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Cytotoxic activity of *Boswellia dalzielii* (Hutch) stem bark extract against head and neck squamous cell carcinoma of the tongue (AW8507 cell line)

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Abstract
Head and neck squamous cell carcinoma (HNSCC) represents the third most common cause of cancer deaths worldwide. In the past, several drugs with anticancer effects have been extracted from plants. The present research was designed to evaluate the cytotoxic potential of *Boswellia dalzielii* Hutch stem bark extract. *Boswellia dalzielii* is indigenous to West Africa and is used in ethnomedicine to treat gastrointestinal disorders and skin diseases among others. Numerous studies have investigated the antiproliferative effects of its congeners, but studies involving the *in vitro* cytotoxic effect of *B. dalzielii* extract are limited. Our objectives were to evaluate the cytotoxicity of the chloroform (CLBD); ethyl acetate (EABD); and petroleum ether (PEBD) fractions obtained from *B. dalzielii* stem bark ethanolic extract and their effect in the cell cycle of head and neck squamous cell carcinoma of the tongue (AW8507 cells). Cytotoxicity of CLBD, EABD and PEBD on AW8507 cells were revealed by MTT and clonogenic assays. Effects of the fractions on AW8507 cell cycle were investigated by flow cytometry. All the three *B. dalzielii* fractions were found to inhibit proliferation and colony formation; and arrested the AW8507 cell cycle in the G2/M phase.

Keywords: *Boswellia dalzielii*; Cytotoxicity; Cell cycle arrest.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) represents the third most common cause of cancer deaths worldwide and the fifth leading cause of death in the world, with an incidence of 500,000 new cases per year [1]. It involves the upper aerodigestive tract anatomic sites. The disease typically appears in the oropharynx, oral cavity, hypopharynx, or larynx. The vast majority (more than 90%) are squamous cell carcinomas [2]. Treatment options include the use of surgery, radiation, and/or chemotherapy [3]. The use of transoral laser assisted surgery accompanied by radiotherapy is a common practice in the treatment of early stage oropharyngeal, hypopharyngeal and supraglottic carcinomas, but radiotherapy is also a good alternative [4]. Primarily, radiochemotherapy is an alternative for patients with advanced head and neck carcinomas. However, emerging cancer drug resistance is a serious problem regarding chemotherapy [5,6]. Therefore, the

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development of novel anticancer drugs with lesser side effects and selective cytotoxicity, while also being cost-effective, has been one of the main thrust of cancer research worldwide.

Plants have formed the basis for treatment of diseases in traditional medicine for many years [7]. Several studies have investigated *Boswellia* genus (family Burseraceae), especially *Boswellia serrata* and *Boswellia carteri* for their anti-inflammatory, anti-leukotriene, anti-acetylcholinesterase, and anticancer activities [8-12]; however there is limited information on *Boswellia dalzielii* Hutch., the West African species of the frankincense genus. Boswellia trees are short, usually of compound leaves, papery bark and star-like flowers [13]. In Nigeria, the Hausa names include "Ararrabi", "Basamu" and "Hanu" [14]. It is used in ethnomedicine to treat gastrointestinal disorders, leprosy, septic sores, skin diseases, rheumatism and various diseases of microbial origin [15-17]. Phytochemical screening of the *B. dalzielii* revealed the presence of saponins, tannins, flavonoids, cardiac glycosides, steroids and terpenes [18,19]. The chemical components of *B. dalzielii* were also investigated by Alemika *et al*, [15] and have been reported to contain beta-sitosterol, resveratrol, gallic acid, and incensole. The cytotoxic activity of the acidic fraction of *B. dalzielii* gum resin was investigated by Alemika and Ojerinde [20]. The acidic fraction, when tested on brine shrimp lethality, showed very high activity (LC_{50} = 0.0013µg/mL) and triterpenoids were shown to be responsible [20]. The cytotoxicity of various extracts of *B. dalzielii* leaves had also been investigated in two ovarian tumour cell lines (OVCAR-3 and IVROV-1). The results showed that the cyclohexane, dichloromethane, and ethyl acetate extracts showed good inhibition, 92.60±1.40, 75.10±2.80, and 64.50±2.80 respectively [21]. The effects of *Boswellia dalzielii* stem bark was also recently investigated [22], from the results obtained; the antimicrobial/antioxidant activity of the stem bark was accounted for by isolated compounds – protocatechuic acid, gallic acid and ethyl gallate with minor contribution from a novel stilbene glycoside and a cembrane diterpenoid (incensole). More interestingly, 3-*O*-Acetyl-11-keto-β-boswellic acid (AKBA) found in other established specie, *B. serrata* [23] was also isolated from the dichloromethane fraction. The extracts were also reported to have demonstrated antifungal, anti-inflammatory, hypoglycemic and cytotoxic effects [22].

