2011

BIOLOGICAL ACTIVITY AND MASS SPECTROMETRIC ANALYSIS OF Vernonia amygdalina FRACTIONS

Winston A Anderson, *Howard University*

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

BIOLOGICAL ACTIVITY AND MASS SPECTROMETRIC ANALYSIS OF *Vernonia amygdalina* FRACTIONS

Daniel A. Oyugi [1], Folahan O. Ayorinde [2,*], Ayele Gugssa [1], Adrian Allen [1], Ernest B. Izevbigie [3], Broderick Eribo [1] and Winston A. Anderson [1]

[1]Department of Biology, Howard University, 415 College Street, NW, Washington DC 20059,
[2]Department of Chemistry, Howard University, 525 College St., NW, Washington, DC 20059,
[3]Department of Biology, Jackson State University, 1400 J.R. Lynch Street,
P.O Box 18540 Jackson,Mississippi, 39217.
[*] E-mail: fayorinde@howard.edu

ABSTRACT

Vernonia amygdalina (VA) is a tropical African plant of the Asteraceae family used as dietary supplement and as a medicinal herb. In this study, fatty acid esters, fatty acid amide, triterpene, diterpene alcohols and phytostanols were identified as the major chemical groups in the aqueous and organic fractions of VA leaf extracts. Their structures were elucidated, on the basis of GC-MS data, as hexanedioic acid, bis (2-ethylhexyl) ester (**2a**), erucamide (**2b**) and squalene (**2c**) in the water fraction; hexadecanoic acid methyl ester (**3a**), 9(Z)12(Z)15(Z)-octadecatrienoic acid, methyl ester (**3b**) and phytol (**3c**) in methanol fraction; phytol (**4a**), squalene (**4b**) and 7,22-ergostadienol (**4c**) in the petroleum ether fraction. Diterpene alcohol and aliphatic acid esters were higher in relative content than triterpene, fatty acid amide, and phytostanol in VA leaf extracts. *In vitro* evaluation of anti-proliferative activities of sample extracts on MDA-MB-231 cells revealed significant growth inhibition by methanol (45-70%, $P < 0.001$) and petroleum ether (70-97%, $P < 0.001$) fractions, compared to aqueous fraction (11-23%, $P < 0.05$).

KEYWORDS:
Cell growth inhibition; Chemical composition; Fragmentation pattern; GC-MS; *Vernonia amygdalina;*

1. INTRODUCTION

Vernonia amygdalina, one of the species of the genius *Vernonia*, belongs to the Asteraceae family. As a perennial shrub, it has been consumed as vegetable food and used in African folk medicine to treat various diseases and infections. In the wild, it has been consumed by Chimpanzees suffering from parasitic infections [1]. Previous studies have showed that VA leaf extracts are effective against amoebic dysentery and gastrointestinal disorders [2-8], microbial and parasitic infections [8,9], hepatoxities [10] and cancer [11-16]. These biological activities may be attributed to various chemical classes [17-22]. Recently, we isolated and identified, in VA, steroid glucosides with anti-proliferative activities in ER+ human breast adrenocarcinoma (MCF-7) cells [23]. Detection and analyses of these groups of compounds have relied on various chromatographic and spectrometric techniques such as TLC, UV, IR, NMR, etc. In our continued investigation of secondary metabolites in VA, we have hereby identified fatty acid esters, fatty acid amide, triterpene, diterpene alcohols and phytostanols in aqueous, methanol and petroleum ether extracts, utilizing a highly sensitive and specific detection technique, GC-MS. Extraction, fractionation and structure elucidation of these compounds involved liquid-liquid extraction, solvent partitioning, and analysis of electron impact (EI) mass fragmentation patterns of their ions.

2. MATERIALS AND METHODS

2.1 General Experimental Procedures

All chromatographic experiments were by GC-MS. An Agilent Technologies 6890 GC (CA, USA) interfaced with an Agilent 5973 Inert Mass Selective Detector was used to generate data. A Supelco fused-silica SPB-1 (30 m, 0.32-mm i.d., 0.25 µm film) column (Bellefonte, PA, USA) was used for high-resolution capillary gas chromatography. Oven temperature was programmed from 40

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

to 300°C at 20°C/min, and helium was used as the carrier gas. RPMI-1640 Medium was supplied by Gibco BRL (Grand Island, NY). Trypsin-EDTA, heat-inactivated Fetal Bovine Serum (FBS), penicillin, streptomycin and fungizone were supplied by Sigma Chemicals Co. (St. Louis, MO). MTT reagent was supplied by Invitrogen Co. (Carlsbad, CA). Sterile 0.45 µm and 0.2 µm filters were purchased from Corning (Corning, NY). All other chemicals were of analytical grade and supplied by Sigma Chemicals Co. (St. Louis, MO, USA).

2.2 Plant Material and Solvent Extraction

Stem cuttings of *Vernonia amygdalina* (family: Asteraceae), identified by Dr. Broderick Eribo were cultivated in the green house, Department of Biology, Howard University, USA. Pesticide-free fresh leaves of the plant were harvested, air-dried and pulverized to a fine powder in a blender to yield 250 g. The dry powder was mixed to a final volume of 800 mL with deionized water and exhaustively extracted by maceration on an orbital shaker at room temperature for 16 hours to afford aqueous extract. The extract was further subjected to liquid-liquid extraction with ethanol and the residue further extracted with methanol (85%) to yield methanol fraction. In an effort to further characterize the chemical constituents in the original sample, we fractionated the methanol sample by solvent partitioning with petroleum ether to yield petroleum ether fraction. All fractions were separately centrifuged for 10 min at 3,000 rpm, and supernatants filtered through 0.45 µm filtration units. The filtrates were then concentrated on a Rotor Vapor (Buchi, Brinkman Inst., NY) and dried to a powder on a Savant Speed Vac (Holbrook, NY).

2.3 GC-MS Analysis.

Identification and analysis of chemical compounds in VA fractions were carried on Gas-Chromatography-Mass Spectrometry (GC-MS). Structural assignments were based on analysis of fragmentation pattern of mass spectra, direct comparison of mass spectral data with profiles in the National Institute of Standards and Technology (NIST2) library, and comparisons of mass spectra with data published in the literature. Lyophilized samples from water, petroleum ether and methanol fractions of VA extracts were separately diluted with respective solvents, filtered through 0.2 µm sterile syringe filters, and 1 uL of each fraction solution injected into a gas chromatograph and analyzed with quadruple mass spectrometric detector.

2.4 Cell Culture

Human breast cancer (MDA-MB-231) cell line was purchased from ATCC. Cells were grown and maintained in 75 cm^2 tissue culture flasks in a humidified atmosphere at 37^oC, 5 % CO_2 in a Fisher Scientific water-jacketed incubator (Pittsburg, PA). Cells were cultured in RPMI-1640 medium supplemented with 10 % heat-inactivated FBS (56^oC, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone.

2.5 Cell proliferation studies (MTT assay)

The anti-proliferative effects of VA on MDA-MB-231 breast cancer cells were determined by MTT assay. MDA-MB-231 cells were seeded at a density of 5.0 x 10^3 per well in 96 well-plates with 100 µL complete RPMI 1640 growth medium supplemented with 10 % Fetal Bovine Serum and antibiotic. After 24 h incubation, cells were treated with various concentrations of aqueous, methanol and petroleum ether fractions of VA (0.01- 1.0 mg/mL) and inhibition of cell proliferation assessed by addition of 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (5mg/mL in PBS), and the reaction mixture incubated at 37^oC, 5 % CO_2 for 4 h. The mixture solution was carefully replaced with 100 µl MTT solvent

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

(isopropanol with 0.04 N HCL) to dissolve the violet formazan crystals formed by mitochondrial reduction of MTT. Absorbance was measured at 570 nm wavelength on a Bio-Tek microplate reader (Winooski, VT). Results were expressed as means ± SD for experiments performed in triplicate. Sample fractions were initially dissolved in DMSO before dilution with PBS to attain required treatment concentrations. Percent DMSO concentration in the final solution was < 0.25%.

2.6 Statistical Analysis

Statistical analysis was carried out using the Analysis of Variance (ANOVA) –single factor model followed by a two-tailed Student's *t*-test on Graphpad Prism, version 5.04., software. Data is presented as means ± SD error of the means. *P*-values < 0.05 were regarded as statistically significant.

