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ORIGINAL ARTICLE

Novel Phytocompounds from *Vernonia amygdalina* with Antimalarial Potentials

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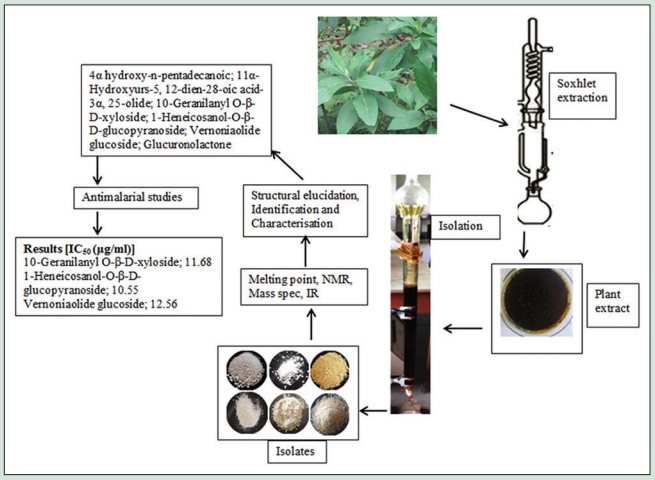
ABSTRACT

Background: Malaria, one of the diseases predominant in the African continent, has been reported to be treated with plants and also some *in vitro* and *in vivo* tests have supported this. *Vernonia amygdalina* belonging to the family of *Asteraceae* is one of the plants widely used in Nigeria and studied for treatment of malaria and some scientific researchers have validated this claim. **Objectives:** In the present study, we aimed at isolation of possible compounds from the methanolic stem-bark of *V. amygdalina*, elucidation and characterization of the isolated compounds, and carry out antimalarial evaluations of the isolated compounds. **Materials and Methods:** Isolation of compounds was done using column chromatography technique, elucidation and characterization were done based on infrared, Mass, ^{1}H, and ^{13}C nuclear magnetic resonance spectra. The *in vitro* antimalarial activity was carried out on the ring stage of the malaria parasite cycle of chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) strains. **Results:** Five novel compounds were isolated; 4α-Hydroxy-*n*-pentadecanoic acid (CC7), 11α- Hydroxyurs-5,12-dien- 28-oic acid-3α, 25-olide (CC10), 1-Hen eicosanol-O-β-D-glucopyranoside (CC19), 10-Geranilanyl-O-β-D-xyloside (AC2A), 6β,10β,14β-Trimethyl heptadecan-15α-olyl-15-O-β-D-glucopyrano syl-1,5β-olide (CC14), and one new compound; Glucuronolactone (CC3). The compounds CC10, CC19, AC2A, CC14, and CC3 recorded schizont inhibition at different percentages. Compounds CC19, AC2A, and CC14 recorded half-maximal inhibitory concentration (IC_{50}) values of 10.55 μg/ml, 12.56 μg/ml, and 11.68 μg/ml, respectively. **Conclusion:** The IC_{50} values obtained are much higher than that of chloroquine, which is 0.02 μg/ml. These compounds showed antimalarial activity at different levels. The presence and effect of these compounds validate the use of this plant for the treatment of malaria in the traditional medicinal practice of Nigeria.

Key words: Antimalarial, half-maximal inhibitory concentration, isolation, phytocompounds, *Vernonia amygdalina*

SUMMARY

- Plants have been serving and still serve as source of medicines and supplements to humankind. This motivated our research on *Vernonia amygdalina*; one of the most commonly used medicinal plant in Nigeria and tropical Africa in general. Based on our antimalarial studies of the different parts of *Vernonia amygdalina*, which revealed that the methanolic stem-bark extract of this plant has the highest percentage of schizont inhibition in comparison to other parts, We decided to carry out phytocompounds isolation present in the methanolic stem-bark of *V. amygdalina*, using column chromatographic technique in the presence of increasing polarity of hexane, ethyl acetate, and methanol as mobile phases. Six compounds were isolated, elucidated, identified, and characterized based on the carbon 13 nuclear magnetic resonance (NMR), proton NMR, Mass and IR spectra of the isolates; 4α-Hydroxy-n-pentadecanoic acid (CC7), 11α-Hydroxyurs-5,12-dien- 28-oic acid-3α, 25-olide (CC10), 1-Hene icosanol-O-β-D-glucopyranoside (CC19), 10-Geranilanyl-O-β-D-xyloside (AC2A), 6β,10β,14β-Trimethyl heptadecan-15α-olyl-15-O-β-D-glucopyranosyl-1,5β-olide (CC14), and one new compound; Glucuronolactone (CC3). The compounds isolated were novel compounds except for the glucuronolactone which is a new compound. The compounds produced different percentages of schizont inhibition for chloroquine-sensitive and chloroquine-resistant strains of malaria parasite. The compounds CC19, AC2A, and CC14 produce IC_{50} values of 10.55 μg/ml, 12.56 μg/ml, and 11.68 μg/ml, respectively.



Abbreviations Used: IC_{50}: Half-maximal inhibitory concentration, HIV/AIDS: Human immunodeficiency virus/acquired immunodeficiency syndrome, DMSO: Dimethyl sulfoxide, CO_2: Carbon dioxide, TLC: Thin-layer chromatography, NMR: Nuclear magnetic resonance, IR: Infrared, CC7: 4α-hydroxy-n- pentadecanoic acid, CC10: 11α-Hydroxyurs-5,12-dien- 28- oic acid-3α, 25-olide, CC19: 1-Heneicosenol O-β-D-glucopyranoside, AC2A: 10-Geranilanyl O-β-D-xyloside, CC14: 6β,10β,14β-trimethylheptadecan-15α-olyl-15-O-β-D-glucopyranosyl-1, 5β-olide (vernoniaolide glucoside), CC3: Glucuronolactone, KBr: Potassium bromide, $CDCl_3$: Deuterated chloroform, ^{1}H NMR: Proton nuclear magnetic resonance, ^{13}C NMR: Carbon-13 nuclear magnetic resonance, ESI: Electrospray ionization, MS: Mass spectrometry, m/z: Mass to charge ratio, rel/int: Relative intensity, MeOD: Deuterated methanol, D_2O: Deuterated water.

