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ISOLATION AND CHARACTERIZATION OF A NOVEL LUPANE-TYPE TRITERPENOID FROM THE LEAVES OF *Piliostigma thonningii*

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ABSTRACT

A novel lupane-type triterpenoid identified as lup-12,20(29)-diene-3,28-diol was isolated from the chloroform extract of the leaves of *Piliostigma thonningii*. The compound gave a retention factor (R_f) value of 0.78 on TLC developed in a solvent system of 100% $CHCl_3$ (100 ml). The structure of the compound was elucidated using spectral methods such as FT-IR, ^{13}C NMR, ^{1}H NMR, DEPT, COSY, HSQC, HMBC and mass spectrometry. This is the first report on the isolation of a novel lupane-type triterpenoid from the leaves of *Piliostigma thonningii*.

Keywords: *Piliostigma thonningii*; Lupane triterpenoid; NMR; Spectral analysis; Chloroform extract

INTRODUCTION

Attention has always been on the discovery and development of new drugs which are functionally selective and efficacious with insignificant and tolerable side effects. Plants still remain a veritable source for novel drug discovery especially the ones used by the locals and natives for the treatment and management of diseases and infections [1]. There is a continuous search for new drugs in medicinal plants from Nigeria and other parts of Africa and of course the therapeutic potentials of these medicinal plants are yet to be fully harnessed [2]. *Piliostigma thonningii* (Schumach.) Milne-Redhead belongs to this category of medicinal plants.

Piliostigma thonningii is a leguminous plant that belongs to the *Fabaceae* family. The Fabaceae, Leguminosae or Papilionaceae commonly known as the legume, pea or bean family, are a large and economically important family of flowering plants [3]. It includes trees, shrubs, and perennial or annual herbaceous plants, which are easily recognized by their fruits (legume) and their compound, stipulated leaves [4]. *P. thonningii* is native to tropical African countries such as the Sudano-Guinean region, Senegal, Namibia, Botswana, Nigeria, Mozambique and South Africa where it is found commonly in the open woodland and wooded grasslands at medium to low altitudes [5]. The plant**,** commonly known as camel's foot tree or monkey bread (English), okpoatu (Igbo), abafe (Yoruba) and kalgo (Hausa), grows up to 4 – 15 m in height with a rounded crown and a short but often crooked bole [5].

P. thonningii leaves and bark have been reported to be used in traditional medicine, especially in the treatment of malaria, wounds, ulcers,

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gastric/heart pain, gingivitis, fever, haemorrhoids and backache [3,6]. The plant has been touted to be used in the treatment of diseases such as cough, gonorrhea, snake bite, HIV, ethno-veterinary diseases, especially across Africa. Pharmacological studies have shown the plant to possess antimalarial, antiviral, antimicrobial and anti-proliferative activities [7]. The fresh leaves and flowers of this tree can be chewed to reduce thirst. Bark infusions are used to treat diarrhoea. The leaf preparations are antiseptic and are used to promote wound healing, treat skin diseases and snake bites [8]. The medicinal uses of *P. thonningii* also include treating loose stool in teething children, worm infestation, arresting bleeding, inflammations, bacterial infections, stomach ache and headache [9]. A decoction of the leaf is drunk and bathed against fever, toothache, and epilepsy. It is also used as a vaginal wash and as an enema to a mother giving birth [8].

Compounds previously isolated from *P. thonningii* are piliostigmin, c-methyl flavonols and quercetin [7]. Afolayan *et al.* [10] reported the isolation of two new compounds; 2β-methoxyclovan-9α-ol and methyl-ent-3β-hydroxylabd-8(17)-en-15-oate together with fourteen other known compounds from the leaves of *P. thonningii*. Previous phytochemical studies on *P. thonningii* also revealed the presence of flavonoids, tannins, kaurane diterpenes, alkaloids, carbohydrates, saponins, terpenes, and volatile oils [11]. D-3-O-methylchiroinositol, which possesses anthelmintic activity has also been isolated from the stem bark of *P. thonningii* [12]. The isolation and characterization of a novel lupane-type triterpenoid is herein reported.

MATERIALS AND METHODS

Sample Collection and Identification

The leaves of *P. thonningii* were collected from the vicinity of the College of Physical and Applied Sciences of Michael Okpara University of Agriculture, Umudike on the 16th of January, 2016. They were authenticated at the Taxonomy Section, Forestry Department, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

Sample Preparation

The sample was air-dried under a shade for thirty days and was ground into small particle sizes using an electric blender. The weight of the ground sample was 378 g which was put in an air-tight container.

Sample Extraction Procedure

About 340 g of the ground leaf sample of *P. thonningii* was weighed into a bottle and 2.5 L of chloroform was introduced into the sample and allowed to stay for seventy-two hours. After that, the mixture was filtered using Whatman No. 1 filter paper. The chloroform extract was subjected to evaporation using a digital Heidolph Rotary Evaporator (4000 series) was set at 45^0C under reduced pressure to get the crude sample. After

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evaporation of the chloroform, the extract was allowed to stand for about 4 hours so that the little chloroform in it could evaporate completely to get the actual weight of the sample [13]. The extract collected weighed 30.42 g and was labeled A1.

Partitioning of the Crude Extract

The chloroform extract labeled A1 was partitioned between 200 ml each of aqueous methanol (methanol: water; 9:1) and hexane in a separating funnel. The layers were collected in different beakers. The methanol layer had water in it which was removed using the rotary evaporator. The beakers were labeled A2 and A3 for the methanol and hexane fractions respectively. The solvents were allowed to evaporate. About 12 g of A2 was partitioned between 200 ml each of chloroform and distilled water in a separating funnel. The chloroform fraction was collected and allowed to dry at room temperature for two days and was labeled A4.