3. RESULTS

3.1 GC-MS Analysis.

Total ion chromatogram of water fraction revealed three major peaks at retention times ($\mathbf{t_R}$) 12.64 min, 14.16 min and 14.26 min (Fig.1a). Mass spectrum of peak at $\mathbf{t_R}$ 12.64 min in the water fraction showed an ion at m/z 129 (base peak), characteristic of adipates. Other major diagnostic ions were at m/z 259, 241 and 112. Fragment ion at m/z 241 suggests loss of a 129 mass alkoxy side chain ($-OC_8H_{17}$), while m/z 112 indicate loss of two alkoxy groups of mass 258. Ion peak at m/z 129 indicates loss of a 241 mass $C_{14}H_{25}O_3$ ion. Fragment at m/z 258 corresponds to loss of a hydrogen and C_8H_{17} ion of 112 mass. Fragment at m/z 223 corresponds to (M+ -146) for the loss of adipic acid ion $[-C_6H_{10}O_4]^+$. At m/z 57(M+-313) an enoate, $C_{18}H_{33}O_4$ ion was eliminated (Fig 1b). These ions, together with a molecular ion at m/z 370 were suggestive of $C_{22}H_{42}O_4$ molecular formula compound, called hexanedioic acid, bis (2-ethylhexyl) ester (**2a**), scheme 1a. Spectrum representing chromatographic peak at $\mathbf{t_R}$ 14.16 min in the water fraction showed an intense ion at m/z 59 (base peak), typical of a long chained fatty acid amide. This peak signifies a McLafferty rearrangement in the alkyl portion of an amide compound. Specific peak ions 72, 114 and 126 are characteristic of primary amide. Other distinct fragments observed included m/z 97, 294, 277, 320 and a molecular ion at m/z 337 (figure 1c). This fragmentation pattern suggested presence of an amide (nitrogen rule), of the formula $C_{22}H_{43}NO$, corresponding to 13Z-Docosenamide (**2b**) [35], scheme 1b.

Analysis of chromatographic peak at $\mathbf{t_R}$ 14.26 min revealed molecular ion at m/z 410(M^+) suggesting a molecular formula $C_{30}H_{50}$ (figure 1d). Diagnostic ions were at m/z 367(M^+-C_3H_7), 341(M^+-C_5H_9) and 69 (base ion). Other significant fragment ions observed were at m/z 81, 95, 107, 121,136, etc. Ion at m/z 341was accompanied by loss of ion bearing a mass of 69, whereas at m/z 367 a 43 mass propyl ion was eliminated. Fragment ion at m/z 83 corresponds to loss of $C_{24}H_{39}$ ion of 327 mass. Ion peak at m/z 121 indicated loss of $C_{21}H_{37}$ ion. In general, this mass ionization pattern indicates a 410 molecular mass compound of $C_{30}H_{50}$ formula, suggestive of Squalene (**2c**), scheme 1c. This fragmentation pattern is confirmed by NIST library and supported by literature reports [24, 25]. Structural representations of various compounds accrued from water fraction are represented in scheme 1d. Three major peaks were detected in the methanol fraction at retention times 10.28 min, 11.17 min and 11.25 min (Fig. 2a).

Identity of compound represented at $\mathbf{t_R}$ 10.28 min was based on major spectral ion peaks. The molecular ion at m/z 270, as well as ion at m/z 239(M^+- 31) representing loss of a methoxy group, are diagnostic of methyl ester. Fragment ion at m/z 227(M^+-43)

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

Figure 1a.
Total ion chromatogram (TIC) of water fraction of *V.amygdalina* with major peaks at retention times (t_R) 12.64 min, 14.16 min, and 14.26 min.

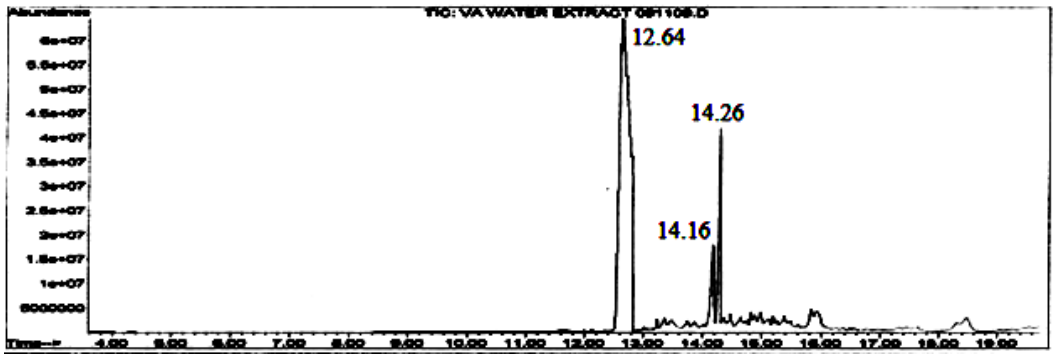
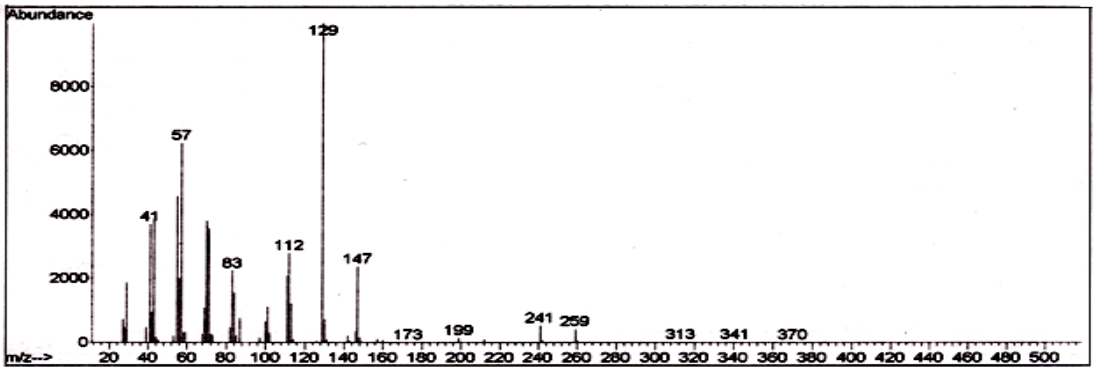
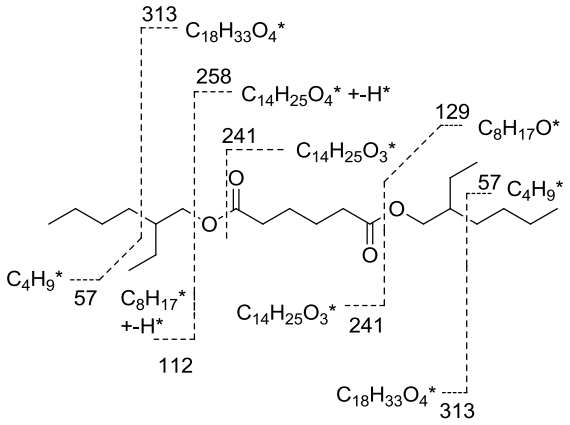


Figure 1b.
GC-MS spectrum of water fraction. Ion fragmentation pattern for spectral peak at t_R 12.64 min was specific to hexanedioic acid, bis (2-ethylhexyl) ester.



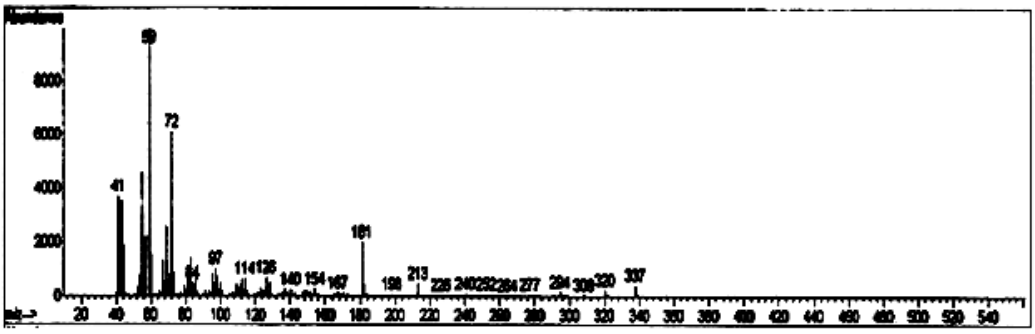
Scheme1a.

Schematic representation of mass fragmentation pattern for spectral peak at **t_R** 12.64 min. The fragmentation pattern was relevant to hexanedioic acid, bis (2-ethylhexyl) ester (**2a**).

