain compound P (0.2519 g). The second fraction was washed with the same solvent mixture to obtain compound Q (0.620 g) with only one spot on TLC analysis. The compounds in the third

fraction were not isolated because the recovery percentage was too low for further separation. All three fractions showed some antibacterial and antioxidant activity by bioautography.

Fourier transform infrared spectrophotometer (FTIR)

FTIR analysis was carried out using a PerkinElmer Frontier MIR/NIR Fourier Transform Infrared spectrophotometer (FTIR) (Perkin-Elmer Inc., Wellesley, MA, USA). The isolated compound (0.01 mg) was ground with 0.02 mg dried potassium bromide (KBr) as a carrier and pressed into a thin, transparent film on an FTIR carrier-nut. The spectra were recorded at room temperature between 4000 and 400 cm^{-1} with 4 cm^{-1} resolutions, running 10 scans.

Nuclear magnetic resonance spectroscopy (^{1}H-NMR)

The nuclear magnetic resonance spectroscopy (NMR) of the isolated compound was run for the proton (^{1}H) as described by Gumel et al. [32].

GC-MS instrumentation for the isolates

The samples were analyzed for their chemical compositions on a Shimadzu GC-MS-QP 2010 Plus instrument (Shimadzu, Japan) equipped with a GC-MS and an Agilent DB-5 ms column (30 m long x 0.25 mm internal diameter x 0.25 µm film thickness). Samples were prepared by dissolving an aliquot in 2 cm^3 of pyridine. We withdrew about 1.5 cm^3 of this solution using a borosilicate glass syringe and filtered it into an amber GC-MS vial (VZS-0209A-100; Cronus, UK) through a 0.02 µm disposable PTFE filter cartridge (Sartorius Stedim, Germany).

The GC-MS method was adapted from Gumel et al. (2012). A sample (1 µl) was automatically injected into the GC-MS at a split ratio of 1:50. The injection temperature was 280 °C. The GC oven temperature ramping was set as follows: 40 °C for 1 min then increased to 120 °C at 15 °C per- minutes; held at 120 °C for 2 min then increased to 250 °C at 10 °C per-minutes; then held at 250°C for 15 minutes. Helium (0.41 bar) was used as the carrier gas at a flow rate of 48.3 cm^3 per minute. Mass spectra were acquired at 1250 scan speed, 50-600 m/z, using an electron impact energy of 70 eV, at a 200 °C ion-source temperature and a 280 °C interface temperature.

RESULTS AND DISCUSSION

Characteristics of compound Q

The isolated compound Q as shown in Table 1 is white amorphous powder with a melting point of 254 ^{o}C with the following R_f values of 0.6133 in cubic meter (CBM) of chloroform: benzene: methanol (10:5:3), 0.4351 in CEF (Chloroform/ethyl acetate/formic acid) 10: 4:1 and 0.7566 in EMW (ethyl acetate /methanol/water (40: 5: 2) eluting solvent mixtures and sprayed with P-anisaldehye/sulphuric acid spray reagent. The molecular formula of compound Q is $C_{30}H_{50}O_2$ based on GC-MS, with a molecular ion peak at 442. The characteristics of compound Q is represented in Table 1.

Table 1: Characteristics of compound Q

Texture	Colour	Melting point (°C)	RF values of isolates		
			EMW	CBM	CEF
Amorphous powder	white	257	0.7566	0.6133	0.4351

Spectra analysis of Compound Q

Fourier Transform Infrared Spectroscopy of Compound Q

The IR spectrum values (KBr) of compound Q are shown in Table 2. The IR spectrum shows characteristic absorption bands from stretching vibrations of different groups at 3301.21 cm-1, 2972.10 cm-1, 1643.20 cm-1, 1430.33 cm-1, 1021.23 cm-1, and 832.13 cm-1.

