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Evaluation of Antimicrobial and Antifungal Activity of Chemical Constituents Isolated from *Vernonia guineensis* Roots (Asteraceae)

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ABSTRACT

The present study focused on the evaluation of the antimicrobial activity and antifungal of chemical constituents isolated from the roots of *Vernonia guineensis*, a plant of the family Asteraceae. *Vernonia guineensis* is used in traditional pharmacopoeia as aphrodisiacs, anti-dysenteric, purgative, anti-gastritis, antimalarial, anti-asthenic, febrifuge, anti-icteric. The chemical study of the CH_2Cl_2-CH_3OH mixture extract (1:1) led to the isolation of five compounds: Betulinic acid **(1)**; alphitolic acid **(2)**; β-sitosterol 3-O-β-D-glucopyranoside **(3)**; scoparone **(4)**; Quercetin-3-O-β-galactoside or isoquercetin **(5)**. Betulinic acid **(1)**, alphitolic acid **(2)** and scoparone **(4)** were isolated from *Vernonia guineensis* for the first time. The structures of the isolated compounds were elucidated based on spectroscopic analysis and comparison of their spectral data with those reported in the literature. The results of the antimicrobial tests on the 11 bacterial strains tested showed that the compounds **(2)** and **(3)** were active on most the bacterial strains tested, whereas the compound **(1)** was active on 4 of the 11 bacterial strains. Tested with an inhibition diameter ranging from 7 to 8 mm, while the compound **(5)** acted only on 1 bacterial strain. On the other hand, the compounds **(2)** and (**3**) tested showed a strong inhibition on *Staphylococcus aureus ATCC BAA 977*, a moderate inhibition on *Staphylococcus aureus ATCC 29213*, a weak inhibition on *Aerococcus viridans ATCC 11563* and a moderate inhibition on *Enterococcus faecalis ATCC 51299*. The MIC (Minimal Inhibitory Concentration), CMB (Concentration Minimal Bactericidal) and CMB/MIC ratios of compounds **(2)** and **(3)** have revealed the highest antibacterial potency. However, the MICs of the compound **(2)** vary from 312.5 to 2500 µg/mL with the smallest value (312.5 µg/mL) observed against *Staphylococcus aureus ATCC BAA 977*, whereas the compound **(3)** MICs vary from 312.5 to 5000 µg/mL with the lowest value (312.5 µg/mL) observed against *Staphylococcus aureus ATCC BAA 977.* The calculation of the CMB/MIC ratio revealed that compounds **(2)** and **(3)** had a bactericidal activity (CMB/MIC<4). This bactericide could justify their use in herbal medicine against bacterial infections.

1. Introduction

Plants provide a natural support of traditional medicine, constitute a means for health care for the world's populations, in general and particularly Africa [1]. Today, demand for these plants is increasing because of the demographic boom. Despite the efforts made by the international community to control the plagues, populations continue to be the target of infectious diseases [2,3]. Some of these medicinal herbs have great potential and have been shown to be very beneficial in the treatment of wounds, promoting the healing rate of wounds with minimal pain, discomfort and scarring in the patient [4]. These plants have their effects on wound healing processes and some on their anti-inflammatory and antimicrobial properties [5]. It is in this context, that in the framework of this work we studied a species of the genus *Vernonia* which is widely used in the traditional pharmacopoeia to cure certain venereal diseases and for these antimicrobial properties [6]. The genus *Vernonia* belongs to the Asteraceae family, it is an herbaceous plant, shrub, more rarely small trees. The leaves are alternate, herbaceous, serrated; the inflorescences are in panicle heads, rarely solitary [7]. *Vernonia guineensis* is defined as a "chamelepy of the low herbaceous layer". The buds are always arranged above the surface of the soil; herbaceous or woody stems do not rise above ground at more than 0.50 m [8]. The roots of *Vernonia guineensis* are used in traditional medicine as aphrodisiacs, anti-dysenteric, purgative, anti-gastritis, antimalarial, anti-asthenic, febrifuge, anti-icteric [6,9]. Previous chemical studies with *Vernonia guineensis* have led to the isolation and characterization of a few secondary metabolites including Terpenes, Flavonoids, Sterols and Stigmastane glycosides [5, 9-13]. Thus, the general objective of this study is to evaluate the antimicrobial activity of secondary metabolites isolated from the Roots of *Vernonia guineensis* (Asteraceae).

2. Experimental Methods

2.1 Apparatus and Equipment

After drying, the roots of *Vernonia guineensis* were crushed using a crushing machine. The maceration of the powder in CH_2Cl_2-CH_3OH (1:1) was done in a tightly sealed 20 L can. A MARQLUTAN GM-300P electronic scale weighed the raw extract and the different masses of the fractions. Flash chromatography was performed using a VELP Scientifica vacuum cleaner, a Buchner and a vacuum flask. A Büchi brand Heidolph WB 200 rotary evaporator made it possible to separate the extract from the solvent. Column chromatographies were carried out in a column 3 cm in diameter and 60 cm long and a small column 2 cm in diameter and 50 cm in length. Stationary phase KIESELGEL 60 type silica (0.04-0.063 mm) was used. The ^{1}H NMR spectrum was obtained through a BRUKER DRX600 spectrometer at a frequency of 600 MHz for the proton and 150 MHz for carbon. This was done at the laboratory of the Institute of Environmental Research, Faculty of Chemistry, Technological University of Dortmund, Germany.

