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Isolation and Identification of β-sitosterol, 7-hydroxystigmast-22-en-3,6-dione and 3β, 24(S)-dihydroxycholesta-5, 25-diene-7-one from stem bark of *Nauclea pobeguinii*

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ABSTRACT

The stem bark of the *Nauclea pobeguinii* was collected, air-dried, and pulverized and was extracted with solvent of varying polarity (n-hexane, ethyl acetate, and ethanol) to obtain the crude extracts. Silica gel column and thin layer chromatographic separation afforded three compounds whose structures were elucidated as β-sitosterol (1), 7-hydroxystigmast-22-en-3.6-dione (2), and 3β, 24(S)-dihydroxycholesta-5, 25-dien-7-one (3) by analysis of their chemical and spectral characteristic from 1D and 2D NMR, FTIR and by comparing of data with those reported in the literature.

Keywords: *lea pobeguinii;* Phytochemical; Isolation; steroids; β-sitosterol.

INTRODUCTION

Over the years, bioactive compounds from plants have been investigated as potential alternative therapeutic options in the management of diseases and infections. The attention on the medicinal plant is due to the presence of a large number of secondary metabolites with their novel mechanism of action and safety compared to synthetic drugs (Ashour *et al*., 2019).

Nauclea species belong to the family Rubiaceae, which is the largest family of woody plants consisting of more than 13,000 species, the family is divided into sub-families including the Cinchonoideae which consist of the genus *Nauclea* that are often found in a tropical area, such as Africa and Asia (Haudecoeur *et al.*, 2018). *Nauclea pobeguinii* (Hua ex Pobég) Merr. locally called Opepe ira in western Nigeria is a deciduous shrub that is endemic to the swamp forest regions of the world. The tree grows to a height of about 30 m high and produces soft yellow sponge-like fruits. It has reportedly been useful in the treatment of jaundice, gonorrhea, fever, stomach ache, and epilepsy (Njoya *et al*., 2017). In Cameroon, the plant is used in the management of hyperglycemia, articular pain, stomach pains, and inflammation (Tsafack *et al*., 2021). Anti-helminthic application of stem bark water decoction of *N. diderrichi* and *N. pobeguinii* were also reported in Congo while its anti-malaria activity was recorded in Mali (Haudecoeur *et al*., 2018). Anti-abortifacient and the ability to treat sexual and reproductive dysfunction properties of *N. pobeguinii* have also been reported in works of literature (Luzakibanza 2012). Methanolic bark and leaf extract of *N. pobeguinii* was also reported to possess anti-proliferative activity on cancer lines.

Phytochemical screening of the plant revealed the presence of alkaloids, saponins, tannins, phenols, flavonoids, and terpenoids (Adepoju *et al.*, 2020). Numerous kinds of literature correlate the ethno-medicinal uses of *N. pobeguinii* to their alkaloid content, including the indole alkaloid-Strictosamide which was responsible for its anti-proliferative and antimalarial activity. Other phytochemicals previously isolated from *N. pobeguinii* include naucleidinal, naucleofficine, kelampayoside, magniflorine, augustine, strictosidine (Xu *et al*., 2012). This study is aimed at further isolation and characterization of bioactive components from *N. pobeguinii* stem bark.

METHODOLOGY

Materials

Organic solvents used (n-hexane, ethyl acetate, chloroform, methanol, and ethanol) were purchased from Sigma-Aldrich Laborchemikalien GmbH. Silica gel for Column Chromatography (Merck Kieselgel 60 PF253 Art No. 7734.1000 and 9385.1000 with the particle size 0.063 - 0.200 mm and 0.040 - 0.063 mm, respectively), and silica gel (60 F_{254}) Aluminium sheet were obtained from Merck, Darmstadt, Germany. Other equipments used include a Rotary evaporator, Ultraviolet lamp (254 and 366 nm) model UVGL-58, San Gabriel, U.S.A. IR spectra were recorded on a Perkin –Elmer FTIR (2000) spectrum BX spectrometer, using Attenuated total reflectance (ATR) and absorption bands were measured in cm^{-1}. Proton Nuclear

