British Biotechnology Journal
16(2): 1-10, 2016, Article no.BBJ.24436
ISSN: 2231–2927, NLM ID: 101616695

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Isolation of a Major Antimicrobial Compound from Stem Bark of *Glossonema boveanum* (Decne)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2016/24436
Editor(s):
(1) Chung-Jen Chiang, Department of Medical Laboratory Science and Biotechnology, China Medical University, Taiwan.
Reviewers:
(1) Isaac Kingsley Amponsah, Knust, Ghana.
(2) Nilutpal Sharma Bora, Defence Research Laboratory, Tezpur, Assam, India.
(3) Yoko Oshima Franco, UNISO, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history/16411

Original Research Article

Received 20th January 2016
Accepted 27th April 2016
Published 1st October 2016

ABSTRACT

Medicinal plants have been used as traditional remedies for the treatments of different ailments, and large number of human population still depends on these medicinal plants for their preventive and curative properties G*lossonema boveanum* is a medicinal plant widely used in a folk medicine in both West and North Africa. Column chromatography of the ethyl acetate extract from stem bark of *Glossonema boveanum* yielded one pentacyclic triterpenoid (betulinic acid) which is reported for the first time in this plant. The structure of the isolated compound was identified on the basis of 1D NMR (^{1}H, ^{13}C and DEPT), 2D NMR (COSY, HSQC, HMBC and NOESY) and by comparison with reported data. The compound was then tested for its antibacterial activity against two Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*) and three Gram-negative bacteria (*Salmonella typhi, Shigella dysenteriae* and *Escherichia coli*) and was found to be effective against the bacteria. The result of the MIC revealed that the lowest concentration of betulinic acid that inhibited the growth of the bacteria was 3.125 µg/ml for *S. aureus* and *S. dysenteriae*. This study

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concluded that the stem bark of *G. boveanum*, used traditionally as a medicinal plant for the treatment of typhoid fever, bacillary dysentery, diarrhoea and stomach pain has antibacterial activities against the causative bacteria.

Keywords: Glossonema boveanum; betulinic acid; antibacterial activity.

1. INTRODUCTION

Plants have been used as traditional remedies for the treatment of different ailments. The herbal health care traditions have evolved over centuries as relevant social traditions with proven safe, efficacious, preventive, promotive and curative health practices [1]. The chemical constituents of medicinal plants, particularly the secondary metabolites have profound pharmacological actions on animal system and organs. Several bioactive compounds were isolated from different plant sources such as digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine, quercetine etc. which have different pharmacological and nutritional properties [2]. It is well documented that plants are the major source of bioactive agents and there is a wealth of drugs potential in the plant kingdom [3].

Betulinic acid is found in many plant species. Its content, however, is usually low. *Menyanthes trifoliate,* a bog plant, is a rare exception. Its underground parts contain marked amounts of free betulinic acid and triterpene saponins with betulinic acid as the aglycone [4]. Betulinic acid has been found in almond hulls [5] and also in other biological sources. Betulinic acid is a naturally occurring pentacyclic triterpenoid and has been shown to exhibit a variety of biological activities including inhibition of human immunodeficiency virus (HIV), antibacterial, antimalarial, anti-inflammatory, antihemintic and antioxidant properties [6,7,8,9,10]. Betulinic acid and its derivatives have been discovered as a new class of compounds that seems to protect the cells of human immunological system in vitro from attack by HIV virus [11].

Glossonema boveanum, a plant used in traditional medicine for the treatment of typhoid fever, bacillary dysentery, diarrhoea and stomach pain, is widely distributed in some countries; Nigeria, Egypt, Niger and Saudi Arabia. *G. boveanum* exist as perennial plants [12,13]. Phytochemical and antibacterial activities of *G. boveanum* have been reported from n-hexane, ethyl acetate and methanol extracts [14].

In this research, a pentacyclic triterpene was isolated from the stem bark extract of *Glossonema boveanum* and antibacterial activity was also determined. This is the first time betulinic acid is been reported from *G. boveanum*.

