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Full Length Research Paper

Cytotoxic compounds from the leaves of *Combretum paniculatum* Vent

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Combretum paniculatum **Vent. (Combretaceae) is a shrub that is widespread in tropical Africa. It is used locally in the treatment of carcinomous tumors. The cytotoxic activity of pheophorbide a and pheophorbide a-methyl ester isolated from the leaves of *C. paniculatum* were investigated. *In vitro* cytotoxicity of the compounds were evaluated against HT-29, MCF-7 and HeLa cancer cell lines using the methyl thiazolyl tetrazolium (MTT) assay and peripheral blood mononuclear cells. Cell cycle analysis was performed using propidium iodide staining and flow cytometry. Pheophorbide a was more toxic to MCF-7 and HeLa cells than its methyl ester, while the opposite result was seen in HT-29 cells. The methyl ester killed all the peripheral blood mononuclear cells from healthy donor blood while less inhibition was observed with pheophorbide a. Both compounds induced cell cycle arrest in HeLa cells in the G_0/G_1 phase, with pheophorbide a-methyl ester yielding a more enhanced G_1/G_0 arrest. After 24 h of exposure, the percentage of HeLa cells in the G_0/G_1 phase was 38.5% in vehicle control cells, 55.9% in pheophorbide a and 70.0% in pheophorbide a-methyl ester treated cells. The *in vitro* effect of *C. paniculatum* on cancer cell lines may be ascribed to the presence of pheophorbide a and its methyl ester. They exert this effect through the induction of cell cycle arrest in the G_0/G_1 phase of the cell cycle.**

Key words: Cytotoxicity, cancer, *Combretum*, pheophorbide a, pheophorbide a-methyl ester.

INTRODUCTION

Combretum paniculatum Vent. (Combretaceae), a scandent shrub or robust liane with vivid scarlet flowers attaining 15 m length is wide spread in tropical Africa. Traditionally, the leaf sap is used externally for gonorrhea in Tanganyika, while the galled leaves are ground with salt and the paste applied to the tongue and inside the mouth of babies with stomatitis in Ivory Coast. The leaves and the aqueous extract of the inflorescence have been reported to have action against carcinomous tumours (Burkill, 1985). The anti-HIV activity of the plant has also been reported (Asres et al., 2001). Antimicrobial compounds such as cholest-5-en-3-ol, 2-phyten-1-ol, gallocatechin and apigenin have been reported from the plant (Samdumu, 2007). There is however no report on the cytotoxic compounds from this plant.

In our earlier investigation of plants used in the management of cancer by traditional medical practitioners in South Western Nigeria, the ethanolic extract of the leaves of *C. paniculatum* showed significant cytotoxic activity against HeLa (cervical cancer), HT-29 (colon cancer) and MCF-7 (breast cancer) cells (Sowemimo et al., 2009,

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2011).

This present work reports the isolation and cytotoxic activity of two compounds (pheophorbide a and pheophorbide a-methyl ester) isolated from the leaves of *C. paniculatum* against colon (HT-29), breast (MCF-7) and cervical (HeLa) cancer cell lines *in vitro*, peripheral blood mononuclear cells (PBMCs) and their effect on the cell cycle.

MATERIALS AND METHODS

Leaves of *C. paniculatum* Vent. (Combretaceae) were collected from the Olokemeji Forest Reserve, Nigeria in July 2008 and authenticated by comparison with corresponding herbarium specimens at the Forestry Research Institute, Ibadan, Nigeria (FRIN) where a voucher specimen (FHI 107980) was also deposited. The plant was air dried for two days followed by drying in a hot air oven at 40°C and ground to powder.