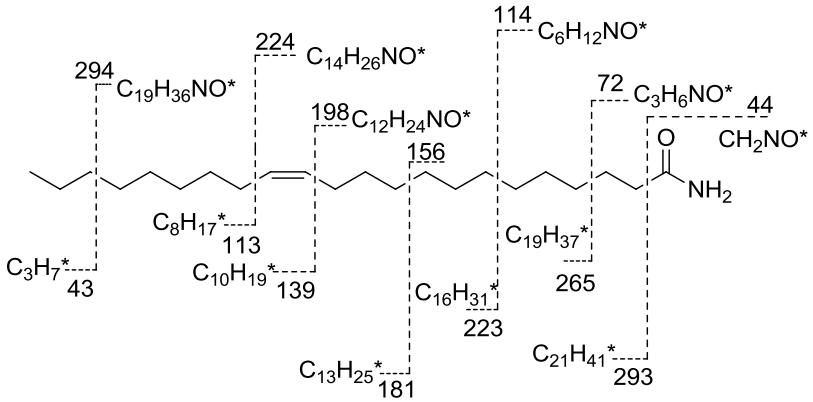


ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

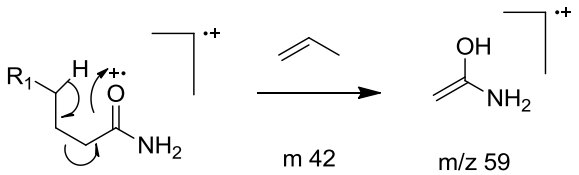
Figure 1c.
Mass spctrum of water fraction. Ion fragmentation pattern for spectral peak at t_R 14.16 min was specific to 13Z- docosenamide.



Scheme 1b.
(i) Schematic representation of mass fragmentation pattern for spectral peak at t_R 14.16 min. The fragments were characteristic of 13 Z-Docosenamide (2b).(ii) Mechanism of McLafferty rearrangement in compound 2b resulted in characteristic peak at m/z 59, typical of unsaturated fatty acid amide.



i m/z 337

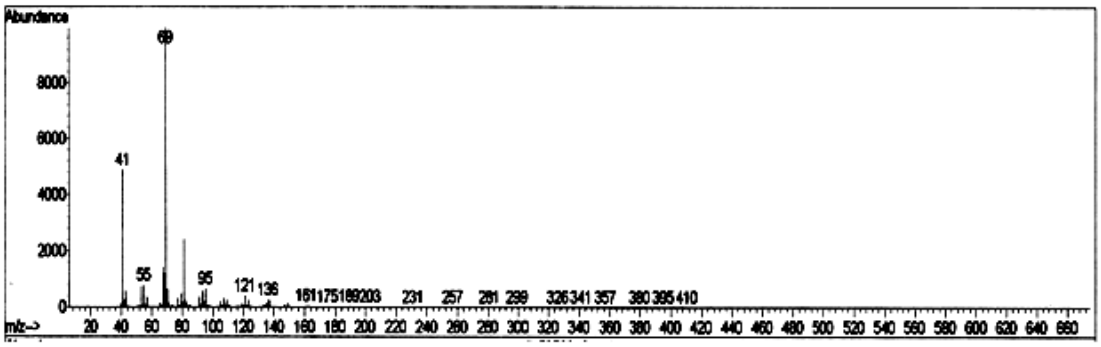


ii

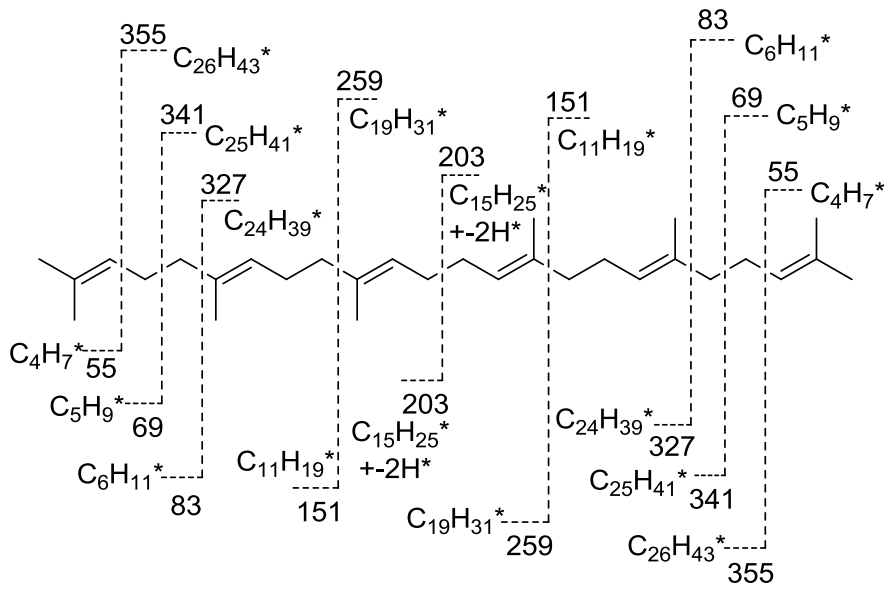
2b

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

Figure 1d.
Mass spectrum of petroleum ether fraction. Ion fragmentation pattern for spectral peak at $\mathbf{t_R}$ 14.25 min was specific to squalene.



Scheme 1c.
Schematic representation of mass fragmentation pattern for spectral peak at $\mathbf{t_R}$ 14.25 min. The fragments were characteristic of Squalene (**2c**).



m/z 410

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

Scheme 1d.
Chemical structures of compounds in *Vernonia amygdalina* water fraction: Hexanedioic acid, bis (2-ethylhexyl) ester (2a), 13- decosenamide (2b) and Squalene (2c).

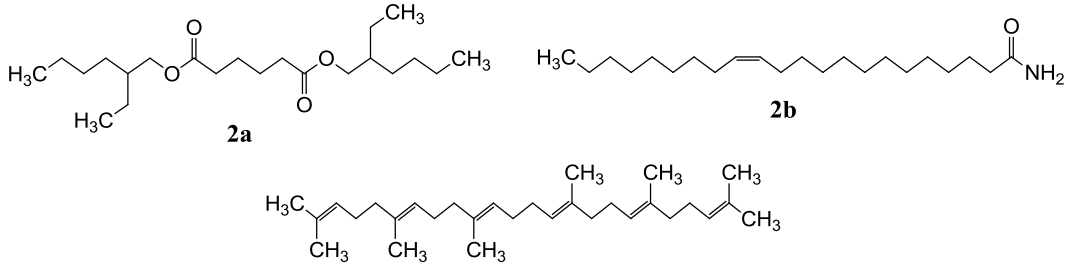


Figure 2a.
Total ion chromatogram (TIC) of methanol fraction of *V.amygdalina* with major peaks at retention times (t_R) 10.28 min, 11.17 min and 11.25 min.

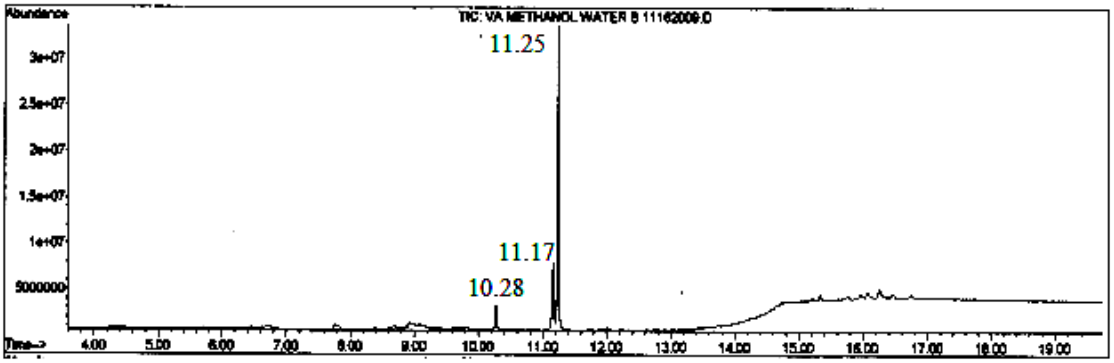
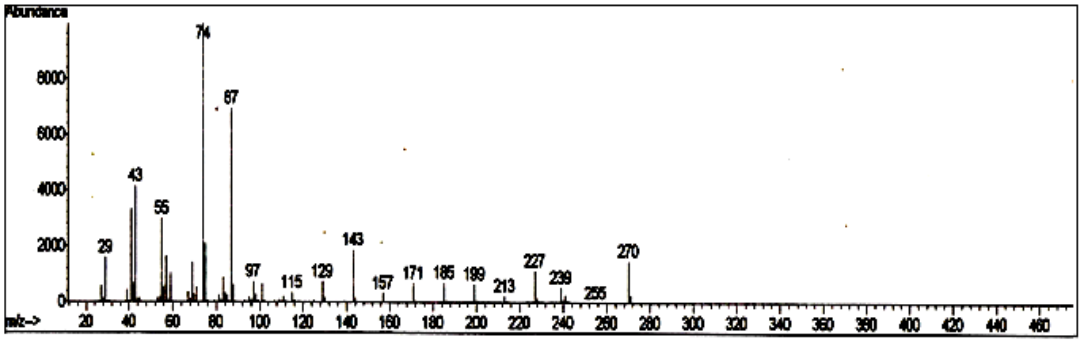


Figure 2b.
Mass spectrum of methanol fraction. Ion fragmentation pattern for spectral peak at t_R 10.27 min was specific to hexadecanoic acid methyl ester.



ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

Scheme 2a.
(i) Mass fragmentation pattern for spectral peak at t_R 10.28 min. The fragments were characteristic of Hexadecanoic acid methyl ester (3a). (ii) Mechanism of γ-Hydrogen shift resulted in characteristic peak at m/z 74, typical of long chain fatty acid methyl ester.

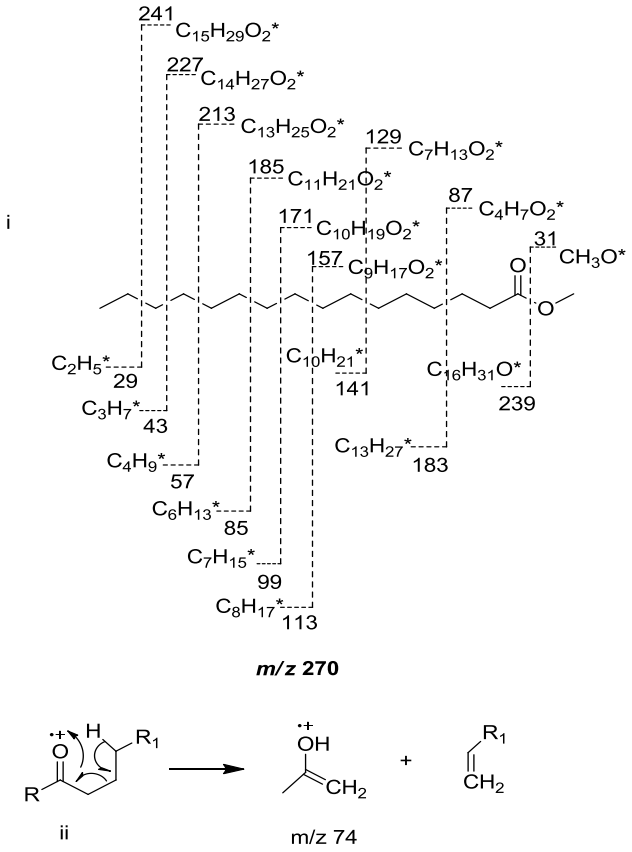
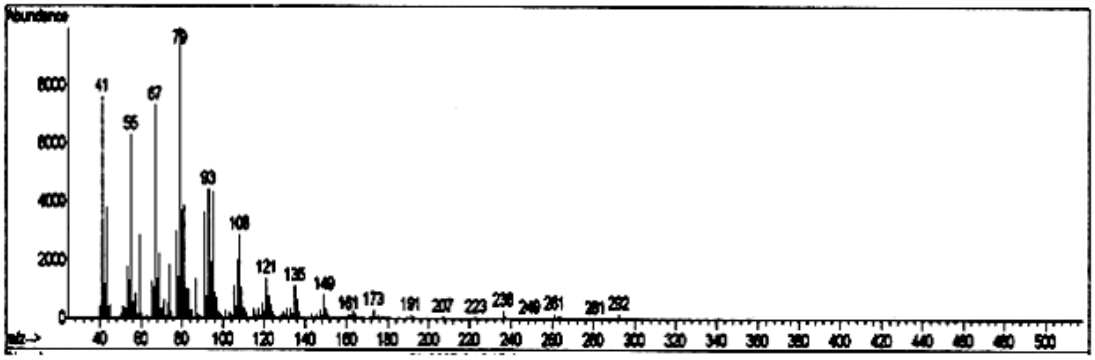


Figure 2c.
Mass spectrum of methanol fraction. Ion fragmentation pattern for spectral peak at t_R 11.15 min was specific to 9(Z)12(Z)15(Z)-Octadecatrienoic acid, methyl ester (α- linolenic acid methyl ester).



ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

Scheme 2b.
Mass fragmentation pattern of 9(Z)12(Z)15(Z)-Octadecatrienoic acid, methyl ester(3b), identified in *V.amygdalina* methanol extract.

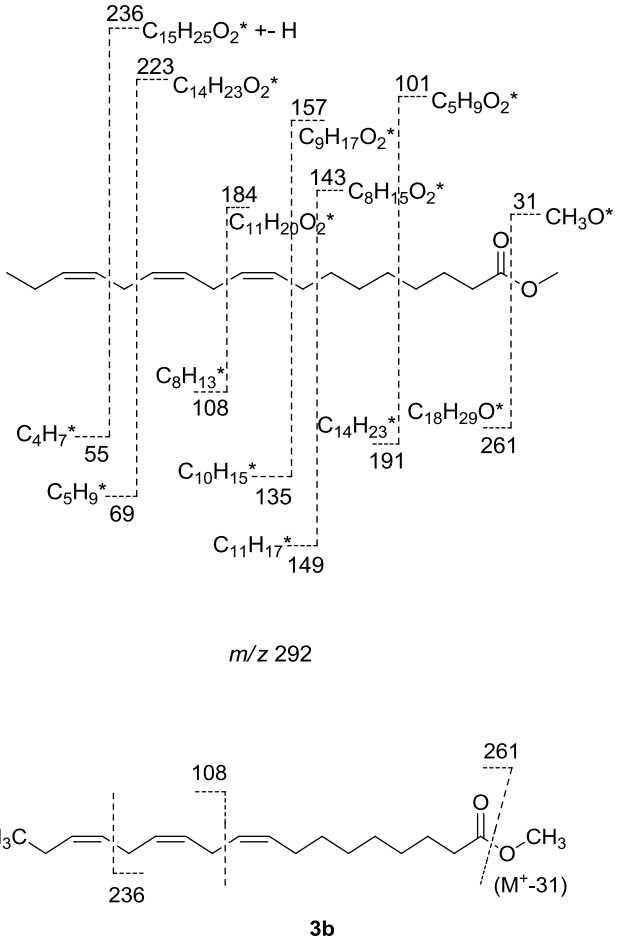
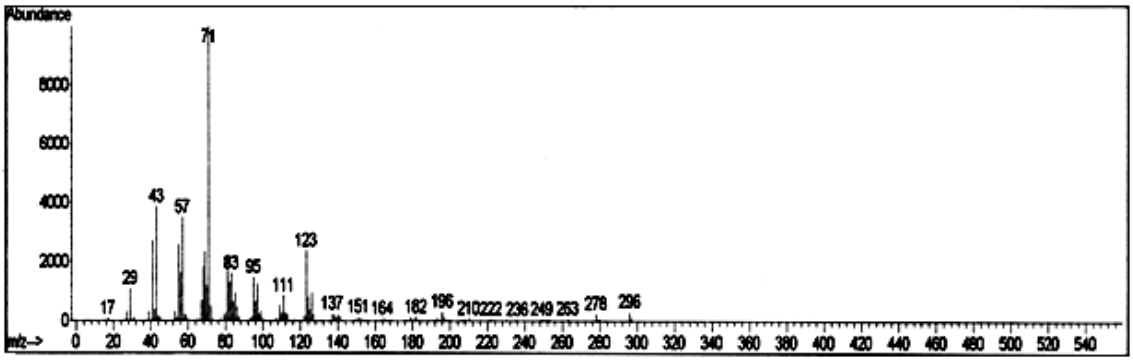
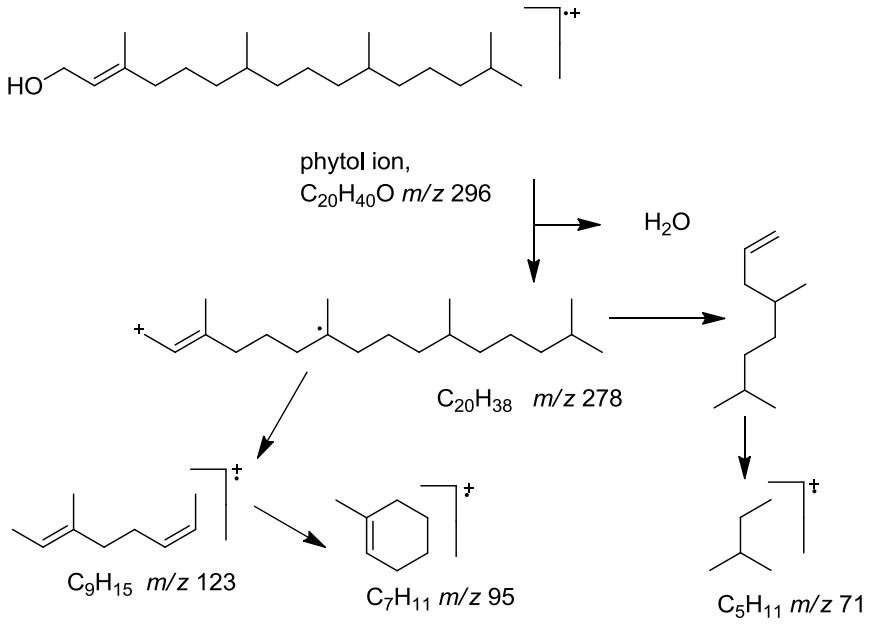


Figure 2d.
Mass spectrum of methanol fraction. Ion fragmentation pattern for spectral peak at t_R 11.24 min was specific to phytol.

