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Acetone Extract of *Dioscorea alata* Inhibits Cell Proliferation in Cancer Cells

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Abstract

Dioscorea spp., White Yam has been shown to exhibit a wide range of nutritional and medicinal properties. However, the compounds associated with its medicinal functions have not been fully examined. The purpose of this study was to generate a chemoinformatic profile of the bioactive compounds present in *Dioscorea. alata* (*D. alata*) and to characterize their putative anti-cancer properties using prostate (DU145) and lung (A549) cancer cells. Chemoinformatic profiling of *D. alata* resulted in five bioactive extracts: hexane (DaJa-1), ether (DaJa-2), acetone (DaJa-3), ethanol (DaJa-4) and water (DaJa-5) were generated. The analytes present in the five bioactive extracts were dissolved in 0.1% DMSO and their anti-cancer activity were determined. We observed that the acetone extract (DaJa3) was the only extract capable of inducing greater than 90% cell death of DU 145 cell at 100 μg/mL. The order of growth inhibition of the extracts in DU-145 cell is DaJa-3 (IC50, 31.45 μg/mL) > DaJa-4 (IC50 60 μg/mL) > DaJa-1 (IC50 > 100 μg/mL) ≥ DaJa-2 (IC50 > 100 μg/mL) ≥ DaJa-5 (IC50 > 100 μg/mL). MTT cell viability, dye exclusion, caspase activity and microscopic assessment of apoptotic cells demonstrated that DaJa-3 displayed cytotoxicity to both lung and prostate cancer cells. The A549 cells were more sensitive toward DaJa-3 with an IC_{50} value of 22.28 μg/mL (CI 28.42 to 36.63 μg/mL), compared to that of DU145 cells with an IC_{50} value of 31.45 μg/mL (CI 27.58 to 35.86 μg/mL). It was also observed that DaJa-3 induces differential anti-proliferative activity in the cancer cells. The apoptotic response induced by DaJa-3 paralleled the level of cell cytotoxicity observed in both cell lines. DaJa-3 induces G_2 phase cell cycle arrest in DU145 cells but G_1 arrest in A549 cells. The level of key apoptotic regulator proteins was upregulated, suggested that DaJa-3 may be mediating its anti-cancer effect through activation of the intrinsic apoptotic pathway. Alto-

gether, our data indicates that DaJa-3 derived from a staple food source (white Yam) contains unique active compounds that have specific biological properties that may prevent certain types of cancer or specific types of cancer.

Keywords

Dioscorea alata, Acetone Extract, Anticancer Activity, White Yam

1. Introduction

Dioscorea spp. is the third most important tuber crop behind cassava and sweet potato in West Africa, Central America, and the Caribbean [1]. Yam is included among the top five food crop species contributing to 99% of the world production and ranks number four in the world after potatoes (46%), cassava (28%), and sweet potato (18%), followed by taro (1%) [2]. *Dioscorea alata* is the Jamaican native of this genus and the roots and rhizomes of this species are known as White Yam. The *D. alata* varieties have been proven to contain a great variety of macro and micronutrients, especially high potassium and low sodium content [3]. Yam has a high nutritional value and is consumed as raw yam, cooked in soups, and as powder or flour in various food preparations. Yam tubers also have a variety of bioactive components, namely: mucin, dioscin, dioscorin, allantoin, choline, polyphenols, diosgenin, and vitamins such as carotenoids and tocopherols [4].

Several studies done using *Dioscorea* spp. have shown that it exhibits a wide range of medicinal properties [1] [3]. *Dioscorea* spp. has the potential to aid in the treatment and control of hypertension and other cardiovascular diseases [3]. The presence of secondary metabolites in the *Dioscorea* spp. also provides an array of possible opportunities for ascertaining effective anticancer compounds. Bioactive compounds such as steroidal saponins, dioscin, terpenoids and steroids, and allantoin have been demonstrated to be present in large quantities in various *Dioscorea* spp. [1]. Studies done on *D. alata* have also revealed the presence of high flavonoid content and significant antioxidant capacity [5].

The medical applications of allantoin, dioscorin, saponins, and dioscin—the dominant constituents of *Dioscorea* spp. [4] [6] [7] have been reported in many studies. Allantoin, an active principle of yam, has been shown to decrease plasma glucose levels in diabetic rats [8] and diosgenin, a steroidal sapogenin, demonstrates antiproliferative activity, mainly in different prostate cancer cells such as PC-3 and DU145 [9]. Dey *et al.*, (2016) demonstrated hormonal immunity, macrophage activity and anti-inflammatory potential of a methanolic extract of *D. alata* [10]. This study, therefore, underscores the importance of *Dioscorea* spp. as a source for medicinally relevant secondary metabolites.