Table 2: Fourier Transform Infrared Spectroscopy of Compound Q

S/N	Frequencies cm^{-1}	Description
1	3301.21	OH stretching of free hydroxyl group
2	2972.10	C-H asymmetrical stretching of CH_3
3	2871.00	C-H stretching of CH_2
4	1643.20	C = C stretching of alkenes
5	1430.33	C-H bending of CH_2
6	1332.31	C-H bending of CH_3
7	1265.06	C-O vibration of CH_2 – OH
8	1021.23	C-O stretching vibration of CH_2 – OH
9	832.13	H-C-H stretching vibration of alkenes

^{1}H-NMR spectroscopy of Compound Q

The ^{1}H-NMR spectrum and data of compound Q are shown in Figure 1 and Table 3. ^{1}H- NMR (500 MHz, $CDCl_3$), δ (ppm),0.68 (d, 1 H), 0.73 (S, 3H, H-24), 0.83 (S, 3H, H-25), 0.93(S, 3H, H-27), 1.00 (S, 3H, H-23), 1.05 (S, 3H, H-26), 1.45 (dd, 3H, H-30), 2.40 (dt, 1H, H-19) doublet of triplet of one proton, 3.20 (dd, 1H, H-3) one oxymethine proton, 3.40 (d, 1H, H-28) gemineal oxomethyle, 3.70 (d, 1H, H-28) gemineal oxomethyle, 4.60 (S, 1Ha, H-29) olefinic protons, 4.80(S, 1Ha, H-29) olefinic protons. Methyl groups at δ_H 0.73, 0.83, 0.93, 1.00, 1.05, 1.45 ppm. A doublet of doublets at δH 3.20 ppm. The olefinic proton signals at δH 4.80 and 4.60 ppm, along with the methyl group.

