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Some Antibacterial and Antifungal Compounds from Root Bark of *Rhus natalensis*

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Abstract Phytochemical studies on *Rhus natalensis* root bark collected from Iten, Kenya led to isolation and characterization of five compounds namely epicatechin (**1**), 3β-sitosterol (**2**), 3β-sitosterol glucoside (**3**), stigmasterol (**4**) and lupeol (**5**). The structures of the isolated compounds were established using spectroscopic techniques as well as by physical methods. The compounds were tested for bacterial and antifungal activities against *Staphylococcus aureus* (ATCC no. 25923), *Bacillus subtilis* (local isolate), *Escherichia coli* (ATCC no. 25922), *Pseudomonas aeruginosa* (ATCC no. 10622), *Candida albicans*, *Penicillium notatum* and *Aspergillus Niger*. All the compounds showed antibacterial and antifungal activities against the test pathogens. Compounds **1**, **3** and **5**, exhibited moderate activity while compounds **2** and **3** showed low activity. These results further confirm the efficacy of extracts of *Rhus species* in treating bacterial and fungal infections.

Keywords *Rhus* species, Isolation and characterization, NMR, Antibacterial, Antifungal, Inhibition zone

1. Introduction

Bacteria and fungi are frequent causes of serious opportunistic infections [1]. This is more so with a wide range of hosts with weakened immunity such as cancer patients, premature infants and human immunodeficiency virus (HIV) victims [2]. Many opportunistic bacteria and fungi are resistant to the antibiotics currently available in the market [3]. The increase in drug-resistance cannot be matched by parallel expansion in the anti-infective agents used to treat infections [4]. In addition, side effects and cost of antibiotics limit their usefulness in disease management.

About 80% of the world's inhabitants still rely on traditional medicines based on herbal plants for their primary healthcare [5,6]. Previous studies have shown that in plants there are bioactive compounds that have gained increasing interest as potential therapeutic agents [7-9]. Anti-infective agents from plant origin are preferred since they have no side effects, have better patient tolerance and are relatively inexpensive [10].

The genus *Rhus* (sumac) which belongs to the family Anacardiaceae, consists of approximately 250 species which occur mainly in the tropics, subtropics and temperate areas, especially in North America and Africa [11]. In traditional medicine, extracts of *Rhus* species are used to manage several ailments including influenza, wounds, diarrhea, abdominal pain, indigestion [12], diabetes, malaria, rheumatism [13], toothaches, swollen legs [14], dog bite, peptic ulcer, kidney stones, skin eruptions, bruises and boils [15]. Root extracts of *R. natalensis* are used to manage venereal diseases, heartburn, cold, cough, diarrhea [16], hernia and stomach ache [17], influenza, abdominal pain and gonorrhea [12]. The leaves are used for treatment of syphilis [18], cough and colds [12].

Previous studied showed that the species posses a wide range of bioactivities including antimicrobial [19-21], antiviral [21], antiinflammation [23], anticancer [24,25], antiplasmodial [26,27] and antioxidant [20,23]. Phytochemical studies revealed that Rhus species are rich in flavonoids [20,23,25,26], biflavonoids [27], anthocyanins and quinones [26], triterpenes [28] sterols and urushiols [20]. We report the antibacterial and antifungal activities of compounds from *R. natalensis.*

2. Materials and Methods

2.1. General Experimental Procedure

Melting points were determined on a Gallenkamp (Sanyo, West Sussex, UK) melting point apparatus and are uncorrected. The UV spectra were run on PyeUnicam SP8-150 UV–vis spectrophotometer (Cambridge, UK) using acetonitrile as a solvent. IR data were recorded on a Perkin-Elmer FTIR 593 series spectrophotometer (Tokyo, Japan) on KBr pellets. The ^{1}H and ^{13}C NMR data were

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recorded in CD_3OD on a Varian Gemini (Illinois, USA) operating at 400 MHz, and 100 MHz, respectively. The mass spectroscopy data were obtained on a Varian MAT 8200A instrument (Bremen, Germany).

2.2. Plant Material

The root bark of *R. natalensis* was collected from Kapkonga Iten, 40 km north east of Eldoret Town in Kenya. The plant materials were identified at the National Museums of Kenya where the voucher specimen, reference numbers PKT/R1, was deposited. The plant materials were dried under the shade at room temperature for one month and then ground into coarse using a mill (Christy and Norris Ltd., Chelmsford, England).

2.3. Solvent Extraction

Powdered root bark of *Rhus natalensis* (1.6 kg) were extracted sequentially with hexane, CH_2Cl_2, EtOAc and MeOH at room temperature for five days with occasional shaking. The macerates were filtered and concentrated at reduced pressure at 45℃ using rotary evaporator to give 12.0, 11.5, 21.5 and 400.0 g of hexane, dichloromethane, ethyl acetate and methanol extracts, respectively. The extracts were stored at 4℃ in brown glass bottles.