2.2 Plant Material

The roots of *Vernonia guineensis* were harvested in Bagangte in the Western Region of Cameroon in February 2017 by Mr. Tchebemou Bakang Bruno. The identification was done by Mr. Nana from the National

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Herbarium of Cameroon, Yaounde where the reference specimen was filed under voucher number (No. 11133SRF/Cam).

2.3 Extraction and Isolation

The roots of *Vernonia guineensis* were dried, crushed and (2.3 kg) powder was obtained. This powder was macerated in 20 L of CH_2Cl_2-CH_3OH (1:1) for 72 h. The filtrate obtained was evaporated to dryness using a rotary evaporator under reduced pressure and (156 g) of crude extract was obtained. (130 g) of this crude extract was cold-fixed on (100 g) of silica gel (SiO_2) (0.063-0.200 mm) and the Buchner was loaded with (120 g) of silica as a stationary phase to undergo flash chromatography. Elution of this extract was done with solvents and gradient solvent systems of increasing polarity such as: hexane, hexane/ethyl acetate, ethyl acetate, ethyl acetate/methanol. After elution, 250 vials of about 400 mL each were collected. The 250 vials were pooled into 4 major fractions (A (5 g), B (45 g), C (35 g), D (15 g)) on the basis of their TLC analysis.

2.4 Purification of the Different Fractions

2.4.1 Chromatography of Fraction B

Fraction **B** (45 g) was fixed on (40 g) of silica gel and then chromatographed under column with (90 g) of silica (0.063-0.200 mm) as stationary phase. Elution was done with hexane and hexane/ethyl acetate mixture by gradient of increasing polarity; we thus collected 134 vials grouped into 5 major fractions (B1, B2, B3, B4, B5) under the base of their TLC analysis.

Fraction B2 led to the isolation of betulinic acid **(1)** (20 mg) in the hexane/acetate system 10% and soluble in methylene chloride.

Fraction B3 led to the isolation of alphitolic acid **(2)** (15 mg) in the hexane/acetate system 20% and soluble in methanol.

Fraction B4 led to the isolation of 3-O-β-D-glucopyranoside from β-sitosterol **(3)** (13 mg) in pure ethyl acetate and soluble in methanol.

2.4.2 Chromatography of Fraction C

Chromatography of fraction C on a silica gel column using CH_2Cl_2-acetone (95:5) as eluent provided a fraction which was further purified by column chromatography with CH_2Cl_2-acetone (9:1) and then (4:1) to give Scoparone **(4)** (12mg).

2.4.3 Chromatography of Fraction D

Fraction **D** was passed through a column of silica gel eluting with CH_2Cl_2- CH_3OH with increasing polarity. The fraction obtained with CH_2Cl_2-ethyl acetate (94:6) was further purified by column chromatography using CH_2Cl_2-ethyl acetate as eluent to give isoquercetin **(5)** (9 mg).

2.5 Physical and Spectral Data of the Compounds (1,2,3,4 and 5).

Betulinic acid (1): ^{1}H NMR (pyridine-d_5): δ 3.47 (1H, brt, *J* = 7.7 Hz, H-3); 3.55 (1H, m, H-18); 4.78 (1H, brs, H-29); 4.96 (1h, brs, H-29b); 1.24, (3H, s, H-23); 1.02 (3H, s, H-24); 0.83 (3H, s, H-25); 1.07 * (3H, s, H-26); 1.08 * (3H, s, H-27); 1.80 (3H, s, H-30) * interchangeable signals;

^{13}C NMR (pyridine-d_5): δ 39.3 (C-1), 28.3 (C-2); 78.2 (C-3); 39.6 (C-4), 56.0 (C-5); 18.8 (C-6); 34.9 (C-7); 41.2 (C-8); 51.0 (C-9); 37.6 (C-10); 21.3 (C-11); 26.2 (C-12); 38.7 (C-13); 42.9 (C-14); 31.3 (C-15); 32.9 (C-16); 56.7 (C-17); 47.8 (C-18); 49.8 (C-19); 152.5 (C-20), 30.3 (C-21); 37.6 (C-22); 28.7 (C-23); 16.4 (C-24); 16.5 (C-25); 16.5 (C-26); 15.0 (C-27); 178.9 (C-28); 110.0 (C-29); 19.5 (C-30).

Alphitolic acid (2): Physical state: White powder; Brute formula: $C_{30}H_{48}O_4$; Calculated mass: m/z: 472: ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.61 (1H, ddd, H-2), 2.89 (1H, d, J = 9.3, H-3), 3.27 (1H, ddd, *J* = 2.27, H-13), 1.46 (1H, t, *J* = 11.3, H-18), 2.96 (1H, m, H-19), 1.01 (3H, s, H-23), 0.92. (3H, s, H-24), 0.97 (3H, s, H-25), 0.99 (3H, s, H-26), 4.71 (1H, d, J = 1.8, H-29α), 1.70 (3H, s, H-30),

^{13}C-NMR (150 MHz, DMSO-d_6) δ 48.60 (C-1), 69.90 (C-2), 84.50 (C-3), 39.56 (C-4), 56.93 (C-5), 19.62 (C-1); -6), 35.56 (C-7), 42.11 (C-8), 52.10 (C-9), 39.77 (C-10), 22.35 (C-11), 26.97 (C-12), 39.62 (C-9), 13), 43.77 (C-14), 31.92 (C-15), 33.49 (C-16), 57.64 (C-17), 50.57 (C-18), 48.53 (C-19), 152.08 (C-20)), 30.89 (C-21), 38.24 (C-22), 29.24 (C-23), 17.34 (C-24), 17.99 (C-25), 16.74 (C-26), 15.18 (C-27), 180.16 (C-28), 110.30 (C-29), 19.64 (C-30).

