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GC-MS Analysis and Antibacterial Effects of *Vernonia glaberrima* n-Hexane Extracts alone and in Combination with Standard Antibiotics

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ABSTRACT:
The occurrence of drug resistant bacteria warrant investigation on herbal plants for effective antibacterial agents. *Vernonia glaberrima* leaf (VGL) and stem (VGS) hexane extracts were subjected to analysis by gas chromatography-mass spectrometry (GC-MS) and subsequently evaluated for antibacterial activity alone and in combination each with Sparfloxacin (SPX) and Ciprofloxacin (CPX) on selected bacteria including resistant species. GC-MS analysis revealed fatty acid esters, triterpenoids and aromatic derivatives largely identified as responsible for the broad-spectrum antibacterial activity. Both the VGL and VGS demonstrated potent antibacterial activities on *P. aeruginosa* (29 mm and 27 mm), respectively. However, combination of SPX with VGL potentiated the effects on *E. coli* and *S. typhi* by synergistic interaction. Similarly, the efficacy of CFX in combination with VGS on MRSA (30 mm) was significantly enhanced by additive action. It was observed that VGS potentiation of CFX on *P. aeruginosa* (32 mm) was the most effective antibacterial inhibition recorded in the study. Thus, combination of SPX and CFX each with the extracts has revealed remarkable properties for alternative or complementary therapeutic strategy. Our findings elicit enormous potentials of *V. glaberrima* hexane extracts as treatment adjuncts for combating drug resistant bacteria. It will be interesting to evaluate *in vivo* effects of extracts in combination with antibiotics against drug resistant bacteria.
Keywords: *Vernonia glaberrima*, hexene extract, GC-MS, MRSA, VRE, drug resistant bacteria

INTRODUCTION

Plants are renewable sources of secondary metabolites for the treatment of human infectious diseases. This is because of multiple therapeutic properties of the structurally diverse phytochemicals such alkaloids, flavonoids, saponins and terpenoids among others [1]). Numerous medicinal plants have been exploited as effective medicines in the fight against drug resistant bacteria [2]. Plant constituents such as essential oils, terpenoids and sesquiterpenoids have been evaluated as potent antibacterial agents on resistant bacteria using novel molecular targets such as inhibitions of efflux pump [3] and quorum sensing [4]. However, drug combination has evolved as a novel strategy to fight resistant bacteria. It involves the combination of standard antibiotics with plant extracts for effective interactions on drug resistant bacteria [5]. Thus, phytochemicals either as pure compounds, semi-pure or component mixtures can be combined with antibiotics as single therapeutic agent. The interactions via synergistic or additive interventions are recognized in recent years as effective complementary medicine against drug resistant bacteria [6-7].

Vernonia glaberrima is a perennial herb distributed in tropical regions of the world especially in Africa and South America [8]. It is found in abandoned fields in Northern Nigeria, and used in traditional medicine against malaria, inflammatory and infectious diseases [9]. Previous phytochemical studies on *Vernonia glaberrima* resulted to isolation of lupeol and coumarins [10]. However, very little information is available on the chemical compositions and their antibacterial efficacies. Hence, *V. glaberrima* leaf and stem hexane extracts were investigated using gas chromatography-mass spectrometry (GC-MS) and evaluated for antibacterial properties alone and in combination with standard antibiotics against selected bacterial strains. This is important to understanding chemical basis of the therapeutic potentials of the plant as source of antibacterial agents especially against resistant bacteria.

MATERIALS AND METHODS

Collection and extraction of plant material

The leaf and stem of *Vernonia glaberrima* were collected (March 2018) in Zaria, Kaduna State, Nigeria. The plant was identified by Umar S. Gallah of the Herbarium, Department of Botany, Ahmadu Bello University, Zaria, Nigeria. A voucher specimen number 215 was deposited there. The plant was air-dried; and leaf and stem samples (100 g each) were subjected to extraction using cold maceration with n-hexane (500 mL) for 12 h on a shaker (Labcon, South Africa). The extracts were filtered and concentrated under reduced pressure on a rotary evaporator (Buchi Rota vapor R-210) at 25ºC. The hexane leaf (VGL) and stem (VGS) extracts weighed 9.50 g and 12.70 g respectively.