Plate 1. *Glossonema boveanum* (Decne)

2. MATERIALS AND METHODS

2.1 General Experimental Procedure

The ^{1}H- and ^{13}C- NMR spectra were measured on Bruker Avance AV 600 spectrometer, using deuterated methanol as an internal standard. Chemical shifts (δ) are expressed in parts per million (ppm).

2.2 Chemicals and Reagents

All the chemicals and reagents including ethyl acetate, n-hexane, methanol, were of Analytical Grade and purchased from Merck® (New Jersey, United States).

2.3 Collection of Plant Material

Fresh leaves, stems and root of *Glossonem boveanum* were collected from Utupko village, Benue State of Nigeria in the month of January, 2014. The plant sample was identified and authenticated by Mallam Musa Mohammed

of Herbarium section, Biological Science Department, Ahmadu Bello Univeristy Zaria – Nigeria, and the Voucher number was given as 4487. The plant sample was deposited for further references.

2.3.1 Extraction of plant material

The stem bark was harvested from the stem, air dried for 7 days under room temperature and crushed to coarse powder. The pulverized plant sample (1000 g) was macerated successively in n-hexane, ethyl acetate and methanol exhaustively until complete extraction. The solvents were removed *in-vacuo* to yield (6.2 g), (14.7 g) and (35 g) of the n-hexane, ethyl acetate and methanol extracts respectively.

2.4 Isolation and Identification of Compound

The ethyl acetate extract (10.80 g) was adsorbed onto Silica Gel G, allowed to dry and directly subjected to chromatography on a silica column (mesh size 60-120, 3 cm dia × 60 cm length) eluted with gradient mixture of n-hexane – Ethyl acetate (9.75:0.25, 9.5:0.5, 9:1, 8.75:0.25, 8.5:1.5, 8:2, 7.5:2.5, 7:3, 1:1, 3:7, 2:8). The flow rate was 2 mL/min and forty of 100 mL collections yielded seven fractions (F_1 – F_7). These fractions were monitored by pre-coated Thin Layer Chromatography (TLC) plates ($60F_{254}$). The TLC of the third fraction, F_3 (52 mg) using a mixture of n-hexane and ethyl acetate (7:3) as developing solvent revealed the presence of 2 compounds (corresponding to 2 spots) having an R_f values of 0.39 and 0.47 respectively. F_3 was further purified using preparative TLC which yielded a compound – compound 1, with R_f value of 0.46 in ethyl acetate: n-hexane (7:3) and the yield value was 11 mg.

2.5 Antibacterial Activity

The isolated compound was tested for antibacterial activity against five pathogens (*Bacillus subtili, Staphylococus aureus, Salmonella typhi, Shigella dysenteriae* and *Escherichia coli)* by agar well diffusion methods using stock concentration of 50 µg/mL. The standardize inocula of the isolates were uniformly streaked unto freshly prepared Mueller Hinton agar plates with the aid of a sterile swab stick. Using sterile cork borer (8 mm in diameter), four appropriately labeled wells were punched into each agar plate. 0.1 mL of the appropriate isolated compound concentration was placed in each well and then allowed to diffuse into the agar, an extra plate was streaked with the inocula isolate and ciprofloxacin standard (5 µg/disc) was placed on it. The plates were incubated at 37℃ for 24 hours [15]. The minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined using the tube dilution method as outlined by the Clinical Laboratory Standard [15]. A plate of nutrient agar was labeled into four regions; respective concentrations were added into the labeled regions and incubate at 37℃ for 24 hrs, MBC was determined in where there were no bacterial.