β-sitosterol 3-O-β-D-glucopyranoside (3): ^{1}H NMR (600 MHz; DMSO-d_6) δ 1.79 (1H,m Ha-1), 0.98(1H,m,Hb-1), 1.82 (1H,m,Ha-2), 1.48(1H,m,Hb-1), 3.46 (1H,m,H-3), 2.36 (1H,dd, *J* = 13.6- 3.4 Hz,Ha-4), 2.12 (1H,dd, *J* = 13.6 - 3.4 Hz,Hb-4), 5.32 (1H,br s,H-6), 1.49 (1H,m,Ha-7), 1.38 (1H,m,Hb-7), 1.92 (1H,m,H-8), 0.87 (1H,m,H-9), 1.47 (1H,m,Ha-11), 1.40 (1H,m,Hb-11), 1.94 (1H,m,Ha-12), 1.13 (1H,m,Hb-12), 0.96 (1H,m,H-14), 1.54 (1H,m,Ha-15), 1.01 (1H,m,Hb-15), 1.79 (1H,m,Ha-16), 1.24 (1H,m,Hb-16), 1.08 (1H,m,H-17), 0.64 (3H,s,H-18), 0.95 (3H,s,H-19), 1.34 (1H,m,H-20), 0.89 (3H,d,J=6.4Hz,H-21), 1.31 (1H,m,Ha-22), 1.01 (1H,m,Hb-22), 1.15 (2H,m,H-23), 0.91(1H,m,H-24), 1.62 (1H,m,H-25), 0.79 (3H,d,J=6.7Hz,H-26), 0.81 (3H, d, *J* = 6.8 Hz ; H-27), 0.81 (2H,m,H-28), 0.81 (3H, t, *J* = 7.2Hz,H-29), 4.21 (d,*J*= 7.5 Hz, H-1′), 3.00 (1H,m,H-2′), 3.04 (1H,m,Ha-3′), 3.15 (1H,m,Hb-3′), 2.89 (1H,m,H-4′),3.04 (1H,m,Ha-5′), 3.15 (1H,m,Hb-5′), 3.64 (dd, *J* = 11.8 – 1.8 Hz,H-6′)

^{13}C NMR (150 MHz; DMSO-d_6) δ 36.9 (C-1), 29.3 (C-2), 76.9 (C-3), 38.4 (C-4), 140.5 (C- 5), 121.3 (C-6), 31.4 (C-7), 31.5 (C-8), 49.6 (C-9), 36.2 (C-10), 20.6 (C-11), 39.3* (C-12), 41.9 (C-13), 56.2 (C-14), 23.9 (C-15), 27.8 (C-16), 55.5 (C-17), 11.7 (C-18), 19.2 (C-19), 35.5 (C-20), 18.7 (C-21), 33.4 (C-22), 25.5 (C-23), 45.2 (C-24), 28.8 (C-25), 19.8 (C- 26), 19.0 (C-27), 22.7 (C-28), 11.8 (C-29), 100.8 (C-1′),70.1 (C-2′),76.8 (C-3′),73.5 (C-4′),76.8 (C-5′),61.2 (C-6′).

Scoparone (4): ^{1}H NMR (600 MHz, DMSO-d_6): δ 6.37 (1H, d, J3, 4 = 9.6 Hz, H-3), 7.93 (1H, d, J4, 3 = 9.6 Hz, H- 4), 6.83 (1H, s, H-5), 3.81 (3H, s, 6-OMe), 3.76 (3H, s, 7-OMe).

^{13}C NMR (150 MHz, DMSO-d_6): 160.1 (C-2), 114.6 (C-3), 144.7 (C-4), 100.2 (C-5), 149.7 (C-6), 56.0 (OMe-6), 140.1 (C-7), 60.5 (OMe-7), 138.5 (C-8), 138.3 (C-9), 114.4 (C-10).

Quercetin-3-O-β-galactoside (5): ^{1}H NMR (600 MHz, DMSO-d_6) 12.62 (1H, s, 5-OH), 10.87 (1H, s, 7-OH), 9.74 (1H, s, 4'-OH), 9.17 (1H, s, 3'-OH), 7.65 (1H, dd, J = 2.1-8.4 Hz, H-6 '), 7.52 (1H, d, J = 2.1 Hz, H -2 '), 6.82 (1H, d, J = 8.4 Hz, H-5'), 6.40 (1H, d, J = 2.1 Hz, H-8), 6.20 (1H, d, J = 2.1 Hz, H -6), 5.38 (1H, d, J = 8.4 Hz, H-1 ''), 5.14 (1H, s, 2''-OH), 4.87 (1H, s, 3''-OH), 4.44 (2H, s, 4 '', 6 ''-OH).

^{13}C NMR (150 MHz, DMSO-d_6) δ 177.5 (C-4), 164.2 (C-7), 161.3 (C-5), 156.2 (C-2), 156.3 (C-9), 148.5 (C) -4 '), 144.9 (C-3'), 133.5 (C-3), 122.1 (C-6 '), 121.1 (C-1'), 115.9 (CH, C-2 '), 115.2 (CH, C-5 '), 103.9 (C-10), 101.8 (CH, C-1' '), 98.7 (CH, C-6), 93.5 (CH, C-8), 75.9 (CH, C-5' 73.2 (CH 3 C 3 '') 71.2 (CH 2 C '2' '), 67.9 (CH 3 C 4'), 60.2 (CH 2, C-6 '').