White Yam has been ascribed several medicinal properties due to the presence of certain bioactive compounds in the tuber [1]. These bioactive compounds in plants are secondary metabolites having pharmacological or toxicological effects

in humans and animals. Several research groups, including ours, have revealed that *Dioscorea* spp. from Jamaica has displayed anti-hypertensive, antidiabetic and anticancer properties [11] [12] [13]. The precise compound or combination of compounds responsible for these activities has not been fully characterized. To our knowledge, there are no known studies that have investigated the anti-cancer effect and mechanism of action of the Jamaican *D. alata*. It is on this premise that we aim to explore the anticancer properties of *D. alata* extracts.

2. Materials and Methods

2.1. Cell Lines and Chemicals

Cell lines: All cell lines used in this study were obtained from The American Type Culture Collection (ATCC; Rockville, MD, USA). The Human prostate cancer cell line DU145, and lung cancer A549 cells were cultured in RPMI-1640 (Invitrogen, Grand Island, NY, USA) with 4 mM L-glutamine and no Phenol Red, adjusted to contain 10% fetal bovine serum and 100 U/mL penicillin-streptomycin. The cells were incubated at 37˚C in a 95% air, 5% CO_2 atmosphere until they approached 80% confluence. All the chemicals used in this study were of HPLC grade and were used without further purification. Assay kits used in all the analyses in this study were purchased through Fisher Scientific Inc.

2.2. Solid-Liquid and Solid-Phase Extraction

The *Dioscorea alata* cv. (White Yam) tubers used were grown in the Parish of St Catherine in Jamaica, harvested in December 2018 then transported to UWI Biotechnology Centre, washed with tap water and sun-dried for 2 hours. The tubers were peeled, cut in pieces and dried at 50˚C to constant weight, milled to a fine powder, and stored in sample bottles at room temperature. The powdered sample (18.802 g) was sequentially extracted in a 120 cm × 500 cm Soxhlet apparatus using 500 mL of hexane, diethyl ether, acetone, methanol them water. The extraction mixture was refluxed for 24 hours at temperatures corresponding to the boiling point of the respective solvents (hexane, 70C; diethyl ether, 35C; acetone, 56C; methanol, 65C and water, 100C). Following extraction, particulate matter was removed by filtering the samples through a 0.45 μm fritted glass filter. The extracts were then purified using an SPE-C18 column (C18-E, 55 um, 70A, 8B-S001-LEG). The SPE column was washed with 10% MeOH and eluted with 25%, 75% and 100% MeOH. The samples were then evaporated to dryness using a combination of simple distillation and rotary evaporation.

2.3. Cell Viability

Cell viability was assayed using CellTiter 96 Aqueous One Solution (Promega, Madison, Wisconsin) according to the manufacture instructions. Cells were seeded in 96 well plates at 5×10^3 cells/well and allowed to grow for 24 hours. They were then treated with different extracts at 5 successive 2-fold dilutions,

ranging from 6.5 µg/mL to 100 µg/mL. The final DMSO content within each extract concentration did not exceed 0.1% (v/v) in the growth medium. Cells were exposed at 37C for 24 and 72 hours after which cell viability and cytotoxicity were assayed using 10% (v/v) CellTiter 96® Aqueous One Solution Reagent. After exposure to the extracts, the effect on cell viability was determined using the CellTiter 96, Aqueous One non-radioactive cell proliferation assay (Promega, Madison, WI) according to manufacturer's instructions. After incubation treated cell with Methyl Thiazolyl Tetrazolium (MTT) reagent for 1hr at 37C, the absorbance at 490 nm was measured using a ELX155 800UV universal microplate reader (Bio-Tech, Inc.). The results were then analyzed using GraphPad Prism 6.

2.4. Acridine Orange and Ethidium Bromide Staining

To determine the effects of apoptosis on cells after the treatment of samples under investigation, the morphology of cells was observed via acridine orange (AO)/ethidium bromide (EB) dual staining [14]. Cells (5.0×10^5) were loaded in a 24 well flask and treated with the compounds of interest. After 24 hours, cells were harvested with Trypsin and resuspended in 500 uL of PBS. Cells (25 µl of cell suspension 5.0×10^5 to 2.0×10^6 cells/ml) were incubated with 1 µl of AO/EB solution. Each sample was mixed prior to microscopy and quantification and was evaluated immediately. The cell suspension (10 µl) was placed on a microscope slide and covered with a glass coverslip. Four fields of view were examined under a fluorescence microscope using a fluorescein filter at 40× and 60× (higher or lower magnification may be desired depending on cell type, nuclear morphology should be discernible). The number of apoptosis cells were counted, and the results were then statistically analyzed.