3. RESULTS AND DISCUSSION

Ethyl acetate and methanol extracts showed the presence of triterpenes in the phytochemical screening and inhibited the growth of *S. aureus, B. subtilis, S. typhii, S. dysentriae and E. coli*. This observed activity might be attributed to the presence of triterpenes in these extracts and can be seen as a potential source of useful drugs [14]. Chromatography separation and purification of ethyl acetate extracts from the stem bark of *G. boveanum* yielded isolation of one compound (compound 1), which showed positive to triterpenes test of Liebermann-Buchard's test. The structure of which was determined by extensive 1D NMR (^1H, ^{13}C and DEPT), 2D NMR (COSY, HSQC, HMBC) and comparison of the spectral data with previously reported values. Compound 1 was isolated as white crystalline, its molecular formula was determined as $C_{30}H_{48}0_3$ on the basis of NMR data (Table 1).

The ^1H NMR (Fig. 1) spectrum revealed the presences of an allylic methyl group at δ 1.69 and terminal methylene protons at δ 4.56 and δ 4.69. It also showed five methyl singlets at δ 0.75, δ 0.88, δ 0.97, δ 1.00 and one singlet of secondary hydroxyl group at δ 3.13.

The ^{13}C NMR (Fig. 2) spectrum in combination with the DEPT (Fig. 3), HMBC (Fig. 4), COSY (Fig. 5) and HSQC spectrum (Fig. 6) revealed thirty signals ranging from 13.7 to 178.9 ppm. The signals between 13.7 to 55.6 ppm corresponds to overlapping methine (-CH), methylene ($-CH_2$) and methyl ($-CH_3$). The signal at 78.4 ppm is due to oxymethine carbon. The signals at 108.1 and 152.5 ppm are typical of olefinic carbons and the signals at 178.9 ppm indicated the presence of a carboxylic carbon. Based on the analysis above and after comparison with data in literature [16,17,18] the structure of compound 1 isolated from the ethyl

acetate extracts of the stem bark of *G. boveanum* was determined to be betulinic acid (Fig. 7).

Betulinic is a pentacyclic triterpene. Pentacyclic triterpenes are secondary plant metabolites widespread in fruits peel, leaves and stem bark. In particular the lupine-, oleanane-, ursane and betulinic acid triterpenes display various pharmacological effects while being devoid of prominent toxicity. These triterpenes are promising leading compounds for the development of new multi-targeting bioactive agents. Depending on the plant material, betulin, betulinic acid, oleanolic acid and ursolic acid are the common pentacylcic triterpenes. They are isolated in low concentration of plants and can be use for development of phytopharmaceutical formulations [19].

Earlier report [14] showed that the ethyl acetate extract had zone of inhibition ranging from 11 – 21 mm. This activity seems to suggest that it was mainly due to betulinic acid. Purification of the ethyl acetate extract led to isolation of betulinic acid whose zone of inhibition was recorded as 14 – 23 mm. The result is presented in Table 2. Betulinic acid showed effective antibacterial activity against all the species tested.

The result of the minimum inhibitory concentration (MIC) in Table 3 revealed that betulinic acid inhibits the growth of the tested microorganisms and was as low as 3.125 µg/mL. The minimum bactericidal concentration (MBC) was also determine and presented in Table 4. The results clearly show that betulinic acid has bactericidal activity on the test organism. The MBC was determined on a plate of nutrient agar.

The plate was labeled into four regions; respective concentrations were added into the labeled regions and incubated at 37℃ for 24 hrs, MBC was determined in where there were no bacterial.

Table 1. The NMR (600MHz) data of the isolated compound 1 using CD_3OD

Position	^{1}H (ppm)	^{13}C (ppm)	DEPT	^{1}H (ppm) [16]	^{13}C (ppm) [16]
C_1		38.6	CH_2		39.1
C_2		26.7	CH_2		27.4
C_3	3.13	78.4	CH	3.03	79.2
C_4		38.8	C		39.4
C_5		55.6	CH		56.2
C_6		18.1	CH_2		18.9
C_7		34.3	CH_2		37.7
C_8		40.6	C		43.1
C_9		50.8	CH		51.3
C_{10}		37.0	C		37.8
C_{11}		20.8	CH_2		21.5
C_{12}		25.7	CH_2		26.2
C_{13}		38.1	CH		39.0
C_{14}		42.2	C		43.3
C_{15}		29.7	CH_2		31.2
C_{16}		30.8	CH_2		32.9
C_{17}		54.2	C		56.9
C_{18}		48.2	CH		47.7
C_{19}		47.2	CH		49.8
C_{20}	4.56	152.5	C	4.68	154.9
C_{21}		35.8	CH_2		30.3
C_{22}		37.0	CH_2		35.0
C_{23}	0.88	27.23	CH_3	0.83	28.3
C_{24}	0.97	14.7	CH_3	0.95	15.8
C_{25}	0.75	15.4	CH_3	0.73	16.3
C_{26}	1.06	16.7	CH_3	1.38	16.5
C_{27}	1.00	13.7	CH_3	0.98	15.1
C_{28}		178.9	C		183.4
C_{29}	4.69	108.1	CH_3	4.78	106.4
C_{30}	1.69	18.3	CH_2	1.68	19.2