Magnetic Resonance (^{1}H-NMR), ^{13}C Nuclear Magnetic Resonance (^{13}C NMR), and 2D-NMR spectra were obtained with Bruker- FT-NMR Spectrometer (500 MHz). Chemical shift (δ) was recorded in ppm relative to tetramethylsilane signal (TMS) and Deuterated ($CDCl_3$) solvents were used. The signals were described in terms of chemical shift with appropriate abbreviations for multiplicities such as *s (singlet), d (doublet), t (triplet), q (quartet),* and *m (multiplet).* Commercial silica gel 60 PF253 Art No 7734.1000 and 9385.1000 with particle size 0.063 - 0.200 mm and 0.040 – 0.063 mm, respectively was applied for column chromatography (CC) and precoated silica gel plates (60 F25 Merck) were used for analytical thin layer chromatography (TLC). Fractions were monitored by TLC and spots were detected under Ultra Violet (UV) light (254 or 366 nm).

Collection and preparation of plant material

The stem bark of *N. pobegunii* was collected from Forest Research Institute of Nigeria (FRIN), Jericho, Ibadan, Oyo State, South West, Nigeria, it was identified and authenticated with the herbarium number FHI 108529. The plant material was air-dried for one month, after which it was pulverized with an electrical blender and stored in a moisture-free container till further analysis.

Extraction of Phytochemicals

Extraction was done by continuously soaking the dried ground stem bark (2 kg) with n-hexane (5.5 L), ethyl acetate (5.0 L), and ethanol (4.5 L). Successive extraction was adopted by using n-hexane first to remove non-polar organic compounds, waxes, and fats. The sample was drained out after one week. This process was followed by extraction with ethyl acetate and ethanol to remove more polar compounds. Each extract obtained was filtered and evaporated to dryness using a rotary evaporator at 40 °C to yield ethyl acetate (10.46 g) and ethanolic (18.54 g) extracts (Hesham *et al*., 2016; Njoya *et al*., 2017).

Isolation of Phytochemicals from *N. pobegunii* crude extracts

Ethyl acetate extract (EAS) (5.0 g) was dissolved in chloroform, it was mixed with 20 g of silica gel, and homogenized in a beaker to form a loose powder. This powder was loaded on a silica gel column and eluted with n-hexane, n-hexane/chloroform, chloroform/methanol, and methanol to yield a total of 70 fractions of 100 mL each. Fraction 7 (EAS 7) ($CHCl_3$ 100%) gave white powder on evaporation but was not pure when viewed under a UV lamp. It was further purified using column chromatography with the mobile phase of increasing polarity as follows; n-hexane 100%, n-hexane/$CHCl_3$ 9:1, n-hexane/$CHCl_3$ 8:2, n-hexane/$CHCl_3$7:3, n-hexane/$CHCl_3$ 6:4, n-hexane/$CHCl_3$ 5:5, n-hexane /$CHCl_3$ 4:6, n-hexane/$CHCl_3$ 3:7, n-hexane /$CHCl_3$ 2:8, n-hexane/$CHCl_3$ 1:9, $CHCl_3$ 100%. A total of 82 fractions (30 mL) each were obtained. Fractions 51 – 55 (Hex.$CHCl_3$ 3:7) formed white powder on evaporation. The isolated compound showed a dark blue spot on the TLC plate when viewed under the UV lamp at 254 nm. It was labeled compound 1 and the spectroscopic data was recorded. Spectroscopic data suggested compound 1 to be β-sitosterol.

Fractions 3-6 (EAS 3-6) were combined and re-chromatographed on a silica gel column eluted with chloroform/methanol mixture to give 50 fractions of 30 mL each. Fractions 11 and 12 gave similar TLC profiles and were therefore combined and concentrated. The resulting yellow amorphous solid was labeled compound 2 and the spectroscopic data was recorded. The data suggested compound 2 to be 7-hydroxystigmast-22-en-3,6-dione.