2.5. Caspase 3/7 Analysis

Caspase protocol was conducted according to manufacturer's instructions [15] [16]. Cells were cultured in a 96-well black plate and treated with increasing concentrations of DaJa-3 sample. Each well contained 100 µL of sample and the control contained 100 µL of growth media only. To each well 100 µL of Apo-ONE® Homogeneous Caspase-3/7 Reagent was added. Plates were gently mixed using a plate shaker at 300 rmp for 30 seconds and then incubated at 37C for 4 hours. The fluorescence of each well was detected using a fluorescence microplate reader at an excitation wavelength range of 485 ± 20 nm and an emission wavelength range of 530 ± 25 nm (Promega, 2018). Results were then analyzed using GraphPad Prism 6.

2.6. Cell Cycle Analysis

Cells were seeded in T75 flasks at 1×10^6 cells/flask and treated with DaJaE3 extract with four, 10-fold dilutions. A549 cells were treated with a concentration close to its IC_{50} value. Cells were then trypsinized and harvested after 24 hours, washed with PBS and fixed with 1 mL 80% cold MeOH. Cells were centrifuged for 5 mins at 1500 rpm, supernatant was removed, and the cell pellet was sus-

pended in 500 µL of propidium iodide (10 µg/ml) containing 200 µg/mL of RNase (Sigma, MO, USA). The cells were then incubated on ice for 1 hour. Cell cycle distribution was determined from 20,000 cells using a Becton Dickinson FACSCalibur flow cytometer [16]. The acquisition data was analyzed using FlowJo V10 software.

2.7. Western Blots

Immunoblotting was carried out according to [17]. Cells were grown to 80% - 85% confluency then treated with varying concentrations of DaJaE3 extract for 24 hours. After treatment, the cells were harvested using trypsin and centrifuged for 5 min at 1500 rpm. The cell pellet was resuspended in RIPA lysis buffer containing 1% protease inhibitor and the protein concentration was determined by BCA assay according to the manufacturer's instructions. An equal amount of protein (20 µg) was separated on 10% SDS PAGE and was transferred to a PVDF membrane. The membrane was blocked with 1% nonfat milk in TBS overnight at 4˚C then incubated with the following antibodies: anti-BAX, anti CDK4, anti-GDPH and anti-cyclin B (Santa Cruz Biotechnology) for 18 hours. The membrane was probed with Horseradish peroxidase-conjugated secondary antibody. The immunogen was detected using a chemiluminescence reagent according to the manufacturer's instructions. The immunoblot signal was detected using an AlphaInnotech Fluorochem HD 9900 (Alpha Innotech, San Leandro, CA) equipped with a Charged -coupled Device camera.

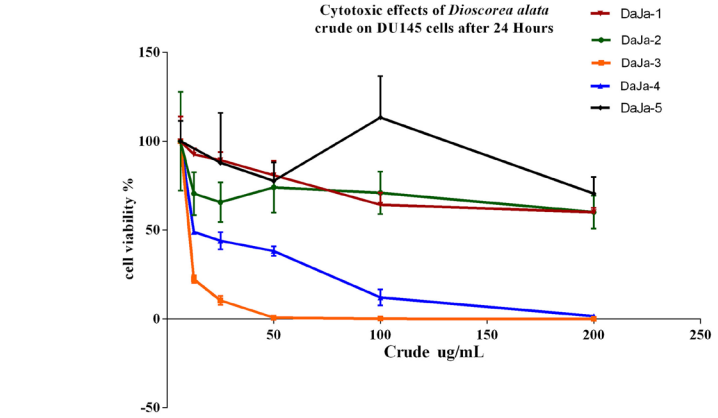
3. Results

3.1. Acetone Extract of *Dioscorea alata c.v* (*D. alata*) Possesses Anti-Cancer Bioactivity

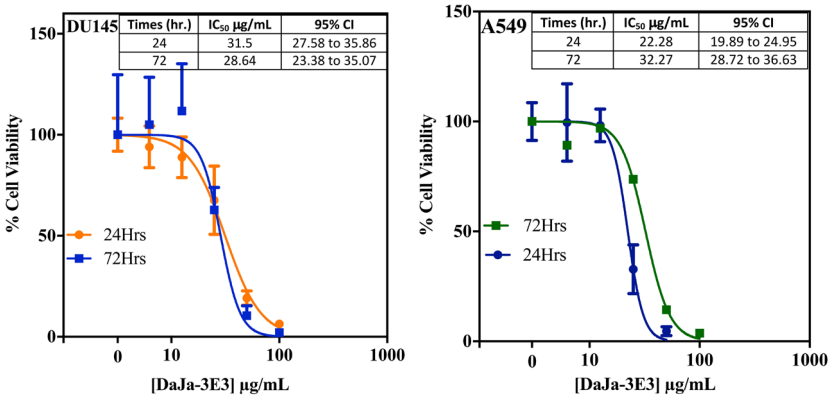
Dioscorea spp. has been studied by many groups for its nutritional and medicinal value [1] [11] [18]. However, the compounds present in *D. alata* that are responsible for its biological activity are not well characterized. Since *D. alata* contains a myriad of active and non-active compounds located in different parts of the tuber, we used solvents of different polarities to solvate the compounds and generate a fingerprint of bioactive analytes present in the yam. To evaluate the bioactive anti-cancer compounds, *D. alata* was extracted with Hexane (DaJa-1), Diethyl ether (DaJa-2), Acetone (DaJa-3), Ethanol (DaJa-4) then Water (DaJa-5) for 24 hours each. Each extract was cleaned-up dissolved in DMSO and analyzed for anti-cancer bioactivity using MTT *In vitro* toxicity assays.