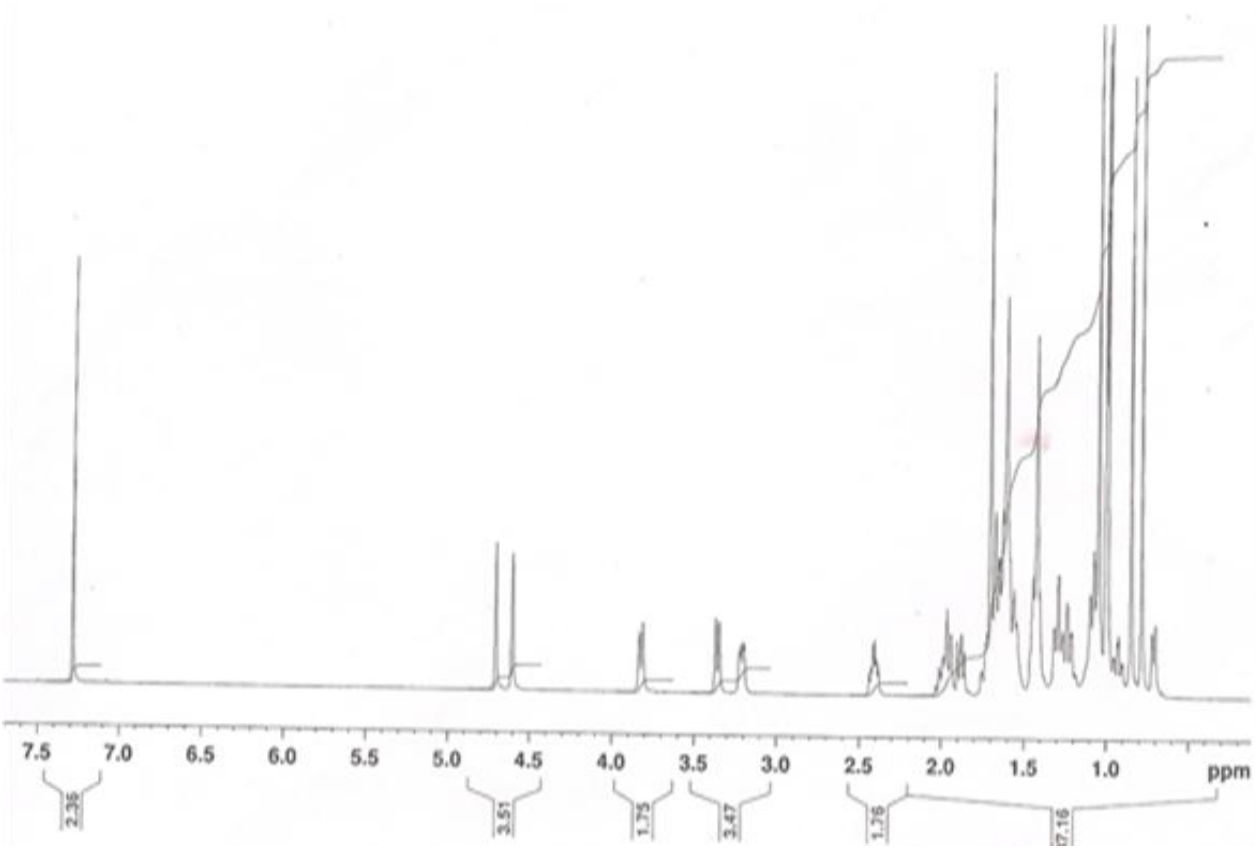


Figure 1: NMR spectra of compound Q

Table 3: ^{1}H-NMR spectroscopy of Compound Q

Position of carbon	δ (ppm),	Number of hydrogen	Coupling
1			
2			
3	3.20	1H	Dd(secondary carbinol)
4			
5	0.68	1H	D
6			
7			
8			
9			
10			
11			
12			
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14			
15			
16			
17			
18			
19	2.40	1H	Dt
20			
21			
22			
23	1.00	3H	S
24	0.73	3H	S
25	0.83	3H	S
26	1.05	3H	S

27	0.93	3H	S
28	3.40	1H	d(germinal hydrogen)
	3.70	1H	d(germinal hydrogen)
29	4.60	1Ha	s
	4.80	1Hb	s
30	1.45	3H	dd

Mass spectroscopy of Compound Q

The low-resolution electron impact mass spectrum of compound Q in Figure 2 shows the molecular ion peak (M^+) at m/z 442, while Table 4 represents fragments, percentage and molecular mass of fragments of Compound Q, and Table 5 reveals bioactivities of Compound Q. The fragment, percentage, and molecular mass in the spectrum of compound Q are shown in Table 4