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INTRODUCTION

Nature as old as human's existence has been a provider of medicines and agents used for the development of medicine. The most prevalent sources have been plants.[1] The development of most of these medicines obtained from plant stemmed from the study of the utilization of the plant in traditional medicinal practices, which gave an insight into the type of activity or likely pharmacological effect for which the products from plants could be developed for.

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Vernonia amygdalina is one of the plants that are widely utilized by traditional medicine practitioners in the tropical region of the African continent for management, treatment, and prevention of certain disease conditions, with some scientific researches to validate these claims. *V. amygdalina* belongs to the Kingdom of Plantae, family of *Asteraceae* and order of Asterales. It is frequently referred to as bitter leaf as a result of its bitter taste. Throughout the cultures of the countries in the equatorial regions of Africa, the leaf of *V. amygdalina* is a staple vegetable used for making soups and stews.[2] One of the most common diseases against which *V. amygdalina* is being used for is malaria.

Malaria is a serious and life-threatening ailment caused by bites of infected female Anopheles mosquitoes which transmit the parasites. It is preventable through the maintenance of hygienic environments or by the use of mosquito nets and cured through the use of medications. In 2016, it was estimated that in 91 countries, there were 216 million cases of malaria. This figure is an increase of five million when compared with that of 2015. In 2015 and 2016, deaths as a result of malaria reached about 446,000 and 445,000, respectively. According to the World Health Organization, African Region carries a high share of the global malaria burden. In 2016, the African region recorded 90% of world malaria cases and 91% of world malaria deaths.[3] According to the Nigeria Malaria Fact Sheet published by the United States Embassy in Nigeria, one of the major public health problems in Nigeria is Malaria, where it is responsible for most health cases and deaths in comparison to other countries of the world. About 97% of the population are exposed to malaria, while the remaining 3% are highland dwellers. According to the report, about 100 million cases and about 300,000 deaths are reported every year. This is compared to deaths from human immunodeficiency virus/acquired immunodeficiency syndrome, which are about 215,000 per year.[4]

Even though their medications currently in use for treatment of malaria, the use of *V. amygdalina* in raw form or in herbal formulations for treatment of malaria and other health conditions in Nigeria is highly persistent. Some studies on *V. amygdalina*, have reported that this plant has an antibacterial property,[5] antibacterial effect towards *Bacillus cereus*, *Bacillus pumilus*, *Bacillus subtilis*, *Micrococcus kristinae*, *Staphylococcus aureus*, *Enterobacter cloacae,* and *Escherichia coli*,[6] amplification of glucose utilization of muscle and liver cell cultures but not on adipose cells,[2] lowers blood glucose levels of both normoglycemic and hyperglycemic rats induced by alloxan,[7] suppresses polymorphonuclear cell and mononuclear cell activity without affecting the cell viability,[8] and other scientifically proven effects. The plant has been reported to contain vernolide, vernodalol, vernolepin, vernodalin, vernomygdin, hydroxyvernolide, vernodalinol, vernomenin, vernolic, 11, 13-dihydrovernodalin, 11, 13-dihydrovernorodeline, 4, 15-dihydrovernodalin,[9-11] and some other phytocompounds.

In our studies on the antimalarial effect of methanolic leaf, stem-bark and root extracts of *V. amygdalina* using *in vitro* antimalarial assay technique, we found that the methanolic leaf extract produced 80.64% ± 4.34% schizont inhibition, methanolic stem-bark produced 71.00% ± 6.10% schizont inhibition, why the methanolic root extract produced no schizont inhibition.

Based on these results, we aimed in the present research at the isolation of the phytocompounds present in the stem-bark part of this plant, structural elucidation and characterization of the isolates, and evaluating through *in vitro* technique the antimalarial potential of the isolates.

MATERIALS AND METHODS

Plant collection and authentication

The plant was collected during the month of August (rainy season), 2015, from Umuono community, Ngodo village, Nise, Anambra state, Southeast Nigeria. The plant was identified and authenticated by a taxonomist of International Centre for Ethnomedicine and Drug Development, Nsukka, Enugu State, Nigeria. The Voucher number is INTERCEDO/041.

Processing the plant parts

The plant was dried in Nigeria under room temperature and the particle size reduced using a manual grinder.

Extraction

The plant (6.2 kg) was subjected to cold maceration using methanol (2.5 L) for 7 days in batches and subsequently subjected to hot extraction in Soxhlet apparatus (2 L per each Soxhlet packing). The Soxhlet extraction was done at the temperature of 45°C. Each packing was run for eight cycles. The extracts were mixed and dried with the aid of a rotary evaporator.

Isolation, elucidation and characterization materials

Methanolic stem-bark extract of *V. amygdalina* (114 g), column chromatography silica gel (60–120 mesh, Spectrochem), chromatography column (1000 mm length and 40 mm inner diameter [I.D]), Erlenmeyer flask 250 ml, thin-layer chromatography (TLC) plate, hexane, ethyl acetate, methanol, and TLC plate (TLC Silica gel 60 F254 [Germany]). Spots were viewed in a CAMAG UV cabinet 3, melting point apparatus (Optic technology), FT-IR Bruker Alpha, FT-NMR (Bruker Avance II, 400 MHz), mass spectrophotometer (Water ZQ-4000), dimethyl sulfoxide (DMSO), incomplete culture medium with the absence of Albumax-II and Hypoxanthine, 96-well flat-bottom microtiter plate, malaria parasites (3D7 strain [chloroquine-sensitive strain] and Dd2 [chloroquine-resistant strain]), blood, Chloroquine diphosphate, an incubator with 5% CO_2 environment at a temperature of 37°C, Giemsa stain, glass slides.

Preparation of the extract for isolation

The extract (114 g) was processed into granules by mix with silica (60–120 mesh) in methanol over a heated water bath and allowed to dry.