Thus, several medicinal uses have been attributed to *Boswellia dalzielii* (Hutch) stem bark, which prompted us to investigate the cytotoxic effect of its extract on an oral squamous cell carcinoma (OSCC) of poorly differentiated tongue cancer cell line AW8507 using MTT assay and colony formation assay. This was followed by cell cycle analysis using propidium iodide (PI) staining and flow cytometry.

EXPERIMENTAL

Plant Source. *Boswellia dalzielii* stem bark (BDB) was collected from Dutsen Hanwa area of Zaria, Kaduna state, Nigeria. The plant was identified and authenticated in Ahmadu Bello University, Biological Sciences Department by Mr. Namadi Sanusi. A voucher specimen was deposited with voucher number 900121.

Extraction and fractionation. Fresh *Boswellia dalzielii* stem bark (BDB) was washed with water, cut into small pieces, air dried at room temperature and powdered. The powdered material (2 kg) was extracted with 70% ethanol (50 g BDB/200 ml ethanol) at room temperature and evaporated to yield a dark-brown jelly (180 g). Further, the crude extract (19.05 g) was suspended in 50 ml of petroleum ether (3 times and the fractions pulled together), followed by chloroform and

lastly ethyl acetate successively. The fractions were concentrated under reduced pressure.

Cell culture. The poorly differentiated tongue cancer cell lines (AW8507) were generously obtained from the Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Kharghar, Navi-Mumbai, India. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics under 5% CO_2 at 37ºC.

Cytotoxicity assay. The cytotoxic effect of fractions obtained from the crude BDB against AW8507 cells was determined according to the method of Mosmann [24], with slight modifications. Briefly, cells were seeded into 96-well microplates at the density of 4 x 10^3cells/well. After 24 h of culture, the medium was removed and replaced with a fresh medium containing 1.6% Ethanol (vehicle control) or various concentrations (0, 5, 10, 20, 40, 80 and 160 µg/mL) of petroleum ether, chloroform and ethyl acetate fractions of BDB (PEBD, CLBD, and EABD respectively). Following 24 h incubation, the media was discarded, cells were washed with PBS once (thus removing the interference of the polyphenols with MTT assay) and 100 µl of MTT solution (0.5 mg/ml in serum and phenol red free DMEM) was added to each well, incubated for 4 h at 37ºC. Afterwards, the solution was discarded and 100µL of DMSO was added to each well and incubated in the dark for 1 hour. Thereafter, absorbance was measured at 570 nm. Three independent experiments were carried out for each fraction. The results were expressed as the mean percentage ± standard deviation (Mean % ± STDEV) of viable cells in comparison with the control cells. MTT test is based on the reduction of yellow soluble compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Chemical, Germany) into an insoluble formazan (blue star-shaped crystals). The reaction takes place in the mitochondrial membrane of living cells. Formazan was dissolved by addition of a strong detergent and color was read spectrophotometrically at a wavelength of 570 nm (cytation5 Cell imaging multi-mode Reader, Reader Serial Number 17081518, BioTek Instruments, Incorporation, Winooski, Vermont 05404-0998 USA). Absorbance of the solution is proportional to the number of living cells. Experiments were performed in triplicates.

Colony formation assay. The method of Franken *et al*, [25] was used. Briefly, cells were plated in 6-well plates, 500cells/well and incubated at 37ºC overnight before treatment with different extracts at 20 µg/ml. The treatments (20 µg/mL of CLBD, EABD and PEBD each, and 0.2% Ethanol vehicle control) were performed before cells began replicating. After treatment, the dishes were placed in an incubator and left for a time equivalent to at least six potential cell divisions. In this case, for 9 days, because the replication time for AW8507 cells is 32 h. This method was used for quick screening of the ability of the different treatments to inhibit colony formation of the cancer cells. After 24 hours treatment, the medium was aspirated and discarded, the wells washed with 1 X PBS and the cells were fixed with absolute ethanol for 20 minutes. The ethanol was removed and replaced with 5 mL 0.5% crystal violet and allowed to stand for a minimum of 30 minutes. The crystal violet was removed and the plates washed with tap water, left to dry overnight and the colonies with >50 cells were counted. The result is presented as surviving fraction (SF), as compared with the control. Experiments were performed in triplicates.