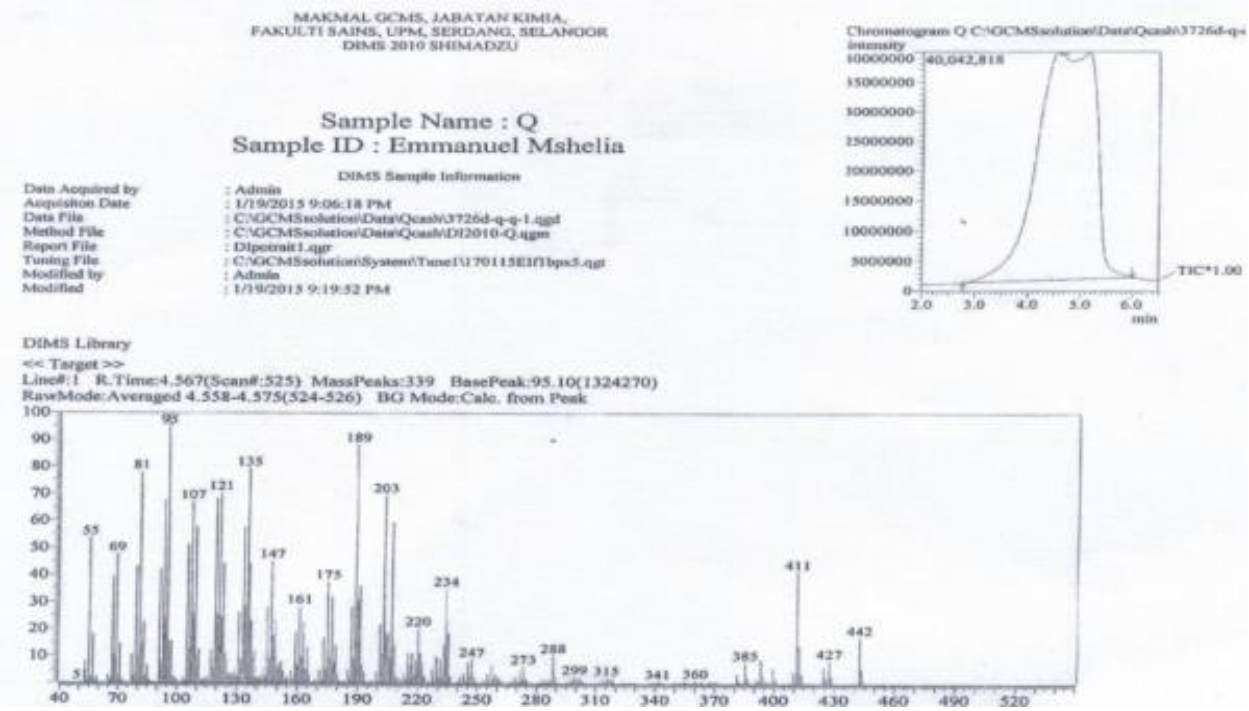


Figure 2: The low-resolution electron impact mass spectrum of compound Q

Table 4: Fragments, Percentage and Molecular Mass of Fragments of Compound Q

S.N	Fragment	Percentage abundance	M/Z	Molecular ion
1	$C_{30}H_{50}O_2$	15	442	442
2	$C_{29}H_{47}O_2$	9	427	
3	$C_{29}H_{47}O$	38	411	
4	$C_{26}H_{26}O$	34	234	
5	$C_{14}H_{23}$	64	207	
6	$C_{14}H_{19}O$	68	203	
7	$C_{14}H_{21}$	90	189	
8	$C_{11}H_{15}$	57	147	
9	$C_{10}H_{15}$	81	135	
10	C_7H_{11}	100	95	
11	C_6H_9	80	81	
12	C_5H_9	49	69	

Table 5: Bioactivities of compound Q

Isolate	LC_{50} (µg/ml)	AO	Antibacterial activity			
			ST	EC	SA	SD
Q	81.92	++	+	++	++	+
Ascorbic acid	ND	+++	ND	ND	ND	ND

Keys: AO = Antioxidant activity, ST= *Salmonella typhii,EC= Escherichia Coli, SA= Staphylococcus aureus, SD= Shigella dysentariae, ND= not determine*

The bioactivities of the isolated compound Q are shown in Table 5. The results showed that the LC50 value of compound Q is 81.92 µg/ml, with moderate antioxidant activity (++) compared with ascorbic acid, which has appreciable activity (+++). The isolated compound showed moderate antibacterial activity, inhibiting the growth of Escherichia coli (EC) and Staphylococcus aureus (SA), while Salmonella Typhii (ST) and Shigella dysenteriae (SD) were fairly inhibited.

Structural elucidation of compound Q

Fourier Transform infrared spectroscopy of compound Q

The IR spectrum values (KBr) of compound Q are shown in Table 2 and indicate the characteristic absorption bands caused by the hydroxyl group at 3301.21cm-1 and olefinic carbon stretching absorption at 1643.20 cm-1, while there are no characteristic absorptions showing the presence of a carbonyl group in the spectra. The absorption at 2972.10 cm-1 indicates asymmetric C-H stretching of the CH_3 methyl group. The absorption at 1021.23cm^{-1} indicates the bending vibrations of the C-O bond of the CH2-OH group, while the absorption at 832.13 cm-1 is due to out-of-plane C-H vibration of the alkene group. The signal at 1430.33 cm-1 is due to –CH3 and –CH2 bending vibrations. The FTIR showed that there was no carbonyl oxygen in the compound, but the C-O was for the –OH group in the alcohol.