2.4. Fractionation of Hexane Extract

Hexane extract (11 g) was dissolved in minimum amount of hexane and adsorbed into silica gel and then evaporation to dryness. The resulting material was loaded on silica gel column (3 cm 60 cm, SiO_2 250 g, pressure = 1 bar) and eluted with hexane, hexane-DCM (4:1 and 1:1) and finally with DCM. A total of 153 fractions (each 20 ml) were sampled and their homogeneity was monitored by TLC. The fractions that showed similar TLC profiles were combined to give three pools (I–III). Pool I (fractions 20-62, 211 mg), a yellowish-brown oil, did not give any major spot and was discarded. Pool II (fractions 65-89, 156 mg) which resulted from elution with hexane-DCM 4:1, was subjected to further column chromatography over silica gel, eluting with hexane: DCM, 2:1 to yield compound **2** (24 mg). Pool III (fractions 90-128, 125 mg) which resulted from elution with hexane - DCM 1:1 solvent system was subjected to further column chromatography eluting with hexane-DCM 4:1, 3:2 and 1:1 solvent systems to give 46 fractions (15 ml each). The fractions were combined into two pools (IIIa and IIIb) depending on their TLC profiles. Pool IIIb (hexane-DCM 1:1 eluates, 85.4 mg) was subjected to PTLC fractionation using hexane-DCM 3:1 solvent system to yield 15 mg of compound **4**.

DCM extract (10.8 g) was fractionated over silica gel column using hexane, gradient hexane-DCM mixture and DCM to give 200 fractions (20 ml each). The fractions were combined into five pools (I-V) depending on their TLC profiles. Pool II (fractions 42-78, 120.0 mg) which resulted from elution with hexane was subjected to further column chromatography eluting with hexane-DCM 2:1 solvent mixture to yield 41 mg of compound **5**. Pool III (fractions 80-146, 214 mg) which resulted from elution with hexane-DCM 2:3 solvent system was subjected to further column chromatography and eluted with hexane-DCM 2:3, 1:1, 3:2 and 1:9 solvent systems to give 67 fractions (15 ml each). The fractions were combined into four pools (IIIa-IIId) depending on their TLC profiles. Pool IIIc (fractions 30-42 91.7 mg) which resulted from elution with hexane-DCM 2:3 solvent system crystallized in MeOH to yield 16.9 mg of compound **3**.

Ethyl acetate extract (20.5 g) was fractionated over silica gel using DCM, DCM containg increaing amounts of EtOAc and finally with EtOAc to give a total of 100 fractions (50 ml each). The fractions were combined into four pools (I-IV) based on their TLC profiles. Pool II (fractions 15-38, 3.0 g) was further fractionated over Sephadex using DC and DCM-MeOH (9:1, 4:1, 3:2 & 1:1) solvent systems to give 32 fractions which were combined into three pools (IIa-IIc) after monitoring their compositions by TLC. Pool IIc (fractions 25-32, 254 mg) was subjected to PTLC fractionation using DCM-acetone 1:1 solvent system to yield 44 mg of compound **1**.

2.5. Physical and Spectroscopic Data

Compound 1: Yellow crystals, mp 115-117℃, R_f 0.68 (acetone: DCM 2:3); ^{1}H-NMR (CD_3OD, 400 MHz) δ ppm: 4.59 (1H, d, *J* = 7.5 Hz), 4.01(1H, m), 2.7 (1H, dd, *J* = 16.0, 7.0 Hz), 2.53 (1H, dd, *J* = 16.0, 8.0 Hz), 5.89 (1H, d, *J* = 2.5 Hz), 5.96 (1H, d, *J* = 2.5 Hz), 6.87 (1H, d, *J* = 1.5 Hz), 6.76 (1H, dd, *J* = 9.5, 1.5 Hz), 6.74 (1H, d, *J* = 0.5 Hz); ^{13}C NMR (CD_3OD, 100 MH_Z) δ ppm (Table 1). EIMS (rel. int) *m/z*: 290, 272, 152 and 139.

Compound 2: White crystalline solid, mp 128-130℃, R_f 0.56 (100% DCM). IR ν_{max} (KBr, cm^{-1}) 3422, 2920, 1640, 1463, 1380, 1048, 1021, 723. ^{1}H NMR (CD_3OD, 400 MHz) δ ppm: 5.37 (1H, d, *J* = 7.2 Hz), 3.53 (m), 1.03 (3H, s), 0.94 (3H, d, *J* = 8.4 Hz), 0.86 (9H, m), 0.70 (3H, s); ^{13}C NMR (CD_3OD, 100 MH_Z) δ ppm (Table 2). EIMS (rel. int) *m/z*: 414, 396, 303, 329 and 213.

Compound 3: White needle-like crystals, mp 156-160℃, R_f 0.65 (DCM: MeOH 3:1). ^{1}H-NMR (CD_3OD, 400 MHz) δ ppm: 5.37 (1H, d, *J* = 7.2 Hz), 3.53 (m), 1.03 (3H, s), 0.94 (3H, d, *J* = 8.4 Hz), 0.86 (9H, m), 0.70 (3H, s), 5.2, 3.8, 3.2; ^{13}C NMR (CD_3OD, 100 MH_Z) δ ppm (Table 2).