Column Chromatography

The column used was 280 mm in height and 35 mm in diameter and was carefully packed with silica gel following standard procedure. The already prepared dry slurry of the sample (A4) was poured into the packed column and then sealed with dry silica gel. The column was clamped to a retort stand. Elution began with hexane (non-polar solvent). The polarity of elution solvent mixture was increased gradually by adding successively increasing quantities of moderately polar chloroform. After elution with hexane-chloroform, chloroform-ethyl acetate was used, followed by ethyl acetate-methanol. Care was taken to run with a flow rate of 12-15 drops per minute. The process of column chromatography lasted for more than 72 hours during which time various fractions were collected in labeled beakers.

Thin-Layer Chromatography (TLC)

Silica gel slurry was made by mixing and stirring one part of silica gel in three parts of distilled water. The surface of the glass plate (5.5 cm x 16 cm) was wiped with clean cotton wool to clean and dry it. One surface of the plate was then coated with the slurry by carefully smearing the surface of the plate with the slurry gel to form a thin layer on the glass plate. The coated plates were left in the open laboratory to dry for three hours at room temperature after which they were activated in the oven at a temperature of 110 ^{0}C. The plates were then used for TLC. The developed TLC plate was allowed to dry in the open laboratory, then treated with iodine (visualizing agent) in a covered tank and left for 30 minutes to allow iodine vapour to saturate the tank. When the spot became visible, the plate was removed from the iodine tank and the spot outlined. Fraction 8A (from column chromatography) gave a single spot on TLC. The retention factor (R_f) value (distance moved by the compound divided by the distance moved by the solvent front) of the fraction was calculated as 0.78 in a solvent system of 100 % chloroform.

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Spectral Analysis

Spectral analyses such as FT-IR, ^{13}C NMR, ^{1}H NMR, DEPT, COSY, HSQC, HMBC and mass spectrometry were carried out on fraction 8A. Nuclear magnetic resonance spectroscopic analysis was done using a 500 MHz Bruker AVANCE NMR spectrometer at the Department of Chemistry, Faculty of Engineering and Physical Sciences, University of Surrey, Guilford, UK. The spectra were recorded in deuterated chloroform.

RESULTS AND DISCUSSION

FT-IR Spectral Result

The FT-IR spectrum of the compound isolated from the chloroform extract of the leaves of *P. thonningii* is shown in Figure 1. Table 1 shows the observable absorption frequencies in wavenumber and the characteristic functional groups and compound type responsible for the absorptions.

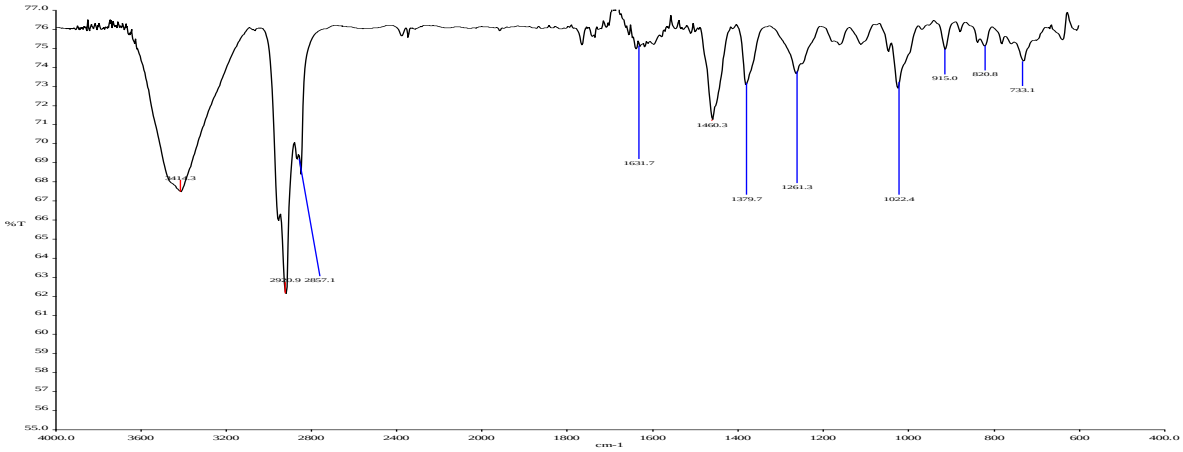

Figure 1: FT-IR spectrum of the isolated compound

Table 1: FT-IR Absorption Frequencies of the Isolated Compound

Absorption frequency (cm^{-1})	Functional group	Compound type
3414.3	OH stretch	Alcohol
2920.9	SP^3 C-H Stretch	Alkyl groups (CH_3, CH_2, CH)
2857.1	SP^3 C-H stretch	Alkyl groups (CH_3, CH_2, CH)
1631.7	C=C stretch	Alkene
1022.4	C-O	Alcohol
1460.3	C-H bend	Alkyl groups (CH_3, CH_2, CH)
1379.7	C-H bend	Methyl (CH_3)
915.0, 820.8	H_2C=C bending vibration	Alkene
733.1	C-C bending vibration	Alkyl groups (CH_3, CH_2, CH)

The strong and broad absorption at 3414.3 cm^{-1} indicates the presence of O-H stretching vibration of an alcohol. The broadness of the band could be due to intermolecular hydrogen bonding that exists between the O-H groups in the compound.

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Absorptions at 2920.9 and 2857.1 cm^{-1} indicate the presence of C-H stretching of $-CH_3$, $-CH_2$ and –CH groups of an aliphatic compound. An intense absorption at 1631.7 cm^{-1} indicates the presence of C=C stretching vibration of an alkene. Absorptions at 1460.3 and 1379.7 cm^{-1} indicate the presence of $-CH_2$ and $-CH_3$ bending vibrations of an aliphatic compound. Also, an absorption at 1022.4 cm^{-1} indicates the presence of a C-O stretching of an alcohol. The weak peaks at 915.0 and 820.8 cm^{-1} were attributed to (=C-H) bond deformation of the alkene functional group while the peak at 733.1 cm^{-1} was as a result of C-C bending vibration of alkyl groups (CH_3, CH_2 and CH). These assignments are in line with the report from Igwe and Okwu [14] and Igwe and Echeme [15].