^{1}H-NMR spectroscopy of compound Q

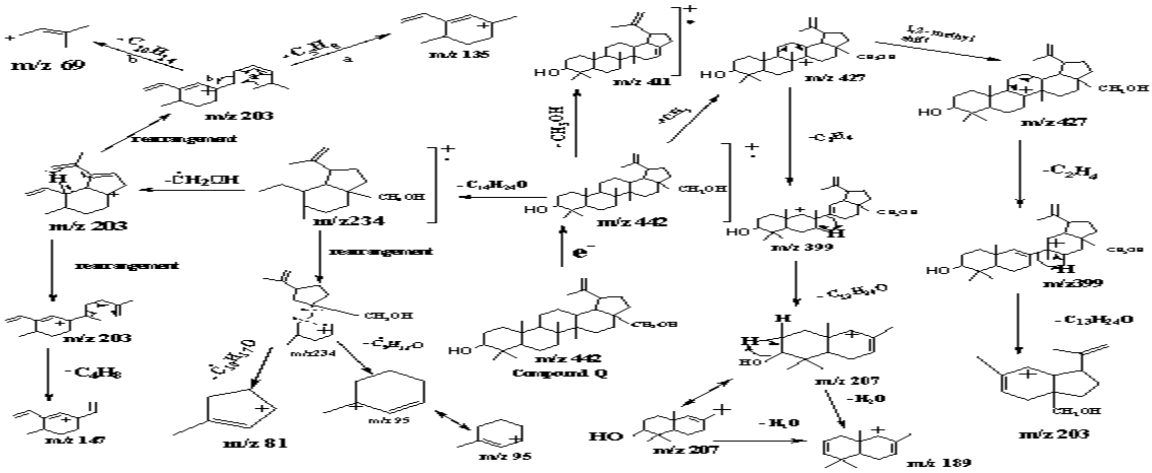
The NMR spectrum and data for compound Q was given in Appendix 1 and Table 3. ^{1}H-NMR (500MHz, $CDCl_3$), δ (ppm),0.68 (d, 1 H), 0.73 (S, 3H, H-24), 0.83 (S, 3H, H-25), 0.93(S, 3H, H-27), 1.00 (S, 3H, H-23), 1.05 (S, 3H, H-26), 1.45 (dd, 3H, H-30), 2.40 (dt, 1H, H-19) doublet of triplet of one proton, 3.20 (dd, 1H, H-3) one oxymethine proton, 3.40 (d, 1H, H-28) gemineal oxomethyle, 3.70 (d, 1H, H-28) gemineal oxomethyle, 4.60 (S, 1Ha, H-29) olefinic protons, 4.80(S, 1Ha, H-29) olefinic protons.

Compound Q showed the presence of six methyl groups at δH 0.73, 0.83, 0.93, 1.00, 1.05, and 1.45 ppm, similar to methyl groups attached to the triterpenoid nucleus. A doublet of doublets of one oxymethylene was present at δH 3.20 ppm, which is characteristic of an α-oriented hydrogen at C-3 of a β-hydroxyl triterpene. The olefinic proton signals at δ_H 4.80 and 4.60 ppm, along with the methyl group at δH 1.45 ppm, suggest that compound Q has a lupeol-type triterpene derivative.

Another pair of doublets at δ_H 3.70 and 3.40 ppm, rather than a seventh methyl singlet around 0.80 ppm, confirms the presence of a second hydroxyl group at C-28. The signals at δ_H 4.60 and 4.80 ppm confirm the pair of olefinic protons as part of an exocyclic methylene group on carbon 29 [24].

Mass spectroscopy of compound Q

The low-resolution electron impact mass spectrum of compound Q (Appendix 2), the fragments in Table 4, and the Fragmentation pattern in Scheme 1 show a molecular ion peak (M+) at m/z 442. The fragmentation pattern strongly indicated a pentacyclic triterpenoid with a lupane skeleton. The fragment ions of compound Q at m/z 427 ($M^+ - CH_3$) and 411 ($M^+ - CH_2OH$) were the major peaks in the upper mass region. The peaks at m/z 234, 203, and 189 were attributed principally to a ring C fission of a lupane-type triterpenoid with charge retention either on the ring A/B or D/E fragment. The further fragmentation and rearrangement of the fragment with m/z 203 lead to the formation of fragments m/z 95 as the base peak, 135, 69, 147, and 81, as seen in Scheme 1 [26].