Compound 3 was obtained from chromatographic fractionation of 6.0 g of ethanolic extract (ETS) of *N. pobeguinii* eluted with n-hexane, n-hexane/chloroform, chloroform/methanol, and methanol to give 114 fractions of 100 mL each. Fractions 46 – 50 ($CHCl_3$ 100%) were pooled together based on their TLC profile, formed white powder, and coded as ETS 46 – 50. Fraction ETS 46 – 50 was further chromatographed with a mini silica gel column using n-hexane and chloroform, 25 fractions of 30 mL each were collected. Fractions 14-17 (ETS 14 – 17) were combined based on their TLC profile. This fraction was further purified with a silica gel column with n-hexane/chloroform 2:8 as the mobile phase. 18 fractions of 30 mL each were collected and fraction 11 appeared as a single spot on the TLC plate. This fraction was concentrated to obtain a white powder labeled as compound 3. The spectroscopic data suggested compound 3 to be 3β, 24(S)-dihydroxycholesta-5,25-dien-7-one.

RESULTS AND DISCUSSION

Three compounds were successfully isolated and characterized from the ethyl acetate and ethanol stem bark extracts of *N. pobeguinii*. Structural elucidation of the compounds was done by spectroscopic techniques such as FTIR, ^{1}H-NMR, ^{13}C-NMR, DEPT-135, COSY, HSQC, and a comparison of spectral data with those reported in literature. The characterizations of the three compounds are therefore described as follows.

Table I. ^{13}C and ^{1}H chemical shift values for β-sitosterol recorded in CDCl3 (500MHz)

Carbon no	Dept	^{13}C	^{13}C *	^{1}H	^{1}H*
C-1	CH_2	33.72	37.28		
C-2	CH_2	31.67	31.69		
C-3	CH(OH)	71.83	71.82	3.48(m)	3.54(m)
C-4	CH_2	42.30	42.33		
C-5	QC(=)	140.77	140.77	-	-
C-6	CH(=)	121.74	121.73	5.30(t)	5.37(overlapping,t)
C-7	CH_2	29.15	31.93		
C-8	CH	33.95	31.93		
C-9	CH	56.06	50.16		
C-10	QC	39.78	36.51		
C-11	CH_2	19.83	21.11		
C-12	CH_2	37.36	39.80		
C-13	QC	42.33	42.34		
C-14	CH	56.77	56.79		
C-15	CH_2	26.07	24.33		
C-16	CH_2	24.31	28.27		
C-17	CH	56.87	56.08		
C-18	CH_3	11.99	11.89	0.60(s)	0.7(s)
C-19	CH_3	21.09	19.42	0.94(s)	1.03(s)
C-20	CH	36.51	36.17		
C-21	CH_3	18.71	18.84		
C-22	CH_2	36.16	33.98		
C-23	CH_3	28.26	26.11		
C-24	CH	45.84	45.86		
C-25	CH	31.91	29.19		
C-26	CH_3	19.41	19.84		
C-27	CH_3	19.04	19.06		
C-28	CH_2	23.07	21.10		
C-29	CH_3	18.79	12.01		

The chemical shift values (δ, ppm) were compared with what was obtained by Ododo *et al*., 2016 previously. Assignments were made based on COSY and HSQC correlations.

Characterization of compound 1 (EAS_{51-55})

Compound 1 (white powder) with an R_f value of 0.5 (3:7, n-hexane/$CHCl_3$) which was positive to the Liebermann- Burchard test gave a violet-blue color that later turned green indicating its steroidal nature. Further analysis by FTIR (Suppl. 1) revealed important absorption bands (cm^{-1}) such as hydroxyl functional group (O - H) at 3430, alkyl chain at 2938 – 2848, olefinic band at 1655, cyclic methylene group $(CH_2)_n$ at 1490, gem-dimethyl (-$CH(CH_3)_2$) isopropyl at 1371 and C-OH of secondary alcohol at 1042. The ^{1}H –NMR spectrum (Suppl. 2a and 2b) revealed the presence of fifty hydrogen atoms; six methyls, eleven methylene, nine methines, and one hydroxyl proton as shown in Table I. The two singlets at δ_H 0.60 and δ_H 0.94 validate the presence of methyl protons H18 and H19 attached to the quaternary carbons C10 and C13, respectively. Multiplet at δ_H 3.48 was assigned to the H3 attached to the carbon (δ_c 71.83) that bears the OH group. Also, the signal at δ_H 5.30 was assigned to the olefinic proton H6. The ^{13}C- NMR spectrum (Suppl. 3) exhibited 29 signals which were classified as six methyls, eleven methylene, nine methine, and three quaternary carbons. DEPT-135 spectrum (Suppl. 4) revealed 26 signals without signals for the three quaternary carbons.