Isolation procedure

The packing of the column was done by dry method. The column was eluted drop wise, first with hexane and then with increasing ratios of polarity using hexane, ethyl acetate, and methanol. During the elutions, a size of 250 ml was collected per fraction and the fractions were compared using a precoated TLC plate. The isolated samples were subject to proton nuclear magnetic resonance (NMR), carbon 13 NMR, mass spectroscopy, and infrared spectroscopy. The spectra obtained were used for the structural elucidation and characterization of the isolates.

In vitro antiplasmodial assay

This was done according to the WHO standard. The isolates were solubilized in DMSO to prepare a solution of 5 mg/ml concentration as the stock solution. A 5 µg/ml and 50 µg/ml concentrations of isolates were prepared from the stock solution using incomplete culture medium with the absence of Albumax-II and Hypoxanthine. A 20 µl volume of each of the concentrations of the isolates was each placed in a well of a 96-well flat-bottom microtiter plate in duplicates and a volume of 180 microliter of malaria parasites (3D7 strain [chloroquine-sensitive strain] and Dd2 [chloroquine-resistant strain]) contained in 3% of the volume of red blood cell to the total volume of blood (hematocrit), having 1% malaria parasite blood infestation (parasitemia) was added to the 20 µl of the isolates dilutions in the microwells of the 96-well flat bottom microtiter plate. A positive control of chloroquine diphosphate was used for the

assay at its known IC_{50} concentration. A negative control containing no test sample was also used. Culture medium of 20 µl was used as negative control. The microwell plates were incubated for 24–30 h (this depends on the maturation of the schizonts) in a modulated incubator which has 5% CO_2 environment at a temperature of 37°C. After the incubation, on a glass slide, a blood smear was made from each of the microwells. Methanol was used for fixing, while Giemsa staining was made on the slides and observation was done under a microscope. If after the assay the schizonts maturation percentage is below 20% in the negative control slide, the assay is taken to be invalid. The number of schizonts found per 200 asexual parasites and the percentage inhibition of the schizonts for each of the isolate dilutions were calculated using the formula given below. The test was performed in triplicate.

Determination of half-maximal inhibitory concentration values for the compounds that were marked "will proceed for half-maximal inhibitory concentration"

For the determination of IC_{50} for the compounds that produced good results from the isolates general evaluation above, the evaluation was repeated at more ranges of concentrations of 4, 8, 16, 32, and 64 µg/ml of the test isolates of AC2A, CC14, and CC19. The results obtained with the above-mentioned ranges of concentration were used for the calculation of IC_{50} for the isolated compounds tested. The IC_{50} values were estimated using GraphPad prism software.

RESULTS

Isolation

4α-Hydroxy-n-pentadecanoic acid (CC7)

Elution of the column with hexane-ethyl acetate (9:1) gave a yellow amorphous mass of Figure 1 (280 mg), *R*f value 0.50 (hexane: ethyl acetate, 9:1) m. p. 61°C–63°C. IR V_{max} (KBr): 3410, 3253, 2928, 1702, 1463, 1374, 1294, 1170, 1051, 938, 723 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.64 (1H, dd, *J* = 6.8, 6.4 Hz, H-4β), 2.34 (2H, t, *J* = 7.2 Hz, H_2-2), 2.03 (2H, m, H_2-3), 1.63 (2H, m, CH_2), 1.32 (2H, m, CH_2), 1.28 (16H, brs, 8 × CH_2), 0.87 (3H, t, *J* = 6.4 Hz, Me-15); ^{13}C NMR ($CDCl_3$): δ 179.03 (C-1), 33.88 (C-2), 31.91 (C-3), 63.10 (C-4), 29.69 (C-5), 29.65 (C-6), 29.58 (C-7), 29.42 (C-8), 29.35 (C-9), 29.23 (C-10), 29.05 (C-11), 25.71 (C-12), 24.68 (C-13), 22.68 (C-14), 14.10 (C-15); ESI MS m/z (rel. int.): 258 $[M]^+$ ($C_{15}H_{30}O_3$) (2.8), 155 (2.4), 103 (18.3), 99 (48.3), 57 (82.7).

11α-Hydroxyurs-5,12-dien-28-oic acid-3α, 25-olide (CC10)

Elution of the column with ethyl acetate produced colorless amorphous crystals of Figure 2 (195 mg), R_f value 0.62 (hexane: ethyl acetate, 4:1), m.p. 243°C–245°C; IR V_{max} (KBr): 3518, 3218, 3278, 2921, 2883, 1767, 1692, 1643, 1442, 1327, 1250, 1138, 1084, 1045, 1003, 945, 897, 839, 794 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.63 (1H, d, *J* = 6.0 Hz, H-12), 5.40 (1H, m, H-6), 4.80 (1H, dd, *J* = 3.2, 4.4 Hz, H-3β), 4.57 (1H, dd, *J* = 4.0, 4.4 Hz, H-11β), 2.83 (1H, d, *J* = 13.2 Hz, H-18β), 2.56 (1H, d, *J* = 4.8 Hz, H2-7α), 2.50 (1H, d, *J* = 6.0 Hz, H2-7β), 2.45 (1H, m, H2-1α), 2.39 (1H, d, *J* = 2.4 Hz, H2-1β), 2.33 (2H, m, H2-2α), 2.30 (1H, m, H2-2α), 2.28 (1H, m, H2-2β), 2.26 (2H, m, H2-15), 2.23 (2H, m, H2-16), 2.20 (1H, m, H-19), 2.18 (1H, m, H-20), 2.04 (2H, m, H2-21), 1.48 (2H, m, H2-22), 1.25 (3H, s, Me-23), 1.11 (3H, s, Me-26), 1.10 (3H, s, Me-27), 1.08 (3H, d, *J* = 9.6 Hz, Me-29), 0.96 (3H, d, *J* = 6.8 Hz, Me-30), 0.59 (3H, s, Me-24); ^{13}C NMR ($CDCl_3$): δ 40.09 (C-1), 28.65 (C-2), 78.89 (C-3), 38.98 (C-4), 144.08 (C-5), 120.14 (C-6), 33.54 (C-7), 39.71 (C-8), 47.72 (C-9), 37.22 (C-10), 76.11 (C-11), 122.37 (C-12), 139.71 (C-13), 42.41 (C-14), 28.39 (C-15), 24.28 (C-16), 45.86 (C-17), 51.17 (C-18), 43.15 (C-19), 41.13 (C-20), 33.18 (C-21), 33.36 (C-22), 29.15 (C-23), 15.73 (C-24), 173.21 (C-25), 16.89 (C-26), 23.67 (C-27), 178.52 (C-28), 18.71 (C-29), 22.53 (C-30); ESI MS m/z (rel. int.): 482 $[M]^+$ ($C_{30}H_{42}O_5$) (1.6), 452 (4.8), 264 (1.2), 219 (1.6), 203 (1.5).