Extraction and isolation

The powdered plant material (1 kg) was macerated with ethanol (3 L) at room temperature for 72 h. The ethanolic extract was concentrated *in vacuo* to get a dark green residue (24.8 g). This residue was suspended in water and partitioned successively with petroleum ether, EtOAc and n-BuOH. The yield was 9.5, 1.2 and 0.8 g, respectively. The EtOAc fraction was subjected to vacuum liquid chromatography (VLC) gradient elution with petroleum ether and ethyl acetate which gave 6 fractions. Fractions 3 (90 mg) and 4 (96 mg) were further subjected to column chromatography over silica gel eluting with petroleum ether : EtOAc (7:3) and (2:1), respectively, to yield C-1 (5 mg), a light green solid and C-2 (4 mg), a light green solid respectively.

Cytotoxicity assay

Cervical (HeLa), colorectal (HT-29) and breast (MCF-7) cancer cells were seeded at 6,000 cells/well in 96-well plates and left to attach overnight at 37°C in a humidified incubator and 5% CO_2. PBMCs (peripheral blood mononuclear cells) were isolated from venous blood of a healthy donor using heparinized Vacutainer® CPTTM cell preparation tubes (Beckton Dickinson, Plymouth, UK) within 30 min of collection. PBMCs were seeded at 100,000 cells/well in round bottomed 96-well plates. The isolated compounds were resuspended in DMSO (0.25%), sonicated for 15 min, and complete RPMI-1640 medium was added to reach concentrations of 62.5 to 250 µg/ml. Cisplatin (10 and 100 µM) was used as the reference drug. The cells were treated for 48 h after which the medium was replaced with 200 µl MTT (Sigma) (0.5 mg/ml in RPMI 1640:10% FBS). After a further 4 h incubation at 37°C, the MTT was removed and the purple formazan product was dissolved in DMSO and absorbance measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). CellTiter-Blue® assay (Promega) was performed according to manufacturer's instructions for the PBMCs and fluorescence was read at $544_{Ex}/590_{Em}$ using a Fluoroskan Ascent FL fluorometer (ThermoLabsystems, Finland). All experiments were performed in quadruplicate.

Cell cycle analysis

HeLa cells were seeded into 10 cm cell culture dishes (Nunc) at 1.15×10^5 cells per ml and incubated in a 37°C incubator supplemented with 5% CO_2 for 24 h. The isolated compounds (62.5 µg/ml) or cisplatin (50 µM) were added and the cells were incubated for a further 24 and 48 h. DNA cell cycle analysis was performed using the Coulter® DNA Prep™ Reagents Kit (Beckman Coulter). The assay was carried out as per kit instructions and the results were analyzed on a Beckman Coulter FC500 flow cytometer. MultiCycle software for cell cycle analysis was used to calculate the percentage of cells in each of the cell cycle phases.

RESULTS AND DISCUSSION

The phytochemical investigation of the leaves of *C. paniculatum* gave two known compounds, pheophorbide a (C-1) and pheophorbide a-methyl ester (C-2) established with the aid of NMR spectroscopic techniques in addition to comparison with data found in literature (Nozawa et al., 1992; Ohsima et al., 1994; Rho et al., 2003).

The comparative growth inhibition results (Figure 1) suggest that at 62.5, 125 and 250 µg/ml, pheophorbide a and pheophorbide a-methyl ester were most effective against MCF-7 breast and HeLa cervical cancer cell lines. In the HT-29 colon cancer cell line, pheophorbide a showed more than 50% inhibition only at 250 µg/ml, while pheophorbide a-methyl ester showed more than 50% inhibition at 125 and 250 µg/ml. Pheophorbide a was however more active than pheophorbide a-methyl ester in the other two cell lines tested. The results from the CellTiter Blue® assay on the PBMCs (Figure 2) suggested that pheophorbide a is less toxic than its methyl ester to normal cells.