Flow cytometric analysis. The method of Ricardo and Nicoletti [26] was used to determine Cellular DNA content by flow cytometric analysis of propidium iodide (PI)-labeled cells. AW8507 cells (3×10^5 cells/well in 6-well plates) were incubated with Chloroform (CLBD), Ethyl Acetate (EABD), and Petroleum Ether (PEBD)

fractions of *Boswellia dalzielii* (20 µg/mL) for 24 h. The cells were then collected by trypsinization and fixed in ice-cold 70% ethanol at −20°C overnight. The cell pellets were re-suspended in 200 µl 1XPBS, 30µl (100 µg/mL) RNAse was added and vortexed briefly, then allowed to stand at room temperature for 20 mins. 300µl of PI solution (100µg/Ml in 1XPBS), was added and vortexed briefly while adding the PI Solution. (Staining was done in the dark). Prepared samples were allowed to stand in the dark for at least one hour. Cells were subjected to flow cytometric analysis using FACS Calibur flow cytometer (BD Biosciences, U.S.A.) and the cell cycle distribution was analyzed using ModFit LT for Mac v 3.0 (BD Biosciences) software. We used 488-nm laser line for excitation. Measured red fluorescence (4600 nm) and side scatter. The results were compared with the negative (untreated) control.

Statistical analysis. All experiments were carried out in triplicate, and results were expressed as mean± standard deviation (mean ± S.D). One-way ANOVA was used to compare differences among the groups. $p< 0.05$ was regarded as significant. Microsoft Excel Graph was used to produce the bar charts.

RESULTS AND DISCUSSION

Search for new anticancer drugs with lesser side effects and selective cytotoxicity has been a main thrust of cancer research worldwide. In this context, natural products, especially those that are derived from plants have stirred scientific and commercial interest. The yields of various chloroform, petroleum ether and ethyl acetate fractions from the ethanolic extract of the *B. dalzielii* stem bark are 9.81%, 9.18% and 6.08% respectively. The effects of three different fractions (Petroleum ether (PEBD), chloroform (CLBD), and ethyl acetate (EABD) fractions) obtained from 70% (v/v) ethanolic extracts of *B. dalzielii* hutch stem bark (BDB) using poorly differentiated tongue cancer cell line, AW8507, of the head and neck squamous cell carcinoma (HNSCC) was investigated. Treatment of cells with fractions at various concentrations inhibited cell viability in dose dependent manner, with median growth inhibitory concentration <35 µg/mL in all the fractions (Figure 1).

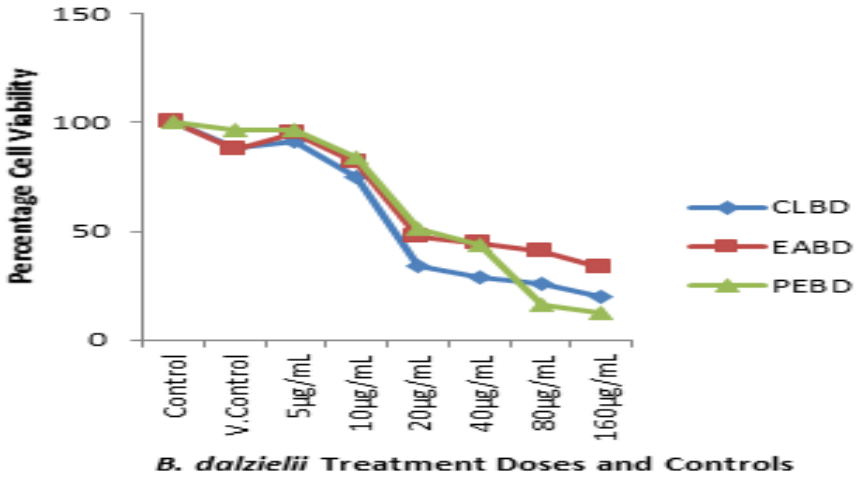

Figure 1: Dose-dependent Growth Inhibition of AW8507 cells by BDB fractions
(Control - Untreated; v.control – vehicle control of 1.6% Ethanol in DMEM, CLBD – Chloroform fraction, EABD – Ethyl acetate fraction, PEBD – Petroleum ether fraction)

