



Original Paper

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Another anticancer elemanolide from *Vernonia amygdalina* Del

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ABSTRACT

A number of antitumor and other bioactive compounds were previously isolated from *Vernonia amygdalina* Del. This study was designed to further isolate and characterize compounds of medicinal value that may also have antitumor activity from this edible and commonly available plant. Bioassay-guided fractionation of the leaf extract of *V. amygdalina* (MEVA) led to the isolation and characterization of a known compound, epivernodalol for the first time in this plant. Its structure was identified by spectroscopic methods including ¹H-NMR, ¹³C-NMR, MS, UV and IR spectra. *In vitro* growth inhibitory and cytotoxic evaluation of MEVA, its fractions and epivernodalol against HT-144 (skin melanoma) cell line was carried out by the Sulforhodamine B (SRB) assay. The results showed that epivernodalol and the dichloromethane fraction of *V. amygdalina* were active against HT-144 (skin melanoma) cell line. *Vernonia amygdalina* Del. leaf extract yielded another cytotoxic which was active against skin cancer.

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Keywords: Epivernodalol, fractionation, characterization, sesquiterpenes, spectroscopy, melanoma.

INTRODUCTION

Globally, current treatment approaches have yielded significant progress in the fight against cancer, though the

incidence of certain types of cancer continues to rise (Izevbigie et al., 2004). The major treatment regime for cancer includes chemotherapy, radiotherapy and surgery.

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However, most of the synthetic chemotherapeutic agents have serious side effects like hair loss, prolonged nausea and vomiting, bone marrow suppression, and even secondary malignancies among others. Hence the search for natural products against cancer, potent, but with fewer side effects, especially those derived from plant becomes more intensified (Abdulaev, 1993; Pezzuto, 1997).

The genus *Vernonia* belongs to the family Asteraceae (Compositae) which has been found to contain sesquiterpenes lactones, glaucolides and elemanolides (Rodriguez et al., 1976; Bingel and Fransworth, 1994), some of which have shown tumor inhibiting activities (Kupchan et al., 1969a, 1969b). A number of elemanolide lactones have been isolated from *Vernonia* species which exhibited cytotoxic activities (Koul et al., 2003; Williams et al., 2005). *Vernonia amygdalina* Del. grows as a small tree in the East, West and South of tropical Africa. It is called "bitter leaf" plant because of the taste of its leaves. The leaves are used as vegetable and spice while the juice serves as a tonic (Igile et al., 1995; Igile et al., 1994).

In the south west of Nigeria, folklore medical practitioners have found its leaves useful in the treatment of various ailments such as malaria, bacterial infections, helminthiasis, sexually transmitted diseases, constipation, diabetes mellitus, anorexia, fungal infections and even in gynaecological applications. Some of these uses have been scientifically verified experimentally and documented by various workers, (Izevbigie et al., 2004; Igile et al., 1994; Ganjian et al., 1983; Akinpelu, 1999; Taiwo et al., 1999; Abosi and Raseroka, 2003; Kambizi and Afolayan, 2001; Alawa et al., 2003; Izevbigie, 2003; Awe et al., 1999; Alabi et al., 2005). Some of the previously isolated constituents in *V. amygdalina* include sesquiterpene lactones (Kupchan et al., 1969a; Erasto et al., 2006), saponins (Igile, 1994), flavonoids (Igile et al., 1995), steroid glycosides and vernoniosides (Igile et al., 1994; Jisaka et al., 1992), etc.

Orthodox medical facilities are relatively expensive and not easily accessible especially in poor countries, so interest in the search for antitumor agents of plant origin, is increasing, especially now that phytochemicals have been noted to play an active role in cancer prevention and treatment (Igile et al., 2004). This study was designed to investigate the leaf extract of *V. amygdalina* with the view to isolate and characterize some yet unidentified constituents for potential biological activity against cancer.