Preliminary screening of *D. alata* extracts demonstrated that the acetone extract (DaJa-3) possesses growth inhibitory properties DU-145 cancer cells (**Figure 1(a)**). The order of growth inhibition of the extracts in DU-145 cell is DaJa-3 (IC50, 31.45 µg/mL) > DaJa-4 (IC50 60 µg/mL) > DaJa-1 (IC50 > 100 µg/mL) ≥ DaJa-2 (IC50 > 100 µg/mL) ≥ DaJa-5 (IC50 > 100 µg/mL). The potency of DaJa-3 extract as an anti-cancer agent, was determined in two cancer cell lines (DU145 and A549) found in the NCI-60 Human Tumor Cell Lines Screen (**Figure 1(b)**, **Figure 1(c)**). The anti-proliferative activity of DaJa-3 in these cells

was performed using the Five-Dose assay method at a concentration of 0, 6.25, 12.5, 25, 50 and 100 µg/mL of DaJa-3. A plot of cell viability as a function of concentration is shown in Figure 1(b), Figure 1(c). The IC_{50} of DaJa-3 was determined by plotting the normalized viability data to a downhill dose–response curve with a variable slope. Maximal inhibition of DU145 and A549 cell growth by DaJa-3 was achieved at 90 - 100 µg/mL. A549 was more sensitive toward DaJa-3 extract with an IC_{50} value of 22.28 µg/mL (CI 19.89 to 24.95 µg/mL), compared to that of DU145 cells with an IC_{50} value of 31.45 µg/mL (CI 27.58 to 35.86 µg/mL) (Figure 1(b), Figure 1(c)). A time dependent effect of DaJa-3 in the lung cells (A549) was noted when compared to the prostate cancer cells (DU145). After a 72-hour exposure to a single dose of DaJa-3, A549 cells appeared to be less sensitive to the extract with an increased IC_{50} value from 22.22 µg/mL to 32.27 µg/mL, suggesting the extract is being metabolized. In contrast, DU145 displayed no changes in sensitivity over the same 72-hour period using a single dose of DaJa-3 (Figure 1(b), Figure 1(c), compare 24 and 72 hours).



(a)



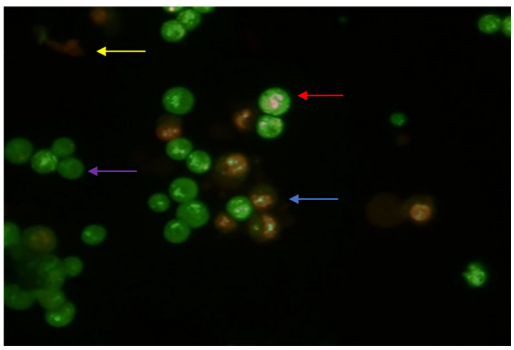
(b) (c)

Figure 1. Cytotoxicity of extracts from *Dioscorea alata* in cancer cells. (a) Toxicity screen of *Dioscorea alata* extracts in DU145 prostate cancer cells. Effect of DaJa-3 in DU145 prostates cancer cell (b) and A549 lung cancer cells (c). Cell were seeded at 1E4 cells per well, treated with *D. alata* samples for a 24-hour and 72-hour at 37C. Cell viability was measured using Cell Titer 96® Non-Radioactive Cell Proliferation Assay (MTS). Data shown are means ± SE n = 4.