NMR Spectral Results

The ^{1}H NMR, ^{13}C NMR and DEPT spectra of the isolated compound are shown in Figures 2, 3 and 4 respectively while Table 2 shows the various chemical shifts observed from the DEPT, ^{1}H and ^{13}C NMR spectra of the compound. Also, the COSY, HSQCDEPT and HMBC spectra of the compound are shown in Figures 5, 6 and 7 respectively.

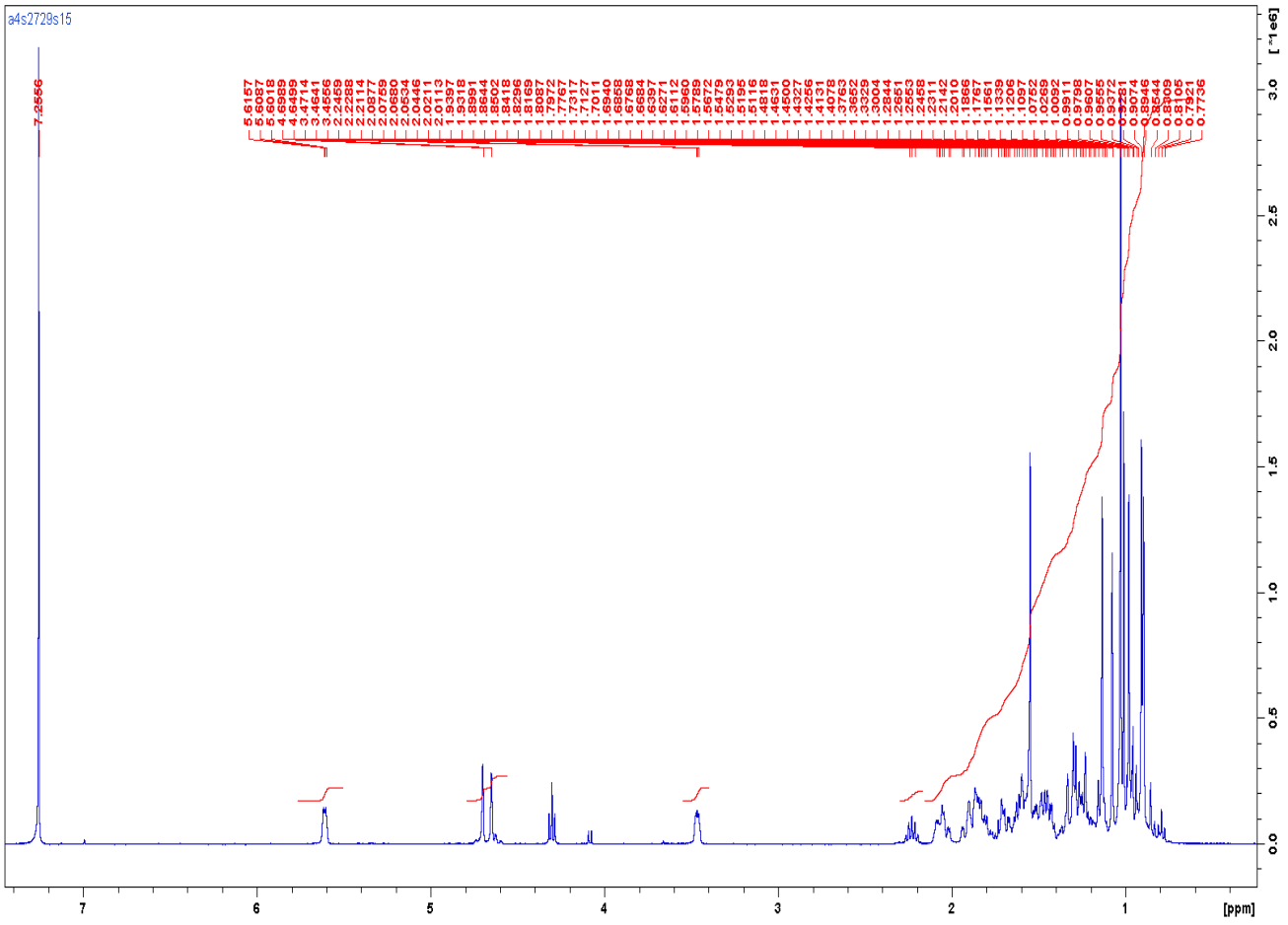

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Figure 2: [1]H NMR Spectrum of the isolated compound