MATERIALS AND METHODS

General

The melting point was recorded on a Butchi 535 (Switzerland) apparatus, and is uncorrected. EI-MS spectra were recorded on JEOL MSRoute mass spectrometer. FAB mass spectra were recorded on a Varian MAT 312 mass spectrometer. Infrared spectra were obtained on Vector 22, Bruker spectrophotometer on KBr pellets. Ultraviolet-Visible spectra were recorded on Hitachi U-3200 (Japan) spectrophotometer. Optical rotations were measured on a digital polarimeter JASCO DIP-360 in methanol. The $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra were recorded on Bruker AV 400 spectrometer operating at 400 ($^1\text{H-NMR}$) and 100 ($^{13}\text{C-NMR}$) MHz.

The 2-D studies namely: COSY, NOESY, HMQC, and HMBC spectra were recorded on Bruker AV 400 spectrometer operating at 400 ($^1\text{H-NMR}$) and 100 ($^{13}\text{C-NMR}$) MHz.

Chemical

Chemicals and reagents were purchased from Sigma Chemical Co. USA. Materials for TLC were obtained from E. Merck, Germany. All other materials used were of the highest analytical grade.

Plant material

The leaves of the plant, *Vernonia amygdalina* Del (bitter leaf) belongs to the Asteraceae family. The leaves used in this study were harvested at a farm in Ibadan, Nigeria, in May, 2006. Botanical

identification and authentication was done at the Forest Research Institute of Nigeria, Ibadan, Nigeria, where the voucher sample number FHI 107408, was deposited for reference. The leaves were rendered pesticide-free, after treatment at the Plant Quarantine Service of the Federal Department of Agriculture, Ibadan, Nigeria. Thereafter, 4.55 kg of the air-dried and pulverized leaves were packed into white polythene bags with the opened end sealed.

Extraction and isolation

The leaves (4.55 kg) were extracted with MeOH (3 x 17 L) at room temperature over a period of three weeks. The cumulated solvent was evaporated with a Rotary Vacuum Evaporator (Eyela N.21, Tokyo) to afford a methanolic extract of *V. amygdalina* (MEVA) weighing 700 g, a yield of 15.4%. 500g of MEVA was then suspended in distilled water (1.5 L) and thereafter successively partitioned with petroleum ether, CH₂Cl₂ (dichloromethane), EtOAc (ethyl acetate fraction), and finally butanol, to give the petroleum ether (VAP), dichloromethane fraction (VAD), ethyl acetate fraction (VAE), and butanolic fractions (VAB) respectively. The extraction procedure is illustrated in Figure 1.

From the VAD fraction, 28.1 g was set aside for further separation and purification using column chromatography. The VAD was diluted with CH₂Cl₂ and silica gel. This mixture was then applied on a glass column (87 cm x 3.5 cm i.d) which was packed with silica gel (E. Merck, 230-400 µm mesh) using gradient mixtures of MeOH in CH₂Cl₂ (0 →100%) as mobile phases. These afforded 149 fractions (F1-F149). Fraction numbers 18 to 21 eluted with 0.2% MeOH in CH₂Cl₂, and was noted to become cloudy a few minutes after collection and concentration on a Rotavapor machine forming a white solid on evaporation of the solvent. This amorphous white solid was further purified by precipitation from petroleum ether and was then filtered with Whatman's filter paper from the mother solutions, and finally exposed to

dry in the fume hood. This procedure afforded a white powdery solid.

On each of the solution of these samples, TLC was carried out on precoated silica gel F₂₅₄ aluminium sheets (Merck, Germany) which were then developed with MeOH/CH₂Cl₂ mixtures of different polarities (1:99, 2:98, 3:97, and 5:95). Spots were detected by exposure of the TLC sheets to UV 254 and 366, and by spraying with ceric sulphate spray reagent followed by heating for about two minutes. The result showed a single spot with R_f: 0.42. This white powdery substance was designated compound VA-I, and it weighed 232.3 mg. VA-I was then subjected to various spectroscopic experiments, and the melting point was also determined.

Anticancer activity of MEVA, the extracts, and epivernodalol

The antiproliferative Sulforhodamine B (SRB) assay was used for the *in vitro* growth inhibitory and cytotoxic evaluation of the *Vernonia amygdalina* methanolic extract (MEVA), and three fractions, namely VAP, VAD, VAB, and epivernodalol against human skin melanoma HT-144 cell line. This colorimetric assay estimates cell number indirectly by staining total cellular proteins with the dye SRB, and the assay was carried out according to the methods earlier described (Skehan et al., 1990; Monks et al., 1990). Doxorubicin was used as positive control.