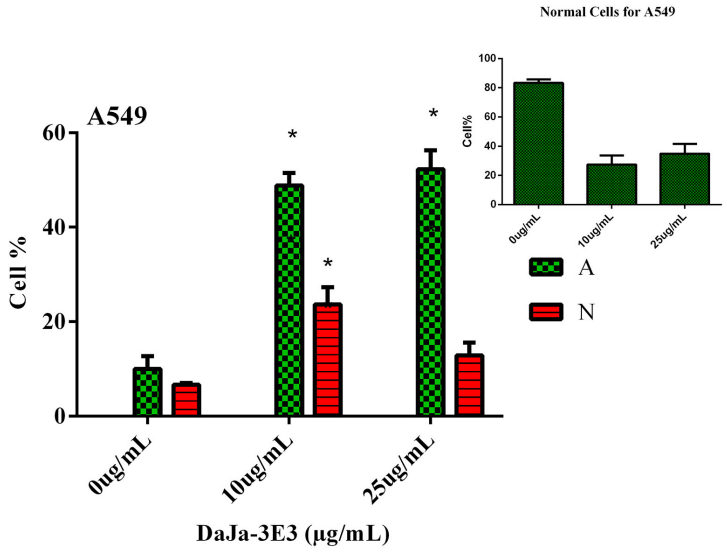
3.2. DaJa-3 Induced Apoptosis and Cell Cycle Arrest in Cancer Cells

Investigations into whether the observed cell cytotoxicity induced by DaJa-3 was a result of chemically induced cellular cytotoxicity or cell-induced apoptosis was carried out. Cells fluorescently stained with acridine orange (AO) and ethidium bromide (EB) were examined following exposure to DaJa-3 to reveal signs of nuclear condensation, nuclei fragmentation, and membrane budding, which are all hallmark features of apoptosis. Examination of both DU145 and A549 cells treated with 25 μg/mL of DaJa-3 for 24 hours revealed the presence of four distinct morphologies associated with the cells in different stages of apoptosis (**Figure 2(a)**). There was a decrease in the number of viable cells (VC) with an intact cell membrane and organized cell structures that fluoresced bright green (**Figure 2(a)**, red**). Cell death was also observed in both early (single arrow), and late (double arrow) apoptotic stages (**Figure 2(a)**) in both cell lines following exposures to DaJa-3.

The apoptotic index was determined for both A549 and DU145 by quantifying the relationship between dose of DaJa-3 and number of apoptotic cells (**Figure 2(b)**, **Figure 2(c)**). The apoptotic index was calculated by averaging the number of apoptotic cells per field (EA + LA) (70 - 60 cells) then dividing by the total number of cells per field (120 - 140 cells). DU145 and A549 cells treated with DaJa-3 and harvested after a 24-hour treatment showed a dramatic increase in apoptotic cells and a decrease in total viable cell number. **Figure 2(b)** shows that A549 cells had a significant increase in apoptotic cells (10% in control versus 52.3% in 10 μg/ml DaJa-3, $p < 0.0001$) after treatment with 10 μg/mL of Daja-3 extract. As seen with the MTT cytotoxicity assay (**Figure 1**), there was a significant decrease in viable cells (85% at 0 μg/ml DaJa-3 versus 35% at 10 μg/mL DaJa-3) and a significant difference in necrotic cells (**Figure 2** insert). The same degree of apoptosis was observed in both A549 and DU145 cells in the presence of DaJa-3 after a 24-hour exposure (Compare **Figure 2(b)**, **Figure 2(c)**). Treatment of DU145 with 25 μg/mL DaJa-3 resulted in a significant increase in apoptotic cells (17.2% in control versus 57.2% in 25 μg/ml DaJa-3, $p < 0.01$), and a significant decrease in viable cells (85% at 0 μg/ml DaJa-3 versus 25% at 10 μg/mL). DU145 cells also showed a significant increase in necrotic cells (7.5% in control versus 35% in 25 μg/ml DaJa-3, $p < 0.01$). However, there was no significant increase in necrotic cells observed in A549 cells at similar concentrations.



(a)



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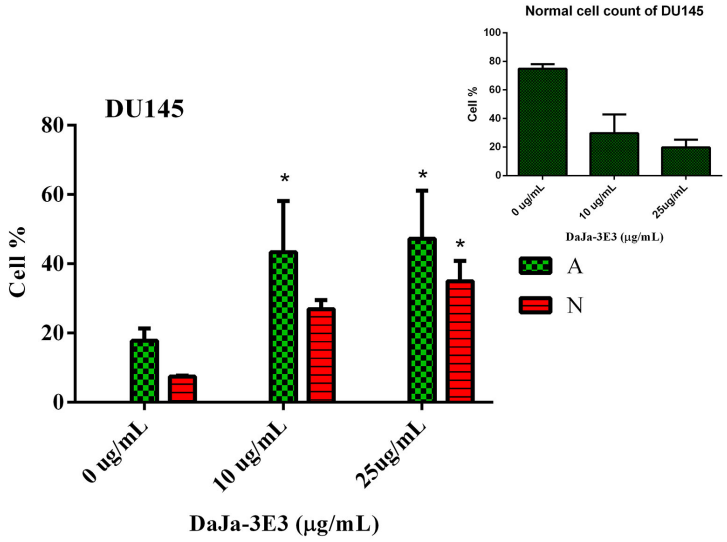