10-Geranilanyl O-β-D-xyloside (AC2A)

Elution of the column with ethyl acetate: methanol (9.5: 0.5) afforded a colorless crystalline mass of Figure 3 (253 mg), *R*f value 0.54 (ethyl acetate: methanol, 7:3), m. p. 219°C-220°C. IR V_{max} (KBr): 3295, 2927, 2869, 1436, 1362, 1259, 1013, 890, 797 cm^{-1}. ^{1}HNMR (MeOD): δ 5.44 (1H, d, *J* = 8.6 Hz, H-1'), 4.41 (1H, m, H-2'), 3.83 (1H, m, H-3'), 3.69 (1H, m, H-4'), 3.32 (2H, m, H2-5'), 3.16 (2H, d, *J* = 8.4 Hz, H2-10), 2.24 (1H, m, H-3), 2.03 (1H, m, H-7), 1.93 (2H, m, H2-4), 1.47 (2H, m, H2-5), 1.38 (1H, m, H2-6a), 1.28 (1H, m, H2-6b), 1.12 (1H, m, H2-2a), 1.03 (1H, m, H2-2b), 0.95 (3H, d, *J* = 6.6 Hz, Me-8), 0.93 (3H, d, *J* = 6.4 Hz, Me-9), 0.61 (3H, t, *J* = 6.5 Hz, Me-1). ^{13}C NMR (MeOD): δ 12.55 (C-1), 30.61 (C-2), 43.37 (C-3), 37.10 (C-4), 36.01 (C-5), 35.05 (C-6), 40.51 (C-7), 19.91 (C-8), 19.94 (C-9), 62.79 (C-10), 102.34 (C-1'), 78.08 (C-2'), 77.86 (C-3'), 75.13 (C-4'), 71.67 (C-5'). ESI MS m/z (rel. int.): 290 $[M]^+$ ($C_{15}H_{30}O_5$) (3.2), 149 (2.8), 157 (2.7).

Heneicosenol O-β-D-glucopyranoside (CC19)

Elution of the column with ethyl acetate-methanol (9:1) gave colorless amorphous powder of Figure 4 (213 mg), *R*f value 0.77 (hexane: ethyl acetate, 1:1), m. p. 104°C–105°C. IR V_{max} (KBr): 3426, 3331, 3218, 2915, 2848, 1616, 1435, 1260, 1115, 1041, 725 cm^{-1}; ^1H NMR ($CDCl_3$): δ 5.39 (1H, m, $w_{1/2}$ = 8.2 Hz, H-13), 5.34 (1H, m, $w_{1/2}$ = 8.6 Hz, H-14), 3.63 (2H, m, H_2-1), 2.17 (2H, m, H_2-12), 2.03 (2H, m, H_2-15), 1.67 (2H, m, H_2-2), 1.50 (2H, m, CH_2), 1.28 (26H, brs, 13 × CH_2), 0.88 (3H, t, *J* = 6.5 Hz, Me-21), 4.85 (1H, d, *J* = 7.2 Hz, H-1'), 4.13 (1H, m, H-5'),

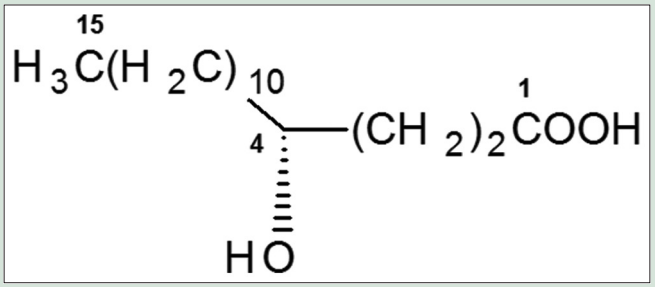

Figure 1: 4α-Hydroxy-n-pentadecanoic acid (CC7)

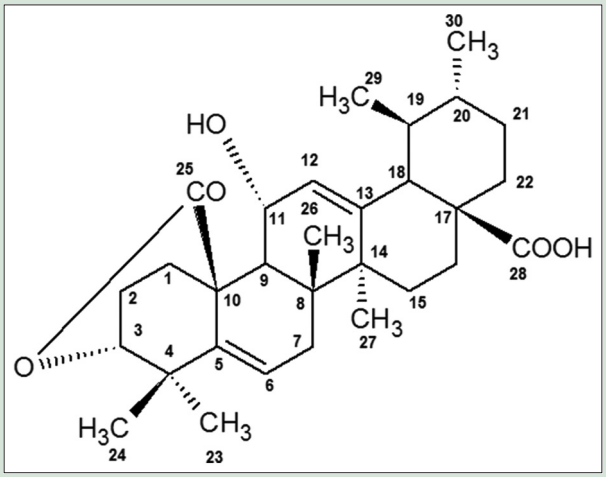

Figure 2: 11α-Hydroxyurs-5,12-dien-28-oic acid-3α, 25-olide (CC10)

3.92 (1H, m, H-2'), 3.79 (1H, m, H-3'), 3.77 (1H, m, H-4'), 3.30 (2H, m, H_2-6'); ^{13}C NMR ($CDCl_3$): δ 132.11 (C-13), 123.58 (C-14), 57.44 (C-12), 48.19 (C-15), 40.02 (C-2), 32.26 (C-3), 29.68 (11 × CH_2), 25.37 (CH_2), 22.68 (C-20), 14.17 (C-21), 63.25 (C-1), 103.37 (C-1'), 73.18 (C-2'), 69.16 (C-3'), 65.43 (C-4'), 85.21 (C-5'), 60.11 (C-6'); ESI MS m/z (rel. int.): 472 $[M]^+$ $C_{27}H_{52}O_6$ (4.8), 293 (2.1), 163 (8.9), 125 (10.2), 99 (1).