HeLa cells were used to establish whether the two purified compounds induced cell cycle arrest. Cisplatin at 50 µM typically caused an accumulation of the cells in the S phase after 48 h of treatment (Table 1). A large increase in the size of the sub-G_1 peak, corresponding to apoptotic cells, was seen after 24 h of cisplatin treatment and the effect was more pronounced after 48 h. The two isolated compounds both induced cell cycle arrest in the G_1/G_0 phase. Although, pheophorbide a was more toxic to HeLa cells, the methyl ester caused a more enhanced G_1/G_0 arrest on the cell cycle after 24 h. The percentage of HeLa cells in the G_1/G_0 phase was 38.5% for the vehicle control, 55.9% for the pheophorbide a and 70.0% for the pheophorbide a-methyl ester treated cells (Table 1). After 48 h of treatment, about 80% of the cells were arrested in G_1/G_0 with both compounds. Interestingly, the sub-G_1 peak did not increase with either of the two compounds, even after 48 h.

Data demonstrating the anti-tumor activity of chlorophyll related compounds are not too common. However, pheophorbide a isolated from *Clerodendrum calamitosum* has been reported to display both strong and broad activity against epidermoid carcinoma of the nasopharynx (KB), ovarian carcinoma (1A9), kidney carcinoma (CA KI-1), malignant melanoma (SK-MEL-2), Ileocecal carcinoma

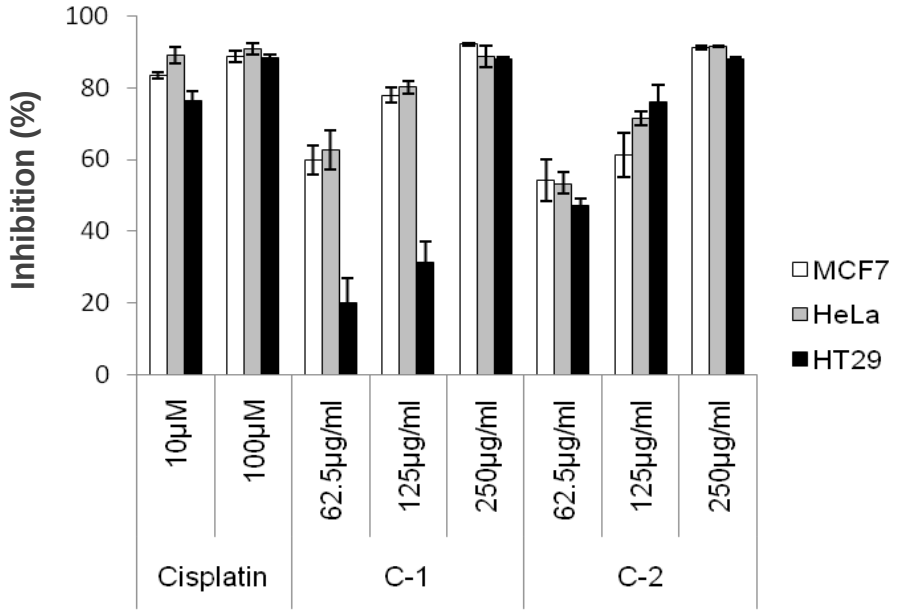

Figure 1. Screening results of compounds in MCF-7, HT-29 and HeLa cancer cells. Results represent the mean ± standard deviation of quadruplicate determinations. Cisplatin is the positive control.