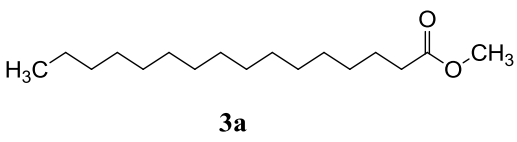


ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

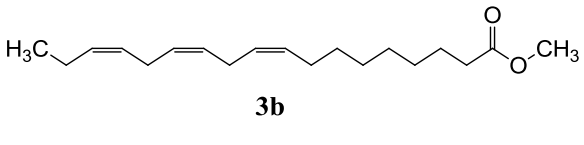
Scheme 2c.
Schematic representation of mass fragmentation pattern of phytol(**3c**).



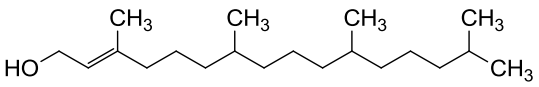
Scheme 2d.
Chemical structures of compounds in *Vernonia amygdalina* methanol fraction: Hexadecanoic acid methyl ester (3a), 9(Z)12(Z)15(Z)-octadecatrienoic acid, methyl ester (α-linolenic acid methyl ester) (3b) and Phytol (3c).



3a



3b



3c

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

Figure 3a.
Total ion chromatogram (TIC) of petroleum ether fraction of *V.amygdalina* with major peaks at retention times (t_R) 11.30 min, 11.60 min, 14.25 min, and 16.47 min.

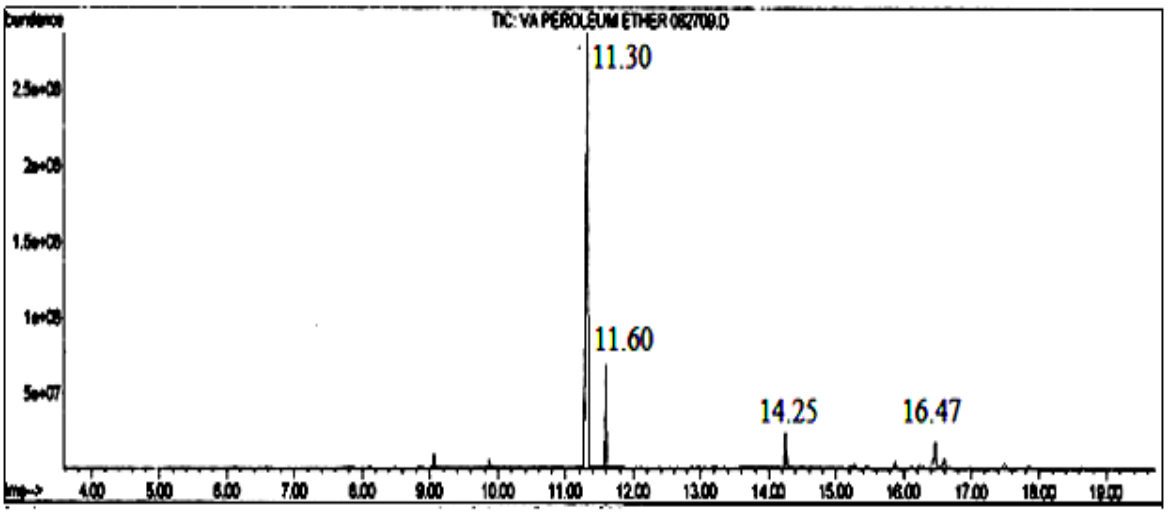
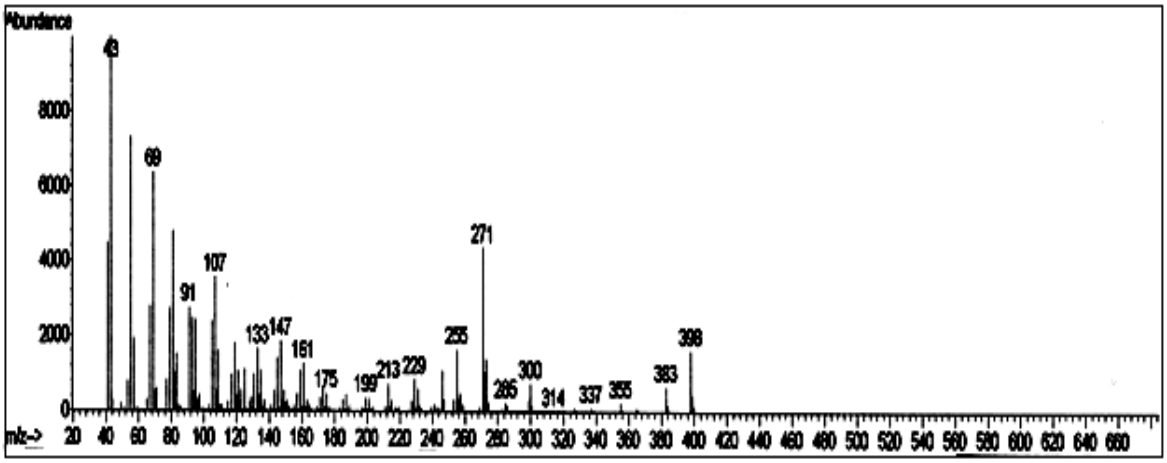
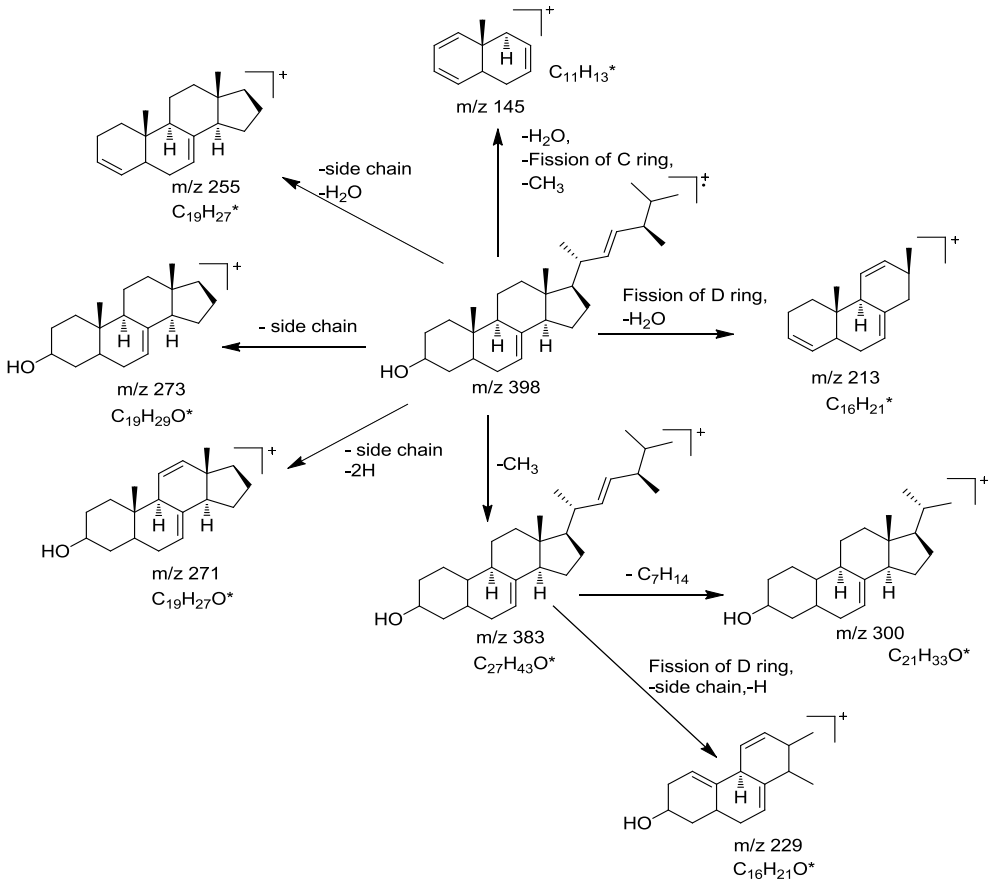


Figure 3b.
Mass spectrum of petroleum ether fraction. Ion fragmentation pattern for spectral peak at t_R 16.47 min was specific to 7,22-Ergostadienol.