6β,10β,14β-trimethylheptadecan-15α-olyl-15-O-β-D-glucopyranosyl-1,5β-olide (Vernoniaolideglucoside) (CC14)

Elution of the column with ethyl acetate-methanol (9:1) produced colorless amorphous powder of Figure 5. (292 mg), *Rf*0.59 (ethyl acetate), m. p. 233°C–235°C. IR V_{max} (KBr): 3445, 2928, 2872, 1765, 1639, 1441, 1386, 1263, 1021, 894, 797 cm^{-1}. ^{1}H NMR (MeOD): δ 5.41 (1H, d, *J* = 7.3 Hz, H-1'), 4.70 (1H, m, H-5'), 4.38 (1H, brm, $w1/_2$ = 14.3 Hz, H-5α), 3.87 (1H, m, H-2'), 3.68 (1H, brm, $w1/_2$ = 14.3 Hz, H-5α), 3.65 (1H, m, H-3'), 3.38 (1H, m, brm, $w1/_2$ = 8.6 Hz, H-15β), 3.31 (1H, m, H-4'), 3.17 (1H, d, *J* = 8.0 Hz, H_2-6'a), 3.13 (1H, d, *J* = 8.0 Hz, H_2-6'b), 2.81 (1H, m, H-6), 2.23 (1H, m, H-14), 2.21 (2H, t, *J* = 8.0 Hz, H_2-2), 2.02 (2H, m, H_2-16), 2.07 (2H, m, H_2-3), 2.05 (2H, m, H_2-4), 1.97 (2H, m, H_2-7), 1.87 (1H, m, H-10), 1.82 (2H, m, H_2-8), 1.62 (2H, m, H_2-9), 1.47 (2H, m, H_2-11), 1.38 (2H, m, H_2-12), 1.35 (2H, m, H_2-13), 1.10 (2H, m, H_2-16), 0.99 (3H, d, *J* = 6.5 Hz, Me-18), 0.96 (3H, d, *J* = 6.3 Hz, Me-19), 0.93 (3H, d, *J* = 6.6 Hz, Me-20), 0.63 (3H, t, *J* = 6.5 Hz, Me-17). ^{13}C NMR (MeOD): δ 178.85 (C-1), 52.71 (C-2), 36.01 (C-3), 42.41 (C-4), 71.66 (C-5), 43.33 (C-6), 35.06 (C-7), 31.88 (C-8), 31.09 (C-9), 37.03 (C-10), 30.60 (C-11), 28.94 (C-12), 24.20 (C-13), 40.49 (C-14), 77.85 (C-15), 20.94 (C-16), 12.57 (C-17), 19.95 (C-18), 17.91 (C-19), 17.03 (C-20), 102.35 (C-1'), 78.99 (C-2'), 77.35 (C-3'), 75.12 (C-4'), 79.96 (C-5'), 62.79 (C-6'). ESI-MS m/z (rel. int.): 488 $[M]^+$ ($C_{26}H_{48}O_8$) (1.3), 389 (2.1), 221 (12.3), 99 (8.6).

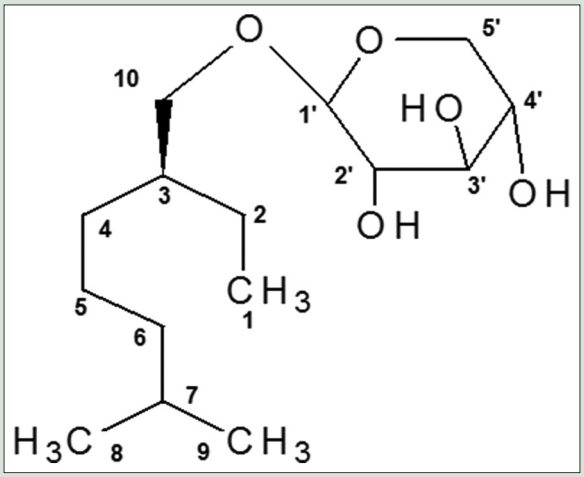

Figure 3: 10-Geranilanyl O-β-D-xyloside (AC2A)

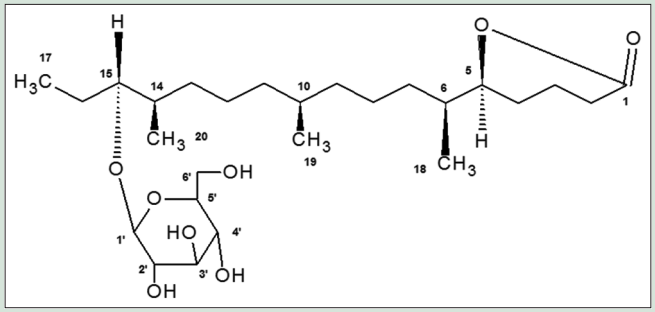

Figure 5: Vernoniaolideglucoside (CC14)

Glucuronolactone (CC3)

Colorless or white crystals or crystalline powder mass of Figure 6 (387 mg), which crystallized from the extract on standing. Melting point 173°C–175°C. IR V_{max} (KBr): 3325, 2916, 2850, 1721, 1643, 1549, 1457, 1369, 1019, 969, 887, 799 cm^{-1}; ^{1}H NMR (D_2O): δ 5.26 (1H, d, *J* = 7.3 Hz, H-1), 4.71 (1H, d, *J* = 7.1 Hz, H-3), 4.68 (1H, m, H-5), 3.84 (1H, m, H-2). ^{13}C NMR (D_2O): δ 103.24 (C-1), 62.11 (C-2), 72.08 (C-3), 166.84 (C-4), 74.29 (C-5), 204.27 (C-6). ESI-MS m/z (rel. int.): $[M]^+$ *m/z* 176 ($C_6H_80_6$).