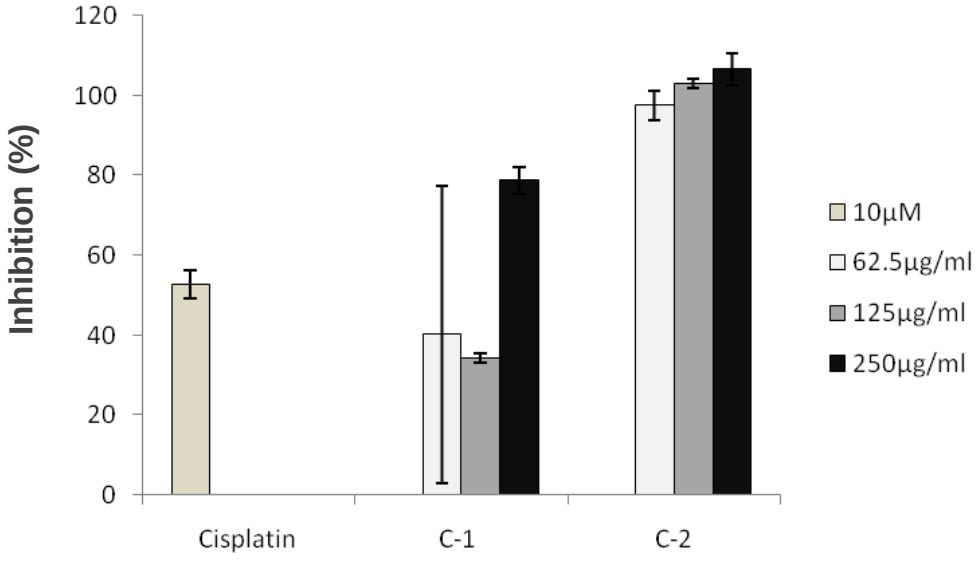

Figure 2. Percentage inhibition of compounds on PBMCs. Results represent the mean ± standard deviation of quadruplicate determinations. Cisplatin is the positive control. Pheophorbide a, C-1; pheophorbide a-methyl ester, C-2.

(HCT-8), human lung cancer (A549) and breast adenocarcinoma (MCF-7) (Cheng et al., 2001), while pheophorbide a-methyl ester from *Garuga pinnata* has been reported to show photo-dependent cytotoxic activity (Kashiwada et al., 1997).

Most previous studies have reported on the photodynamic activities of pheophorbide a and its derivatives (Tang et al., 2006) and not on its cytotoxic activity and mechanism of action in the absence of photo-irradiation. In a recent study by Mohan et al. (2010), fractions of an extract from *Typhonium flagelliforme* were shown to induce G_1/G_0 arrest and apoptosis in CEMss human T4-lymphoblastoid cells. The most active fraction contained, amongst other compounds, four pheophorbide related

Table 1. Effect of pheophorbide a and its methyl ester on the cell cycle distribution of HeLa cells after 24 and 48 h of exposure.

Exposure time (h)	Treatment	G_1/G_0 (%)	S (%)	G_2/M (%)
24	Vehicle control	38.5	48.6	12.9
	Cisplatin	34.4	41.8	23.8
	C-1 (pheophorbide a)	55.9	38.0	6.1
	C-2 (pheophorbide a-methyl ester)	70.0	6.2	23.9
48	Vehicle control	59.0	21.5	19.5
	Cisplatin	42.7	35.1	22.2
	C-1 (pheophorbide a)	78.2	13.8	2.0
	C-2 (pheophorbide a-methyl ester)	83.1	9.0	7.9

Vehicle control, cells treated with 0.125% DMSO; 50 µM cisplatin is the positive control; G_1/G_0, cells in the G_1 and G_0 phase of the cell cycle; S, cells in the synthesis phase of the cell cycle; G_2/M, cells in the G_2 and M phase of the cell cycle.

compounds including pheophorbide a and its methyl ester. The cell cycle arrest corresponds to the results from our study. The observation of apoptosis by Mohan et al. (2010) on CEMss cells and not on HeLa cells as in the present study could indicate that the compounds do not have identical effects in all cell types or the presence of other compounds in their extract were responsible for apoptosis induction. Chan et al. (2006) also reported induction of apoptosis by pheophorbide a in Hep3B hepatoma cells while being non-toxic in the normal human liver cell line WRL-68.

The cell cycle analysis results revealed that the two compounds act in a very similar way through induction of cell cycle arrest in the G_1/G_0 phase. No increase was observed in the sub-G_1 (apoptotic) peak and therefore, it appeared that the reduction in viable cell numbers as compared to vehicle control treated cells was due to inhibition of proliferation rather than cytotoxicity. This is the first report on the isolation of the two compounds, pheophorbide a and its methyl ester from *C. paniculatum* and the induction of cell cycle arrest in HeLa cells in the absence of photoactivation. Our results further suggested that pheophorbide a is a more potent inhibitor of proliferation than its methyl ester in at least two of the three cell lines investigated. Interestingly, the methyl ester derivative was more cytotoxic to PBMCs isolated from normal human blood.