2.6 Assessment of Antimicrobial Activity

For the demonstration of the antimicrobial activity, several bacterial and fungal strains were tested vis-a-vis the compounds 1, 2, 3, 4 and 5 isolated from *Vernonia guineensis*.

2.6.1 Bacterial Strains

In this study, eleven bacterial strains including 9 referenced strains and 2 strains provided by the bacteriology laboratory of the University Hospital Center of Yaounde (CHUY). Among these strains, we have 7 Gram-negative (*Escherichia coli ATCC 25922, Escherichia coli ATCC 11775, Klebsiella pneumoniae ATCC 700603, Neisseria gonorrhoeae ATCC 49226, Pseudomonas aeruginosa ATCC 10145, Salmonella choleraesuis, Proteus mirabilis*) and 4 Gram-positive strains (*Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC BAA 977, Staphylococcus aureus ATCC 29213, Enterococcus faecalis ATCC 51299*). These strains were stored at 37 °C on agar nutrient in the galenic pharmaceutical laboratory microbiology section of the Department of galenic pharmacy and legislation of the Faculty of Medicine and Biomedical Sciences, University of Yaounde I.

2.6.2 Preparation of Samples of Different Compounds (1-5) and Disks

100 mg of the various compounds to be tested were diluted in 1 mL of dimethylsulfoxide (DMSO) at 10%. Whatman No.39 sterile paper discs, 6 mm in diameter, were impregnated with 2 µL, 10 µL and 20 µL of each stock solution, corresponding to 200 µg, 1000 µg and 2000 µg of compounds per disc respectively. Discs impregnated with DMSO were also prepared. These disk categories served as controls. All the disks thus prepared were dried in an oven at 37 °C for 30 minutes to 1 hour. Ciprofloxacin and gentamicin discs were also used as reference antibiotics.

2.6.3 Dilution Method

The antibacterial activity of the various compounds showed activity on most the strains tested by the diffusion method, which was evaluated by the double dilution method in liquid medium. In a series of eight hemolysis tubes numbered from T1 to T8, we introduced 1 mL of Mueller Hinton Broth. Then we added in the T1 tube, 1 mL of plant extract prepared at a concentration of 80 mg/mL with DMSO. Subsequently, the T2 tube received 1 mL of 40 mg/mL and so on until the T8 tube which received the 0.625 mg/mL solution. The concentration range of each compound will then be halved and spread as follows: 40, 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 mg/mL. We also prepared for each bacterial strain, an inoculum whose turbidity was adjusted to 0.5 Mc Farland (i.e., 10^8 ufc/mL) and diluted 10 µL of this inoculum in 10 mL of Mueller-Hinton broth. Then, in tubes T1 to T8, 1mL of prepared bacterial inoculum was added. The concentration range of each extract is then diluted by half and is as follows: 20; 10; 5; 2.5; 1.25, 0.625, 0.3125 and 0.15625 mg/mL. We also prepared a growth control tube containing 1 mL of sterile distilled water and 1 mL

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of inoculum; then a sterility control tube containing 1 mL of sterile distilled water and 1 mL of sterile broth, as well as both controls are incubated at 37 °C for 24 hours. After incubation, bacterial growth is examined in each tube, which results in turbidity. The MIC of a compound against a given strain will be the smallest of the concentrations showing no visible growth of germ.

2.6.4 Determination of Minimal Inhibitory Concentration (MIC) and Concentration Minimal Bactericidal (CMB)

To determine the CMB, a bactericidal control is carried out 24 hours earlier by streaking on a Petri dish agar, the dilutions 10^{0}, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of the starting inoculums, corresponding to 100%, 10%, 1%, 0.1% and 0.01% of survivors, respectively. After reading the MIC, streaking is done on new agar, tubes without visible growth. These subcultures are then incubated at 37 °C and 24 hours later, the streaks are compared with the bactericidal control. The CMB will be the smallest concentration where transplantation shows germination of less than or equal to 0.01% survivors [14]. The calculation of the CMB/MIC ratio made it possible to determine the bactericidal effect (CMB/MIC <4) or bacteriostatic effect (CMB/MIC ≥ 4) of the substances tested [15]. The classification of plant material compounds based on MICs is as follows: strong inhibition: MIC below 500 µg/mL; moderate inhibition: MIC varies from 500 µg/mL to 1500 µg/mL; weak inhibition: MIC greater than 1500 µg/mL [16].

3. Results and Discussion

The roots of *Vernonia guineensis* (Asteraceae) were cut, dried and crushed; (2.3 kg) of powder thus obtained were macerated in 20 L of CH_2Cl_2- CH_3OH 1:1 for 72 hours. The filtrate was concentrated on a rotary evaporator, which gave (156 g) of crude extract. This extract was subjected to a Phytochemical Screening and fractionation to evaluate the antimicrobial activities of different fractions obtained on different strains (*Escherichia coli* ATCC 25922, *Escherichia coli ATCC 11775, Klebsiella pneumoniae ATCC 700603*, *Neisseria gonorrhoeae ATCC 49226, Pseudomonas aeruginosa ATCC 10145*, *Salmonella choleraesuis, Proteus mirabilis*) and Gram positive 4 (*Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC BAA 977, Staphylococcus aureus ATCC 29213, Enterococcus faecalis ATCC 51299*), and then isolate the secondary metabolites in these fractions.

