FULL-LENGTH ARTICLE

Tetracyclic Triterpenes from the Stem Bark of *Vernonia biafrae* and Evaluation of its Antimicrobial Activity

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ABSTRACT
In the search for bioactive metabolites from Ethiopian medicinal plants, two tetracyclic triterpenes; spinasterol (**1**) and shionone (**2**) along with cetyl alcohol (**3**) were isolated from the acetone extract of the stem bark of *Vernonia biafrae*, a medicinal plants widely used for the treatment of infectious diseases. Column chromatographic separations and Sephadex LH-20 gel filtration were employed for the isolation of the pure compounds. The compounds were characterized by spectrometric (NMR and mass) analyses and comparison with literature data. This is the first report of compound **2** and **3** from the genus *Vernonia*. The extracts and the isolated compounds were evaluated for their antimicrobial activities against both bacterial and fungal test strains and exhibited comparable activities with the standard antibiotics. The highest activity was observed for acetone extract (23.73± 0.10 mm) and compound **2** (23.72± 0.30 mm) against *Staphylococcus aureus.* Whereas, the rest showed moderate to low inhibitory activities against the test strains. The finding could be used for comprehensive evaluations of the phytochemicals for their microbial activities and also support the claim that the plant *V. biafrae* is used for the treatment of microbial diseases.

Keywords: Antimicrobial; Cetyl alcohol; Medicinal plan; Shionone; Spinasterol; *Vernonia biafrae*

INTRODUCTION

Vernonia biafrae is a medicinal plant belonging to Asteraceae family, which consists of more than 1000 species widely distributed in tropical and sub-tropical Africa, South America and South-East Asia (Hua et al., 2012; Turak et al., 2015). The family is highly diversified in Ethiopia with 133 genera and 472 species and subspecies of which 103 are endemic to Ethiopia (Kelbessa and Demissew, 2014). While the family has a wide distribution in Ethiopia, *V. biafrae* is well documented from Illubabor and Sidamo Floristic Regions of Ethiopia. The species in the family have been known for the treatment of different ailments as they have an antibacterial, anti-fungal, anti-diabetic, anthelmintic, anti-inflammatory, antitumor and venereal disease (Buskuhl et al., 2010; Hua et al., 2012; Aliyu et al., 2015; Alara et al., 2018), insecticidal (Stevensona et al., 2017) and anti-plasmodia (Toyang et al., 2013) activities. Preliminary phytochemical investigation and isolation of different species of *Vernonia* revealed the presence of terpenoids, steroids, flavonoids, alkaloids, phenols, saponins, glycosides, tannins, steroidal glycosides, triterpenoids, sesquiterpene lactones and other classes of compounds, with a wide range of biological activities (Kuo et al., 2003; Anthoney et al., 2013; Albejo et al., 2015; Alara et al., 2017).

Even though *V. biafrae* is widely distributed throughout Ethiopia (Turak et al., 2015) and commonly visited by traditional healers for the treatments of different ailments including microbial infections, the phytochemical information and biological activities pertaining to this plant have not been addressed. Therefore, this study was initiated to isolate, characterize, and evaluate the bioactivity of the crude extracts and pure compounds, isolated from the plant under investigation.

MATERIALS AND METHODS

General Information

Chemicals such as petroleum ether, chloroform, acetone, methanol and ethyl acetate were used for extraction and column elution; Silica gel (60-120 mm size (Mark, Darmstadt, Germany) and Sephadex LH-20 (Mark, Darmstadt, Germany) were used for column chromatography. Analytical thin-layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} plate (Mark, Darmstadt, Germany). Iodine vapour was used for detection of spots on TLC. Dimethyl sulfoxide (DMSO), Mueller Hinton agar and Nutrient broth were used as growth media; while standard antibiotics including chloramphenicol and ketoconazole were used as positive controls for bacterial and fungal cultures, respectively. All the chemicals and reagents were of analytical grade. Rotary evaporator (Heidolph, USA) was used to concentrate the extracts. Visualization of the spots was performed under UV/Vis 254 & 365 nm detector cabinet. NMR spectra were recorded on an Avance 500 MHz spectrometer (Bruker, Billerica, MA, USA) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C at 298 K using the residual solvent peaks as a reference.