The results obtained in this work further support the traditional use of the plant in the treatment of cancer.

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REFERENCES

Asres K, Bucar F, Kartnig T, Witvrouw M, Pannecoupe C, De Clercq E (2001). Antiviral activity against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) of ethnobotanically selected Ethiopian medicinal plants. Phytol. Res. 15: 62-69.

Burkill HM (1985). The useful plants of West Tropical Africa. 2nd Edn. Richmond, UK, Kew Royal Botanical Garden, London, p. 404.

Chan JY, Tang PM, Hon PM, Au SW, Tsui SK, Waye MM, Kong SK, Mak TC, Fung KP (2006). Pheophorbide a, a major antitumor component purified from *Scutellaria barbata,* induces apoptosis in human hepatocellular carcinoma cells. Planta Med. 72: 28-33.

Cheng H, Wang H, Ito J, Bastow KF, Tachibana Y, Nakanishi Y, Xu Z, Luo T, Lee K (2001). Cytotoxic Pheophorbide-Related Compounds from *Clerodendrum calamitosum* and *C. cyrtophyllum.* J. Nat. Prod. 64(7): 915-919.

Kashiwada Y, Fujioka T, Mihashi K, Chen IS, Katayama H, Ikeshiro Y, Lee KH (1997). Antitumor Agents. 180.[1] Chemical studies and cytotoxic evaluation of cumingianosides and cumindysoside A, Antileukemic triterpene glucosides with a 14,18-cycloapotirucallane skeleton. J. Nat. Prod. 60(11): 1105-1114.

Mohan S, Abdul AB, Abdelwahab SI, Al-Zubairi AS, Sukari MA, Abdullah R, Mohammed M, Taha E, Ibrahim MY, Syam S (2010). *Typhonium flagelliforme* induces apoptosis in CEMss cells via activation of caspase-9, PARP cleavage and cytochrome *c* release: Its activation coupled with G0G1 phase cell cycle arrest. J. Ethnopharmacol. 131: 592-600.

Nozawa T, Nishimura M, Hatano M (1992). Cross Polarization Characteristics in Solid State High Resolution ^{13}C NMR of Chlorophyll a and Pheophorbide a. Chem. Lett. 12: 2371-2374.

Ohsima T, Hirata M, Oda T, Sasaki A, Shiratsuchi M (1994). Pheophorbide a, a Potent Endothelin Receptor Antagonist for Both ET_A and ET_B Subtypes. Chem. Pharm. Bull. 42: 2174-2176.

Rho M, Chung MY, Song HY, Kwon OE, Lee SW, Baek JA, Jeune KH, Kim KK, Lee HS, Kim YK (2003). Pheophorbide A-methyl ester, Acyl-CoA: Cholesterol Acyltransferase Inhibitor from *Diospyros kaki*. Arch. Pharm. Res. 26: 716-718.

Samdumu FB (2007). Characterization of antimicrobial compounds from *Combretum paniculatum*, a plant with proven anti-HIV replication activity (Doctoral thesis, University of Pretoria, South Africa.

Sowemimo AA, Van de Venter M, Baatjies L, Koekemoer T (2009). Cytotoxic activity of selected Nigerian plants. Afr. J. Trad. Comp. Alt. Med. 6(4): 526-528.

Sowemimo AA, Van de Venter M, Baatjies L, Koekemoer T (2011).

Cytotoxicity evaluation of selected Nigerian plants used in traditional cancer treatment. J. Med. Plants Res. 5(11): 2442-2444.
Tang PM, Chan JY, Au SW, Kong SK, Tsui SK, Waye MM, Mak TC, Fong WP, Fun KP (2006). Pheophorbide a, an active compound isolated from *Scutellaria barbata*, possesses photodynamic activities by inducing apoptosis in human hepatocellular carcinoma. Cancer Biol. Ther. 5(9): 1111-1116.