The main signals in the ^{13}C-NMR spectrum are the signals at δ_c 140.77 and 121.74 for the vinylic carbons C5 and C6 (C5 = C6), respectively. The signal at δ_c 71.83 was assigned to the carbon that is bonded to the hydroxyl group (C3 - OH). The two angular methyl carbons C18 and C19 resonated at δ_C 11.99 and 21.09, respectively. COSY spectrum (Suppl. 5) revealed the ^{1}H – ^{1}H correlation between H3/H1, H3/H2, H3/H4, H6/H7, and H6/H4 while the HQSC spectrum (Suppl. 6) shows the correlation between carbon and protons attached to it. There are correlation

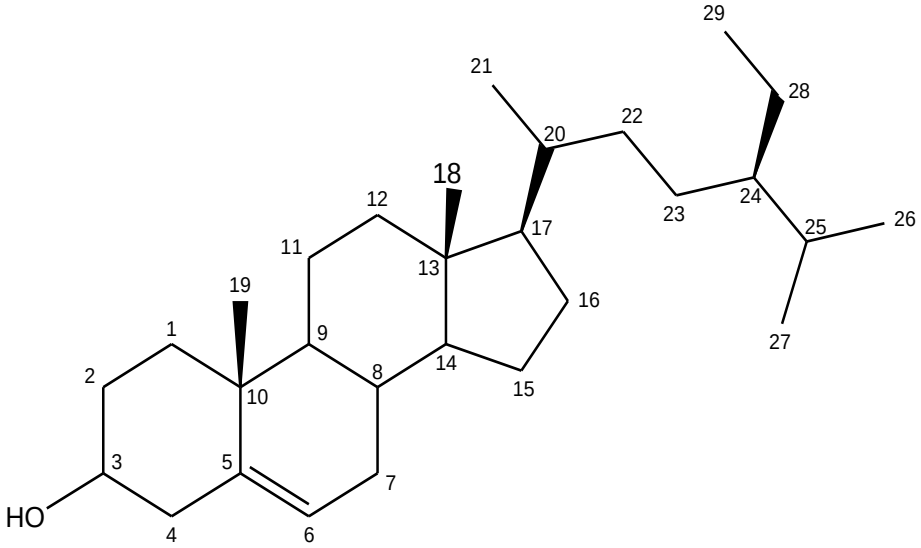

Figure 1. Structure of β-sitosterol

Table II. ^{13}C -NMR and ^{1}H -NMR spectral values of 7-hydroxystigmast-22-en-3,6-dione compared with related stigmastane-3,6-dione in literature recorded in $CDCl_3$ (500MHz)

Carbon no	Dept	^{13}C	^{13}C *	^{1}H	^{1}H*
C-1	CH_2	38.35	38.20		
C-2	CH_2	37.09	37.45		
C-3	QC	210.34	211.20		
C-4	CH_2	37.02	37.06		
C-5	CH	56.50	57.61		
C-6	QC	208.17	209.08		
C-7	CH	64.02	46.70	4.1	1.99
C-8	CH	35.03	38.12		
C-9	CH	52.46	53.61		
C-10	QC	41.98	41.31		
C-11	CH_2	22.99	21.76		
C-12	CH_2	36.37	39.48		
C-13	QC	44.77	43.10		
C-14	CH	55.59	56.71		
C-15	CH_2	27.02	24.08		
C-16	CH_2	28.11	28.11		
C-17	CH	54.99	56.12		
C-18	CH_3	11.54	12.09	0.60(s)	0.69(s)
C-19	CH_3	13.09	12.62	0.85(s)	0.95(s)
C-20	CH	35.98	36.12		
C-21	CH_3	18.79	18.77	0.82(d)	0.92(d)
C-22	CH	128.64	33.93	5.30	1.04
C-23	CH	128.64	26.19	5.30	1.15
C-24	CH	45.61	45.91		
C-25	CH	30.90	29.26		
C-26	CH_3	21.67	19.86	0.76(d)	0.84(d)
C-27	CH_3	20.65	19.1	0.75(d)	0.81(d)
C-28	CH_2	23.86	23.16		
C-29	CH_3	10.99	12.04	0.78(t)	0.83(t)

The chemical shift values (δ, ppm) were compared with what was obtained by Wei *et al*., 2004 previously. Assignments were made based on COSY and HSQC correlations.