Statistical analysis

All data are reported as mean ± SD of the mean, and the Student *t*-test was used to determine the difference between test and control preparations. Significance was attributed to probability values $P \leq 0.05$. The IC₅₀ values were calculated using Excel based program.

RESULTS

General

In this study, the dried and pulverized pest-free leaves of *Vernonia amygdalina* Del, collected from Ibadan, Nigeria, were

exhaustively extracted with methanol to give the methanolic extract of *Vernonia amygdalina* Del, (MEVA). Further partitioning produced the fractions namely: petroleum ether gave VAP, dichloromethane gave VAD, ethyl acetate gave VAE, and n-BuOH gave VAB fractions. From the VAD fraction, the compound VA-1 was isolated through column chromatography by gradient elution.

Identification of compound VA-1

The melting point was 131.5 – 132.5 °C. The optical rotation was $[\alpha]_D^{27} + 76.6^\circ$ (c 0.128, MeOH). The compound gave an EI-MS molecular ion peak at m/z 392, while its FAB-MS +ve exhibited the $(M-H)^+$ ion at m/z 393. Both data are consistent with molecular formula $C_{20}H_{24}O_8$. Its IR absorptions displayed characteristic bands which implied the presence of vinylic (1630 cm^{-1}) and hydroxyl ($3482, 3433\text{ cm}^{-1}$) functionalities (Koul et al., 2003; Ganjian et al., 1983; Loudon, 2002). Other prominent bands were the presence of terminal methylene (1691 cm^{-1}), methoxycarbonyl (1711 cm^{-1}) and α, β -unsaturated δ -lactone (1727 cm^{-1}) functionalities (Loudon, 2002). The UV-Vis spectra exhibited a λ_{max} at 205 nm. All the structures were determined on the basis of the spectral data ($^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and 2D COSY, NOESY, HMQC, HMBC) and, for identification, they were compared with published data of Koul et al. (2003), which is epivernodalol. Epivernodalol is the C-10 epimer of vernodalol, an elemanolide, first isolated by Asaka et al. (1977) from *V. amygdalina*. While epivernodalol was first isolated in *Vernonia lasiopus* by Koul et al. (2003) in India utilizing the ethanolic extract, **VA-1** was isolated from the leaves of *V. amygdalina*, native to Ibadan, Nigeria, in West Africa, utilizing the dichloromethane fractions. According to current literature search and to the best of our knowledge, **epivernodalol** has been isolated from *V. amygdalina* for the first time by this present study. The elucidated structure of compound VA-1 is shown in Figure 2.

Anticancer activity of MEVA, the extracts, and epivernodalol

The anticancer studies on the above samples showed that the VAB fraction exhibited 37% growth inhibition, and was not evaluated further. The extract MEVA, VAD and VAP fractions, as well as epivernodalol which showed better growth inhibitory and cytotoxic activities against the HT-144 (skin melanoma) cell line, and were further tested at 5 doses to obtain their GI_{50} , TGI and LC_{50} values. Table 1, shows that both VAD and VAP fractions and epivernodalol exhibited highly significant growth inhibitory as well as cytotoxic activities when compared with the methanolic extract respectively, ($p \leq 0.01$). Also shown in Table 1, is the evidence that the VAD fraction and epivernodalol demonstrated similar growth inhibitory activity. However, the VAD fraction demonstrated significant cytotoxic activity when compared with epivernodalol which was isolated from the VAD fraction ($p < 0.05$) as shown in Figure 3.

DISCUSSION

The isolation of the compound, *epivernodalol* from the dried and pulverized pest-free leaves of *Vernonia amygdalina* Del, demonstrated the observation of the presence of sesquiterpene lactone from the *Vernoniae* family (Rodriguez, 1977). Epivernodalol has been reported only in the *Vernonia lasiopus* (Koul et al., 2003), this represents the first report of the compound in *V. amygdalina* according to current literature search.