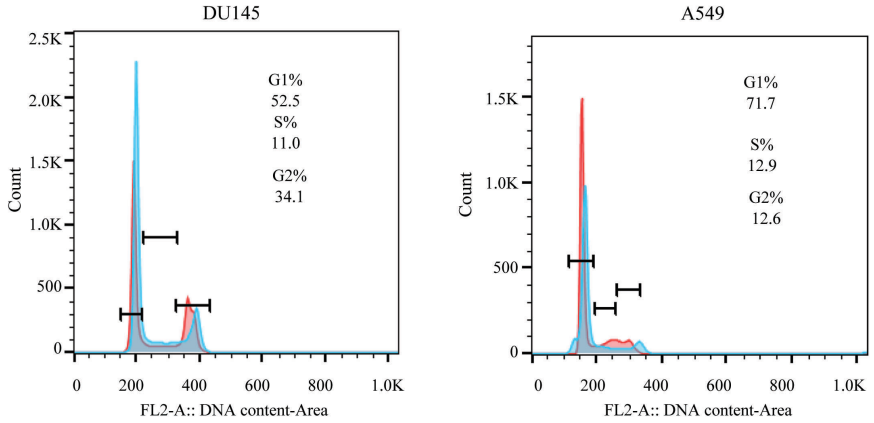
(c)

Figure 2. Morphological assessment of apoptotic and necrotic cells in DU145 and A549 treated with DaJa-3. Cells were treated with DaJa-3 then stained with Ethidium bromide and Acridine Orange. Images were taken at X40; (EA) early apoptosis and (LA) late-stage apoptosis cells are identified by the arrows. Changes in the number of apoptotic cells were determined after 24 hours and the results are presented as mean ± SEM, n = 6. Two-tailed-t-tests revealed significant difference at $p < 0.001$ compared to the control (no treatment). (a) Microscopic image of fluorenone-stained DU145 cells; (b) Apoptotic index of DU145 cells; and (c) Apoptotic index of A549 cells. Inserts in B and C represent percent of viable Cells in A549 and DU145 respectively.

3.3. DaJa-3 Inhibits Cell Cycle Progression in DU145 and A549 Cancer Cells

The mechanism by which DaJa-3 induces anti-proliferative activity was next examined using flow cytometry analysis (**Figure 3(a)**). DaJa-3induced prolifera-

tion blockage was determined on unsynchronized DU145 and A549 cells which were treated with 25 μg/mL of extract for 12 or 24 hours. Analysis of untreated DU145 cells, showed that the G_1 phase contained 64.4% of cells, S phase 13.8% and G_2 phase 20.0%. Upon treatment of DU145 cells with DaJa-3, there was a notable decrease (22%) in G_1 cells, which was accompanied by an increase (41%) of cells in the G_2/M phase (**Figure 3**). When these experiments were performed on A549 cells, no concentration dependent changes were observed in the different phases of the cell cycle (**Figure 3(b)**). However, a high concentration of DaJa-3 (25 μg/mL extract for 24 hours) produced a sharp (26%) decrease in cells in the G_2/M phase (**Figure 3(b)**). A time-dependent change in cell accumulation for A549 cells in different phases of the cell cycle was also observed. Treatment of A549 for 12 hours resulted in a 21% accumulation of cells in the G1 phase with a corresponding 61% decrease of cells in the S-phase and 52% decrease in the G_2/M phase of the cell cycle. The data suggests that DaJa-3 induces G_2 phase cell cycle arrest in DU145 cells and G_1 arrest in A549 cells. These results therefore suggest that DaJa-3 affects multiple pathways in inducing its antiproliferative effects, depending on the tumor cell type.



(a)

	G1		S		G2/M	
	0.0ug/mL	25ug/mL	0.0ug/mL.	25ug/mL	0.0ug/mL.	25ug/mL
DU145	64.2%	52.5%	13.8%	11%	20%	34.1%
A549	75.6%	71.7%	11.9%	12.9%	9.36%	12.6%

	G1		S		G2/M	
	0hrs	12hrs	0hrs	12hrs	0hrs.	12hrs
DU145	66.4%	65.4%	18%	17.6%	14.7%	17.1%
A549	55.7%	70.9%	21.9%	13.6%	21.3%	14%

(b)

Figure 3. Dosage dependent effects of DaJa-3 on cell cycle progression of DU145 and A549 cancer cells. Cells were cultured in complete medium and treated with either 10 or 25 μg/mL of extract (0.05% DMSO in medium). After the indicated time of treatment, cells were harvested, washed with cold PBS buffer, and digested with RNase. Cellular DNA was stained with propidium iodide and flow cytometric analysis was done to determine the cell cycle distribution as described previously. (a) Histogram of flow cytometry analysis in the presence (blue) and absence (red) of DaJa-3. (b) Quantification of cell cycle progress in DU145 and A549 cells at 25 μg/ml and 12 hours exposure to DaJa-3.