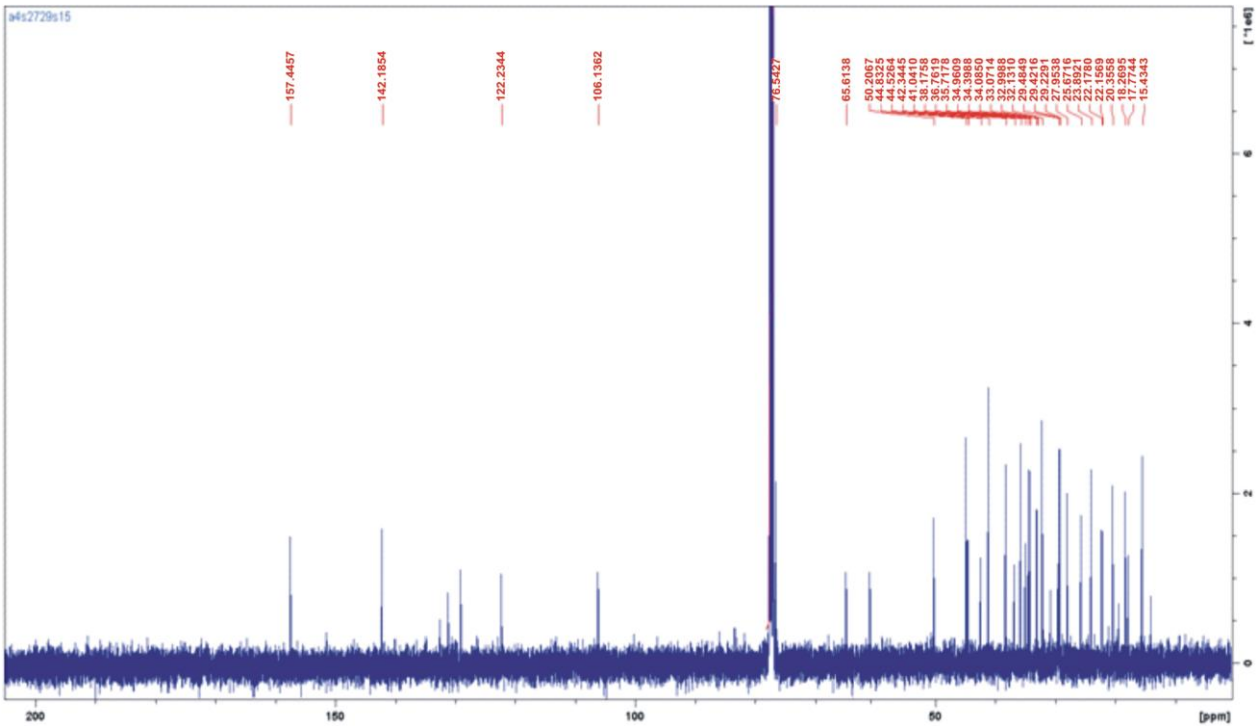

Figure 3: [13] C NMR Spectrum of the isolated compound

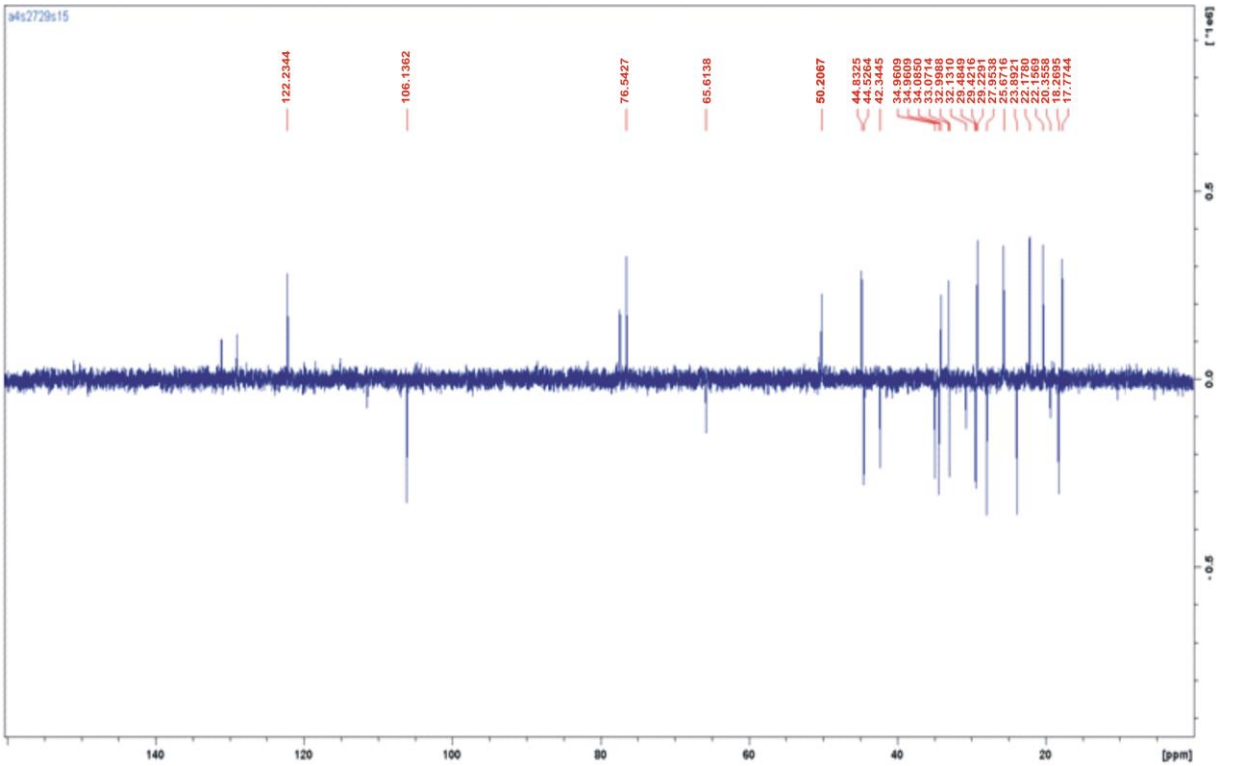

Figure 4: DEPT NMR Spectrum of the isolated compound

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Table 2: DEPT, ^{13}C NMR and ^{1}H NMR Chemical Shifts of the Isolated Compound