Plant Material collection

The stem bark of *V. biafrae* was collected from Jiren Kebele (geographical coordinate: 7°40′33.7″N 36°50′3.4″ E and altitude of 1779 meters above sea level), Jimma Zone, Oromia Regional State in July 2019. Identification of the plant species was done using botanical keys as indicated in Volume 4 Part 2 of Flora of Ethiopia and Eritrea (Tadesse, 2004) and a voucher specimen (CH1-JUH) has been deposited at Jimma University Herbarium.

Extraction and Isolation

The air-dried and powdered stem bark of *V. biafrae* (1 Kg) was sequentially extracted with petroleum ether, chloroform, acetone and methanol three times each for 24 h at room temperature. The extracts were then concentrated under reduced pressure using a rotary evaporator.

About 10 g of acetone extract was adsorbed with silica gel and subjected to column chromatography packed with silica gel (105 g) following its better antibacterial activity (as indicated below in result discussion section) and a number of spots on TLC profile. The column was eluted with petroleum ether containing an increasing amount of ethyl acetate to give 310 fractions, each of *ca*. 30 mL. The fractions 50-65, collected with 2% ethyl acetate in petroleum ether was combined due to similarity in TLC profile, which was then further purified by Sephadex LH-20 (eluting with CH_2Cl_2/MeOH; 1:1) to give compound **3** (10 mg). Similarly, the late fractions (79-86) from 7% ethyl acetate in petroleum ether gave a white powdered compound **2** (7.5 mg), while fractions 90-112 (12% EtOAc in petroleum ether) contained a mixture of compounds which was further purified by column chromatography on silica gel (eluent: increasing gradient of EtOAc in petroleum ether) followed by further purification using Sephadex LH-20 (eluting with CH_2Cl_2/MeOH; 1:1) that gave compound **1** (12 mg).

Antimicrobial activity test

Test strains

For this research, four standard bacteria strains and two standard fungi strains were used for the evaluation of antimicrobial activities. These microbial strains are among the human pathogenic bacteria and fungi strains (Girish and Satish, 2008; Rahman et al., 2015). Two of the strains were Gram-positive, namely *Staphylococcus aureus* (ATTC 25923) and *Bacillus subtilis* (ATTC 6633), while the other two were Gram-negative including *Escherichia coli* (ATCC 25922) and *Salmonella typhi* (ATTC 14028), whereas the fungi strains were *Candida spp* and *Fusarium spp*. The bacteria strains were obtained from microbiology laboratory, Department of Biology, Jimma University and the fungi strains were from Medical Microbiology Research Laboratory, Jimma University, Specialized Hospital. The bacteria and fungi strains were reactivated by sub-culturing on nutrient broth at 37 °C and maintained on nutrient agar slant at 4 °C for further use.

Antimicrobial Evaluation (Disk diffusion assay)

The extract and isolated compounds were tested for antibacterial and antifungal activities by the disk diffusion method according to the procedure of the Clinical and Laboratory Standards Institute (Patel et

al., 2015). The standard inocula were prepared as 0.5 McFarland turbidity standard and the standardized culture suspension spread on Muller-Hinton agar following the disc diffusion method according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) (NCCLS, 2002). The filter paper discs (6 mm in diameter), was individually impregnated with 25 µL of the crude extract and pure compounds at a final stock solution concentration of 40 mg/mL and 30 µg/mL, respectively in DMSO were placed on the agar plates previously inoculated with test microorganisms. Similarly, each plate carried a blank disc by adding DMSO solvent alone to serve as a negative control and antibiotic discs (6 mm in diameter) of 30 µg/mL of chloramphenicol (Sigma, Germany; for bacteria), and 30 µg/mL of Ketoconazole (Sigma, Germany; for fungi), the most commonly used antimicrobial drugs were used as positive controls. All the plates were incubated at 37 ℃ for 24 h for bacteria and 30 ℃ for 48 h for fungi. The diameters of the inhibition zones were measured in millimetres. The sensitivity of the microorganisms to the extract and compounds were determined by measuring the size of the zone of inhibition (mm) on the agar surface around the discs. All the tests were performed in triplicate.