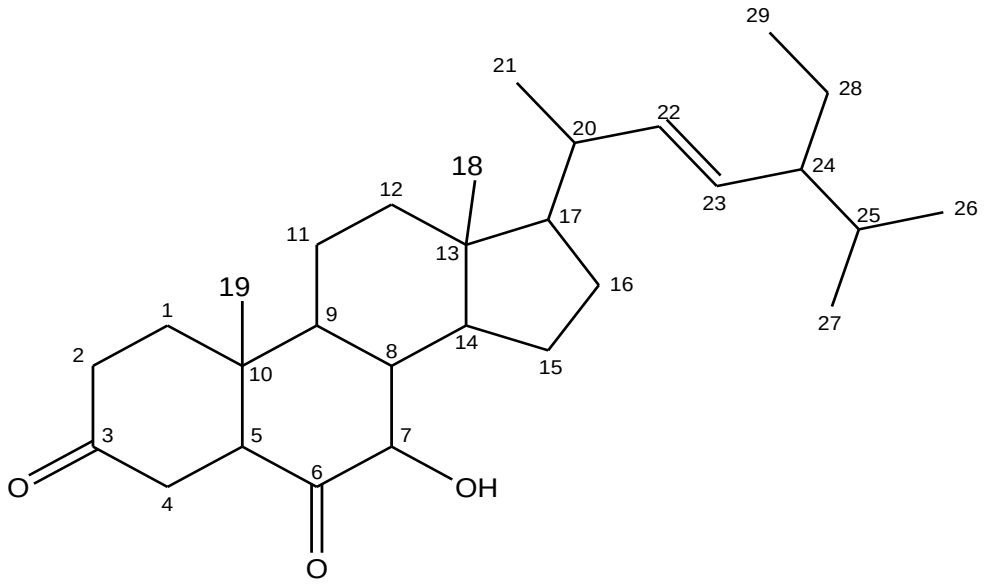

Figure 2. Structure of 7-hydroxystigmast-22-en-3,6-dione

Table III. ^{13}C-NMR and 1H-NMR spectral values of 3β, 24(S)-dihydroxylcholesta-5,25-dien-7-one compared with literature recorded in $CDCl_3$ (500MHz)

Carbon no	Dept	^{13}C	^{13}C *	^{1}H	^{1}H*
C-1	CH_2	36.17	36.36		
C-2	CH_2	30.79	31.22		
C-3	CH	71.02	70.55	3.46 (1H, m)	3.67 (1H, m)
C-4	CH_2	41.31	41.83		
C-5	QC	170.88	165.32		
C-6	CH	122.92	126.94	5.65(1H, s)	5.69 (1H,s)
C-7	QC	198.92	202.15		
C-8	CH	49.14	45.41		
C-9	CH	54.87	49.93		
C-10	QC	38.56	38.29		
C-11	CH_2	22.08	21.23		
C-12	CH_2	38.72	38.71		
C-13	QC	44.78	43.13		
C-14	CH	55.00	49.98		
C-15	CH_2	27.16	26.30		
C-16	CH_2	28.04	28.49		
C-17	CH	55.76	54.56		
C-18	CH_3	10.97	11.98		
C-19	CH_3	18.84	18.90		
C-20	CH	35.45	35.53		
C-21	CH_3	18.41	18.32		
C-22	CH_2	31.94	31.72		
C-23	CH_2	31.02	31.22		
C-24	CH	74.02	76.74		
C-25	QC	139.95	147.54		
C-26	CH_2	120.82	111.40	5.28 (1H) 5.27(1H)	4.93(1H) 4.83(1H)
C-27	CH_3	17.66	17.23		

The chemical shift values (δ, ppm) were compared with what was obtained by Yang *et al*., 2011 previously. Assignments were made based on COSY and HSQC correlations.

between H18 – C18 at δ_C 11.99, H19 – C19 at δ_C 21.09, H6 – C6 at δ_C 121.74 and H3 – C3 at δ_C 71.83. When all these spectra data were put together and compared with the literature (Ododo *et al*., 2016), compound 1 was characterized as β-sitosterol with molecular formula $C_{29}H_{50}O$ (Figure 1).