The results of the anticancer studies showed that although the VAD fraction as well as epivernodalol demonstrated similar growth inhibitory activity, the VAD fraction demonstrated better cytotoxic activity against the HT-144 (skin melanoma) cell line when compared with epivernodalol which was isolated from the VAD fraction ($p < 0.05$). This finding suggests the presence of yet unidentified cytotoxic in the VAD fraction.

The significance of the findings in this study is that although other antitumour compounds have previously been isolated

from *V. amygdalina* Del. including *vernodalol* and *vernomygdin* (Kupchan et al., 1969b), this present study has demonstrated the possibility of the presence of yet another unidentified cytotoxic (epivernodalol) in the plant for the first time. Although a previous study had reported the activity of epivernodalol against colonic, cervical and breast cancer (Koul et al., 2003), this appears to be the first report of this additional information of the demonstration of epivernodalol's activity against human skin cancer (HT 144, melanoma cell line), according to current literature search. This report is also significant since plant-derived products are currently enjoying consideration as sources for the discovery and development of cancer chemoprotective and chemotherapeutic agents (Abdulaev, 1993). The result of this study may be beneficial to skin cancer studies when cognizance is taken of the results of Jemal et al. (2008), who reported that the estimated

new skin cancer cases by sex in United States in 2008 was 5.1% in males and 4.3% in females. Similarly, the estimated deaths in the same period for skin cancer by sex were 2.5% in males and 1.4% in females. Research findings that find application to practical solution in skin cancer management are indeed welcome.

The mechanism of this anticancer activity demonstrated by MEVA and its fractions were not specifically studied in this work. However, Izevbigie et al. (2003) and Izevbigie (2004) had reported that the water soluble extract of *Vernonia amygdalina* leaves showed anti-cancer activity against human breast tumor cells (MCF-7). This activity was reported to have been due to concentration-dependent inhibition of DNA synthesis and of cell proliferation. Since DNA synthesis is required for cancer cell growth, this ability to inhibit DNA synthesis by *Vernonia amygdalina* leaf extract may be

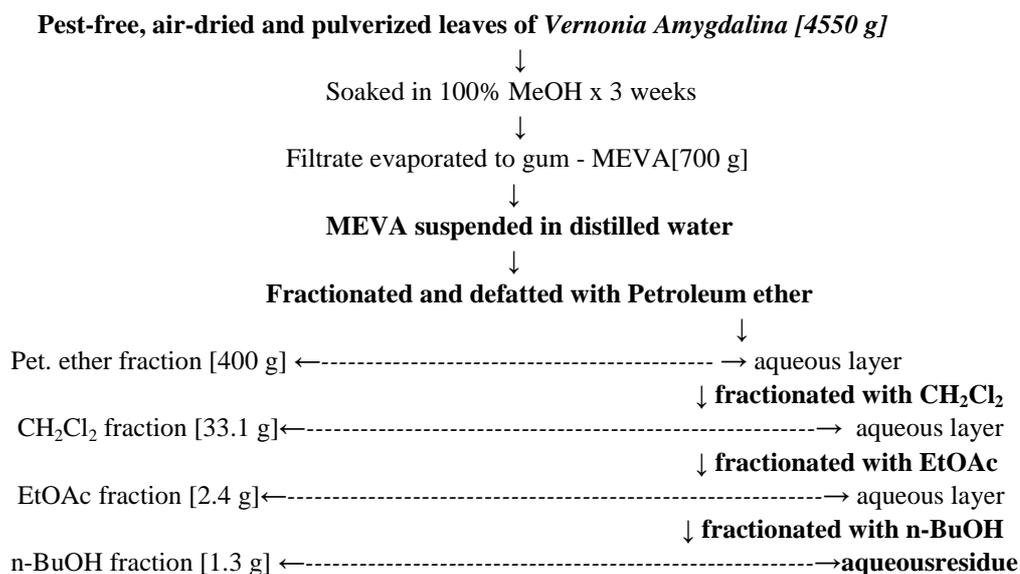


Figure 1: Extraction of the leaves of *Vernonia Amygdalina* and fractionation.