Compound 4: White feather-like crystals, mp 168-170℃, R_f 0.66 (100% DCM). ^{1}H NMR (CD_3OD, 400 MHz) δ ppm: 5.15 (1H), 5.09 (1H, d, *J* = 1.5, 8.5 Hz), 5.35 (1H, d, *J* = 3.15 H_Z), 3.51(1H, m) 1.01, 0.91, 0.85, 0.81, 0.77, 0.68; ^{13}C NMR (CD_3OD, 100 MH_Z) δ ppm (Table 2). EIMS (rel. int): *m/z* 412, 394, 369, 351, 300, 271 and 255.

Compound 5: White needle-like crystals, mp 126-128℃, R_f 0.57 (*n*-hexane: DCM 1:1). ^{1}H NMR (CD_3OD, 400 MHz) δ ppm: 4.6, 4.5, 3.15, 2.3, 1.66, 1.01, 0.95, 0.92, 0.81, 0.77 and 0.74 ppm; ^{13}C-NMR (CD_3OD, 100 MH_Z) δ ppm (Table 2). EIMS (rel. int): *m/z* 426, 411, 385, 355, 220, 218, 207, 189.

2.6. Antibacterial Activity Test

Antibacterial test was done using paper disc diffusion method [29]. *Staphylococcus aureus* (ATCC no. 25923), *Bacillus subtilis* (local isolate), *Escherichia coli* (ATCC no. 25922) and *Pseudomonas aeruginosa* (ATCC no. 10622) used were obtained from the Department of Botany, Kenyatta University in Kenya. Sterile nutrient agar (28 g/l in distilled water, 15 ml) was dispensed in 90 mm diameter sterile petri dishes to yield a uniform depth of 4 mm. Bacteria from stock cultures were spread on the nutrient agar surface using sterile wire loop and incubated at 37℃ for 24 hrs. One loop-full of the bacterial strain from the culture was added to sterile nutrient broth medium and incubated at 37℃ in a rotary shaker. After 24 hrs, the broth bacteria culture (0.1 ml) was pipetted into nutrient agar media in petri dishes and spread evenly. 100 µl of the isolated compounds at a concentration of 1 mg/ml in DMSO were dispensed on 6 mm sterile paper disc [29]. The discs were oven died at 50℃ for 1 hr and then firmly placed on the inoculated petri dishes using sterile forceps. The petri-dishes and their contents were then incubated at 37℃ in inverted position. The zones of inhibition were measured after 24 hrs [20,30].

2.7. Anti-fungal Activity Test

Agar-well diffusion method was used as described by Opiyo *et al* [31]. Three fungal species, *Candida albicans*, *Penicillium notatum* and *Aspergillus Niger* were obtained from the Department of Botany, Kenyatta University in Kenya. 15 ml of sterile potato dextrose agar prepared by dissolving 39 g in 1L of water was dispensed in petri dishes under sterile conditions and left to solidify. Pure cultures of test fungi were introduced in the petri dishes from stock cultures and incubated at 30℃ for 7 days to produce a good crop of spores. The fungal inoculums were prepared by harvesting the spores from the crop of spores using a spores-harvesting needle and transferred into a sterile tube containing sterile distilled water. The spore suspension (0.5 ml) was transferred to PDA surface in petri-dishes using a pipette and the petri-dishes were tilted several times to spread the inoculum. After 10 minutes four wells were cut out from the inoculated PDA medium using a sterile cork-borer (6 mm). 100 µl of suspension of the isolated compounds at concentration of 1 mg/ml in DMSO were pipetted into the wells and then incubated at 30℃ for 24 hours. Diameters of zones of inhibition were measured as described by [31].