Characterization of compound 2 (EAS_{11-12})

Compound 2 was isolated as a yellow amorphous solid with an R_f value of 0.4 (100 % $CHCl_3$) which was also positive for the Liebermann-Burchard test. The IR absorption band (cm^{-1}) (Suppl. 7) at 3489 is due to O – H stretching vibration, bands at 2922 – 2848 are due to stretching vibration of – CH_3 and – CH_2, 1714 is for the stretching vibration of carbonyl, band at 1655 showed the presence of carbon-carbon double bond (C = C), band at 1446 is due to cyclic methylene groups $(CH_2)_n$ and band at 1371 are due to the presence of gem-dimethyl (- $CH(CH_3)_2$) group. The ^{1}H- NMR spectrum (Suppl. 8) of compound 2 showed the presence of 46 protons, six high-intensity peaks which correspond to the methyl protons H18, H26, H27, H29, H21 and H19 at δ_H 0.6, 0.75, 0.76,0.78, 0.82 and 0.85, respectively. The proton at H7 corresponds to the proton attached to the carbon that bears the hydroxyl group and resonated at δ_H 4.1. At δ_H 5.30 are the protons at H22 and H23 which revealed the presence of symmetric olefinic protons. ^{13}C- NMR spectrum (9) of compound 2 showed a total of 29 signals classified as methyl, methylene, methine, and quaternary carbons. DEPT-135 spectrum (10) showed the presence of 24 signals instead of 25. This is because of the two olefinic carbons C22 and C23 that overlapped at δ_C 128.64. The important signals in the ^{13}C-NMR spectrum are at δ_C 210.47 and 208.15 which corresponded to C3 and C6, respectively. The signal at δ_C 128.64 for the olefinic carbons C22 and C23. The signal at δ_C 64.06 is due to C7 that bears the hydroxyl group. Signal at δ_C 11.54 and 13.09 are for the two angular methyl carbons C18 and C19, respectively. COSY spectrum (Suppl.11) shows the correlation between H7/H8, H22/H24, H22/H20 through cross peak. More so, the HSQC spectrum (Suppl. 12) reveals the correlation between carbons and protons. There is correlation between H7 and C7 (64.02), H22 and C22 δ_C 128.64, H18 and C18 δ_C 11.54, H19 and C19 δ_C 13.09, H5 and C5 δ_C 56.50. The HSQC spectrum also showed that C6 δ_C 208.17 and C3 δ_C 210.34 have no correlation with any proton which established the presence of two carbonyl carbons that are quaternary in the structure of compound 2. Based on the spectra data of compound 2 and comparison with spectra data of stigmastane -3,6-dione from literature (Wei *et al*., 2004), compound 2 is proposed to be 7-hydroxystigmast-22-en-3,6-dione. The major differences in the spectra data of compound 2 and that of published data for stigmastane-3,6-dione are the chemical shifts for hydroxyl group at C7 and the double bond at C22 of the side chain. Therefore, compound 2 was elucidated to be 7-hydroxystigmast-22-en-3,6-dione with molecular formula $C_{29}H_{46}O_3$.

Characterization of compound 3 (EAS_{11})