RESULTS AND DISCUSSION

Isolation of pure compounds

Phytochemical investigation of acetone extract of stem barks of *V. biafrae* gave three compounds; two tetracyclic triterpenoids named Spinasterol (**1**) and shionone (**2**), and aliphatic alcohol, cetyl alcohol (**3**) (Fig. 1).

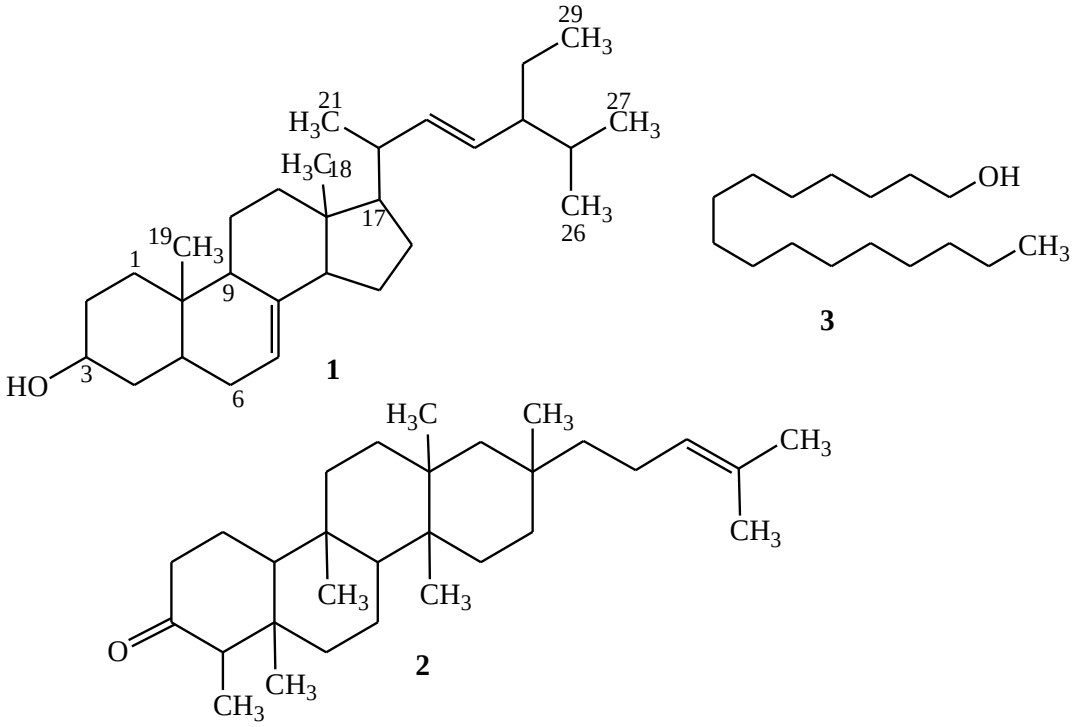


Figure 1: Compounds isolated from the stem bark of *V. biafrae*

Compound **1** was isolated as a white powder from 12% of ethyl acetate in petroleum ether fractions. The ESI-MS ion peak at *m/z* 413 for $[M+H]^+$ along with the NMR data corresponds to the molecular formula $C_{29}H_{48}O$, indicating six indices of hydrogen deficiency. The ^1H NMR spectrum (Table 1) showed the presence of three olefinic protons resonated at δ_H 5.16 (1H, *dd*, *J* = 12.1, 6.0 Hz), 5.21 (1H, *dd*, *J* = 10.8, 5.2 Hz, and 5.04 (1H, *dd*, *J* = 15.2, 8.5 Hz) with the latter two are *trans* configured (deduced from the vicinal coupling constant) and were assigned to H-7, H-22 and H-23, respectively.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz,) NMR data of compounds**1** and **2** (in $CDCl_3$)