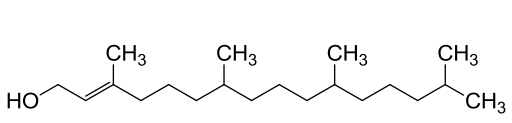


ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

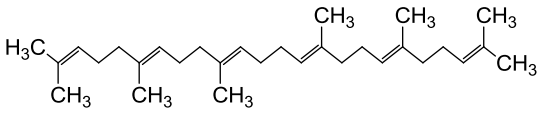
Scheme 3a.
Schematic representation of mass fragmentation pattern of 7,22-Ergostadienol (4c)



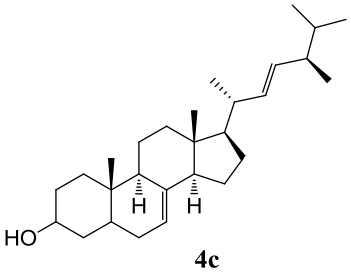
Scheme 3b.
Chemical structures of compounds in *Vernonia amygdalina* petroleum ether fraction: Phytol (4a), Squalene (4b) and 7, 22 ergostadienol (4c).



4a



4b



4c

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

Table 1. Chemical composition of *Vernonia amygdalina* leaf extracts

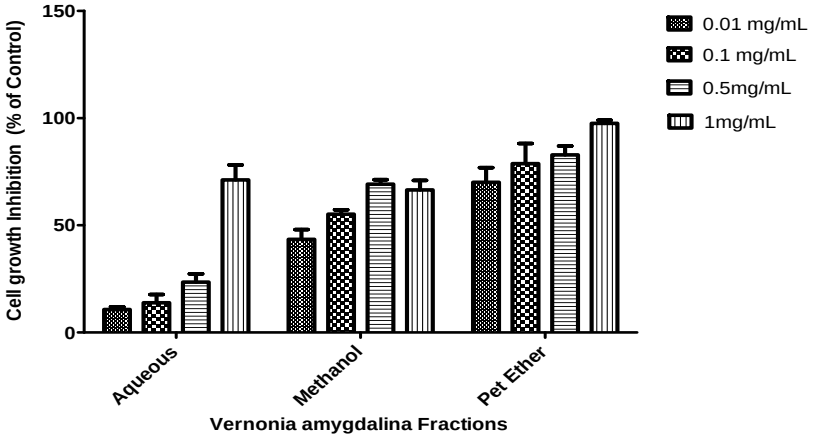
Fraction	*Compound*	t_R *(min)*	*Mol. Ion m/z*	*Fragment ions m/z*
Aqueous	Hexanedioic acid, bis (2-ethylhexyl) ester (**2a**)[i]	12.64	370	241, 129
	13- decosenamide (**2b**)	14.16	337	294, 72,114,126,277,59
	Squalene (**2c**)	14.26	410	341 [M-69],367,83, 69
Methanol	Hexadecanoic acid methyl ester (**3a**)[ii]	10.28	270	239 [M-OCH_3],241,227,213 143,129, 87, 74
	9(Z)12(Z)15(Z)-octadecatrienoic acid, methyl ester (**3b**)[iii]	11.17	292	261 [M-OCH_3],108,236,79
	Phytol (**3c**)	11.24	296	278 [M-H_2O], 71
Pet ether	Phytol (**4a**)	11.30	296	278 [M-H_2O], 71
	Squalene (**4b**)	14.25	410	341 [M-69], 69
	7,22-ergostadienol (**4c**)	16.47	398	383 [M-CH_3], 355, 271,255,43

i, compound is also known as Bis(2-ethylhexyl) adipate. **ii**, compound is also known as Methyl palmitate. **iii**, compound is also known as alpha-linolenic acid methyl ester.

Retention time (**tR**) obtained by chromatogram

Fragment ions were determined by mass spectrometry (Agilent Tech)

Figure 4.



Inhibition of MDA-MB-231 by 0.01-1 mg/mL aqueous, methanol and petroleum ether extracts of VA was concentration dependent. Results are expressed as means ± SD for experiments performed in triplicate. VA methanol and petroleum ether extracts significantly inhibited cell growth by 45-70 % ($P < 0.001$) and 70-97% (*P*

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

< 0.001), respectively, while aqueous extract inhibition was between 11-23 % ($P < 0.05$), except at 1mg/mL with 71% ($P < 0.001$) inhibition. Cells numbers are expressed as a percentage of cells relative to 100% control.

represents loss of a C3 unit (C_3H_7) ion. Prominent fragment ion at m/z 74(base peak) is diagnostic of a long chain aliphatic fatty acid of methyl ester, confirming that the formation of the compound was as a result of site-specific rearrangement of atoms, in which *γ*-hydrogen from potion 4 of aliphatic chain is transferred to the carbo-methoxy group, through a sterically- favored six-membered transition state (McLafferty rearrangement) followed by Cα-Cβ bond cleavage. Fragment at m/z 87 indicates loss of a 183 mass $C_{13}H_{27}$ ion; m/z 129 (M^+- $C_{10}H_{21}$) of 141 mass; m/z 157 (M^+- C_8H_{17}) of 113 mass; m/z 171(M^+- C_7H_{15}); m/z 185(M^+- C_6H_{13}); m/z 213(M^+- C_4H_9); m/z 227(M^+- C_3H_7); m/z 241(M^+- C_2H_5). Other homologous series of related ions at m/z 87, 101, 115, 129, 143, 157,171,199 etc, formed by losses of neutral aliphatic radicals, of the general formula $[(CH_2)n\ COOCH_3)]^+$ indicate no occurrence of other functional groups in the chain (figure 2b). In general, the ions at m/z 270, 239, 227, 74, and 241 were the major product ions identifying the compound as Hexadecanoic acid methyl ester (**3a)**, of formula $C_{17}H_{34}O_2$, as shown in scheme 2a. This fragmentation pattern is consistent with interpretation by other authors [26, 27].

Identity of compound represented by peak at $\mathbf{t_R}$ 11.15 min in methanol fraction was determined by diagnostic ion peaks at m/z 79, 93, 173, 236, and 292. Ion at m/z 79 was the base peak. Ion at m/z 108 is an omega ion which defines methyl esters of polyunsaturated fatty acids with an *n*-3 terminal group**.** Fragment ion at m/z 236 is an alpha ion defining Δ9, 12 carbons as the position of first double-bond. This ion is formed by a cleavage at the carboxyl end of unsaturated fatty acid to produce a fragment containing two double bonds and a second methylene group (minus a proton). The molecular ion peak at m/z 292 together with (M^+-31) for the loss of methoxyl group elements, and a hydrogen atom, was diagnostic for polyenoic fatty acid with methylene-interrupted unsaturation (figure 2c). These distinct ions were typical for an *n*-3 homo-allylic unsaturated fatty acid of molecular formula $C_{19}H_{32}O_2$, called 9(Z)12(Z)15(Z)-Octadecatrienoic acid, methyl ester (**3b**), (Scheme 2b).This data was in agreement with NIST library and literature reports [28].

Analysis of GC peak at $\mathbf{t_R}$ 11.25 min revealed fragment ion at m/z 278 in which H_2O is eliminated, thereby yielding a phytadiene ($C_{20}H_{39}$) ion. Ion at m/z 123 is typical of an acyclic-type hydrocarbon of the formula C_9H_{15}. Ion at m/z 69 (C_5H_9) is a cyclopentyl. Ion peak m/z 81(C_6H_9) is a 1-pentene-3- yl ion. These ions are characteristic of isoprene side chain. Fragment at m/z 95 (C_7H_{11}) corresponds to cyclohexene-1-methyl- ion. Intense ions at m/z 43, 57, 71 (base peak, C_5H_{11}), and 85 are characteristic of saturated alkanes. The molecular ion appeared at m/z 296, agreeing with the condensed formula $C_{20}H_{40}O$ (figure 2d). Together, this fragmentation pattern is typical of an acyclic C_{20} diterpene alcohol called phytol (**3c)** (scheme 2c). These mass features were consistent with library data, as well as literature reports [29]. Complete structures of compounds realized in the methanol fraction are as presented in Scheme 2d below.