Carbon atom	**DEPT**	**^{13}C NMR (ppm)**	**^{1}H NMR (ppm)**
C-1	CH_2	35.00	1.53
C-2	CH_2	29.23	1.23
C-3	CH	76.50	3.43
C-4	C	35.72	-
C-5	CH	50.20	2.00
C-6	CH_2	18.30	0.83
C-7	CH_2	32.99	1.41
C-8	C	36.80	-
C-9	CH	44.53	1.64
C-10	C	34.40	-
C-11	CH_2	25.70	1.08
C-12	CH	122.2	5.62
C-13	C	142.2	-
C-14	C	38.18	-
C-15	CH_2	29.50	1.30
C-16	CH_2	34.10	1.48
C-17	C	41.04	-
C-18	CH	44.83	1.84
C-19	CH	42.34	1.58
C-20	C	157.5	-
C-21	CH_2	32.13	1.37
C-22	CH_2	33.10	1.45
C-23	CH_3	23.90	0.97
C-24	CH_3	22.20	0.93
C-25	CH_3	17.80	0.79
C-26	CH_3	15.40	0.77
C-27	CH_3	28.00	1.33
C-28	CH_2	65.00	2.30
C-29	CH_2	106.1	4.70
C-30	CH_3	20.40	0.89

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In the ^{1}H NMR ($CDCl_3$, ppm), ∂H 5.62(t, H, J = 2.80, 5.56 Hz) was assigned to the methane proton on C-12, the doublet proton signal at 4.70 (d, 2H, J = 19.6 Hz) was assigned to the vinylic protons on C-29 while the triplet peaks at 3.47(t, H, J = 2.92, 6. 32 Hz) were characteristic of methane protons assigned to C-3. The signals ranging from 0.78 to 2.24 ppm are mostly due to methyl, methylene and methine signals characteristic of highly saturated molecules like terpenes and steroids. These assignments are in line with the report from Igwe and Okwu [16].

^{13}C NMR ($CDCl_3$, ppm) has shown ten methylene (CH_2) carbons (at C-1, 2, 6, 7, 11, 15, 16, 21, 22 and 28), five tertiary carbons (CH) (at C-3, 5, 9, 18 and 19), five quaternary carbons (C) (at C-4, 8, 10, 14 and 17), four methene carbons (C=C) (at C-12, 13, 20 and 29) and six methyl carbons (CH_3) (at C-23, 24, 25, 26, 27 and 30). The carbon signals were assigned as shown in the Table 2. Diagnostic signals are 76.5 ppm oxy-methine carbon assigned to C-3 position; the signals at 106.1 and 157.5 ppm were assigned to C-29 and C-20 respectively. These assigned signals are characteristic of a pentacyclic triterpene (lupane) ring. The signals at 122.2 and 142.2 ppm were assigned to the carbon-carbon double bond at position C-12 and C-13 respectively. The DEPT 135 NMR Spectrum showed 1.1 CH_3, 1.1 CH_2 and CH groups.