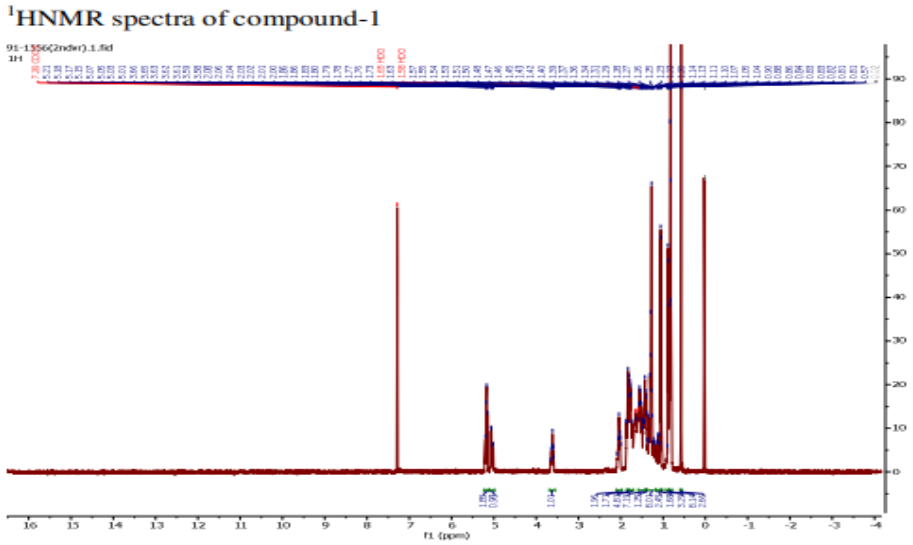
No	Compound 1		Compound 2	
	δ_H (m, J in Hz)	δ_C	δ_H (m, J in Hz)	δ_C
1	1.04, 1.76 (2H, *m*)	37.2	1.98, 1.73 (2H, *m*)	22.3
2	1.36, 1.73 (2H, *m*)	31.5	2.39, 2.40 (2H, *m*)	41.6
3	3.61 (1H, *td*, 10.8, 5.2 Hz)	71.1	-	213.1
4	1.29, 1.40 1.52 (2H, *m*)	38.1	2.26 (1H, *d*, 6.5)	58.2
5	1.40 (1H, *m*)	40.3	-	42.3
6	1.25, 1.53 (2H, *m*)	29.7	1.24, 1.73 (2H, *m*)	41.3
7	5.16 (1H, *dd*, 12.1, 6.0 Hz)	117.5	1.35 (2H, *m*)	18.1
8	-	139.6	1.32 (1H, *dd*, 10,10)	50.1
9	1.63 (1H, *m*)	49.5		38.5
10	-	34.8	1.59 (1H, *m*)	59.6
11	1.47 (2H, *m*)	21.6	1.36 (2H, *m*)	35.4
12	1.26, 1.83 (2H, *m*)	39.5	0.92, 1.60 (2H, *m*)	32.5
13	-	43.3	-	37.0
14	1.77 (1H, *m*)	55.2	-	38.8
15	1.39, 1.40 (2H, *m*)	23.0	1.34, 1.27 (2H, *m*)	29.4
16	1.26, 1.73 (2H, *m*)	28.5	1.36, 1.59 (2H, *m*)	34.8
17	0.81 (1H, *m*)	55.9	-	31.9
18	0.57 (3H, s)	12.2	1.22, 1.15 (2H, *m*)	44.6
19	0.81 (3H, *s)*	13.1	1.18, 1.74 (2H, *m*)	43.7
20	0.90 (1H, *m*)	40.8	1.77, 2.00 (2H, *m*)	23.4
21	1.05(3H, *d*, 6.6)	21.4	5.12 (1H, *dd*, 7.5, 5)	125.4
22	5.21 (1H, *dd*, 15.2, 5.2)	138.2	-	130.9
23	5.04 (1H, *dd*, 15.2, 8.5)	129.5	0.88 (3H, *d*, 10)	7.0
24	1.53 (3H, *s*)	51.2	0.73 (3H, *s*)	14.7
25	1.42 (1H, *s*)	31.9	0.94 (3H, *s*)	19.6
26	0.86 (3H, *d*, 6.5)	21.1	0.90 (3H, *s*)	15.3
27	0.84 (3H, *d*, 6.5)	19.0	1.15 (3H, *s*)	20.6
28	1.04 , 1.39 (2H, *m*)	25.4	0.90 (3H, *s*)	33.0
29	0.82 (3H, *t*, 6)	12.1	1.71 (3H, *s*)	25.9
30			1.60 (3H, *s*)	17.9