GC-MS analysis

The GC-MS analysis was carried out on an Agilent Technologies (6890 Series) GC coupled with a (5973 Series) Mass Selective Detector. It was equipped with an Agilent HP-5MS capillary column (0.25 µm film thickness) with dimensions 30 m (length) × 0.25 micron I.D). The sample ionization energy of 70eV for GC-MS detection was used. Helium was used as the carrier gas at a pressure of 60 kPa, with the oven temperature programming at 100°C (for 2 min) to 280°C (for 30 min) at a ramping rate of 4°C per min. Diluted sample (2.0 µl) was manually injected while the injection temperature was 280°C with a split ratio of 1:50. The system software was driven by Agilent Chemstation software. The relative percentage of each component was calculated by comparing its average peak to the total areas. The identification of the various compounds was carried out by comparison of their mass spectra with those of authentic samples or those obtained from isolated pure compounds in our laboratory. The NIST/NBS 2005 mass spectral database of the GC-MS system was also used to identify some compounds whose structures were confirmed by published data [11].

Test microorganisms

The bacterial strains: *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, Methicillin resistant *Staphylococcus aureus* (MRSA) and *Vancomycin resistant Enterococci* (VRE) used in this study were obtained as clinical isolates, from the Department of Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika. The isolates were purified on nutrient agar (OXOID) plates and characterized using standard microbiological and biochemical procedures as previously described [12-13].

Antibacterial susceptibility testing

In vitro antimicrobial activity of *V. glaberrima* leaf (VGL) and stem (VGS) hexane extracts was determined by standard agar well diffusion assay as reported [14]. Molten Mueller Hinton agar were seeded with the inoculum (1×10^8 CFU/ml, 200 µl) and poured into petri dishes and allowed to solidify. The wells were prepared in the seeded agar plates with the help of a cork borer (6 mm). The sample extract each was dissolved in DMSO (5 mg/ml) and extract solution (100 µl) was then introduced into the 6 mm diameter well. The plates were incubated at 37 °C for 24 h. Testing was done in duplicate and Sparfloxacin (30 µg/ml) and Ciprofloxacin (30 µg/ml) discs (Oxoid, UK) were used as standard antimicrobial agent controls and DMSO was used as a negative control. Antibacterial activity was determined by measuring the diameter of the inhibition zone (clear zone) formed around the well in millimeters and classified as follows: Resistant (R): $\leq$ 10 mm; Intermediate (I): 11-14 mm; Sensitive (S): $\geq$ 15 mm [15].

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was carried out using micro broth dilution in accordance with Clinical Laboratory Standards Institute [16]. Serial dilution of sample extract (0.1 mg/ml to 6.50 mg/ml) was prepared. The tests tubes were inoculated with the suspension of the standardized inocula and incubated at 37 ºC for 24 h. MICs were recorded as the lowest concentration of extract showing no visible growth of the broth.

Antibacterial combination studies

In vitro antimicrobial combination studies of *V. glaberrima* leaf (VGL) and stem (VGS) hexane extracts each with Sparfloxacin and Ciprofloxacin was determined by standard agar well diffusion assay as reported [17]. Molten Mueller Hinton agar were seeded with the inoculum (1×10^8 CFU/ml, 200 µl) and poured into petri dishes and

allowed to solidify. The wells were prepared in the seeded agar plates with the help of a cork borer (6 mm). The sample extracts were dissolved in DMSO (5 mg/ml) and the standard drugs each (30 µg/ml). The combined solution of extract with standard drugs (30 µl each) was then introduced into the 6 mm diameter well. The plates were incubated at 37 °C for 24 h. DMSO was used as a negative control. Antibacterial activity was assayed by measuring the diameter of the zone of inhibition formed around the well in millimeters. The experiment was done in triplicate and the average values were calculated for antibacterial activity.

RESULTS AND DISCUSSION

The GC-MS analysis of VGL hexane extract revealed the presence of twenty-one (21) compounds with the abundance of fatty acid/esters such as 9, 12-octadecadienoic acid (Z, Z)-2-hydroxy-1-(hydroxy methyl) ethyl ester- (23.7%), hexadecanoic acid (11.6%), hexacosanoic acid, 2-methyl-methyl ester- (8.6%), tetra tetracontane (7.89%), hexadecanoic acid, 1-(hydroxy methyl)-2, 2-ethane diyl ester (5.29%), ethyl tetracosanoate (4.45%) and glycidol stearate (3.90%) which accounted for 68.2% of the total identified compounds. However, the VGS hexane extract contains twenty-three (23) components largely triterpenoids together with aromatic derivatives such as olean-18-ene (15.6%), β-amyrin (14 %), 1, 3, 5-trimethyl benzene (11.21%), 2-methylhexacosane (6,80%), ethanol, 2-(1, 12-octadecadienyloxy)- (Z, Z)- (6,28%), lupeol (5.74%), 1, 4-diethyl benzene (3.40%) and 1, 2, 4, 6-tetramethyl benzene (3.24%). These represent 66.3% of the total identified components. The retention times (RT) and relative percent (%) composition of identified components of both VGL and VGS are presented in Table 1. The compounds identified in *V. glaberrima* (VGL/VGS) are similar to previous GC-MS analysis on *V. calvoana* leaf ethyl acetate extract [18] and *V. cinerea* methanol extract [19]. Furthermore, fatty acid esters and aromatic derivatives were identified from the extract of *V. arborea* as reported [20]. In this study, the GC-MS analysis has revealed numerous bioactive constituents of *V. glaberrima* leaf and stem hexane extracts, as fat-soluble mixtures of aggregate compounds of different structural motifs with variable content and compositions which may indicate interesting therapeutic properties [21-22].