3. Results and Discussion

3.1. Characterization of Compounds

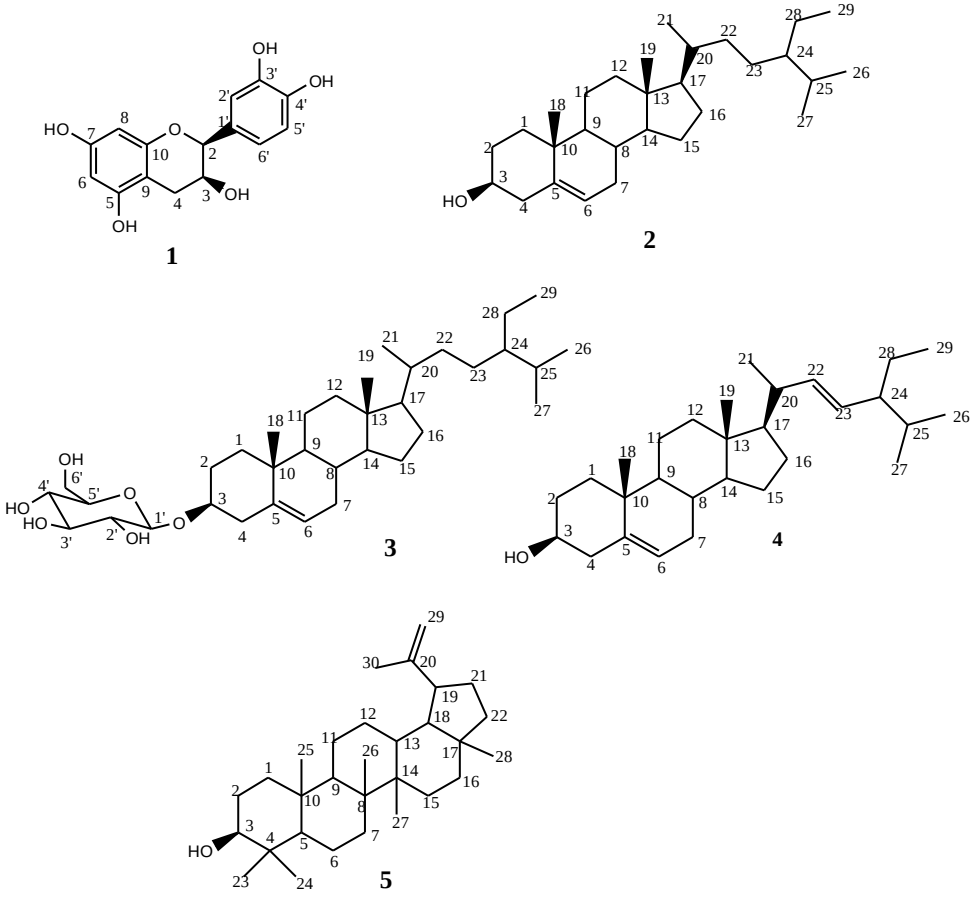

Figure 1. Structures of the isolated compounds

Chromatographic fractionation of root bark extracts of *R. natalensis* lead to isolation of five compounds (Figure 1). The structures of the compounds were determined using spectroscopic as well as physical methods. Compound **1** was isolated as yellow crystals having R_f value of 0.68 (DCM-acetone 3:2). On spraying with anisaldehyde the spot turned red and then yellow suggesting the compound to be a flavonoid. ^{1}H-NMR data (Table 1 and Figure 2) showed aromatic peaks at δ 6.87 (1H, d, *J* = 1.5 Hz), 6.76 (1H, dd, *J* = 9.5, 1.5 Hz) and 6.74 (1H, d, *J* = 0.5 Hz) representing protons of a trisubstituted benzene ring, and δ 5.96 (1H, d, *J* = 2.5 Hz) and δ 5.89 (1H, d, *J* = 2.5 Hz) representing protons of a tetra-substituted benzene ring [32,33].

The relatively small coupling constant (*J* = 2.5 Hz) between the two protons indicated *meta* substitution [32,33]. The pattern of the proton peaks strongly suggested a flavonoid skeleton with hydroxyl groups at positions C5, C7, C3′ and C4′ [32-34]. A doublet at δ 4.59 (*J* = 7.5 Hz) was assigned to H-2 while a multiplet centered at δ 4.01 was assigned to C-3 [32,33,35]. Two additional signals centered at δ 2.87 (1H, dd, *J* = 16.0, 7.0 Hz) and 2.53 (1H, dd, *J* = 16.0, 8.0 Hz) were assigned to the germinal protons at C4. ^{13}C NMR peaks at δ 28.5, 68.8 and 82.8 (Table 1 and Figure 3) were assigned the methylene carbon at C4, hydroxylated carbon at C-3 and oxymethine carbon at C-2 [33]. Signals at δ 157.5, 156.9 146.2 and 146.2 ppm were assigned to the four hydroxylated quaternary carbon atoms at C5, C7, C3' and C4' respectively. The connectivity and orientation of the protons were supported by the HMBC, COSY and NOESY experiments (Table 1). EIMS data of the compound gave the molecular ion peak at *m/z* 290 which corresponded to $C_{15}H_{14}O_6$ formula. Other diagnostic peaks occurred at m/z 272 $[M\text{-}H_2O]^+$, 152 $[C_8H_8O_3]^+$ and 139 $[C_7H_7O_3]^+$. The spectral data were in agreement with those published for epicatechin [32-35].