3.4. DaJa-3 Modulation of Apoptotic Pathway in DU145 and A549 Cancer Cells

The morphological analysis and flow cytometry assessment of DaJa-3-induced apoptosis was validated by ascertaining if the activity of the downstream apoptotic protein Caspase-3/7 had been modulated in the presence of DaJa-3. The level of active Caspase-3/7 was measured by proteolytic cleavage of rhodamine 110, from the bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD R110) substrate (**Figure 4**). There was a dose-dependent increase in DaJa-3-induced caspase activity in DU145 cells and a bi-phase response in A549 cells. There was a significant difference with the Caspase 3/7 activity in DU145 cells at 50 μg/mL of DaJa-3E3, when compared to the control. In A549 cells, the greatest activity observed was at 25 μg/mL of DaJa-3, followed by a decrease in the Caspase-3/7 activity to below 25% at 50 μg/mL (**Figure 4**).

The expression levels of select apoptosis and cell cycle proteins in the absence and presence of DaJa-3 was also examined. **Figure 5** demonstrated inhibition of CDK4 protein levels in DU145 cells following exposure to DaJa-3 for 24 hours. However, DaJa-3 showed no modulation of CDK4 expression in A549 lung cancer cells. The levels of Bax, a pro-apoptotic regulator protein, showed a significant increase by DaJa-3 in the A549 lung cancer cells but was undetected in the DU145 prostate cancer cells. Analysis of cyclin B, a G_2/M cell cycle check protein indicated that DaJa-3 decreased its expression in DU145 prostate cancer cells and induced a slight increase in A549 lung cancer cells (**Figure 5**).

4. Discussion

Dioscorea alata (*D. alata*) is consumed worldwide as a major nutritional food, but is increasingly being recognized as a nutraceutical, containing compounds that may provide medicinal or health benefits to prevent and treat diseases. In the present study, data was provided that identified major anticancer components in *D. alata* as evidence of its nutraceutical properties. Studies on the effects of *D. alata* in cancer treatment are emerging. However, they are limited in that the toxicity of *D. alata* has not been thoroughly assessed and in other instances the extracts used are not relevant to a specific cancer type [9]. The bioactive fraction of *D. alata* was identified containing putative anticancer compounds with biological activity in two epithelial derived cancer cell lines. The use of solid-liquid extraction with solvents of different polarity, made it possible to solubilize potent anticancer activity (DaJa-3) from *D. alata* using acetone. DaJa-3 showed potent toxicity towards lung carcinoma cells (A549) and androgen insensitive prostate cancer cells (DU145) with an IC_{50} value of 22.3 μg/mL and 31.5 μg/mL, respectively. DaJa-3 displayed differential biological activity dependent on the cell phenotype. The potency of DaJa-3 in A549 cells decreased by 49% at 72 hours of exposure, while DU145 cells had relatively no significant changes. This data suggests that the mechanism of toxicity induced by DaJa-3 is cell type specific.

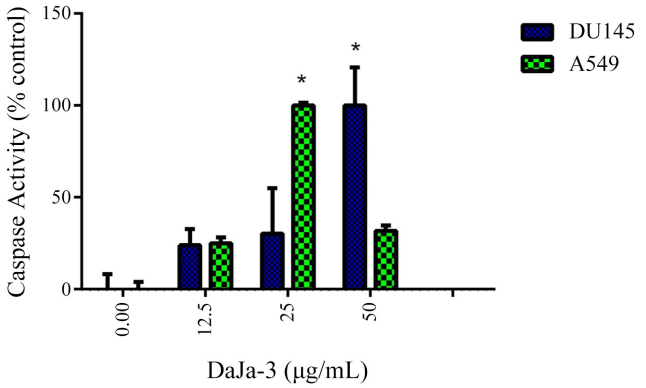


Figure 4. Increased caspase activity in the presence of DaJa-3. A549 and DU145 cells were treated with various concentrations of DaJa-3E3 and incubated overnight. Apo-ONE® Caspase-3/7 reagent (100 µl) was added to each well. Cells were incubated for 5 hours prior to recording ($485_{EX}/520_{EM}$). Caspase 3/7 activity normalized to control = 0% and greatest activity = 100%. All data are expressed as mean ± SEM, N = 4
* $p < 0.05$ compared with corresponding controls.