Table 1: Chemical composition of *V. glaberrima* leaf (VGL) and stem (VGS) hexane extracts

Chemical constituents	RT (min)	VGL	VGS
1, 4-dimethyl benzene	4.13	-	1.62
1-ethyl, 2-methyl benzene	4.94	-	3.00
1, 3, 5-trimethyl benzene	5.05	-	11.21
1-methyl-3-propyl benzene	6.16	-	1.45
1, 4-diethyl benzene	6.24	-	3.40
2-ethyl-1, 4-dimethyl benzene	6.52	-	1.35
1-ethyl-3, 5-dimethyl benzene	6.57	-	2.79
1, 2, 4, 6-tetramethyl benzene	7.14	-	3.24
1, 2, 4, 5-tetramethyl benzene	7.67	-	1.12
Naphthalene	8.30	-	1.06
Eicosane	15.8	2.08	-
2-pentadecane	16.2	2.43	-
Hexadecanoic acid	17.2	11.6	-
L-(+)-ascorbic acid, 2, 6-dihexadecanoate	17.6	1.26	2.21
Eicosane	17.9	2.34	1.00
ε-11-hexadecanal	19.3	-	1.61
9, 12-octadecadienoic acid	19.4	2.05	-

(E)-9-octadecanoic acid, ethyl ester	19.5	3.23	-
Heptadecanoic acid, ethyl ester	19.7	2.26	-
Tetracosane	19.8	2.97	-
Phytol, acetate	19.9	2.53	-
Hexadecanoic acid, 1-(hydroxy methyl)-2, 2-ethanediyl ester	20.7	5.29	1.81
4, 8, 12, 16-tetramethylheptadecan-4-olide	21.2	1.18	-
9, 12-octadecadienoic acid (Z, Z)-2-hydroxy-1- (hydroxy methyl) ethyl ester-	22.1	23.7	-
Ethanol, 2-(1, 12-octadecadienyloxy)- (Z, Z)-	22.2	-	6.28
Tridecanoic acid, 3-hydroxy-ethyl ester	22.3	2.38	-
Glycidol stearate	22.4	3.90	1.46
Hexacosanoic acid	22.7	1.14	-
Tetracosenal	26.1	-	1.08
Ethyl tetracosanoate	25.4	4.45	-
Hexatriacontane	26.9	2.63	1.00
Stigmasta-5, 22-dien-3-ol-acatate (3β-22 Z)	27.2	-	2.15
Hexacosanoic acid	27.5	1.27	-
Lupeol	28.3	-	5.74
Hexacosanoic acid, 2-methyl-, methyl ester-	28.6	8.16	-
β-amyrin	30.0	-	14.0
Olean-18-ene	30.5	-	15.6
2-methylhexacosane	30.9	-	6.80
Tetra tetracontane	31.0	7.89	-
Total components identified (%)		**94.7**	**91.0**

The results of antibacterial activity on the VGL and VGS hexane extracts are presented in Table 2. Both extracts demonstrated effective antibacterial activity especially on *P. aeruginosa*, MRSA, *E. coli* and *S. aureus*. The VGL was more effective on *P. aeruginosa* (29 mm, MIC 0.125 mg/ml). Similarly, the effects of VGL on MRSA and *S. aureus* (27 mm, MIC 0.125 mg/ml) can be attributed to the therapeutic potentials of fatty acid esters as largely identified constituents from the VGL. This finding is consistent with previous report on the antibacterial activity of fatty acid esters of lipophilic extract of *Pavetta corymbosa* [23]. The susceptibility of MRSA and *P. aeruginosa* to the VGL extract may indicate a broad-spectrum antibacterial potency of the hexane extracts. This is not surprising as *V. ambigua, V. blumeoides* and *V. oocephala* have previously been reported with broad-spectrum antibacterial activities [24].