3.1 Elucidation of Structures

Extracts of *Vernonia guineensis* (Asteraceae) were fractionated by column chromatography on silica gel to give five compounds **(1-5)** (Fig. 1).

Compound **1** was isolated in hexane/ethyl acetate 10% as a white powder and soluble in methylene chloride. The analysis of its ^{1}H NMR spectrum (600MHz, $CDCl_3$) towards the strong fields shows the presence of six singlets having three protons each at 0.72 ppm (H-24); 0.79 ppm (H-23); 0.90 ppm (H-25); 1.23 ppm (H-27); 1.34 ppm (H-26) and 1.66 ppm (H-30), a hydroxyl proton at 3.40 ppm (H-3) and two olefinic protons appearing as singlets at chemical shifts of 4.56 ppm (H-29a) and 4.72 ppm (H-29b). Its ^{13}C NMR spectrum (150 MHz, $CDCl_3$) in accordance with its ^{1}H NMR spectrum indicates the presence of two olefinic carbons at 109.4 ppm (C-29) and 152.0 ppm (C-20), these data are characteristic of the presence of a lup-20(29)-ene backbone [17]. 179.31 ppm (C-28) a characteristic carboxyl signal of the carboxylic acids from which this compound is an acid. These physical and spectroscopic data compared to those described in the literature made it possible to identify the compound **1** as being betulinic acid, also called 3β-hydroxylup-20(29)-en-28-oic acid [17-19]. This compound was isolated for the first time from *Vernonia guinensis*.

Compound **2** was obtained in the form of a white powder in the hexane/ethyl acetate 20% system. It is soluble in methanol and gives a purplish pink coloration to the Liebermann Burchard test characteristic of triterpenes. Its ^{1}H NMR spectrum (600MHz, MeOD) shows five singlets resonant at δH 0.78, 0.92, 0.97, 0.99 and 1.01 ppm attributable to the angular methyls of the pentacyclic triterpenes and a sixth methyl group that is deshielded at 1.70 ppm indicating an electronic environment different from the other 05. Resonances of isopropenyl protons are also observed at δH 4.71 (d, J = 2.0 Hz) and 4.60 ppm (dd, J = 2.0-1.4 Hz) and a methyl (δH 1.70) suggesting a lupane or hopane skeleton. It also has a resonant doublet at 2.89 ppm (J = 9.6 Hz) attributable to proton H-3. On the cosy spectrum, the H-3 proton correlates with the unshielded H-2 proton (δH 3.61, ddd, J = 11.3-9.6-4.6 Hz). The chemical shift of C-2 carbon (δC 70.0) confirms the presence of a hydroxyl group in position 2. The ^{13}C APT NMR spectrum of compound **2** reveals the presence of 30 carbons from which we can distinguish the following characteristic carbons: A signal resonant at δC 180.1 ppm indicating the presence of a free carboxyl function in the molecule. HMBC correlations between this carbon and protons H-16, H-18, suggest that the carbonyl is placed in position 28. A carbon mass of between 14-50 ppm attributable to CH_2, CH and quaternary carbon. Two oxygenated carbons at 84.7 and 70.0 ppm. The two ethylenic carbons detected, including a quaternary carbon located at 152.2 and a methylene carbon at 110.5 ppm. Thanks to the analysis of the direct hetero-nuclear correlations ^{1}J H-C observed on the J-modulated HSQC spectrum 2 combined with the ^{13}C APT spectrum, we can count 7 quaternary carbons, 7 methines, 10 methylenes and 6 methyls for the compound **2**. From the protons identified on the cosy spectrum, the corresponding carbons C-1 (δC 48.6), C-2 (δC 69.9) and C-3 (δC 84.5) are assigned to ring A, C.-11, C-12 (δC 25.4) and C-13 (δC 39.62) to ring C, C-18 (δC 50.57), C-19 (δC 48.53), C-21 (δC 30,5) and C-22 (δC 37,1) to the ring E. This analysis also makes it possible to identify the degree of substitution of the double bond deduced by the fact that the two ethylenic protons are carried by the same carbon (110.5 ppm) which confirms the presence of an exocyclic isopropenyl double bond (Δ^{20}). Correlations observed between proton H-18 (δH 1.66) and C-13, C-14, C-17, the quaternary carbon of the isopropenyl group (δC 150.2) and the carboxyl at 181.2 ppm make it possible to unambiguously place the isopropenyl group at position C-19 and the carbonyl at position 28 as in the lupane skeleton. The axial orientation of proton H-3 and axial proton H-2 is established from the coupling constant values between protons H-2 and H-3 greater than 9 Hz. These physical and spectroscopic data compared to those described in the literature have identified the compound **2** to the alphitolic acid [20, 21], isolated from *Vernonia guineensis* for the first time.

Compound **3** has been isolated as a white powder; it crystallizes in ethyl acetate. It is Soluble in methanol and gives a greenish coloration to the characteristic Liebermann-Burchard test of steroids. The ^{1}H NMR spectrum of compound **3** exhibits the characteristic signals of β-sitosterol, to which are added signals corresponding to the presence of a saccharide unit. A signal at 5.38 ppm corresponding to the ethylenic proton H_6, signals between δH 3.20 and δH 4.44 ppm characteristic of the protons of a sugar. ^{1}H NMR spectrum of this compound also shows 6 methyl signals between δ_H 0.72 and 1.03 ppm and many multiplets between δ_H = 1.32 and 2.41 ppm attributable to methylenes and methynes. The ^{13}C APT NMR spectrum of compound 3 reveals the presence of 35 carbons which confirms the presence of β-sitosterol substituted by a glucose molecule. We have identified 06 methyl groups, 08 methyl carbons, one of which has one hetero atom, 11 methylenes and four quaternary carbons have been identified. We can distinguish the following characteristic carbons: Two ethylenic carbons C-5 and C-6 resonating respectively at 142.0 and 122.8 ppm. Anomeric carbon at 102.5 ppm, six hydroxylated carbons at 62.8-79.9 ppm suggesting a hexose, six methyls at 12-20.3 ppm, a cluster of carbons at 22.3-43.7 ppm. The set of all these physical and spectroscopic data compared to those described in the literature made it possible to identify compound **3** as β-sitosterol 3-O-β-D-glucopyranoside [22,23].