MEVA = methanolic extract of *Vernonia Amygdalina*

CH₂Cl₂ = dichloromethane

EtOAc = ethyl acetate

n-BuOH = butanol

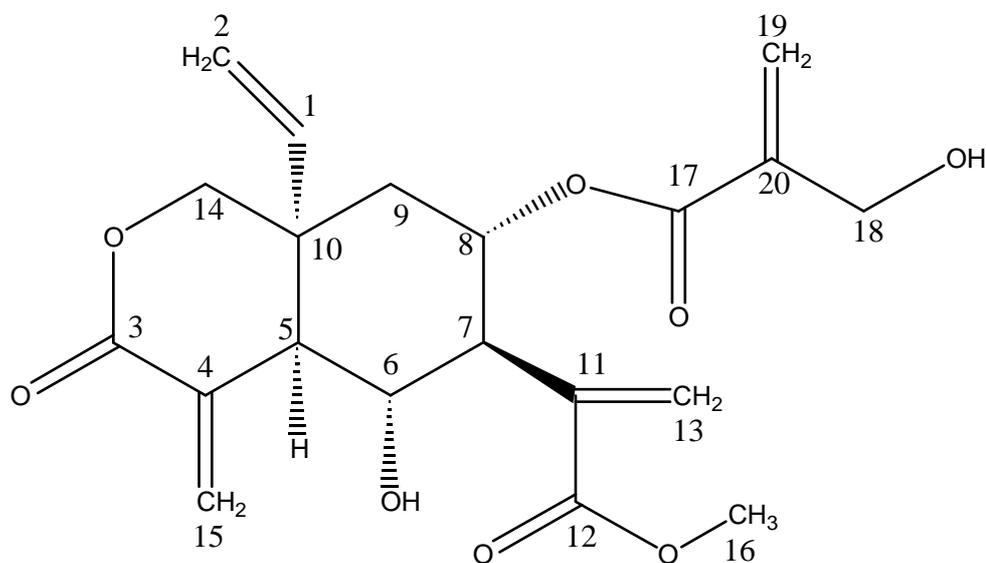


Figure 2: The elucidated structure of compound VA-1.

Table 1: Activity of *Vernonia amygdalina* Del. extract, fractions, epivernodalol and doxorubicin against HT-144 (skin melanoma) cell line.

Code	GI ₅₀ (µg/ml)	TGI (µg/ml)	LC ₅₀ (µg/ml)
MEVA (extract)	86 ± 1.3	141.3 ± 4.7	199 ± 10.8
VAD (fraction)	3.3 ± 0.3**	6.3 ± 0.3**	10.6 ± 0.8**
VAP (fraction)	9.7 ± 2.3*	21.2 ± 5.4*	37.7 ± 4.4*
Epivernodalol	1.76 ± 0.3**	7.33 ± 0.55**	22 ± 1.2**
Doxorubicin	0.01 ± 0	0.07 ± 0.03	0.48 ± 0.1

Each value represents the means ± SD of three independent experiments

* Significantly different from MEVA (p < 0.05).

** Significantly different from MEVA (p < 0.01).

GI₅₀ = Growth inhibition of 50 % of the cells; TGI = Total growth inhibition

LC₅₀ = Lethal concentration of the compound / extract that kills 50% of the cells.

MEVA: methanolic extract of *Vernonia amygdalina*; VAP: petroleum ether fraction; VAD: dichloromethane fraction.