Compound 3 with R_f value 0.5 (2:8, n-hexane/$CHCl_3$) was isolated as a white powder which was positive for Liebermann- Burchard reagent confirming its steroidal or triterpenoid nature. This was then validated by NMR spectra that the compound contains steroidal nucleus. The IR spectrum (Suppl. 13) showed the important absorption bands (cm^{-1}). Band at 3415 revealed O – H functional group, 1670 revealed the presence of conjugated carbonyl functional group (C=O), 1550 also revealed the presence of conjugated double bond, 2938 correspond to aliphatic or C-H stretching (CH_3), 1400 indicates the presence of C-O in compound 3. The proton NMR spectrum (Suppl. 14) showed the presence of 42 protons in the compound. This spectrum also revealed four groups of methyl protons attached directly to the ring and the side chain (tertiary methyl) of compound 3 at δ_H 0.68 (H18), δ_H 0.97 (H19), δ_H 1.75 (H27) and one doublet at δ_H 1.20 for H21. A multiplet at δ_H 3.46 is assigned to H3 that is directly attached to the carbon that bears the oxygen atom (δ_C 70.81). Also, a singlet at δ_H 5.65 corresponds to olefinic proton H6. The Signal at δ_H 4.05 is also for the second oxygenated methine H24 while the doublet at δ_H 5.30 is assigned to the olefinic proton H26. ^{13}C- NMR spectrum (suppl. 15a and 15b) showed the presence of 27 signals which include four methyl, eight methine, ten methylene, and five quaternary carbons. ^{13}C spectrum revealed the signal of two carbons attached to oxygen (oxymethine) at δ_C 71.02 (C3) and δ_C 74.02 (C24), two double bonds at δ_C 170.88, 122.92 (C5, C6) and 139.95 (C25), 120.82 (C26), one ketone δ_C 198.92 (C7), two tertiary methyl δ_C 10.97 (C18) and 18.84 (C19) and two secondary methyl δ_C 18.41 (C21) and 17.66 (C27). DEPT-135 (Suppl. 16) revealed a total of 22 carbon atom signals. Twelve signals on the positive side of the spectrum correspond to eight methine and four methyl carbons. Ten signals were shown on the negative side corresponding to the number of methylene carbon. COSY spectrum (Suppl. 17) revealed the correlation between H3/H2, H3/H4, H5/H4, H24/H23, H26/H27. HSQC spectrum (Suppl. 18) also revealed the correlation between carbons and protons in compound 3 as follows; H18 and C18 at δ_C 10.97, H19 and C19 δ_C

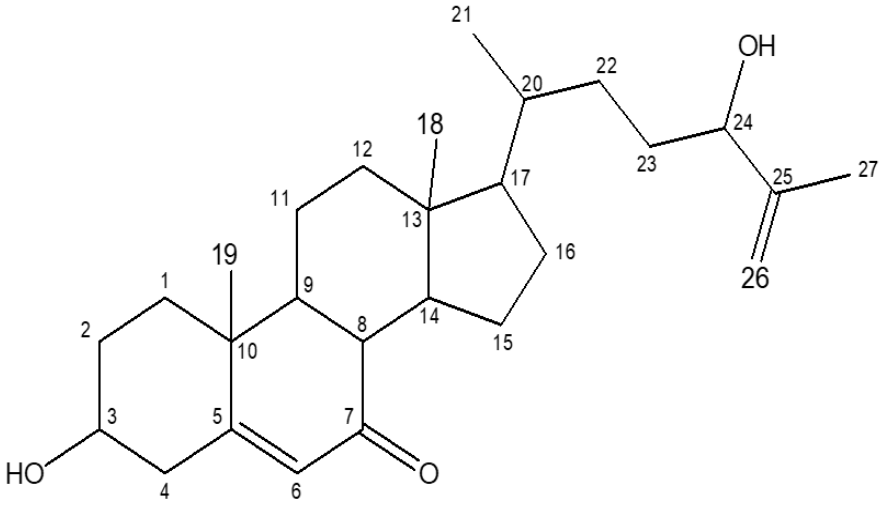

Figure 3. Structure of 3β, 24(S)-dihydroxycholesta-5,25-dien-7-one

18.84, H3 and C3 δ_C 71.02, H6 and C6 δ_C 122.92, H26 and C26 δ_C 126.82. The spectrum also showed olefinic quaternary carbons without any correlation with protons C5, C7, and C25 at δ_C 170.88, 198.92, and 139.95, respectively. The combined spectra data suggested that compound 3 is 3, 24-dihydroxycholesta-5,25-diene-7-one with molecular formula $C_{27}H_{42}O_3$ which was further validated by comparison with the literature (Yang *et al*., 2011) to be 3β, 24(S)-dihydroxycholesta-5,25-dien-7-one.

CONCLUSION

Based on the chemical and spectral evidence and comparison of obtained results with literature data, the isolated compounds are identified as β-sitosterol (1), 7-hydroxystigmast-22-en-3,6-dione (2), and 3β, 24(S)-dihydroxycholesta-5,25-dien-7-one (3) and to the best our knowledge compound 2 and 3 are being reported for the first time in *N. pobeguinii.*

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