It also exhibited oxygenated proton signals at δ_H 3.61 (1H, *td*, *J* = 10.8, 5.2 Hz, H-3) and six set of protons signals at δ_H of 0.57 (3H, *s*, H-18), 0.81(3H, s, H-19), 1.05 (3H, *d*, J = 6.6 Hz, H-21), 0.86 (3H, *d*, *J* = 6.5 Hz, H-26), 0.84 (3H, *d*, *J* = 6.5 Hz, H-27) and 0.82 (3H, *t*, *J* = 6.1 Hz, H-29) for six methyl groups. These and the ^{13}C NMR spectral data (Table1), which showed the presence of olefinic carbons resonated δ_C117.5 (C-7), 138.1 (C-22) and 129.5 (C-23) with the hydroxyl bearing sp^3 hybridized carbon at 71.1 (C-3) were observed. In addition, the six methyl carbons resonated at 12.2 (C-18), 13.3 (C-19), 21.4 (C-21), 21.1 (C-26), 19.0 (C-27) and 12.1 (C-29) is in agreement with the structure of α-spinasterol (Ragasa and Lim, 2005; Lee et al., 2014; Meneses-Sagrero et al., 2017; Khan et al., 2019). α-Spinasterol is a stigmastane-type phytosterol found in a variety of plants(Mozirandi et al., 2019), which was reported to have antiproliferative effect in the cervical cancer cell line HeLa and murine macrophage cancer cell line RAW 264.7.

The second compound (**2**) was isolated as a white solid from 7% ethyl acetate in petroleum ether fractions. Its ESI-MS data revealed a peak for a sodium adduct ion at m/z 449 $[M+Na]^+$ and a dehydrated ion at 408 $[M-H_2O]^+$, both corresponding to a molecular formula of $C_{30}H_{50}O$. The ^{1}HNMR (Table 1) showed one olefin proton resonated at δ_H 5.12 (1H, *dd*, *J* = 7.5, 5 Hz, H-21) and eight methyl protons resonated at δ_H 0.88 (3H, *d*, *J* = 10 Hz, H-23), 0.73 (3H, s, H-24), 0.94 (3H, s, H-25), 0.90 (3H, s, H-26), 1.15 (3H, s, H-27), 0.90 (3H, s, H-28), 1.71 (3H, s, H-29), and 1.60 (3H, s, H-30). The ^{13}C NMR (Table 1) showed the presence of ketone functional group revealed from a signal at δ_C 213.1 (C-3) and two olefinic carbons at 125.4 (C-21) and 130.0 (C-22). Based on these and including the 2D NMR spectral

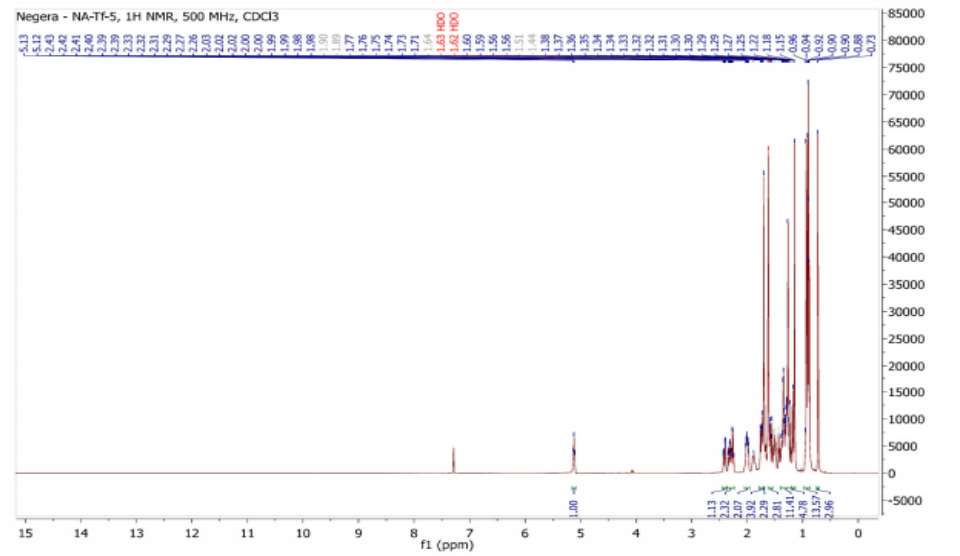
data and comparison with the related literature(Akihisa et al., 1998; Zhou et al., 2010; Wang et al., 2012; Zhou et al., 2013), the structure of compound **2** was concluded to be shionone. This is the first report of the isolation of rare tetracyclic skeleton with oxidized a C-3, shionone in the genus *Vernonia.*