Three major peaks were detected in the petroleum ether fraction at retention times 11.30 min, 11.60 min, 14.25 min, and 16.47 min. (Fig. 3a).

GC peak at $\mathbf{t_R}$ 16.47 min in petroleum ether fraction displayed a molecular ion at m/z 398, suggesting a structural formula

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

$C_{28}H_{46}O$. Loss of CH_3 from the molecule resulted in a 383 mass $C_{27}H_{43}O$ fragment. Other characteristic ions include 300(M^+-C_7H_{14}) generated for allylic cleavage with transfer of hydrogen, typical of unsaturated sterols in C_{22}; 273(M^+-side chain), 271(M^+-side chain-2H), 255(M^+-side chain -H_2O),229(M^+-fission of D ring-side chain-H) diagnostic for side chain in C_{17}, 213(fission of D ring-H_2O) and 145(M^+-H_2O- fission of C ring- methyl group) (figure 3b). In addition, the minor ion peak at m/z 246 is characteristic of Δ7-3-hydroxyandrostene templates, which, together with peaks at m/z 300 and 229 corresponding to unsaturation in C_{22}, confirm the identity of the compound as 7,22-ergostadienol (**4c)**, Scheme 3a. Overall structures of compounds in the petroleum ether fraction are represented as shown in scheme 3b, below.

Examination of GC elution order of VA compounds reveals some patterns that influence their behavior on GC. First, most low molecule weight compounds eluted earlier than high molecular weight compounds. For example, at 12.64 retention time ($\mathbf{t_R}$), compound **2a** (C_{22}, 370MW) eluted 1.62 min earlier than compound **2c** (C_{30}, 410MW). Compound **2b** (C_{22}, MW 337) eluted 0.1 min earlier than **2c**. This observation is the same for all compounds in the water extract, except for compound **2b** (C_{22}, MW 337) which eluted 1.52 min later than **2a,** despite its low molecular weight. This difference may be due to polar property of **2b**.

At 10.28 min, compound **3a** (C_{17}, 270 MW) in methanol fraction eluted 0.89 min and 0.97 min earlier than compounds **3b** (C_{19}, 292MW) and **3c** (C_{20}, 296 MW), respectively. A similar linear relationship exists for compounds in petroleum ether fractions, with compound **4a** (C_{20}, MW 296) at 11.30 min retention time, eluting 2.95 min and 5.17 earlier than compounds **4b** (C_{30}, 410MW) and **4c** (C_{28}, 398MW), respectively. Despite the lower molecular weight of **4c,** relative to **4b**, compound **4c** eluted at a higher retention time than **4b**. This difference may be attributed to low unsaturation of its side chain. Secondly, low molecular weight saturated compounds **2a** and **3a** eluted earlier than high molecular weight unsaturated compounds **2b- 4c**.These observations generally indicate a direct relationship between molecular weight or carbon length, and compound's retention time on GC. Straight chain fatty acid alcohols **3c** and **4a** eluted earlier than esters and terpenes of analogous structures **3b** and **4b**. Thirdly, unsaturated polycyclic compound **4c** was retained on GC longer than straight chain unsaturated terpenes **4a**, and **4b**, table 1. This may be attributed to the nature of its side chain groups.

3.2 Chemical composition

The chemical composition of VA fractions was studied by gas chromatography coupled to mass spectrometry. Constituent compounds were detected and their composition recorded as shown in Table 1. Our results showed the presence of aliphatic acid esters, terpene, diterpene alcohol and phytostanol in the fractions. Diterpene alcohol was the major chemical group in VA fractions. Phytol **3c, 4a** were the main diterpene alcohols in methanol fraction, and petroleum ether fraction. Terpenes, fatty acid amide and phytostanol were the least abundant groups in the fractions. Compound **2c**, a terpene, was detected in the aqueous fraction and **4b** in the petroleum ether fraction. Compound **2b**, a fatty acid amide, was found in aqueous fraction. Finally, compound **4c**, a phytostanol, was detected in petroleum ether fraction. These data demonstrate similarities and differences in the occurrence of compounds in *Vernonia amygdalina* fractions. The aliphatic acid

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

esters occurred in both aqueous and methanol fractions. Terpenes occurred in both aqueous and petroleum ether fractions but absent in the methanol fraction, with higher quantities present in the water fraction than in the petroleum ether fraction. Diterpene alcohols were obtained in both methanol and petroleum ether fractions with higher levels in petroleum ether fraction than in methanol fraction. Fatty acid amide and phytostanol occurred in the water and petroleum ether fractions.

3.3 Cell growth Inhibition

MDA-MB-231 human breast cancer cells exhibited significant VA-induced suppression of proliferation. A concentration-dependent inhibition of cell growth, relative to 100% control, was observed for all fractions. At equal concentration of 0.01 mg/mL aqueous, methanol and petroleum ether fractions inhibited cell growth by 11%, 45%, and 70%, respectively. At 0.1 mg/mL, the fractions inhibited growth by 13.7%, 55.2% and 79.4%, respectively, while at 0.5 mg/mL cell growth inhibition was by 23.5%, 69.7%, and 83.6%, respectively. At the highest concentration (1mg/mL), fractions induced growth inhibition by 71.2%, 70.0% and 97.1%, respectively. In general, at equal concentration of 0.01-1.0 mg/mL, aqueous fraction elicited lower inhibition rate, representing 11-23 % ($P < 0.05$) of total inhibition, except at 1 mg/mL (71% ;$P < 0.001$). In contrast, methanol and petroleum ether fractions elicited greater inhibition, representing 45-70% ($P < 0.001$) and 70-97% ($P < 0.001$), respectively, at equal concentrations (0.01-1mg/mL), figure 4.

4. DISCUSSION

As a source of anti-cancer agents, plants and other natural products with medicinal properties continue to be active areas of research, offering leads to the development of conventional drugs [30, 31]. Improved interest and growth of natural product-based drug discovery has been influenced by high costs, side effects, and therapeutic implications of conventional medicine [32, 33].

Sample analyses by GC-MS showed compounds such as squalene (**2c**) represented by peak at 14.26 min in the water fraction. Squalene is a terpenoid and a biological precursor of steroids with activities against colon, lung and skin cancers. It is also a potent cytoprotective agent against chemotherapeutic toxicities [34-38]. 13(Z)-decosenamide (erucamide) (**2b**), also detected in the water fraction is a fatty acid amide known to enhance neovascularization in regenerating skeletal muscle [39, 40], and modulate water balance in the visceral organs and in the cerebrospinal fluid [41].

Phytol (**3c**) in the methanol and petroleum ether fractions is a diterpene alcohol which functions as a precursor for Vitamins E and K1 and an antioxidant and a preventive agent against epoxide-induced breast cancer carcinogenesis [42]. It's also an effective vaccine adjuvant with no adverse auto-immune effects [43]. Hexadecanoic acid methyl ester (**3a**), also known as Methyl palmitate, in the methanol fraction is an aliphatic acid ester reported to cause growth inhibition and apoptosis induction in human gastric cancer cells [44]. 9(Z)12(Z)15(Z)-octadecatrienoic acid, methyl ester (**3b**), also known as α- linolenic acid methyl ester, is an aliphatic acid ester known to inhibit proliferation of ER-positive and ER-negative breast cancer cells [45]. It is also a potent antiangiogenic agent in colorectal cancer and in HUVEC cells [46]. 7, 22 ergostadienol (**4c**) detected in petroleum ether fraction is a phytostanol of the brassicasterol family reported to inhibit

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

promotion of carcinogenesis in urinary bladder [47].

Extraction and sequential fractionation of VA water extract by solvent partitioning significantly increased inhibition rate of cancer cell growth. The original extract (water) exerted growth inhibition ($P < 0.05$) of 11-23 % at 0.01mg/mL. Further fractionation of water extract to obtain methanol fraction improved the rate of inhibition by 45-70% ($P < 0.001$) at similar concentrations. Successive fractionation and purification of methanol fraction to afford petroleum ether fraction yielded greater inhibition rate of 70-97% ($P < 0.001$) at same concentrations. We may conclude that petroleum ether and methanol can differentially extract active molecules capable of acting synergistically to elicit stronger cell growth inhibition compared to water-soluble extract. It is also likely that variation in quantity, quality and activity of chemical components in VA fractions may account for the differences observed in growth-inhibitory activities of the fractions.