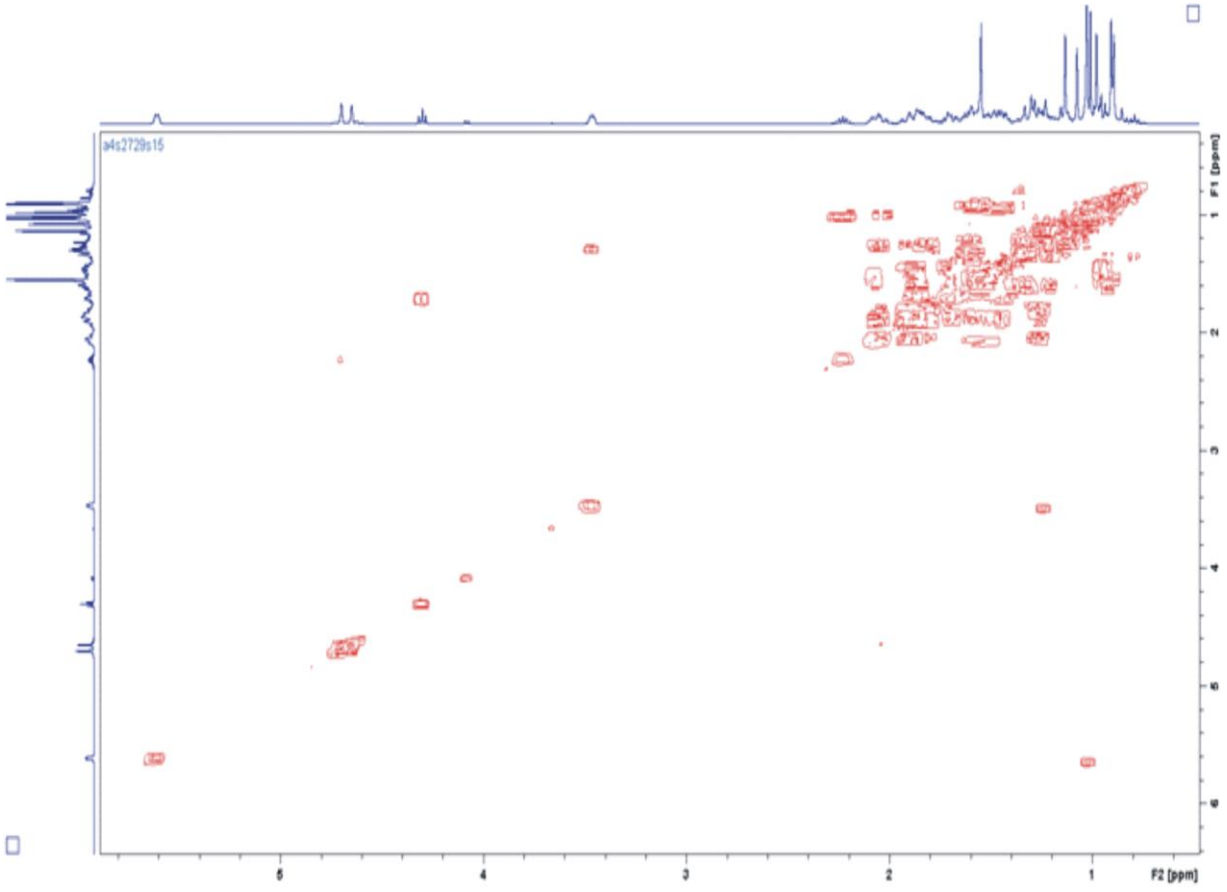

Figure 5: COSY NMR Spectrum of the isolated compound

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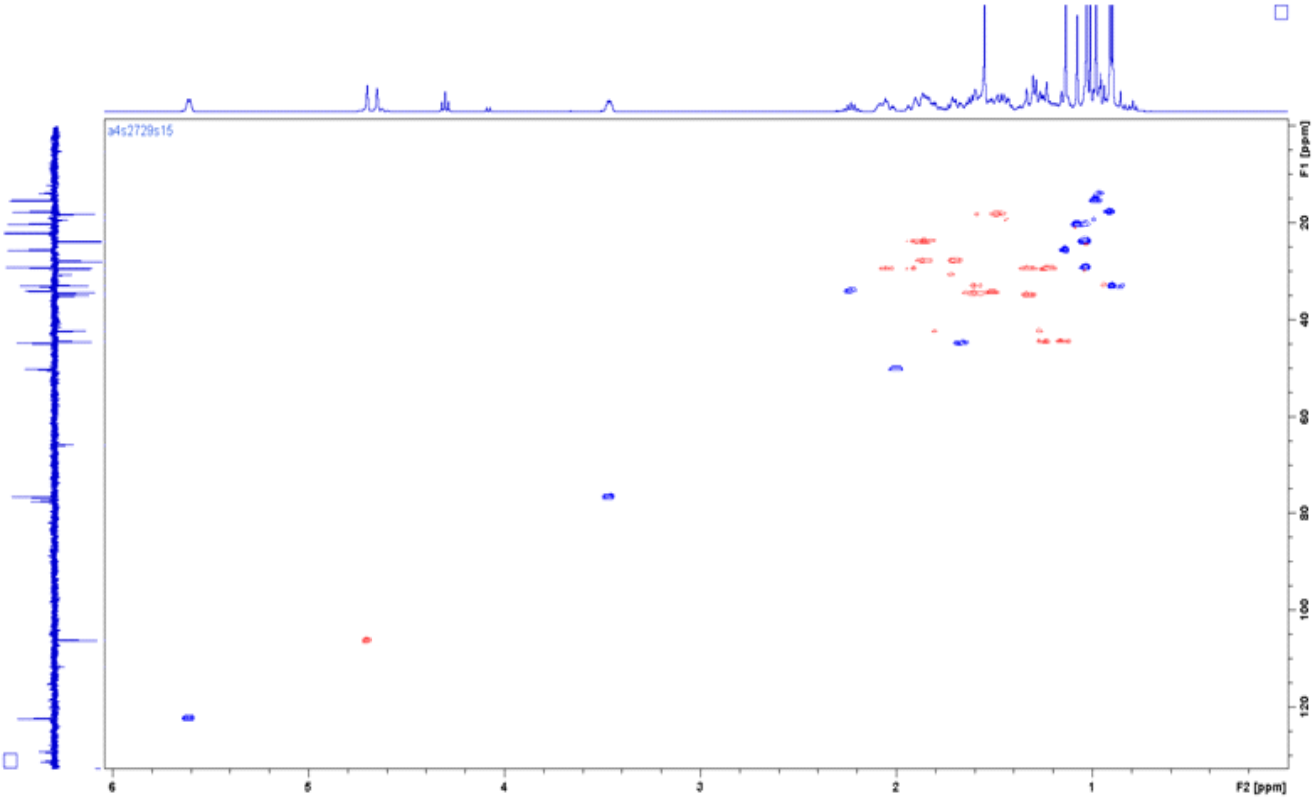