Cetyl alcohol (**3**) was isolated as a white solid compound from 2% ethyl acetate in petroleum ether fractions. The ^{1}H NMR ($CDCl_3$, 400MHz) showed proton signals δ_H 3.66 (2H, *t*, *J* = 6.1 Hz, H-1) and 5.37 (1H, *bs*, OH, H-1), 2.79 (2H, *m*, H-2), 0.81 (3H, *t*, H-16) and other protons resonated from 2.38-0.81(26H, *m*) for a long aliphatic chain. The ^{13}C NMR ($CDCl_3$, 100Hz) showed carbon signals for sixteen carbon atoms including the OH bearing carbons at δ_H 63.1 (C-1) and for fourteen methylene carbons at δ_C 34.0 (C-2), 32.8 (C-3), 31.9 (C-4), 29.7 (C-5 and C-6), 29.6 (C-7), 29.4 (C-8 and C-9), 29.3 (C-10), 29.0 (C-11), 27.2 (C-12), 25.7 (C-13), 24.7 (C-14 and C-15) and 14.1(C-16). From these spectroscopic data, it is evident that the compound is aliphatic alcohol with sixteen carbon chain (cetyl alcohol). Cetyl alcohol has been reported from *Whale oil* (Gupta et al., 2012) and has good pharmaceutical and biological properties (Gupta et al., 2012). The occurrence of cetyl alcohol is reported for the first time from the Asteraceae family (*Vernonia*). The NMR spectra data of the isolated compounds are given by Fig. 2 (A-C).



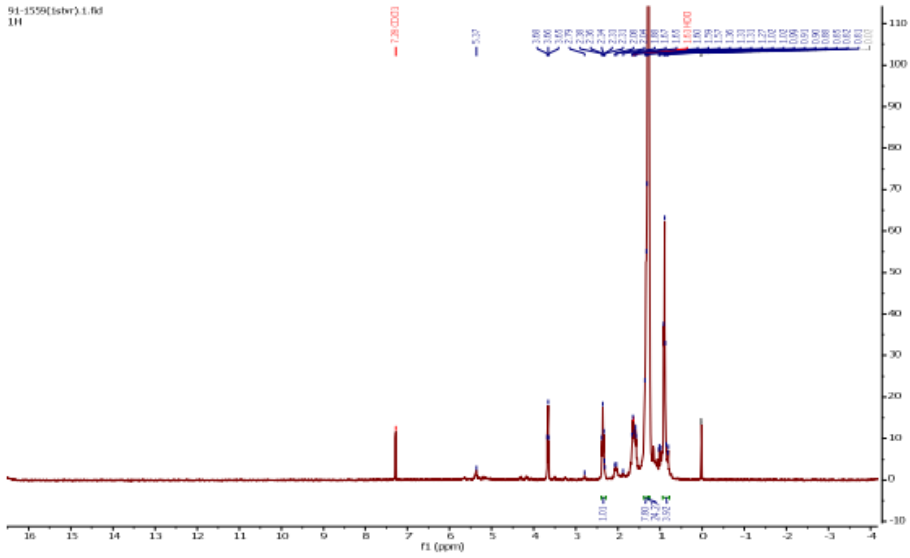
(**A**)

^{1}HNMR spectra of compound-2



(B)

The ^{1}HNMR Spectrum of compound-3



(C)

Fig. 2. ^{1}H NMR spectra data of compound-1(**A**), compound-2(**B**), and compound-3(**C**).