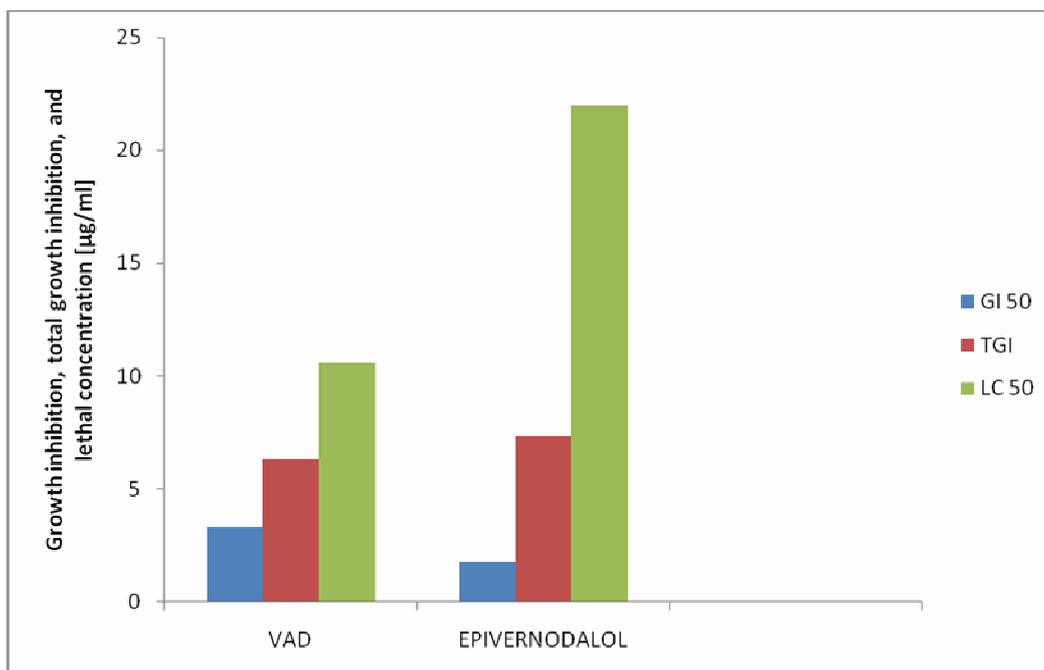


Figure 3: Comparison of the potency of activity of VAD and Epivernodalol as measured by the growth inhibition (GI_{50}), total growth inhibition (TGI), and lethal concentration (LC_{50}) against HT-144 (skin melanoma) cell.

Each value represents the means \pm SD of three independent experiments.

GI_{50} = Growth inhibition of 50 % of the cells.

TGI = Total growth inhibition.

LC_{50} = Lethal concentration of the compound / extract that kills 50% of the cells.

VAD: dichloromethane fraction.

considered an anticancer activity. That epivernodalol exhibited anticancer activity could be explained by its being a sesquiterpene lactone. Rodriguez et al., (1976) described the mechanism by which sesquiterpene lactones exhibit growth inhibition properties which depended on the structural configuration of the compound, namely the presence of an exocyclic methylene conjugated to the gamma-lactone, and the presence of a functional group such as hydroxyl or unsaturated ketone among other groups. A review of the epivernodalol molecule in Figure 2 shows that, epivernodalol has attached to its lactone an exocyclic methylene, and hydroxyl groups on carbons 6 and 18, in addition to the ketone groups attached to carbons 12 and 17. These properties might have qualified epivernodalol

to behave as a growth inhibitor, as demonstrated in its activity against human skin cancer in this experiment. The inhibitory action of sesquiterpene lactones results from the presence of highly electrophilic functional groups, which according to Rodriguez et al. (1976) selectively alkylate by Michael-type addition to sulphhydryl proteins, specifically thiol groups in preference to other nucleophiles.

Taken together, these results demonstrate not only the presence and anticancer activity of epivernodalol in *V. amygdalina* leaf extract against human melanoma skin cancer (HT-144), but also the observation of a better cytotoxic activity in the dichloromethane fraction as compared with the pure compound (epivernodalol). This suggests that this fraction be further,

investigated as it seems to possess other cytotoxic components besides epivernodalol.

ACKNOWLEDGEMENTS

The financial support given by the Faculty of Basic Medical Sciences and the College of Medicine, University of Ibadan, Nigeria to Dr. Olatunde Owoeye to visit the laboratories of H.E.J. Research Institute of Chemistry, and Dr Panjwani Center for Molecular Medicine and Drug Research, (I.C.C.B.S.), University of Karachi, Pakistan, is hereby gratefully acknowledged.

The authors hereby declare that there is no conflict of interest, be it actual or potential or financial or personal or other relationships with other people or organizations that could have inappropriately influenced this work.

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