Figure 6: HSQCDEPT NMR Spectrum of the isolated compound

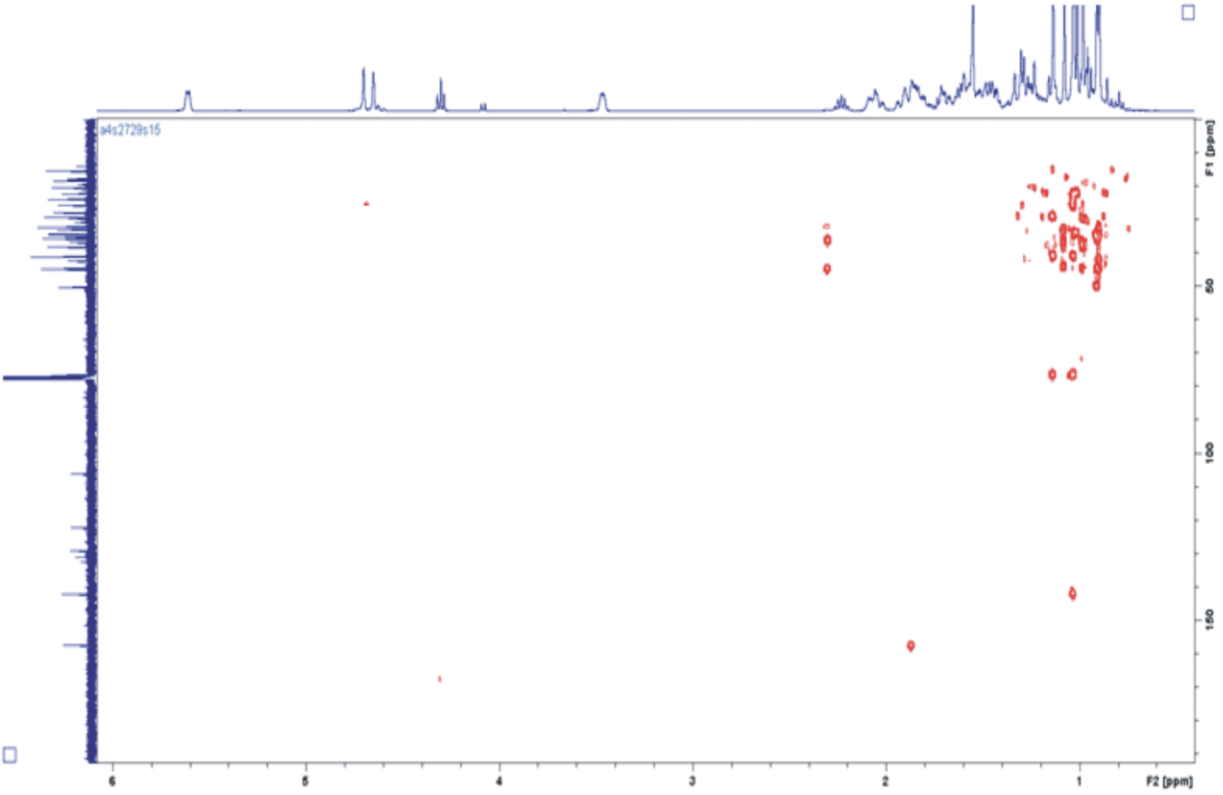

Figure 7: HMBC NMR Spectrum of the isolated compound

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The COSY NMR Spectrum showed correlation between proton (H-12) at 5.62 ppm and proton (H-11) at 1.08 ppm. Also there was correlation proton (H-3) at 3.45 ppm and proton (H-2) at 1.23 ppm. The COSY NMR Spectrum also showed correlation between proton (H-22) at1.45 ppm and proton (H-23) at 0.97 ppm.

The HSQCDEPT Spectrum showed single bond correlation between carbon atom (C-12) at 122.2 ppm and the proton (H-12) at 5.62 ppm. There were also correlations between carbon atom (C-29) at 106.1 ppm and proton (H-29) at 4.70 ppm, carbon atom (C-3) at 76.5 ppm and proton (H-3) at 3.45 ppm, carbon atom (C-5) at 50.2 ppm and proton (H-5) at 2.0 ppm, and carbon atom (C-9) at 44.53 ppm and proton (C-9) at 1.64 ppm respectively.

In HMBC NMR Spectrum, there was multiple bond correlation between carbon atom (C-30) at 20.4 ppm and proton (H-29) at 4.7 ppm. Also, there was multiple bond correlation between carbon atom(C-20) at 157.5 ppm and proton (C-18) at 1.84 ppm, and between carbon atom (C-15) at 29.5 ppm and proton (H-27) at 1.33 ppm respectively. There was also multiple bond correlation between carbon atom (C-22) at 33.1 ppm and proton (H-28) at 2.3 ppm.

Mass Spectral Result

The mass spectrum of the isolated compound is shown in Figure 8. The molecular ion peak for the compound is seen at 440.3 amu while the base peak is found at 187.0 amu. From all the spectral data interpretations, the structure of the compound is hereby proposed to be Lup-12,20-(29)-dien-3,28-diol (Figure 9) with a molecular formula of $C_{30}H_{48}O_2$. The mass spectrometry molecular ion peak of 440.3 (m/z) was very close to the calculated molecular ion peak of 440.7 g/mol. Other peaks due to the daughter ions which are typical of the isolated compound are shown in its fragmentation pattern in Figure 10.

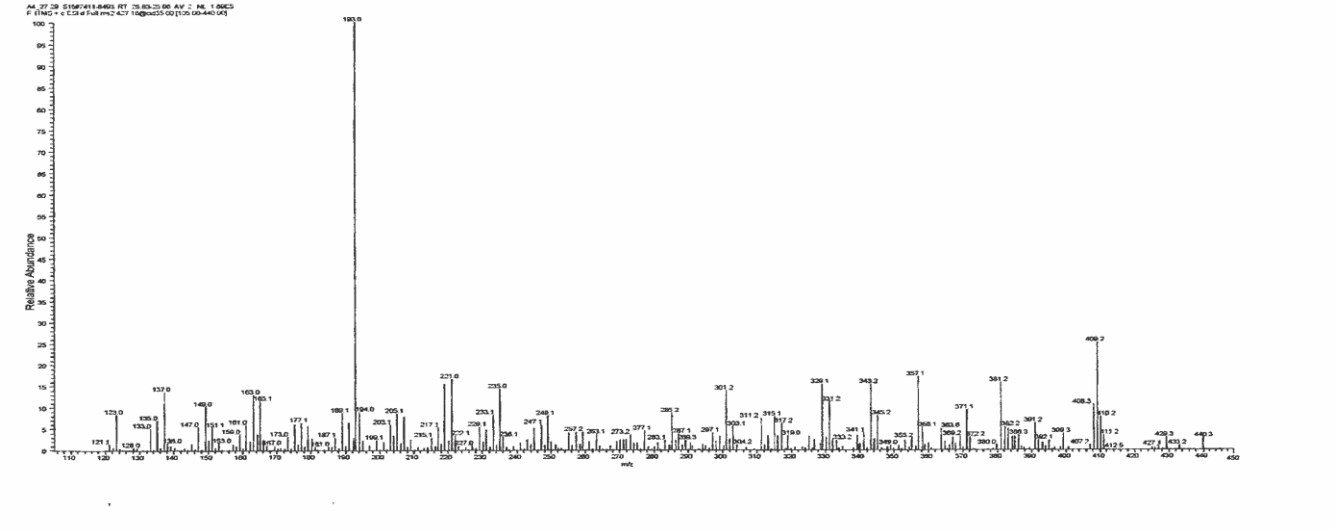

Figure 8: Mass spectrum of the isolated compound

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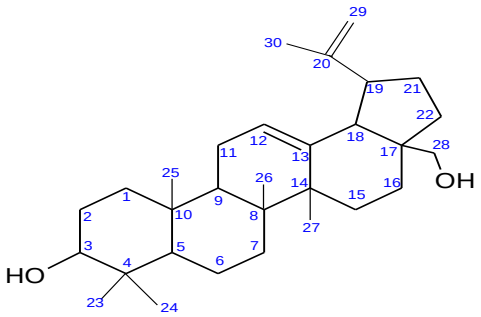