Compound **2** was obtained as white crystals having a melting point of 128-130℃ and R_f value of 0.56 in 100% DCM.^{1}H-NMR signal at δ 5.37 (1H, d *J*=7.2 Hz) suggested presence of a trisubstituted double bond while a multiplet centered at δ 3.53 was characteristic of a proton geminal to a hydroxyl group at C-3 in terpenoids (Table 2) [33,36,37]. Six signals representing the methyl groups were observed at δ 1.03 (3H, s), 0.94 (3H, d, *J* = 8.4 Hz), 0.86 (9H, m) and 0.70 (3H, s) which are characteristic of a modified triterpenoid [38]. ^{13}C-NMR and DEPT data (Table 2) confirmed presence of the six methyl (at δ 11.8, 12.0, 18.7, 19.4, 19.4 & 19.7 ppm), 11 methylene (at δ 21.1, 23.1, 24.3, 26.7, 28.2, 31.7, 31.9, 31.9, 37.3, 39.5 & 42.3 ppm), nine methine (at δ 29.2, 31.9, 33.9, 36.5, 45.9, 50.2, 56.1, 56.7 & 121.7 ppm) and three quaternary (at δ 36.5, 42.3, 140.8 ppm) carbons. ^{13}C-NMR data further confirmed the presence of olefinic carbons at δ 140.8 and 121.7 ppm with the former peak representing a quaternary carbon atom. The signal at δ 71.8 was assigned to oxymethine carbon at C-3. ESI-MS spectrum of compound **2** showed a molecular ion peak at *m/z* 437 $[M+Na]^+$ corresponding to the formula $C_{29}H_{50}O$. EIMS spectrum showed other diagnostic peaks at m/z 396 $[M\text{-}H_2O]^+$, 329 $[M\text{-}C_6H_{13}]^+$, 273 $[M\text{-}C_{10}H_{21}]^+$ and 255 $[M\text{-}C_{10}H_{21}\text{-}H_2O]^+$. Comparison of spectral data of compound **2** literature data confirmed **2** to be β-sitosterol [36-38].

Table 1. 1D and 2D NMR data of compound **1**

Position	^{1}H (δ_H, mult, *J* in Hz)*	^{13}C (δ_C, mult)*	^{1}H–^{1}H COSY	HMBC	NOESY
2	4.59 d (7.5)	82.8 d	H-3	C-3, C-10, C-1′, C-2′, C-6′	H-3, H-4a, H-2', H-5' H-6'
3	4.01 m	68.8 d	H-2, H-4a, H-4b		H-2, H-4b, H-5', H-6'
4a	2.53 dd (16.0, 8.0)	28.5 t	H-3, H-4a	C-2, C-3, C-9, C-10	H-2, H-4b
4b	2.87 dd (16.0, 7.0)		H-3, H-4B	C-2, C-3, C-9, C-10	H-2, H-3, H-4a
5		157.5 s			
6	5.89 d (2.5)	96.2 d		C-8, C-10	H-8
7		156.9 s			
8	5.96 d (2.5)	95.5 d		C-2, C-10	H-6
9		100.8 s			
10		157.8 s			
1′		132.2 s			
2′	6.87 d (1.5)	115.2 d	H-2, H-6'	C-2, C-4', C-6'	H-5',
3′		146.2 s			
4′		146.2 s			
5′	6.74 d (9.5)	116.1 d	H-6'	C-2, C-1', C-3' C-4′	H-2, H-2'
6′	6.76 dd (9.5, 1.5)	120.3 d	H-5'	C-1', C-2′, C-4′	H-2, H-2'

* ^{1}H and ^{13}C NMR spectra were run at 400 and 100 MHz, respectively in CD_3OD

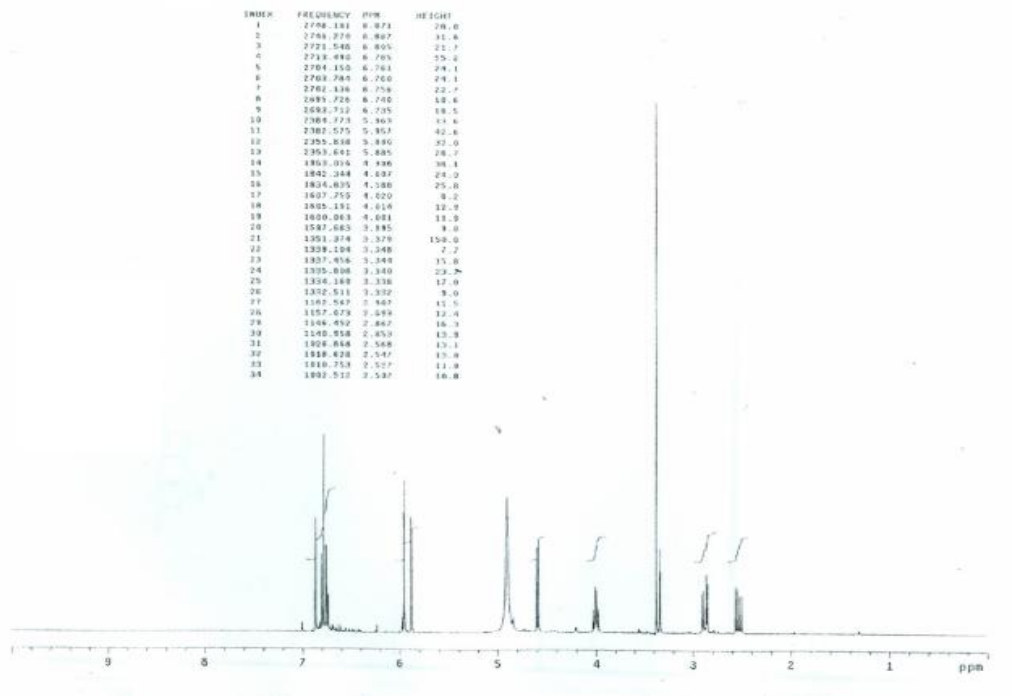

Figure 2. ^{1}H-NMR spectrum of compound **1** (CD_3OD, 400 MHz)