Compound **4** crystallizes from the CH_2Cl_2-acetone (95:5) mixture as a greenish powder. It is soluble in chloroform and gives 366 nm UV fluorescence of a blue color characteristic of simple coumarins [24]. On its IR spectrum, there is an absorption band at 3033 cm^{-1} and at 1618-1660 cm^{-1} attributable respectively to the C-H and C=C groups. A band between 1094 cm^{-1} and 1138 cm^{-1} characteristic of the C-O function. Band at 1702 cm^{-1} characteristic of the C=O conjugation function (lactone carbonyl). An aromatic system absorbing between 1557 and 1513 cm^{-1}. All of these bands seem to confirm the presence of a coumaric ring [24]. On the ^{1}H NMR spectrum Two doublets at 7.62 and 6.86 ppm attributable to protons (H-3) and (H-4) respectively. The similarity of their coupling constant and the value of their chemical shift suggest the existence, of a cis-disubstituted AB-spin system in compound 4 and vicinal ethylenic group to an attractor group with which it participates in a mesomerism. There are also two singlets at 3.93 ppm and 3.95 ppm attributable respectively to 6-OCH_3 and 7-OCH_3 protons. Finally, two singlets of an aromatic proton each respectively at 6.30 and 7.21 ppm. On the broadband-decoupled ^{13}C NMR spectrum, an attractor group is identified by virtue of the 161.41 ppm characteristic signal of the lactone carbonyls already mentioned above. This spectrum also shows 10 other carbon atoms resonating between 152.8 and 56.6 ppm, of which 4 attributable to aromatic quaternary carbons (152.81, 150.04, 146.31, 111.40 ppm). This suggests that compound 4 has a single tetrasubstituted aromatic nucleus of which two of the substituents are visibly two OCH_3 groups. All of these physical and spectroscopic data compared to those described in the literature have identified compound **4** as being Scoparone [6, 24], isolated from *Vernonia guinensis* for first time.

The UV spectrum of compound **5** in methanol (354 nm, band I) indicated a Flavonol of Flavonol substituted with 3-O. These data were confirmed by the ^{1}H and ^{13}C spectra. ^{1}H and ^{13}C NMR spectra indicated the presence of a quercetin fragment and a sugar motif [25]. The proton at 5.33 ppm was assigned to the hexose anomeric proton and suggested in a

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β-glycosidic bond. Acidic hydrolysis of compound **5** yielded glucose and quercetin, which were identified by co-chromatography with an authentic laboratory sample. All of these physical and spectroscopic data compared to those described in the literature made it possible to identify compound **5** as quercetin-3-O-β-galactoside (5) [26].

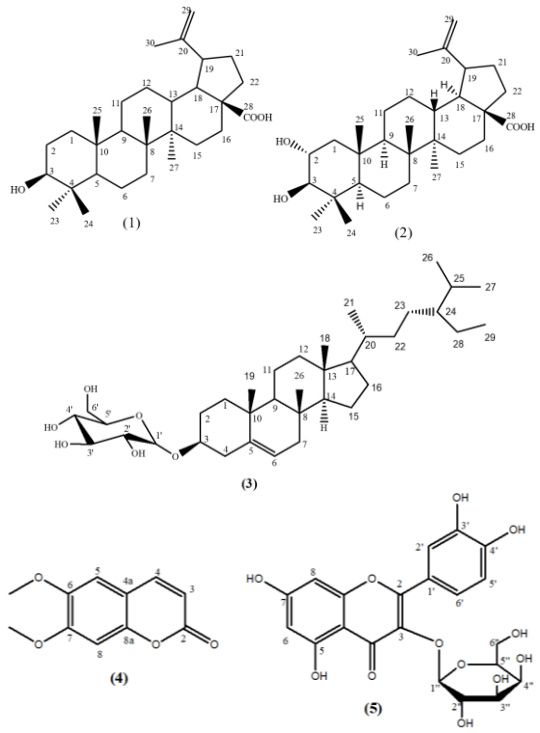

Fig. 1 Structure of compounds **1** to **5** isolated from *Vernonia guineensis*

Table 1 Diameters of zones of inhibition of compounds isolated from the roots of *Vernonia guineensis*

Strain	Quantity of compounds on disk (µg)	Diametre of inhibition zones (mm)					
		(1)	**(2)**	**(3)**	**(4)**	**(5)**	Antibiotic
E coli ATCC 25922	200	-	-	7	-	-	
S aureus ATCC 25923	200	-	-	-	-	-	
K pneumoniae ATCC 700603	1000	-	-	-	-	-	
E coli ATCC 11775	1000	-	-	-	-	-	
S aureus BAA 977	2000	-	-	-	-	-	
S aureus ATCC 29213	2000	-	-	-	-	-	
Salmonella choleraesuis	2000	-	-	-	7	-	40 C
Proteus mirabilis	2000	-	-	-	-	-	18 G
Enterococcus faecalis ATCC 51229	2000	-	-	-	7	-	
Neisseria gonorhae ATCC 49226	2000	15	18	10	8	8	
Pseudomonas aeruginosa ATCC 10145	2000	9	7	8	8	7	
Candida albican	200	-	7	7	-	-	