Figure 9: Lup-12,20 (29)-dien-3,28-diol

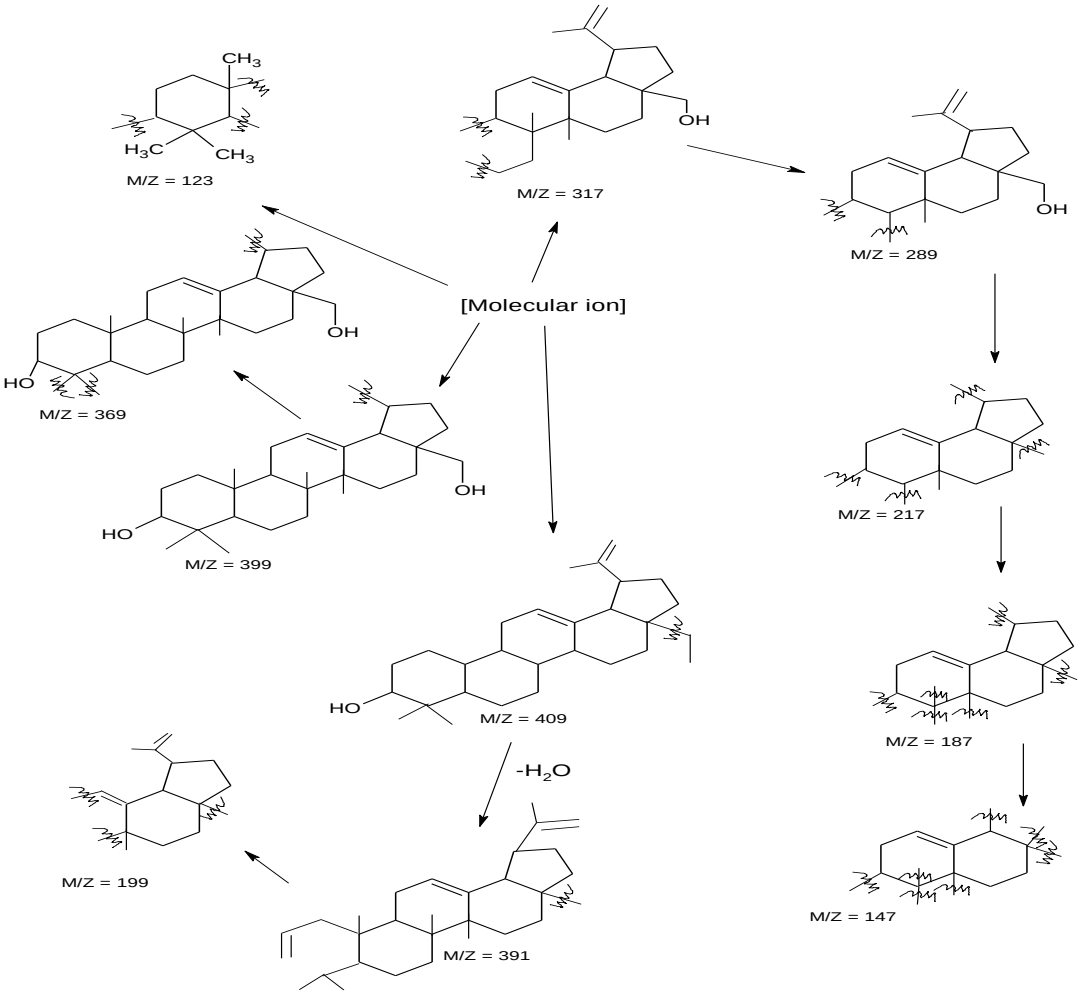

Figure 10: Fragmentation pattern of the isolated compound

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The most thoroughly studied lupane-type pentacyclic triterpenes are betulin, betulinic acid and lupeol which had been reported as strong chemopreventive and anticancer agents against a large variety of cancer cells [17, 18]. The compound isolated herein is a pentacyclic triterpenoid and a novel derivative of Lupane or Lupeol or Lup-20(29)-en-3H-ol which is a dietary triterpene found as an active constituent of various medicinal plants, fruits and vegetables [19]. Lupeol possesses strong antioxidant, antiinflammatory, antiarthritic, antimutagenic, antimalarial and anticancer activities [19]. The ability of lupane-type triterpenes in apoptosis and cell cycle regulation is highly important in the quest for new therapeutic agents for treatment of cancer. Recent approaches have found that lupeol possesses anti-gastric cancer activities [20]. Some lupane triterpenes have been identified in various plant species and have been reported to be active against Mycobacterium tuberculosis [18]. The isolated compound, Lup-12,20 (29)-dien-3,28-diol, may share similar pharmacology properties with lupane since they have similar molecular scaffold, however, further studies are required.

CONCLUSION

The isolated compound is proposed to be Lup-12,20 (29)-dien-3,28-diol with a molecular formula of $C_{30}H_{48}O_2$. The structure of the compound suggests that it is a triterpenoid, having a lupeol skeleton with a hydroxyl substitution at C_3 and C_{28} and an unsaturation at C_{12} and C_{13} as its distinguishing properties which were confirmed by the FTIR, ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQCDEPT, HMBC and Mass spectral results obtained for the isolated compound. This is the first isolation of a lupane-type triterpenoid from the leaves of *P. thonningii*. This compound could not be found anywhere in the literature. Therefore, this research presents the isolation of a novel compound from the chloroform extract of the leaves of *P. thonningii*. Further studies are required on the compound to ascertain its pharmacological properties.

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