In vitro antiplasmodial assay

The Results of the *in vitro* antiplasmodial assay is presented in Table 1.

DISCUSSION

Compound CC7 gave effervescences with sodium bicarbonate solution and showed IR absorption bands for the hydroxyl group (3410 cm^{-1}), carboxylic function (3253, 1702 cm^{-1}), and long aliphatic chain (723 cm^{-1}). Its mass spectrum displayed a molecular ion peak at m/z 258 corresponding to a molecular formula of saturated hydroxyl aliphatic carboxylic acid, $C_{15}H_{30}O_3$. Generation of a prominent ion peak at m/z 103 [C_4-C_5 fission, CH (OH)-$(CH_2)_2$-COOH]+ and at m/z 155 [M−103, CH_3-$(CH_2)_{10}$]$^+$ indicated the location of the hydroxyl group at C-4. The ^{1}H NMR spectrum of 1 exhibited a one-proton double doublet at δ 3.64 (*J* = 6.8, 6.4 Hz) assigned to β-oriented carbinol H-4 proton. A two-proton triplet at δ 2.34 (*J* = 7.2 Hz) was ascribed to methylene H_2-2 protons adjacent to the carboxylic group. The other methylene protons resonated as two-proton multiplets at δ 2.03, 1.63, and 1.32 and as a broad singlet at δ 1.28 (16H). A three-proton triplet at δ 0.87 (*J* = 6.4 Hz) was accounted to terminal C-15 primary methyl protons. The ^{13}C NMR spectrum of CC7 displayed signals for carboxylic carbon at δ 179.03 (C-1), carbinol carbon at δ 63.10 (C-4), and methylene carbons

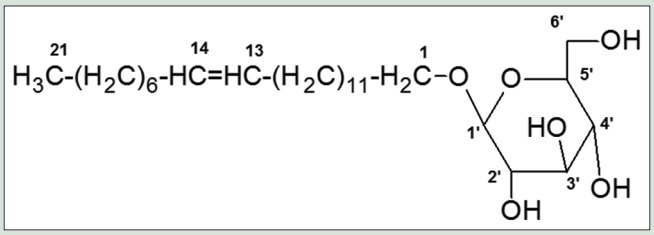

Figure 4: 1-Heneicosenol O-β-D-glucopyranoside (CC19)

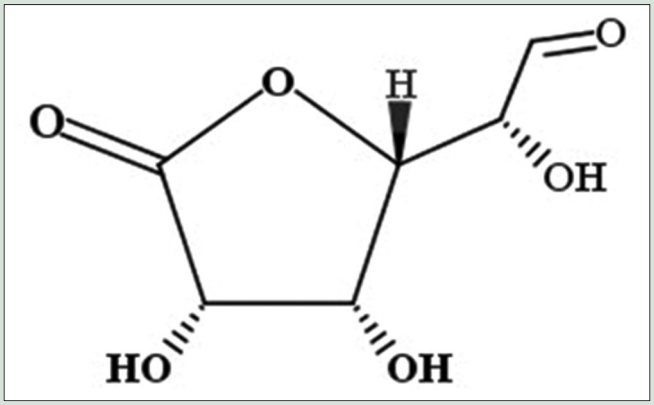

Figure 6: Glucuronolactone

between δ 33.88–22.68 and methyl carbon at δ 14.10 (C-15). The absence of any signal beyond δ 3.64 in the ^{1}H NMR and between δ 179.03–63.10 in the ^{13}C NMR spectra supported the saturated nature of the molecule. On the basis of these evidence, the structure of 1 was elucidated as 4α-hydroxy-*n*-pentadecanoic acid, a new alkyl carboxylic acid.

Compound CC10 responded to Liebermann–Burchard test positively for triterpenoids and exhibited characteristic absorption bands for hydroxyl group (3518 cm^{-1}), lactone ring (1767 cm^{-1}), carboxylic function (3218, 1692 cm^{-1}), and unsaturation (1643 cm^{-1}). The mass spectrum displayed characteristic ion fragments at m/z218 and 264 generated due to retro-Diels–Alder fragmentation suggesting a vinylic linkage at C-5 in ring B another vinylic bond at C-12 in ring C,[12] and ion fragments appearing at m/z 452 $[M-2 \times Me]^{+}$, 203 $[218-Me]^{+}$, and 219 $[264-COOH]^{+}$ supported the existence of the carboxylic group in the ring D/E. The ^{1}H NMR spectrum of CC10 displayed two one-proton downfield signals as a doublet at δ 5.63 (*J* = 6.0 Hz, H-12) and as a multiplet at δ 5.40 assigned to vinylic H-12 and H-6 protons, respectively, two one-proton double doublets at δ 4.80 (*J* = 3.2, 4.4 Hz) and 4.57 (*J* = 4.0, 4.4 Hz) ascribed correspondingly β-oriented carbinol H-3 and H-11 protons, methylene and methine protons in the range from δ 2.83–1.48, four three-proton singlets at δ 1.25 (Me-23), 1.11 (Me-26), 1.10 (Me-27) and 0.59 (Me-24), and two three-proton doublets at δ 1.08 (*J* = 9.6 Hz, Me-29) and 0.96 (*J* = 6.8 Hz, Me-30) attributed to methyl protons of ursene-type compound, all located on the saturated carbons. The ^{13}C NMR spectrum of two displayed signals for thirty carbons including for lactone carbon at δ 173.21 (C-25), carboxylic carbon at δ 178.52 (C-28), vinylic carbons atδ 144.08 (C-5), 120.14 (C-6), 122.37 (C-12), and 139.71 (C-13), oxymethine carbon at δ 78.89 (C-3), carbinol carbon at δ 76.11 (C-11), and methyl carbons resonated from δ 29.15–15.73. The assignments of the carbon chemical shift were made by comparison with δ values of the corresponding carbons of urs-12-enes.[12-14] On the basis of the above discussion and literature values, the structure of CC10 was elucidated as 11α-Hydroxyurs-5,12-dien-28-oic acid-3α, 25-olide, a new ursenoic acid olide.