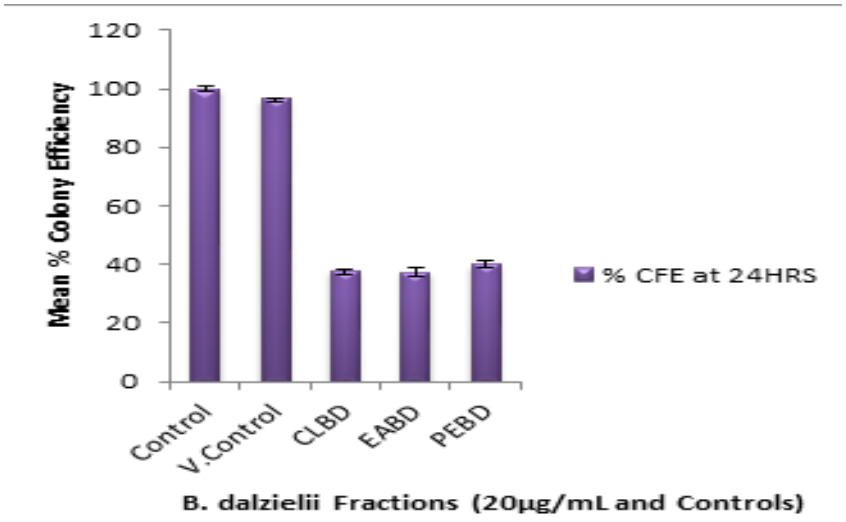

Figure 2: Inhibition of AW8507 Cells Colony Formation Efficiency by BDB fractions
(Control: Untreated; V.Control – vehicle control of 0.2% ethanol in DMEM, CLBD – Chloroform fraction, EABD – Ethyl acetate fraction, PEBD – Petroleum ether fraction)