Evaluation of antimicrobial activity

The activities of the extracts and the isolated compounds were comparatively assessed by the diameter of the zone of inhibition in millimetres and zones of inhibition more than 6 mm were taken into consideration. The extracts and pure isolates demonstrated positive results for both bacterial and fungal

strains (Table 2) as compared to standard drugs; chloramphenicol for bacteria strain and ketoconazole for fungi strains.

Table 2. Zone of growth inhibition (mm) of crude extract and isolated compounds.

Samples	Bacteria strains				Fungi strains	
	S. aureus	*S. typhi*	*E. coli*	*B. subtilis*	*Candida spp*	*Fusarium spp*
PE	21.39 ± 0.15	21.90 ± 0.15	21.51 ± 0.15	19.11 ± 0.25	21.69 ± 0.21	20.4 ± 0.10
CE	18.81 ± 0.25	20.19 ± 0.15	18.90 ± 0.20	18.09 ± 0.21	21.51 ± 0.06	18.81 ± 0.15
AE	23.73 ± 0.10	21.69 ± 0.30	21.90 ± 0.10	19.11 ± 0.15	21.39 ± 0.15	21.51 ± 0.21
ME	22.20 ± 0.20	19.41 ± 0.21	19.29 ± 0.31	19.80 ± 0.20	19.11 ± 0.30	18.69 ± 0.15
1	20.01 ± 0.06	21.99 ± 0.30	20.61 ± 0.21	20.91 ± 0.21	21.81 ± 0.30	18.30 ± 0.00
2	23.72 ± 0.30	22.69 ± 0.21	22.59 ± 0.11	21.90 ± 0.20	22.79 ± 0.15	21.60 ± 0.10
3	23.03 ± 0.15	23.03 ± 0.23	23.40 ± 0.20	22.71 ± 0.25	21.78 ± 0.21	22.50 ± 0.10
Chl	25.71 ± 0.25	29.43 ± 0.00	28.11 ± 0.25	26.58 ± 0.20	-	-
Ket	-	-	-	-	27.00 ± 0.20	26.58 ± 0.15
DMSO	-	-	-	-	-	-

Where, ´´-´´: Not active, PE: Petroleum ether extract, CE: Chloroform extract; AE: Acetone extract; ME: Methanol extract; Chl: Chloramphenicol; positive control for bacteria; Ket: Ketoconazole; positive control for Fungi strains, DMSO, Dimethyl sulfoxide: Negative control. Each group represents the mean ± SD for both bacterial and fungal strains.

The extracts showed good inhibitory activities against the tested bacterial strains, with the highest activity being observed for acetone extract (AE) with a zone of growth inhibition of about 23.73 ± 0.10 mm against *S. aureus*. Whereas compound **2** demonstrated the highest growth inhibition among the isolated compounds with the highest activity (23.72± 0.30) observed against *S. aureus* and (22.79± 0.10) against *Candida spp*. It would be worth to point out that gram-positive bacterial strains were more susceptible than that of gram-negative. This trend was the case also in fungal strains; where *candida spp* was more susceptible than that of *Fusarium spp.* It worth pointing out that this is the first report on antimicrobial activities of *V. biafrae* crude extracts and isolated compounds. The positive results of extracts and pure isolates to all bacteria and fungi strains confirmed the claims on the traditional medicinal values of *V. biafrae*.

CONCLUSION

Phytochemical investigation of acetone extract of stem barks of *V. biafrae* gave three compounds; spinasterol (**1**), shionone (**2**) and cetyl alcohol (**3**). The occurrences of **2** and **3** were reported for the first time from the genus *Vernonia*. The crude extracts (PE, HE, AE and ME) and the three pure compounds (**1**, **2** and **3)** showed comparable activity against all tested bacterial and fungal strains and the values are close to that of the reference drugs. Thus, the observed antibacterial activities of the extracts and pure compounds supported the claims on the use of the plant in traditional medicine and could give insight about the potential of traditional medicinal plants as good sources for lead compounds in the development of antibacterial drugs.

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