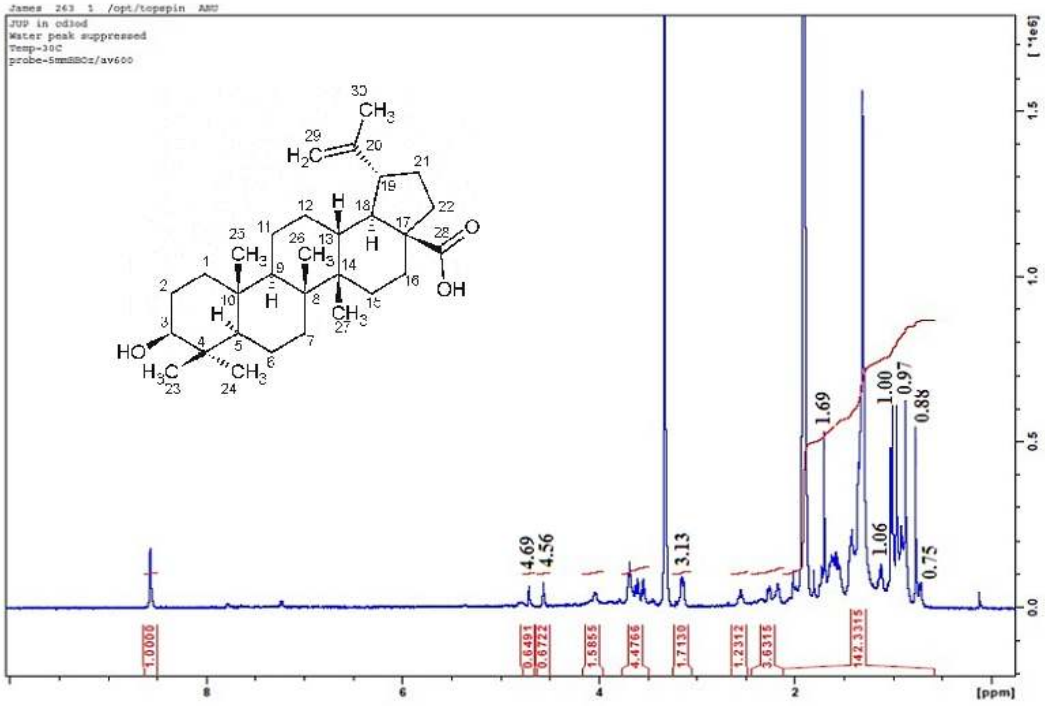

Fig. 1. ^{1}H NMR spectrum of isolated compound 1

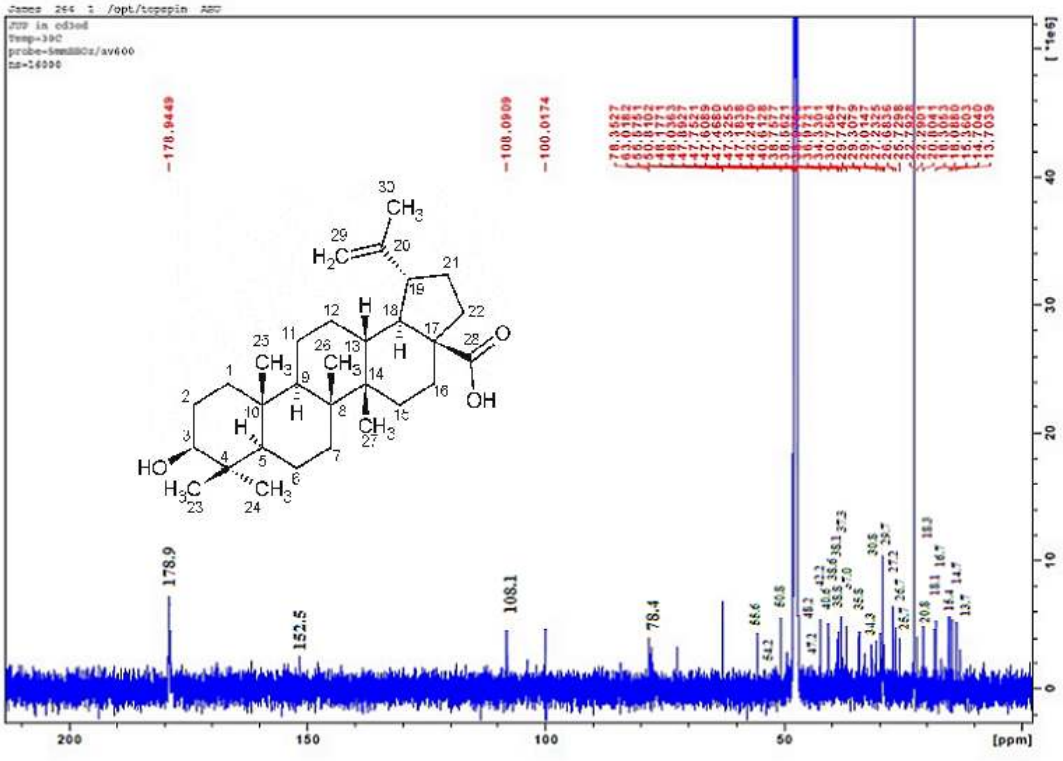

Fig. 2. ^{13}C NMR spectrum of isolated compound 1

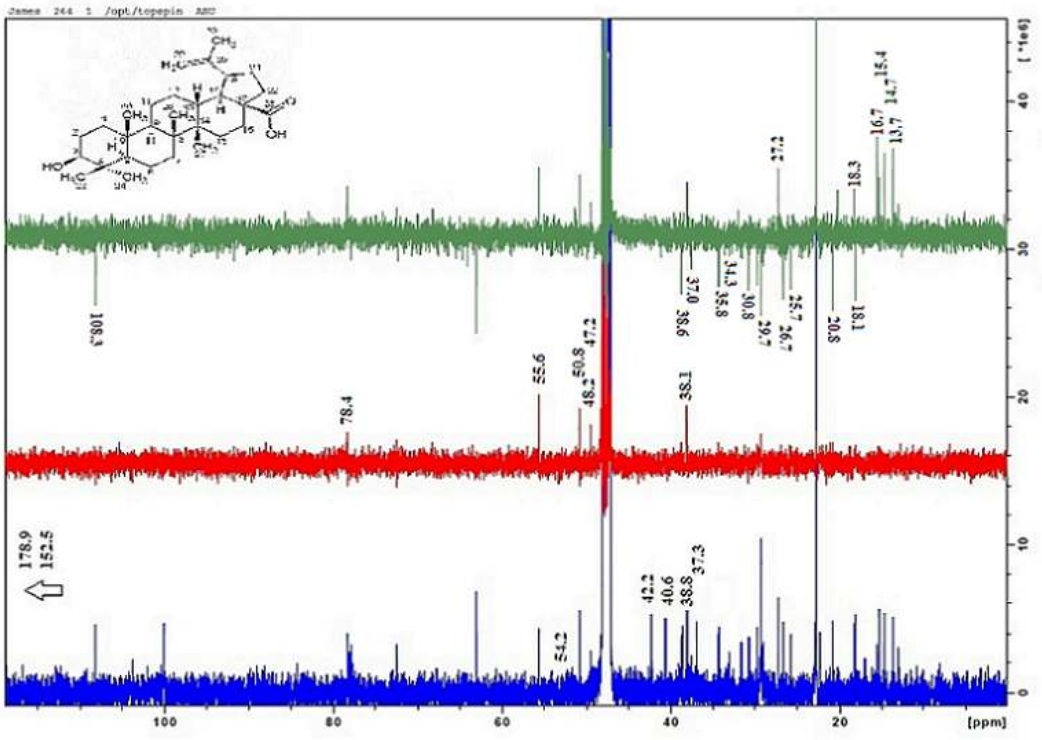

Fig. 3. DEPT spectrum of isolated compound 1

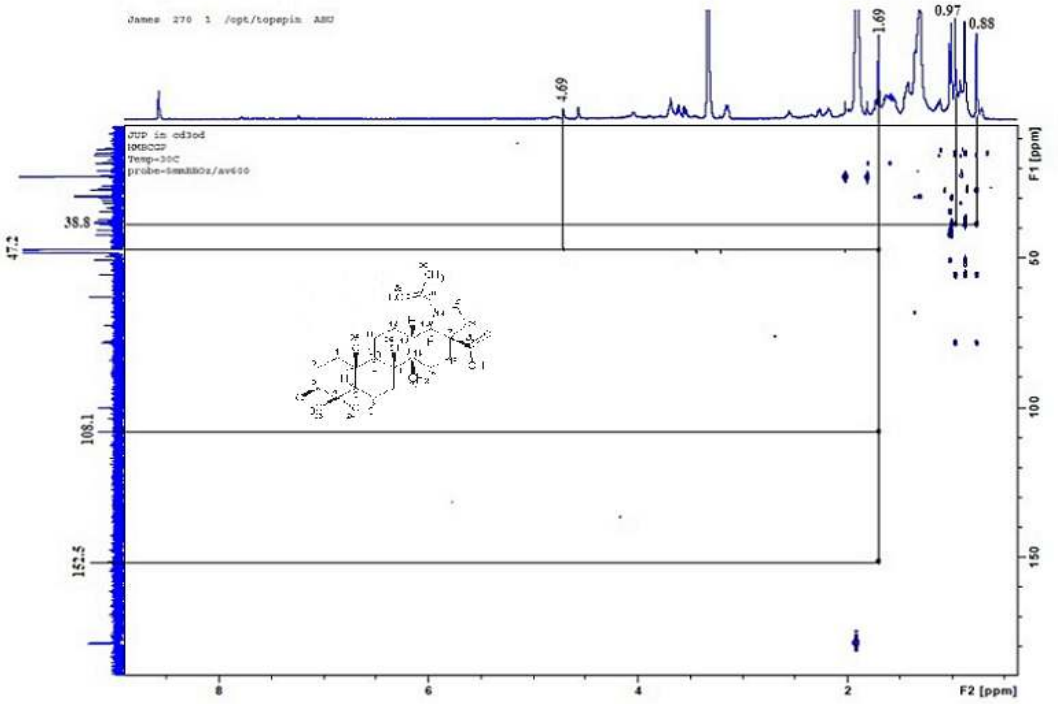

Fig. 4. HMBC spectrum of isolated compound 1

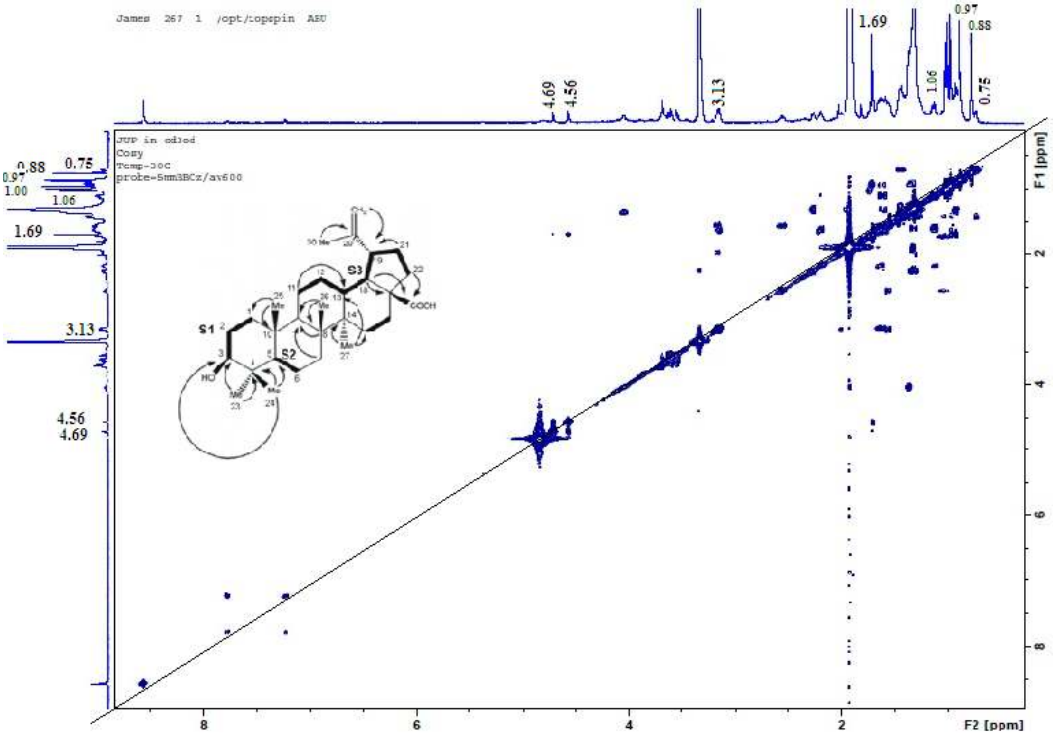

Fig. 5. COSY spectrum of compound 1

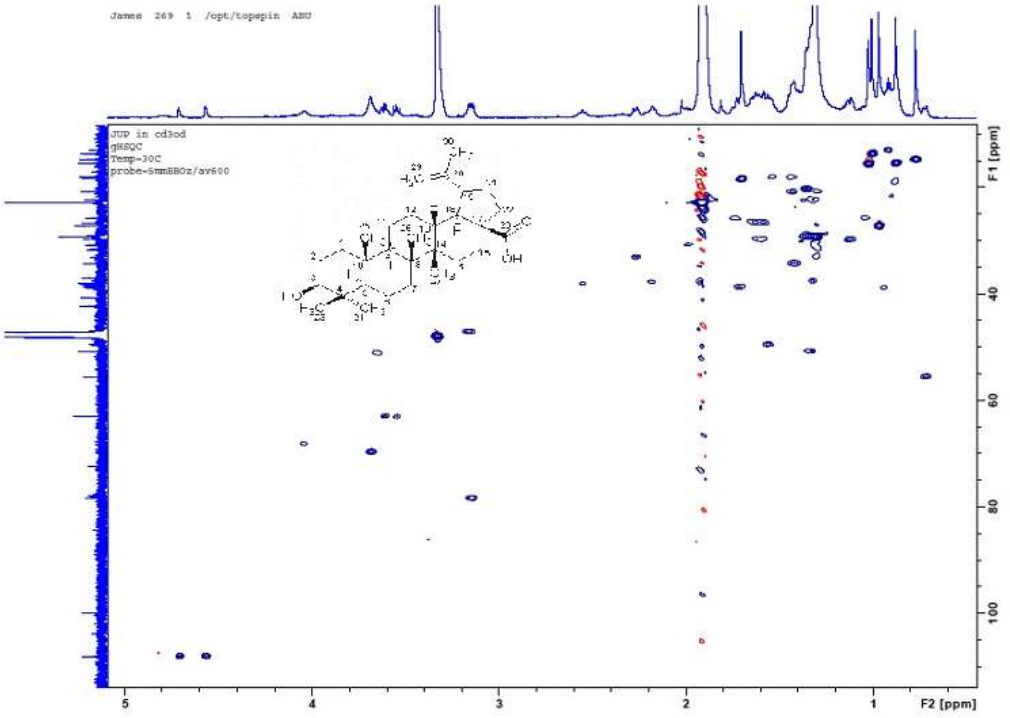

Fig. 6. HSQC spectrum of compound 1

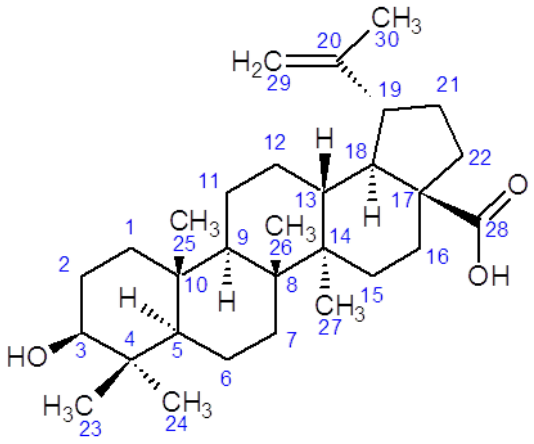

Fig. 7. Chemical structure of betulinic acid [16]

Table 2. Antimicrobial results of compound 1