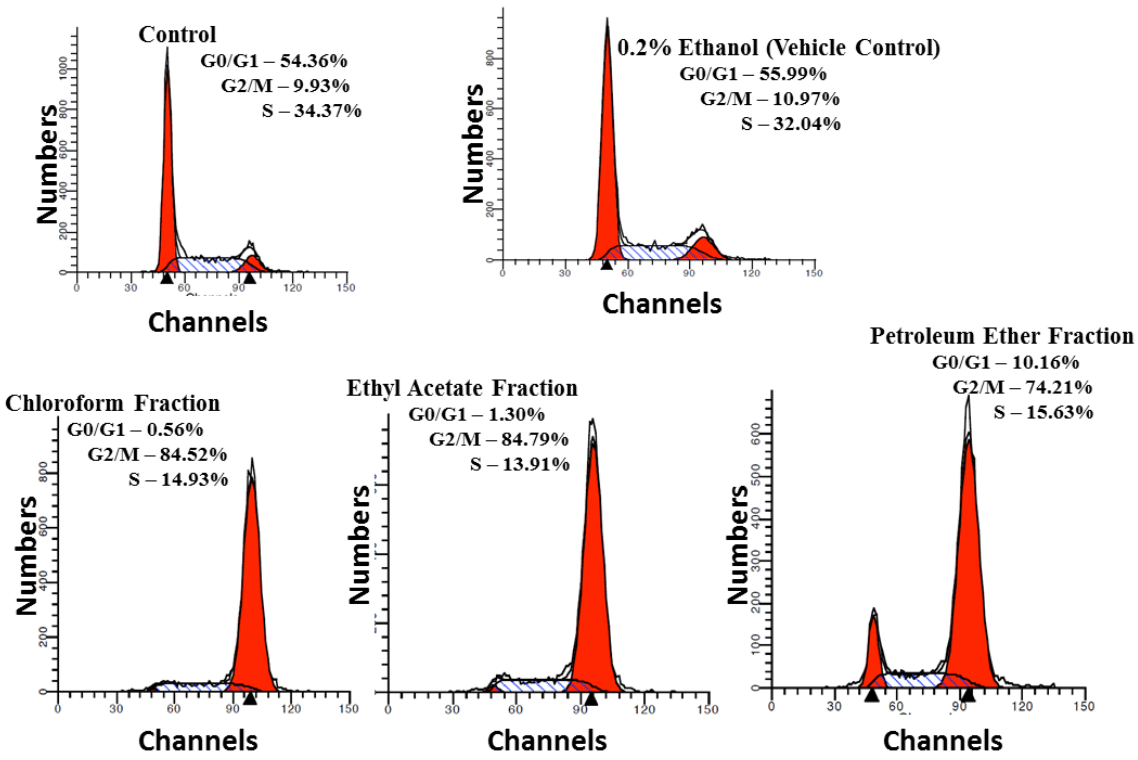

Figure 3: Flow cytometric analysis of *Boswellia dalzielii* Stem Bark Fractions (20µg/ml)

The cytotoxic effect of the extracts were further determined and verified via anchorage-dependent colony formation assay. Thus measuring the ability the fractions, CLBD, EABD and PEBD, to inhibit the ability of AW8507 cells to grow and form foci. As shown in Figure 2, during the 9-day culture period, AW8507 cells treated with BDB fractions had significant reduction in the number of growing colonies. Colony formation efficiency (CFE) of AW8507 cells were ≥60% inhibited after 24-hour treatment with 20 μg/ml of BDB fractions (CLBD, EABD and PEBD) (Figure 2). This assay measures the capability of tumor cells to grow and form foci in a manner unlimited by growth contact inhibition as is typically found in normal, untransformed cells. Thus, clonogenicity provides a secondary assessment of the susceptibility of tumor cells to undergo neoplastic transformation.

Perturbation of cell cycle progression in cancer cells is a useful strategy to arrest cancer growth [27]. Furthermore, cell cycle arrest also provides an occasion for cells to undergo either repair or programmed cell death. Based on the preliminary assays where we observed a strong growth inhibitory effect of *B. dalzielii* stem bark fractions in AW8507 cells, we then determined the possible mechanism of anti-proliferative activity of *B. dalzielii* stem bark fractions. For this purpose the effect of *B. dalzielii* stem bark fractions on cell cycle progression in AW8507 cells was determined following 20 μg/ml doses of chloroform, ethyl acetate and petroleum ether fractions (CLBD, EABD and PEBD respectively) treatment for 24 h. As summarized in Figure 3, at the concentration of 20μg/mL, CLBD, EABD and PEBD significantly increased G2/M phase cell population at the expense of S and G1/Go phases, a sharp contrast to the untreated control and the vehicle control (0.2% ethanol in DMEM). It is widely accepted that control of cell cycle progression in cancer cells is an effective strategy to halt tumor growth [28, 29].

Molecular analyses of human cancers have shown that cell cycle regulators are often deregulated in most of the common malignancies [30,31]. Our data suggested that treatment of AW8507 cells with *B. dalzielii* stem bark fractions induced G2/M phase arrest of the Head and neck squamous cell carcinoma of the tongue (AW8507) cell cycle progression indicating that one of the mechanisms by which *B. dalzielii* stem bark fractions inhibit the proliferation of AW8507 cells is inhibition of cell cycle progression. The arrest of the cell cycle at G2/M phase induced by BDB fractions can be credited to the activity of the phyto-constituents present in the fractions [15,22,32]. A downregulated spindle or mitotic checkpoint (G2/M phase) have been reported to provide survival and growth advantage and promotes progression, enabling cells to tolerate aneuploidy and to escape from apoptosis [33-38]. Several regulators of the mitotic checkpoint have been identified and most of them are localized to the kinetochore, which is connected to both the chromosome and the spindle [39]. Further molecular mechanistic studies would be needed to suggest the disruption of the uncontrolled cell cycle progression of human poorly differentiated squamous cell carcinoma of the tongue (AW8507 cells).

Conclusion. This study was the first to investigate the cytotoxic and antiproliferative properties of *Boswellia dalzielii* extract on a HNSCC cell line. Our study suggests that the chloroform, ethyl acetate and petroleum ether fractions obtained from 70% ethanolic stem bark extract of *Boswellia dalzielii* inhibit proliferation and colony formation of AW8507 cells, and arrest the cells in G2/M phase of the cell cycle. Further studies are required using other methods, also the isolation and identification of individual bioactive compounds in the extract.

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