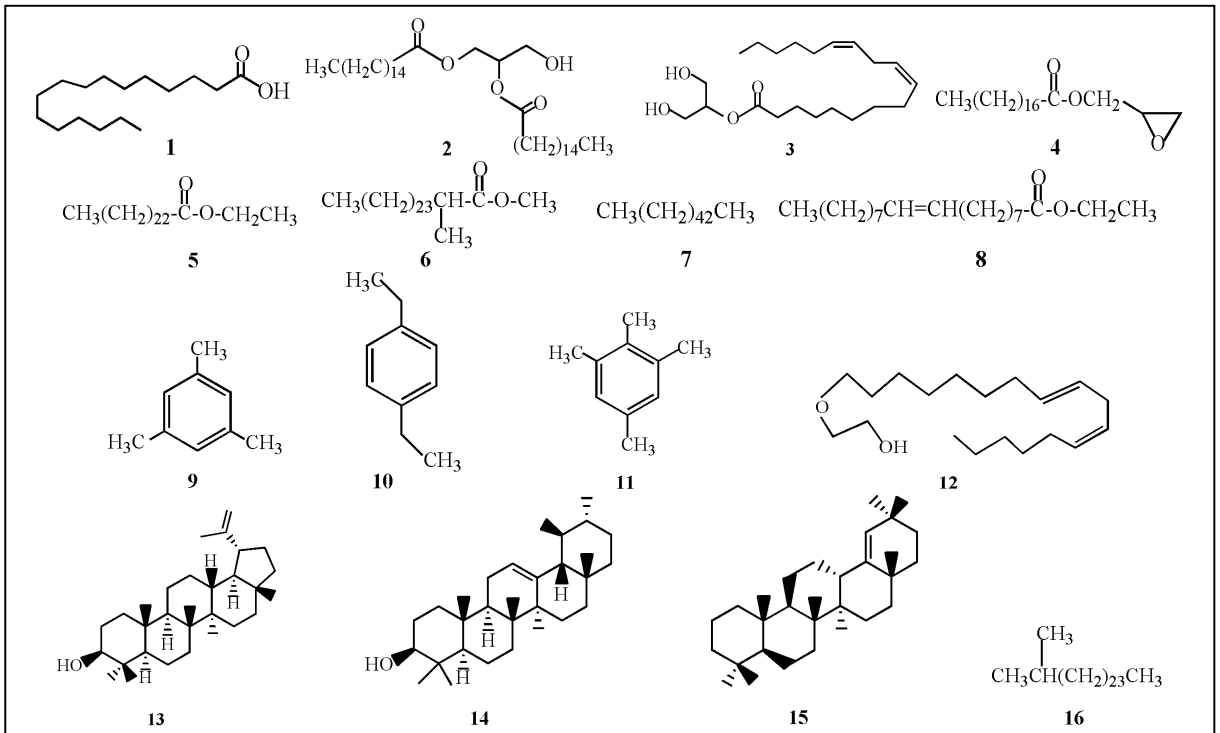
Table 2: Antibacterial activity of VGL and VGS hexane extracts alone

	Zone of inhibition (mm)				**MIC mg/ml**	
Test organisms	**VGL**	**VGS**	**Sparfloxacin**	**Ciprofloxacin**	**VGL**	**VGS**
MRSA	27	25	22	0	0.125	0.125
S. aureus	27	26	27	25	0.125	0.125
P. aeruginosa	29	27	26	24	0.125	0.125
E. coli	24	26	22	25	0.25	0.125

S. typhii	0	0	23	29	NT	NT
VRE	0	0	0	26	NT	NT

MRSA= Methicillin resistant *Staphylococcus aureus*, VRE= Vancomycin resistant enterococcus
VGL=*Vernonia glaberrima* leaf, VGS=*Vernonia glaberrima* stem, NT=not tested

The VGS hexane extract also showed similar effects as VGL in terms of potent activity on Gram-negative bacteria *P. aeruginosa* (26 mm, MIC 0.125 mg/ml) and *E. coli* (26 mm, MIC 0.125 mg/ml). This indicates the influence of aromatic and sterols components identified (Fig. 1). Previous study of plant extracts containing largely lupeol and β-amyrin demonstrated antibacterial activity with MIC (0.5 mg/ml) on *S. aureus* [25]. In this study, both VGL and VGS have shown potent activity on Gram-positive and Gram-negative bacteria including resistant MRSA indicating broad-spectrum antibacterial activity of the extracts.