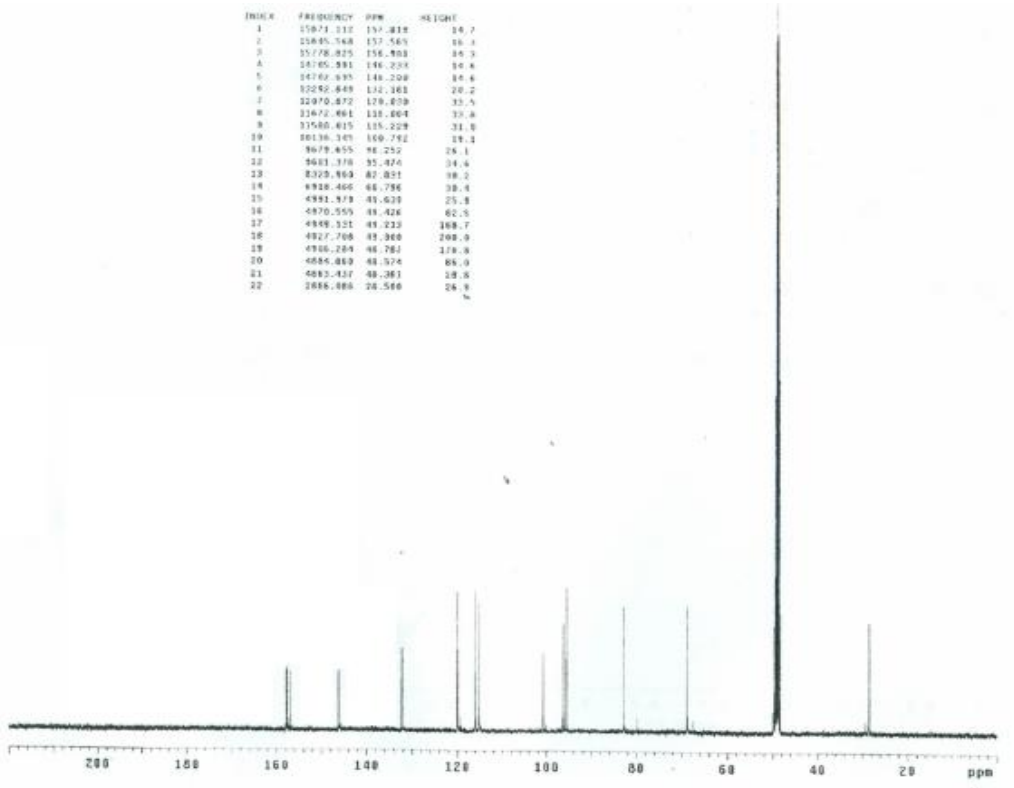

Figure 3. ^{13}C-NMR spectrum of compound **1** (CD_3OD, 100 MHz)

Compound **3** was isolated as white needle-like crystals, mp 156-160°C and R_f value of 0.65 (DCM-MeOH 3:1). The spot turned blue then green on spraying with anisaldehyde suggesting **3** to be a terpenoid [38]. ^{1}H-NMR data (Table 2) strongly suggested a triterpenoid structure [37,39]. A doublet at δ 5.37 ppm (1H, *J* =7.2 Hz) suggested the presence of olefinic proton on trisubstituted alkene. A multiplet centered at δ 3.53 ppm was characteristic of oxymethine proton at C-3 [37]. Methyl proton peaks at δ 1.03 (3H, s), 0.94 (3H, d, *J* = 8.4 Hz), 0.86 (9H, m) and 0.70 (3H, s) were characteristic of a modified triterpenoid [38]. ^{1}H-NMR spectral data closely compared to the spectral data of compound **2**. However,

there appeared additional peaks between δ 3.0 and 5.0 suggesting presence of a sugar moiety [40]. The olefinic carbon peak appeared at δ 140.8 and 121.7 ppm while the signal due to the C-3 oxymethine carbon appeared at δ 71.8 ppm. ^{13}C NMR data was similar to those of β-sitosterol except for the six peaks at δ 102.0, 78.5, 77.0, 71.7, 68.8 and 62.0 ppm associated with a sugar moiety [40]. The identity of β-sitosterol glucoside was confirmed by Co-TLC with authentic specimen.