Compound AC2A named 10-geranilanoyl O-β-D-xyloside, gave positive tests for glycosides and showed IR absorption bands for hydroxyl groups (3295 cm^{-1}). Its molecular ion peak was determined at m/z 290 on the basis of mass and ^{13}C NMR spectral data consistent with a molecular formula of a monoterpenic pentoside, $C_{15}H_{30}O_5$. The ion peaks generating at m/z 157 [O-C1' fission, $C_{10}H_{21}O]^{+}$ and 149 [C10– O fission, $C_5H_9O_5]^{+}$ indicated that an acyclic monoterpenic unit was linked with a pentose moiety. The ^{1}H NMR spectrum of AC2A exhibited a one-proton deshielded doublet at δ 5.44 (*J* = 8.6 Hz) ascribed to anomeric H-1' proton, other sugar protons between δ 4.41–3.32, a two-proton doublet at δ 3.16 (*J* = 8.4 Hz) due to oxymethylene H2-10 protons, two three-proton doublets at δ 0.95 (*J* = 6.6 Hz), 0.93 (*J* = 6.4 Hz) attributed correspondingly to secondary C-8 and C-9 methyl protons, a three-proton triplet at δ 0.61 (*J* = 6.5 Hz, Me-1) accounted to primary C-1 methyl protons and the remaining methine and methylene protons between δ 2.24 and 1.03. The ^{13}C NMR spectrum of AC2A displayed signals for an anomeric carbon at δ 103.34 (C-1'), other sugar carbons between δ 78.08 and 71.67, a oxymethylene carbon at δ 62.79 (C-10), methyl carbons at δ 12.55 (C-1), 19.91 (C-8) and 19.94 (C-9) and the remaining methylene and methine carbons from δ 43.37 to 30.61. The absence of any carbon signal beyond δ 102.34 in the ^{13}C NMR spectrum supported saturated nature of the molecule. Acid hydrolysis of AC2A yielded D-xylose, R_f 0.2 (n-butanol: acetic acid: water; 4:1:5, top). On the basis of above discussion, the structure of AC2A was elucidated as geranilan-10-olyl-10-O-β-D-xylop yranoside, a new acyclic monoterpenic xyloside.

Compound CC19 showed positive tests for glycosides and IR absorption bands for hydroxyl groups (3426, 3331, 3218 cm^{-1}), unsaturation (1616 cm^{-1}), and long aliphatic chain (725 cm^{-1}). On the basis of mass and ^{13}C NMR spectra, the molecular ion peak of CC19 was deduced at m/z 472 corresponding to a molecular formula of an alkyl alcohol, $C_{27}H_{52}O_6$. The generation of ion fragment peaks at m/z 163 $[C_6H_{11}O_5]^{+}$, 293 [C_1– O fission, $C_{21}H_{41}]^{+}$, 125 [C_{12}–C_{13} fission, $C_9H_{17}]^{+}$, and 99 [C_{14}–C_{15} fission, $C_7H_{15}]^{+}$ indicated the presence of a hexose unit linked to and a C_{21}-aliphatic alcohol heneicosenol with a double bond at C-13. The ^{1}H NMR spectrum of CC19 displayed two one-proton multiplets at δ 5.39 and 5.34 with half-width of 8.2 Hz and 8.6 Hz, respectively, ascribed correspondingly to cis-oriented vinylic H-13 and H-14 protons. A two-proton multiplet at δ 3.63 was accounted to oxymethylene H_2-1 protons. The other methylene protons appeared as two-proton multiplets at δ 2.17, 2.03, 1.67, and 1.50 and as a broad singlet at δ 1.28 (26H). A three-proton triplet at δ 0.88 (*J* = 6.5 Hz) was assigned to C-21 primary methyl protons. A one-proton doublet at δ 4.85 (*J* = 7.2 Hz) was accounted to anomeric H-1' proton. The other sugar oxymethine protons resonated as one-proton multiplets at δ 4.13 (H-5'), 3.92 (H-2'), 3.79 (H-3'), and 3.77 (H-4') and hydroxymethylene protons as a two-proton multiplet at δ 3.30 (H_2-6'). The ^{13}C NMR spectrum of CC19 exhibited important signals for vinylic carbons at δ 132.11 (C-13) and 123.58 (C-12), oxymethylene carbon at δ 63.25 (C-1), other methylene carbons between δ 57.44 and 22.68, methyl carbon at δ 14.17 (C-21), anomeric carbon at δ 103.37 (C-1'), and the remaining sugar carbons between δ 73.18 and 60.11. Acid hydrolysis of CC-19 yielded D-glucose, R_f 0.26 (*n*-butanol-acetic acid-water, 4:1:5). On the basis of above discussion, the structure of CC19 was determined as (*Z*)-heneicos-13-en-1-ol O-β-D-glucopyranoside, a new alkyl glucoside.