5. CONCLUSION

Vernonia amygdalina is a dietary medicinal plant used for many diseases and infections. Phytochemical analysis by GC-MS revealed presence of fatty acid esters, fatty acid amide, terpenoids, diterpene alcohols and phytostanol as major compound groups in the aqueous, methanol and petroleum ether fractions. Compositional variation in quantities, qualities and structural features may influence compounds' behavior on GC-MS, as well as bioactivities of their precursor fractions. These fractions displayed concentration-dependant growth inhibition activities on hormone-independent MDA-MB-231 breast cancer cells, *in Vitro*, with organic fractions eliciting higher growth inhibition than aqueous fraction, further indicating a direct relationship between serial fractionation and activity. The identified compounds in this study have important pharmacological functions against various neoplastic diseases. Studies aimed at further isolation to identify additional components and determine mechanism of action of *Vernonia amygdalina* compounds would be necessary.

6. ACKNOWLEDGEMENT

This research was supported in part by HHMI professors grant and conducted in the Howard Hughes Research Core Facility, Department of Biology, and the Environmental Chemistry Core Facility, Department of Chemistry at Howard University.

7. REFERENCES

[1] Huffman, M.A., Seifu, M., *Primates*. 1989, 30, 51-63.

[2] Moundipa, F. P., Kamini, G., Melanie, F., Bilong, F. C., Bruchhaus, I, *African J Trad CAM*. 2006, 2, 113-121.

[3] Akah, P. A., Ekekwe, R. K., *Fitoterapia*.1995,66, 352-355.

[4] Akah, P. A., Okafor, C. L., *Phytother Res*.**1992**, 6, 171-173.

[5] Huffman, M. A., Page, J. E., Sukhdeo, M. V., Gotoh, S., Kalunde, M. S., Towers, G.H., *International J of Primatology*.**1996**, 72, 475–503.

[6] Hamill, F.A., Apio, S., Murbiru, N. K., Masango, M., Bukenya-Ziraba, R., Maganyi, O. W., Soejarto, D. D., *J. Ethnopharmacology*. **2000**, 70, 281-300.

[7] Olajide, O. A., Makinde, J. M., Awe, S. O., *Fitoterapia*. **1999**, 70, 161-165.

[8] [Akinpelu, D. A., *Fitoterapia*. **1999**, 70, 432-234.

[9] Hladik, C., Krief, S., Haxaire, C., *Journal of Ethnopharmacology*. **2005**, 101,1-5.

[10] Iwalokun, B. A., Efedede, B. U., Alabi-sofunde, J. A., Oduala, T., Magbagbeola, O. A., Akinwande, A. I., *J. Med. Food*. **2006**, 4, 524-130.

[11] Kupchan, S. M., Hemingway, R. J., Karim, A., Werner, D., *Journal of Organic chemistry*. **1969**, 34, 3908-3911.

[12] Jasaka, M., Ohigashi, H., Takagaki, T., Nozaki, H., Tada, T., Hiroto, M., Hirie, R., Huffman, M.

ISSN:0976-0172
Journal of Bioscience And Technology
www.jbstonline.com

A., Nishida, T., Kaji,, K. K., *Tetrahedron*.**1992**, 4, 625-632.

[13] Izevbigie, E. B., *Exp. Biol. Med.* **2003**, 228, 293-298.

[14] Oyugi, D. A., Luo, X., Lee, K. S., Hill, B., Izevbigie, E. B., *Exp. Biol. Med.* **2009**, 4,410-417.

[15] Izevbigie, E. B., Bryant, J. L., Walker, A., *Exp. Bio. Med.* **2004**, 229, 163-169.

[16] Opata, M., Izevbigie, E. B., *Int. J. Environ. Res. Public Health.* **2006**, 2, 174-179.

[17] Sayed, M. D., Zaki, A. Y., El-Marzabai, M. M., Doss, S. L., *Egypt J Pharmaceut Sci*. **1980**, 21, 125-130.

[18] Cimanga, R. K., Tona, L., Mesia, K., Musuamba, C. T., De Bruyne, T., Apers, S., Hernans, N., Miert, V. S., Pieters, L., Totte, J., Vlietink, A. J., *Journal of Ethnophamacology*. **2004**, 93, 27-32.

[19] Harborne, J. B., *Phytochemical method*, Chapman and Hall, London, **1973**; pp. 278.

[20] Monroe, E. W., Mansukh, C. W., Govindranjan, M., Philip, A., Harold, T., Thomas, J. H., Warner, J., MacGivney, R. *Journal of natural products.* **1988**, 5, 1084 -1091.

[21] Lin, Y. L., Juan, I. M., Chen, Y. L., Liang, Y. C., Lin, J. K., *J. Agric. Food. Chem.* **1996**, 44, 1387-1394.

[22] Wanzel, U., Kunz, S., Brendel, M. D., Daniel, H., *Cancer Res*. **2000**, 60, 3823-3831.

[23] Luo X, Oyugi D, Lin C, Izevbigie B, Lee S., *Exp.Biol. Med.* **2010**. 235(12):1472-8.

[24] [24] Sun, S., Gao, Y., Ling, X., Lou, H., *Analytical Biochemistry*.**2005**, 336, 39-45.

[25] Auwarter, V., Kiebling, B., Pragst, F., *Forensic science international*. **2004**, 145, 149-159.

[26] Lin, H.W., Liu, C., Cao, D., Chen, P., Chen, M., Lin, S., Mozayan, M., Chen, A.F., Premkumar, L. S., Torry, D.S., Lee, T.J., *PNAS.* **2008**, 105, 19526-19531.

[27] Burdi, D.K., Hasan,M., Ahmad, V.U., *Pakistan Journal of Pharmaceutical Sciences*.**1990**, 3(2)33-37.

[28] Kaya, K., Morrison, L.F., Codd, G.A., Metcalf, J.S., Sano, T., Takagi, H.,Kubo, T., *Molecule*.**2006**, 11, 539-548.

[29] Ekeberg, D., Jablonska, A.M., Ogner, G., *Environmental pollution*.**1995**, 89, 55-58.

[30] Cragg, G. M., Newman, D. J., *Journal of ethnopharmacology.* **2005**, 100, 72-79.

[31] Mans, D. R., Da Rocha, A. B., Schwartsmann, G., *The Oncologist.* **2000**, 5, 185-198.

[32] Calixto, J. B., *Braz J Med Biol Res.* **2000**, 2, 179-189.

[33] MacLennan, A. H., Wilson, D. H., Taylor, A. W., *Preventive medicine*. **2002**, 35, 166 173.

[34] Rao, C. V., Newmark, H. L., Reddy, B. S., *Carcinogenesis.* **1998**, 2, 287-290.

[35] Smith, T. J., Yang, G., Seril, D. N., Liao, J., Kim, S., *Carcinogenesis.* **1998**, 4, 703-706.

[36] Das, B., Antoon, R., Tsuchida, R., Lotfi, S., Morozova, O., Farhat, W., Malkin, D., Koren, G., Yeger, H., Baruchel, S., *Neoplasia.* **2008**, 10, 1105-1119.

[37] Murakoshi, M., Nishino, H., Tokuda, H., Iwashima, A., Okuzumi, J., kitano, H., Iwasaki, R., *Int. J. Cancer.* **1992**, 6, 950-952.

[38] Senthilkumar, S., Devaki, T., Manohar, B. M., Babu, M. S., *Clinica Chimica Acta.* **2005**, 364, 335-342.

[39] Mitchel, C., Davies, M., Grounds, M., Mc Geachie, J., Crawford, G., Hong, Y., Chirila, T., *Journal of biomaterials application*.**1996**, 3, 230-249.

[40] Wakamatsu, K., Masaki, T., Itoh, F., Kondo, K., Sudo, K., *Biochem. Biophys. Res. Commun.* **1990**, 168, 423-429.

[41] Hamberger, A., Stenhagen, G., *Neurochem. Res*. **2003**, 28, 177-185.

[42] [42] Yu, F., Gapor, A., Bender, W., *Cancer Detection and Prevention.* **2005**, 29, 383-388.

[43] Lim, S., Meyer, M., Kjonaas, R. A., Ghosh, S. K., *Journal of Immune Based Therapies and Vaccines*. **2006**, 6, 1476-8518.

[44] Yu, F., Lian, X., Guo, H., Mc Guire, P., Li, R., Wang, R., Yu, F., *J. Pharm. Pharmaceut. Sci.* **2005**, 3, 528-535.

[45] Grossmann, M., Mizuno, N., Dammen, M., Schuster, T., Ray, A., Cleary, M., *Cancer. Prev. Res.* **2009**, 2, 879-886.

[46] Tsuzuki, T., Kawakami, Y., *Carcinogenesis.* **2008**, 4, 797-806.

[47] Yazawa, Y., Yokota, M., Sugiyama, K., *Bio Pharm. Bull*. **2000**, 23, 1298-1302.