Compound **4** was isolated as white feather-like crystals having melting point of 168-170℃ and R_f value of 0.66 in DCM. On spraying with anisaldehyde, the spot turned blue/purple, suggesting compound **4** to be a terpenoid [38]. ^{1}H-NMR data (Table 2) displayed several peaks between δ 1.01 and 0.68 ppm which are characteristic of triterpenoid parent. A broad doublet at δ 5.35 ppm suggested presence of a double bond at a quaternary carbon atom. Two signals at δ 5.17 and 5.01 ppm suggested presence of a double bond on the side chain [8,9,33,41]. The multiplet at δ 3.51 ppm represented a proton attached to oxymethine carbon [33]. The diagnostic chemical shift values of the angular methyl protons at C-18 and C-19 appeared as singlets at δ 0.68 and 1.01 ppm, respectively. The protons peaks of the remaining four methyl groups appeared at δ 0.77, 0.81, 0.85 and 0.91 ppm. ^{13}C-NMR and DEPT data showed twenty-nine carbon atoms consisting of six methyl (at δ 12.0, 12.2, 19.0, 19.4, 21.0 & 21.2), nine methylene (at δ 21.1, 24.4, 25.4, 28.9, 31.7, 31.9, 37.3, 39.7 & 42.2), eleven methane (at δ 31.7, 31.9, 40.4, 50.2, 51.2, 56.0, 56.9, 71.8, 121.7, 129.3 & 138.7) and three quaternary (at δ 36.5, 42.3 & 140.7) carbons atoms, which further confirmed the presence of a sterol. Signals at δ 140.7 and 121.7 ppm were assigned to the olefinic carbons with the deshielded signal assignable to the quaternary carbon at the bridge. Signal at δ 138.7 and 129.3 were assigned to the olefinic carbon on the side chain while δ 71.8 ppm was assigned to the oxymethine carbon at C3 [33,38]. EIMS spectrum gave a molecular ion peak at m/z 412 corresponding to $C_{29}H_{48}O$. Other diagnostic peaks were observed at m/z 394 $[C_{29}H_{46}]^+$, 369 $[M\text{-}C_3H_7]^+$, 300 $[C_{21}H_{32}O]^+$, 271 $[C_{19}H_{27}O]^+$ and 255 $[C_{19}H_{27}]^+$. Based on the spectral data as well as comparison with literature data [8,9,33,38], compound **4** was identified to be stigmasterol.

Compound **5** was isolated as white needle-like crystals having m.p. of 126-128℃ and R_f value of 0.57 in hexane-DCM (1:1). On spraying with anisaldehyde, the spot turned blue/purple then green suggesting compound **5** to be a terpenoid. ^{1}H-NMR data displayed two singlets at δ 4.67 and 4.58 ppm which suggested presence of a terminal olefinic group [9,41,42,44] while a multiplet at δ 3.16 suggested presence of oxymethine carbon at C3 (Table 2). Seven singlets associated with methyl groups of a pentacyclic terpene appeared at δ 1.66, 1.01, 0.95, 0.92, 0.81, 0.77 and 0.74 ppm [9,44]. The ^{13}C-NMR data and DEPT experiment showed the presence of 30 carbon atoms which further suggested compound **5** to be a triterpenoid. Signals at δ 150.8 and 109.3 ppm were assigned to C_{20}-C_{29} double bond while the signal at δ 79.0 ppm was assigned to the oxymethine carbon atom at C-3 [42]. The seven methyl peaks at δ 14.6, 15.4, 16.0, 16.1, 18.0, 19.3 & 28.0 ppm were assigned to C_{23}-C_{28} and C_{30} carbon atoms. EIMS data showed a molecular ion peak at m/z 426 $[M^+]$ for the formula $C_{30}H_{50}O$. Other diagnostic peaks were observed at *m/z* 411 $[M\text{-}Me]^+$, 355 $[M\text{-}Me\text{-}3H_2O]^+$, 220 $[M\text{-}C15H26]^+$, 207 $[M\text{-}C16H27]^+$, 189 and $[M\text{-}C16H29O]^+$. Based on comparison with literature data [9,42-44], compound **5** was confirmed to be lupeol.

Table 2. ^{13}C NMR data of compounds **2-5**

	^{13}C (δ_C, multplicity)*			
Carbon	**2**	**3**	**4**	**5**
1	37.3 t	37.3 t	37.3 t	38.7 t
2	31.9 t	31.9 t	31.7 t	27.4 t
3	71.8 d	71.8 d	71.8 d	79.0 d
4	42.3 t	42.3 t	42.2 t	38.9 s
5	140.8 s	140.8 s	140.7 s	55.3 d
6	121.7 d	121.7 d	121.7 d	18.3 t
7	31.7 t	31.7 t	31.9 t	34.3 t
8	33.9 d	34.0 d	31.7 d	40.8 s
9	50.2 d	50.2 d	50.2 d	50.4 d
10	36.5 s	36.5 s	36.5 s	37.2 s
11	21.1 t	21.1 t	21.1 t	20.9 t
12	39.5 t	39.5 t	39.7 t	25.1 t
13	42.3 s	42.3 s	42.3 s	38.1 d
14	56.7 d	56.7 d	56.9 d	43.0 s
15	24.3 t	24.3 t	24.4 t	27.4 t
16	28.2 t	28.2 t	28.9 t	35.6 t
17	56.1 d	56.1 d	56.0 d	43.1 s
18	12.0 q	12.0 q	12.0 q	48.3 d
19	19.0 q	19.3 q	19.4 q	48.0 d
20	36.5 d	36.5 d	40.4 d	151.0 s
21	19.3 q	19.4 q	21.0 q	29.8 t
22	31.9 t	31.9 t	138.7 d	40.0 t
23	26.7 t	26.7 t	129.3 d	28.0 q
24	45.9 d	45.8 d	51.2 d	15.4 q
25	29.2 d	29.2 d	31.9 d	16.0 q
26	18.7 q	18.7 q	19.0 q	16.0 q
27	19.8 q	19.7 q	21.2 q	14.6 q
28	23.1 d	23.1 t	25.4 t	18.0 q
29	11.8 q	11.8 q	12.2 q	109.3 t
30				19.3 q
1'		102.0 d		
2'		78.5 d		
3'		77.0 d		
4'		71.7 d		
5'		68.8 d		
6'		62.0 t		