Compound CC14, named **v**ernoniaolide glucoside, responded positive tests for glycosides and showed IR absorption bands for hydroxyl functions (3445 cm^{-1}) and lactone ring (1765 cm^{-1}). Its molecular ion peak was determined at m/z 488 on the basis of mass and ^{13}C NMR spectra corresponding to a molecular formula of an acyclic diterpenic glycoside $C_{26}H_{48}O_8$. The ions peaks arising at m/z 99 [C_5–C_6 fission, $C_5H_7O_2]^{+}$ and m/z 389 $[M-99]^{+}$ indicated that a δ-lactone ring was present at one of the end of the diterpenic glycoside. The generation of a prominent ion fragment at m/z 221 [C_{14}–C_{15} fission, $CH_3CH_2CH\text{-}O\text{-}C_6H_{11}O_5]^{+}$ indicated the existence of the glycosidic unit at C-15 carbon. The ^{1}H NMR spectrum of CC14 exhibited a one-proton doublet at δ 5.41 (*J* = 7.3 Hz) ascribed to anomeric H-1' proton, other sugar proton as one-proton multiplets at δ 4.70 (H-5'), 3.87 (H-2'), 3.65 (H-3'), and 3.31 (H-4') and as one-proton doublets at δ 3.17 (*J* = 8.0 Hz, H_2-6'a) and 3.13 (*J* = 8.0 Hz, H_2-6'b), two one-proton broad multiplets at δ 4.38 with half-width of 14.3 Hz, and at δ 3.38 with half-width of 8.6 Hz were accounted to oxymethine H-5α and H-15β protons, respectively, three doublets at δ 0.99 (*J* = 6.5 Hz), 0.96 (*J* = 6.3 Hz), and 0.93 (*J* = 6.6 Hz) and triplet at δ 0.63 (*J* = 6.5 Hz) integrating for three protons each were associated correspondingly with secondary C-18, C-19, and C-20 and primary C-17 methyl protons, all attached to saturated carbons. The remaining methine and methylene protons resonated as multiplets from δ 2.81 to 1.10 and as a two-proton triplet at δ 2.21 (*J* = 8.0 Hz) due to methylene H_2-2 adjacent to the lactonic carbon C-1. The ^{13}C NMR spectrum of CC14 displayed signals for lactone carbon at δ 178.85 (C-1), anomeric carbon at δ 102.35 (C-1'), other sugar carbons between δ 79.96 and 62.79, oxymethine carbons at δ 71.66 (C-5) and 77.85 (C-15), methyl carbons at δ 12.57 (C-17), 19.95 (C-18), 17.91 (C-19), and 17.03 (C-20), and the methine and methylene carbons in the range of δ 52.71–20.94. Acid hydrolysis of CC14 yielded D-glucose, R_f 0.26 (*n*-butanol-acetic acid-water, 4: 1: 5). On the basis of these evidence, the structure of CC14was established as 6β,10β,14β-trimethyl heptadecan-15α-olyl-15-O-β-D-glucopyrano syl-1,5 β-olide, a new acyclic diterpenic glucosidic lactone.

Table 1: Antimalaraial effects of the isolates

Compounds	Percentage of schizont inhibition (control-treated/control×100)				IC_{50} (µg/ml)
	3D7		Dd2		
	5 (µg/ml)	50 (µg/ml)	5 (µg/ml)	50 (µg/ml)	
CC10	0	17.6	0	7.5	-
CC19	48	97.7	5	98	10.55
AC2A	29	96.3	0	100	11.68
CC14	20	100	0	100	12.56
CC3	0	41	0	2.5	-

Each of the percentage results presented was calculated from the average of triplicate number of life schizont. CC10: 11α- Hydroxyurs-5,12-dien- 28- oic acid-3α, 25-olide; CC19: 1-Heneicosenol O-β-D-glucopyranoside; AC2A: 10-Geranilanyl O-β-D-xyloside; CC14: 6β,10β,14β-Trimethyl heptadecan-15α-olyl-15-O-β-D-gluco pyranosyl-1,5β-olide; CC3: Glucuronolactone; 3D7: Chloroquine sensitive; Dd2: Chloroquine resistant; IC_{50}: Half maximal inhibitory concentration

Compound CC3 was obtained as white crystalline mass from the extract during storage, prior to isolation. Its IR spectrum displayed important signals for hydroxyl (3325 cm^{-1}) and carbonyl (1721, 1643 cm^{-1}) functionalities. On the basis of ^{13}C NMR and mass spectra, the molecular weight of CC3 was established at 176 corresponding to the molecular formula $C_6H_8O_6$. The formula indicated the presence of three double-bond equivalents, two of which were adjusted in two carbonyl functionalities and one in a ring structure. ^{13}C NMR spectrum of CC3 exhibited signals for six carbons consisting of a carbonyl carbon at δ204.27 (C-6); a lactone carbon δ166.84 (C-1) and four oxygenated carbons at δ72.11 (C-2), 81.08 (C-3), 78.61 (C-4), and 74.29 (C-5). The ^1H NMR spectrum of CC3 displayed two one-proton doublets at δ5.26 (1H, d, J = 7.3 Hz) and 4.71 (1H, d, J = 7.1 Hz) were assigned to H-5and H-2, respectively. Two one-proton multiplets at δ4.68 and 3.84 were ascribed correspondingly to H-4 and H-3. Based on above discussion, the compound CC3 was identified to be glucuronolactone.

The results presented in Table 1 show the activity of the evaluated isolated compounds against chloroquine-sensitive and chloroquine-resistant strains of malaria parasite. As can be observed, the compounds (CC10 and CC3), recorded no inhibition for both the chloroquine-sensitive and chloroquine-resistant strains at a lower concentration and hence were excluded in the determination of IC_{50}. Compound CC14, CC19, and AC2A were evaluated for IC_{50} using chloroquine-sensitive strain.

When evaluating the activities of different compounds against a particular disease condition *in vitro*, the compound that produces more effect at a much lower concentration is considered to be more effective. This means that a more active compound should produce more effect at the lowest concentration tested. Taking this assumption to Table 1, we can say that the isolated compound CC19 is the most active. Further, the IC_{50} of compounds AC2A, CC19, and CC14 were determined, and the results presented in Table 1. The compound CC19 having the least IC_{50} value, makes it relatively most effective of the entire isolated compounds. However, according to the World Health Organization protocol for *in vitro* antimalarial screening model, if a compound has IC_{50} value >5 µg/ml, the compound is classified as inactive. If the IC_{50} value is between 0.5 and 5 µg/ml, the compound is classified as moderately active. If the IC_{50} is <0.5 µg/ml, the compound is classified as active and further evaluated using other strains. IC_{50} which stands for half-maximal inhibitory concentration is the estimation or estimated effectiveness of a drug molecule in hindering a particular biological or biochemical activity.

The significance of the result obtained is that since our previous research on the methanolic stem-bark extract of *Vernonia amygdalina* against chloroquine-resistant strain of malaria parasite recorded positive result, the result can be attributed to the presence of these isolated compounds, which could have acted either additively or synergistically.

CONCLUSION

Even though six compounds were isolated, five of the compounds which showed different levels of antimalarial activity (schizont inhibition), more work needs to be done toward the validation of the isolation process, *in silico* structural modifications of these structures for better activity, and study to check for possible synergistic effects among these compounds and other possible pharmacological effects. The study also validates the use of this plant in the treatment of malaria in Nigeria traditional medicinal system.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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