Scheme 1: Fragmentation pattern of Compound Q

All the above physical properties and spectral data presented for compound Q were in agreement with the data reported in literature for Lup-20(29)-ene-3,28-diol (Betulin) as has been isolated previously from numerous plant sources [27].

Direct comparison with authentic data confirmed the identity of compound Q as betulin. They had similar IR, NMR and mass spectra with those of an authenticate sample of betulin isolated from literatures [28-46]. The structure of compound Q is shown in Figure 3

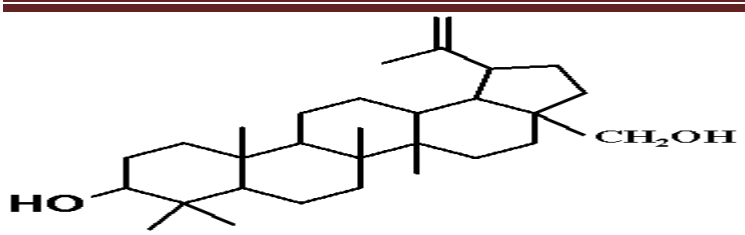


Figure 3: compound Q: Lup-20(29)-ene-3,28-diol (Betulin)

Bioactivity of the isolates

Medicinal plants have been reported to possess a wide range of properties, including anti-carcinogenic, anti-inflammatory, antioxidant, antitumor, antimicrobial, antihelminthic, antiviral, and antibiotic activities [47-48], medicinal plants are the best source for a variety of drugs for cancer and antimicrobial use, since cancer cells and microorganisms are developing resistance to current chemotherapies. Plant components possessing antioxidant, antimicrobial, and cytotoxic activities have been identified, and their possible uses for treatment of diseases are studied [49]. In the present study, the isolated compound belongs to the triterpenoid class of phytochemicals.

The results showed the presence of triterpanes and moderate antioxidant, cytotoxic, and antimicrobial activities. Triterpanes represent a varied class of natural products. Pentacyclic triterpenes are based on a 30–carbon skeleton comprising five six-membered rings (Ursanes and lanostanes) or four six-membered rings and one five-membered ring (Lupanes and nopanes), as seen in our structural elucidation discussed, and are produced by arrangement of squalene epoxide molecules [38]. Some plant terpenoids serve as basic shapers of antiseptic, expectorant, gastrointestinal, pain-relieving, immune-stimulating, and antioxidant activities. Previously reported biological activities of triterpenes include anti-AIDS activity for betulinic acids, herbicidal activity for lupenone, and fibrinolytic activity for betulin [46].

The suppressive effect of betulin on superoxide generation in the human neutrophiti and inhibiting ROS (reactive Oxygen species such as superoxide) agents produced by Hep G2 cells and ethanol activated hephtic satellite cells is similar to the result obtained in our study for compound Q which may be responsible for the use of the plant *Grewia mollis* for treatment of cuts, wound and in controlling bleeding and lubrication in childbirth [34]. The results for the antimicrobial, antioxidant, and anticancer activities of isolated compound Q (betulin) agree well with the reported literature [45-47]. For example, Betulin antimicrobial activity was previously

reported to be active against *Fusarium oxysporium*, it also displayed anticancer activity against walker carcinoma – 256 as well as antiviral activity against herpes simplex virus and anti-inflammatory activity. Betulin has been reported to exhibit anti-human immune deficiency virus (anti-HIV), anticarcinogenic, antiflu, anti-inflammatory, antiallergen, detoxicant (detoxicating agent) and antioxidant activities which are some of the properties exhibited by plant [49].

CONCLUSION

Bioactive compounds occur in different parts of plants and consist of various compounds. The combination of several chromatographic techniques and various purification methods were used to isolate the bioactive compound. The bioactive compound was characterized using GC-MS, FTIR, and 1H NMR, and the isolated compound showed some antibacterial activity against certain organisms and antioxidant activity. The proposed structure of the bioactive compound, based on the information acquired, proved it is a pentacyclic triterpenoid, lup-20(29)-ene-3,28-diol (betulin), and showed some degree of bioactivities in vitro.

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