VGL: (**1**) Hexadecanoic acid, (**2**) Hexadecanoic acid, 1-(hydroxy methyl)-2, 2-ethanediyl ester, (**3**) 9, 12-octadecadienoic acid (Z, Z)-2-hydroxy-1- (hydroxy methyl) ethyl ester-, (**4**) Glycidol stearate, (**5**) Ethyl tetracosanoate, (**6**) Hexacosanoic acid 2-methyl-, methyl ester-, (**7**) Tetra tetracontane, (**8**) (E)-9-octadecanoic acid, ethyl ester **VGS:** (**9**) 1, 3, 5-trimethyl benzene, (**10**)1, 4-diethyl benzene, (**11**) 1, 2, 4, 6-tetramethyl benzene, (**12**) Ethanol, 2-(1, 12-octadecadienyloxy)- (Z, Z)-, (**13**) Lupeol, (**14**) β-amyrin, (**15**) Olean-18-ene, (**16**) 2-methylhexacosane

Figure 1: Structures of major chemical compounds identified by GC-MS

The combinations of plant extracts with several classes of antibiotics showed various therapeutic properties on drug resistant bacteria by synergistic or additive interactions [7]. In this study, the antibacterial effects of VGL and VGS in combination each with Sparfloxacin (SFX) and Ciprofloxacin (CFX) are presented in Table 3. The potentiation of SFX when combined with VGL was observed on *E. coli* and *S. typhi*. Similar trend of potentiation was observed when SFX combined with VGS on MRSA, *P. aeruginosa* and *S. typhi* (Table. 3). This implies that both extracts can improve the therapeutic efficacy of Sparfloxacin on Gram-positive and Gram-negative bacteria. SFX and CFX are broad spectrum antibiotics of the fluroquinolone family that target bacterial DNA gyrase or the topoisomerase IV enzymes leading to formation of tertiary complexes which

inhibits DNA replication and transcription [26]. It was observed that SFX in combination with VGS remarkably enhanced the antibacterial potency on VRE which otherwise was resistant to the SFX alone (Table 2 and 3). This modulation of SFX activity by VGS suggest the possibility of developing effective phytomedicines against drug resistant bacteria.

The combination of CFX with VGL also demonstrated enhanced activity on MRSA and *E. coli*, which could be attributed to the fatty acid esters largely identified (Fig. 1). However, the interaction of CFX with VGS on MRSA (30 mm) showed significant enhancement of the antibiotic effects (Table 2 and 3). The resistance of MRSA to CFX was modified by additive interaction of the VGS (Table 2 and 3). It was also observed that the potentiation of CFX by the VGS on *P. aeruginosa* (32 mm) demonstrated the most effective antibacterial efficacy in the whole study. Previous study showed the enhancement of Ciprofloxacin against multi-drug resistant bacteria by *Carum copticum* extracts of aromatic constituents [27]. Thus, our findings have indicated that phytochemicals from *V. glaberrima* hexane extracts could significantly potentiate the antibacterial effects of Ciprofloxacin antibiotics especially against resistant pathogens

Table 3: Antibacterial activity of VGL and VGS hexane extracts combined with antibiotics

Test organisms	VGL+SFX	VGS+SFX	VGL+CFX	VGS+CFX
MRSA	23	29	25	30
S. aureus	24	0	23	0
P. aeruginosa	27	30	28	32
E. coli	30	22	29	20
S. typhii	28	29	32	31
VRE	27	27	27	27

MRSA= Methicillin resistant *Staphylococcus aureus*, VRE= Vancomycin resistant enterococcus, VGL=*Vernonia glaberrima* leaf, VGS=*Vernonia glaberrima* stem, SFX= Sparfloxacin, CFX= Ciprofloxacin

CONCLUSION

The GC-MS analysis of *V. glaberrima* leaf (VGL) and stem (VGS) hexane extracts revealed fatty acid esters, triterpenoids and aromatic derivatives. The extracts have demonstrated broad spectrum antibacterial activity alone and in combination with antibiotics. The combinations of Ciprofloxacin (CFX) with VGS showed significant enhancement of antibiotic effects on MRSA. These findings elicit enormous potentials of *V. glaberrima* hexane extracts as therapeutic adjuncts for combating drug resistant bacteria. It will be interesting to evaluate *in vivo* effects of extracts in combination with antibiotics against drug resistant bacteria.

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