Test organism	*B. subtili*	*S. aureus*	*S. typhi*	*S. dysenteriae*	*E. coli*
Average zone of Inhibition (mm)	23	14	22	20	15
Sensitivity test	S	S	S	S	S
Ciprofloxacin (5 µg/disc)	37	35	42	40	35

Table 3. Minimum inhibitory concentration (MIC) of compound 1

Test organism	*B. subtili*	*S. aureus*	*S. typhi*	*S. dysenteriae*	*E. coli*
Compound 1 (µg/mL)	12.500	3.125	6.250	3.125	6.250

Table 4. Minimum bactericidal concentration (MBC) of compound 1

Test organism	*B. subtili*	*S. aureus*	*S. typhi*	*S. dysenteriae*	*E. coli*
Compound 1 (µg/mL)	25.000	6.250	12.500	6.250	12.500

In general the accumulation and concentration of secondary metabolites are responsible for antimicrobial activity and varies according to the plant extracts base on their polarity [20]. The ethyl acetate extract and Isolated Betulinic acid showed activities against *S. typhii*, the bacteria responsible for salmonellosis and typhoid fever, the sensitivity of *E. coli*, *S. aureus* to the extracts and betulinic acid implies that chemical compounds can be further developed for the fight against this microorganism and the use of the plant for the treatment of diarrhea, stomach pain and skin itching is justified since this bacteria are responsible for such illness [14]. The extract and betulinic acid also showed activities against *S. dysenteriae* the bacteria responsible for bacillary dysentery and were more active compared to the previous reported antimicrobial activities of betulinic acid [9,10]. Therefore these could serve as source of compounds that may be effective in the management of the ailments.

4. CONCLUSION

The research established the presence of triterpene and some other phytochemicals through preliminary phytochemical screening from stem bark extract (ethyl acetate extracts) of *Glossonema boveanum*. An active substance, betulinic acid was also isolated and characterized from the ethyl acetate extract. The betulinic acid is responsible for the exhibited antibacterial activity seen against some bacteria (*Staphylococcus aureus, Bacillus subtilis, Salmonella typhii, Shigella dysenteriae,* and *Escherichia coli*).

This research has thrown light upon the use of *Glossonema boveanum* for the treatment of infectious disease such as typhoid fever, bacillary dysentery, diarrhoea and stomach pain has been achieved.

ACKNOWLEDGEMENT

The authors would like to express their gratitude and thanks to Mr. Ochigbo Celestine for providing the plant, Mallam Musa Mohammed for identifying and authenticating the plant. We are also thankful to Mallam A. M Shittu and Mallam Mubarak Dambatta for their valuable technical assistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/16411