(1): *Betulinic acid;* ***(2):*** *alphitolic acid* ***(3);*** *β-sitosterol 3-O-β-D-glucopyranoside;* ***(4)*** *Scoparone ;* ***(5)*** *: Quercetin-3-O-β-galactoside; (-): Absence*

3.2. Antimicrobial Activities

3.2.1 Diameters of the Inhibition Zones

The antibacterial activity of the compounds (**1**, **2**, **3**, **4** and **5**) resulted in the presence of the inhibition zones around Whatman No.39 paper disks impregnated with the various compounds to be tested. Table 1 below shows these zones of inhibition.

The observation of this Table 1 shows that compound **3** exhibited the highest antibacterial potency. Indeed, the growth of 4 strains (*Salmonella choleraesuis, Enterococcus faecalis* ATCC 51299, *Neisseria gonorrhoeae ATCC 49226*, *Pseudomonas aeruginosa ATCC 10145*) was inhibited by compound **3** with inhibition diameters varying from 7 to 8 mm. Compound **4** inhibited the growth of 3 strains (*Escherichia coli ATCC 25922*, *Neisseria gonorrhoeae ATCC 49226*, *Pseudomonas aeruginosa ATCC 10145*) with inhibition diameters ranging from 7 to 10 mm. 2 strains (*Neisseria gonorrhoeae ATCC 49226*, *Pseudomonas aeruginosa ATCC 10145*) were sensitive to all compounds. Inhibition diameters on *Neisseria gonorrhoeae ATCC 49226* range from 8 to 18 mm and compound **2** was the most active with an inhibition diameter of 18 mm. Thus, the inhibition diameters range from 7 to 9 mm for *Pseudomonas aeruginosa ATCC 10145* and compound **1** exhibited the highest inhibition diameter. We also note that 6 strains (*Staphylococcus aureus ATCC 25923*, *Klebsiella pneumoniae ATCC 700603*, *Escherichia coli ATCC 11775, Staphylococcus aureus ATCC BAA 977*, *Staphylococcus aureus ATCC 29213*, *Proteus mirabilis*) were all non-sensitive to all compounds. The fungal strain (*Candida albican*) was sensitive to the compounds **(2)** and **(3)**.

The observation in Table 1 shows that compounds **(2)** and **(3)** were active on most the strains tested, whereas compound **1** was active on 4 strains and compound **5** only acted on one strain. In fact, the growth of 8 strains (*Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC BAA 977, Staphylococcus aureus ATCC 29213, Salmonella choleraesuis, Proteus mirabilis, Enterococcus faecalis ATCC 51299, Neisseria gonorrhoeae ATCC 49226, Pseudomonas aeruginosa ATCC 10145*) for compound **2**, 9 strains (*Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Staphylococcus aureus ATCC BAA 977, Staphylococcus aureus ATCC 29213, Salmonella choleraesuis, Proteus mirabilis, Enterococcus faecalis ATCC 51299, Neisseria gonorrhoeae ATCC 49226, Pseudomonas aeruginosa ATCC 10145) for compound **3** and of 7 strains (Staphylococcus aureus ATCC BAA 977, Staphylococcus aureus ATCC 29213, Salmonella choleraesuis, Proteus mirabilis, Enterococcus faecalis ATCC 51299, Neisseria gonorrhoeae ATCC 49226, Pseudomonas aeruginosa ATCC 10145*) for compound **4** out of the 11 strains tested was inhibited; with the diameters of the zones of inhibition going from 7 to 15mm for the compound **2**; 7 to 15 mm for compound **3** and 9 to 14 mm for compound **4.**

The highest antibacterial potency of compound **2** was observed against 2 strains (*Staphylococcus aureus ATCC BAA 977, Staphylococcus aureus ATCC 29213*) with a 15 mm inhibition diameter; that of the compound **3** was observed against *Staphylococcus aureus ATCC 29213* with an inhibition diameter of 15 mm and that of the compound **4** was observed against *Staphylococcus aureus ATCC 29213*, *Salmonella choleraesuis* with an inhibition diameter of 14mm.

Pseudomonas aeruginosa ATCC 10145 was found to be the least sensitive strain with respect to Compound **2** with an inhibition diameter of 7 mm; *Staphylococcus aureus ATCC 25923*, *Pseudomonas aeruginosa ATCC 10145* were found to be the least sensitive strains to compound **3** and *Staphylococcus aureus ATCC 29213*, *Salmonella choleraesuis, Neisseria gonorrhoeae ATCC 49226, Pseudomonas aeruginosa ATCC 10145* were found to be the least sensitive to the compound **4** with an inhibition diameter of 9 mm.

Klebsiella pneumoniae ATCC 700603 and *Escherichia coli ATCC 11775* were non-sensitive to all compounds. Compound **5** has the least antibacterial potency with activity on a strain (*Pseudomonas aeruginosa ATCC 10145*) and a diameter of 8 mm. The fungal strain (*Candida albican*) was sensitive to compound **2**.