* ^{13C} NMR spectra were run at 100 MHz, in CD_3OD

3.2. Antibacterial Activity of Isolated Compounds

Isolated compounds were tested for antibacterial activity against *P. aeruginosa, B. subtilis, S. aureus* and *E. coli* using paper disc diffusion method. All isolated compounds were active against all the gram-positive bacteria tested (Table 3). Compounds **1**, **3** and **5**, exhibited moderate activity while **2** and **3** showed low activity. *Bacillus subtilis* and *S. aureus* were most susceptible ($P \leq 0.05$) to compound **1** with zones of inhibitions of 14 ± 0.2 and 15 ± 0.3 mm respectively. Stigmasterol (**4**) showed equal antibacterial activity against the two gram-positive bacteria. *S. aureus* was more susceptible to compound **2** than *B. subtilis*. Only compounds **1**, **3** and **5** were active against the gram-negative bacteria. *Escherichia coli* was most susceptible ($P \leq 0.05$) to epicatechin (**1**) while *P. aeruginosa* was only susceptible to epicatechin (**1**). All isolated compound showed lower antibacterial activity compared to standard drugs. These results were consistent with previous studies which reported antibacterial activities of 3β–sitosterol (**2**), stigmasterol (**4**) [36], 3β-sitosterol glucoside (**3**) [45] lupeol (**5**) and epicatechin **(1)** [46].

Table 3. Antibacterial activities of isolated compounds

Compounds	Diameter of zone of inhibition *			
	B. subtilis	*S. aureus*	*P. aeruginosa*	*E. coli*
Epicatechin (**1**)	14 ± 0.2	15 ± 0.3	10 ± 0.2	11 ± 0.3
3β–Sitosterol (**2**)	7 ± 0.3	8 ± 0.2	-	-
3β–Sitosterol glucoside (**3**)	13 ± 0.3	14 ± 0.2	-	10 ± 0.2
Stigmasterol (**4**)	8 ± 0.2	8 ± 0.2	-	-
Lupeol (**5**)	11 ± 0.2	14 ± 0.4	-	8 ± 0.3
Contrimoxazole 25 µg/disc	19 ± 0.2	24 ± 0.3	22 ± 0.3	18 ± 0.1
Streptomycin 10 µg/disc	20 ± 0.3	22 ± 0.2	20 ± 0.3	20 ± 0.2
LSD, $P \leq 0.05$	0.3			

* mm ± SD; *n* = 3; (-) no activity

3.3. Antifungal Activity of Compounds

Table 4. Antifungal activity of isolated compounds

Antifungal agents	Diameter of zone of inhibition*		
	C. albicans	*P. notatum*	*A. niger*
Epicatechin (**1**)	14 ± 0.3	12 ± 0.2	12 ± 0.2
3β–Sitosterol (**2**)	-	-	-
3β–Sitosterol glucoside (**3**)	10 ± 0.3	11 ± 0.3	9 ± 0.3
Stigmasterol (**4**)	-	8 ± 0.2	-
Lupeol (**5**)	-	9 ± 0.2	-
Kenamycin 30 µg/disc	20 ± 0.2	22 ± 0.4	24 ± 0.1
LSD, $P \leq 0.05$	0.2		

* mm ± SD; *n* = 4; (-) no activity

Isolated compounds were tested for antifungal activity against *C. albicans, P. notatum* and *A. niger* (Table 4). Epicatechin (**1**) recorded the highest activity against all the testes fungi having inhibition zones of 14 ± 0.3, 12 ± 0.2 and 12 ± 0.2 mm against *C. albicans, P. notatum* and *A. niger* respectively. Lupeol (**5**) and stigmasterol (**4**) were only active against *P. notatum* with inhibition zones of 9 ± 0.2 and 8 ± 0.2 mm, respectively. β-Sitosterol (**2**) showed no activity against the test fungi. Antifungal activity of β-sitosterol (**2**) [36,47], stigmasterol (**4**) [36], lupeol (**5**) [48] and β-sitosterol glucoside (**3**) [45] were earlier reported.

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