3.2.2 Inhibition Parameters-Minimum Inhibitory Concentrations (MICs) and Concentration Minimum Bactericidal (CMB) and the CMB/MIC Ratios of Compounds (2) and (3)

Table 2 below shows the MICs, CMBs and CMB/MIC ratios of the compounds **(2)** and **(3)** because they have revealed the highest antibacterial powers. We able to note that the MICs of compound **2** vary from 312.5 to 2500 µg/mL with the lowest value (312.5 µg/mL) observed against *Staphylococcus aureus ATCC BAA 977*. As for compound **3**, the MICs vary from 312, 5 to 5000 µg/mL with the lowest value (312.5 µg/mL) observed against *Staphylococcus aureus ATCC BAA 977*. This is in line with the results of the previous test where these strains proved to be the most sensitive towards of these compounds. The calculation of the CMB/MIC ratio showed that compounds **2** and **3** have a bactericidal action.

According to the Aligiannis' classification [16] stated in this work, these 2 compounds exhibited a strong inhibition on *Staphylococcus aureus ATCC BAA 977*, a moderate inhibition on *Staphylococcus aureus ATCC 29213*, a weak inhibition on *Aerococcus viridans ATCC 11563*, moderate inhibition on *Enterococcus faecalis ATCC 51299* for compound **2** and low for compound **3**, finally a weak inhibition on *Salmonella choleraesuis* for compound **2** compared to ciprofloxacin which has a strong inhibition on this strain.

Plants are widely used in traditional medicine by people in Africa to treat vast majority of diseases. The extraction of *Vernonia guineensis* roots by increasing polarity solvents resulted in the isolation of 5 compounds. The results show that the different compounds studied have a high solubility in hydroalcoholic solvents. A study conducted by Penchev [27] showed that the polarity of a solvent can be increased by adding polar co-solvents, which improves the affinity and potency of the solvent over polar molecules. The antimicrobial activity of several molecules belonging to the different secondary metabolites revealed during the phytochemical analysis has already been reported by several authors [28]. According to

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the classification of ponce stated in this work, the test results of antibacterial study have revealed that the compounds isolated from the roots of *Vernonia guineensis* have more or less important activities with respect to the gram negative and gram-positive bacterial species tested. These observed antibacterial activities could be due to the presence of these different secondary metabolites in our extracts. Indeed, Marjorie [29] reported that phenolic compounds, alkaloids and saponins inhibited the growth of microorganisms. It is also apparent from our results that the isolated compounds have greater antibacterial potency than root extracts on the majority of strains tested. This difference is due to the fact that the sensitivity of a microorganism to a plant extract depends not only on the functional groups, but also on the microorganism itself [30]. The non-effectiveness of the compound's vis-a-vis other bacterial strains can be explained either by the absence of compounds likely to inhibit their growth, or by their low concentration in the extracts. According to the Aligiannis [16] classification stated in our results, compounds **2** and **3** tested showed strong inhibition on *Staphylococcus aureus ATCC BAA 977*, moderate inhibition on *Staphylococcus aureus ATCC 29213*, low inhibition on *Aerococcus viridans ATCC 11563*, moderate inhibition on *Enterococcus faecalis ATCC 51299* for compound **2** and low for compound **3**, finally a weak inhibition on *Salmonella choleraesuis* for compound **2** compared with ciprofloxacin which has a strong inhibition on this strain. The results obtained corroborate with the work of Donfack (2012) [31] who showed that there are compounds within *Vernonia guineensis* that are responsible for antibacterial activities against many microbial strains. Calculation of the CMB/MIC ratio revealed that compounds **2** and **3** had bactericidal activity (CMB/MIC<4). This bactericide could justify their use in herbal medicine against bacterial infections.

Table 2 Minimal inhibitory concentrations (MICs) and concentration bactericidal minimum (CMB) and the CMB/MIC reports of compounds **2** and **3**

Strain	Parameters	compounds		
	(µg/mL)	**(2)**	**(3)**	Ciprofloxacine
Staphylococcus aureus ATCC 29213	CMI	1250	1250	
	CMB	2500	2500	
	CMB/CMI	2	2	
Staphylococcus aureus ATCC BAA 977	CMI	312,5	312,5	
	CMB	625	625	
	CMB/CMI	2	2	
Aerococcus viridans ATCC 11563	CMI	2500	2500	
	CMB	5000	5000	
	CMB/CMI	2	2	
Enterococcus faecalis ATCC 51299	CMI	625	5000	
	CMB	1250	10000	
	CMB/CMI	2	2	
Salmonella choleraesuis	CMI	2500	nd	0,195
	CMB	5000	nd	0,39
	CMB/CMI	2		2

(2): *Alphitolic Acid;* ***(3):*** *3-O-β-D-glucopyranoside of β-sitosterol;* ***nd*** *= not determined*

4. Conclusion

In the present work, we have investigated the *in vitro* antimicrobial effects of compounds isolated from the roots of *Vernonia guineensis* belonging to the Asteraceae family, widely used in the Cameroonian pharmacopoeia. The evaluation of the antimicrobial activity of these compounds shows that they have inhibitory properties vis-a-vis the growth of most of the microbial strains tested. This explains their various uses in traditional therapy in the treatment of several infectious diseases. A study of the phytochemical composition of the extracts revealed the presence of numerous bioactive chemical compounds known for their biological activities. Our preliminary results show that the compounds tested possess *in vitro* antimicrobial activities. From this study, we can conclude that the mixture CH_2Cl_2- CH_3OH (1:1) extracted from *Vernonia guineensis* bark roots and the isolated compounds possess interesting antibacterial and antifungal properties.

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