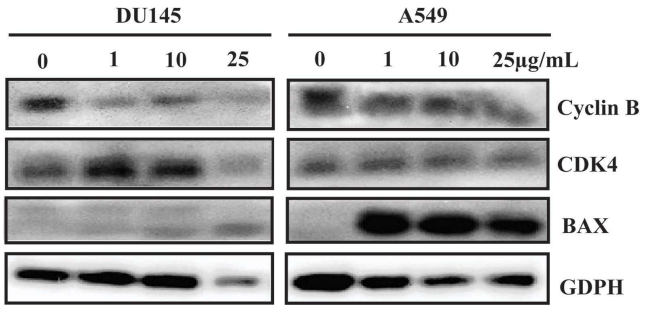


Figure 5. Regulation of cell cycle proteins by DaJa-3E3. The DU145 cells were cultured in complete medium and treated with increasing concentrations of DaJa-3E3 for 24 hours, then subjected to SDS-PAGE followed by Western blot analysis, as described previously. Blots were probed with antibodies for cyclin B, CDK4, BAX and GDPH.

The uses of natural products such as yam for the treatment of various ailments are attractive for several reasons. Incorporating natural medicine into the cancer treatment regime provides lower cost of treatment, lower possibility of severe side effects, synergizes the potentiation of therapeutic efficacy and alleviates toxicity [6] [19] [20]. DaJa-3 induction of cell toxicity is dose-dependent, cell specific and is likely caused by DNA damage in cancer cells. The mechanism of DaJa-3 cell toxicity in lung and prostate cancer cells was investigated using morphological examinations. The AO/EB staining confirmed the apoptosis inducing effect of DaJa-3 on both cell lines. DaJa-3 produced a 4 - 5-fold increase in apoptosis after a single dose for 24 hours. AO/EB staining revealed apoptotic structures such as chromatin condensation and membrane blebbing in cells exposed to DaJa-3. It appears that DaJa-3 may have several different compounds with mixed modes of action. A significant increase in the necrotic cells in DU145 at treatments with 25 µg/mL was observed. The high level of necrosis seen in

DU145 cell culture may signal autophagy [21]. The ability of DaJa-3 to potentially induce apoptosis and possible autophagy makes it a potential beneficial anticancer agent, and would provide less harmful side effects, which is common amongst natural compounds [22].

Control of cell cycle progression is considered to be a potentially effective strategy for the control of tumor growth by anticancer compounds [19] [23]. Thus, understanding the mechanism of DaJa-3 anti-proliferative activity, flow cytometry was used to determine where in the cell-cycle proliferation blockage was induced. Analyzing any changes to the cell cycle after treatment allows for a better understanding of how the extract disrupts the cell cycle by inducing apoptosis, which results in the accumulation of cells in different phases of the cycle [8]. Analysis of cell cycle progression in DU145 cells following DaJa-3 exposure resulted in a G_1/G_0 cell cycle arrest as revealed in a decrease of cells in G_1 and a corresponding increase in cells in the G_2/M phase. This G_1 blockage is indicative of the presence of anti-proliferative agent(s) present in DaJa-3 which resulted in apoptosis in the cells. The G_1/G_0 checkpoint is controlled by Cyclin D in the form of a Cyclin D/CDK4 complex [24] [25]. Activation of this complex by phosphorylation suppressor protein retinoblastoma from the pRb family makes it inactive, thus allowing for the progression of the cell from G_1 into S phase [26]. It was observed that the level of protein kinase CDK4, which activates this process was significantly reduced in response to an increased concentration of DaJa-3 in DU145 cells (**Figure 5**). This observation corroborates our toxicity data and indicates a reduction in cell progression through the G_1 checkpoint. When these experiments were extended to the A549 cells, a G_2/M cell cycle arrest was observed. This suggests that our DaJa-3 extract contains multiple antiproliferative compounds that target different cellular pathways depending on cell type.

To corroborate our observations, the mechanism by which DaJa-3 induces apoptosis was examined. Apoptosis is mediated through two distinct pathways: an intrinsic and an extrinsic pathway. Extrinsic mediated apoptosis involves activation from external stimuli through the binding of death receptors (DRs) which belong to the superfamily of tumor necrosis factor (TNF) [27]. Whereas the intrinsic apoptosis pathway relies on the release of factors from the mitochondria as a result of cellular stress. The levels of down-stream apoptotic executor Caspase 3/7 were analyzed in both cell lines in the presence and absence of DaJa-3. The data showed an increased expression of effector Caspase 3/7 in response to increased dosage of DaJa-3 confirming that DaJa-3 induces apoptosis in both DU145 and A549 cells. An increased activity of Caspase 3/7 coupled to an increased expression of Bax protein suggests that DaJa-3 triggers an intrinsic apoptosis in DU145 cells and A549 cells. It appears that the intrinsic apoptotic stimuli present in DaJa-3 were more potent in A549 cells as compared to the DU145 cells (**Figure 4** and **Figure 5**). However, further investigations are needed to elucidate the direct apoptosis mechanism induced by DaJa-3.

5. Conclusion

There is an urgent need for alternative methods of treating several cancers that involve the use of natural compounds extracted from medicinal plants. The experiments conducted provide evidence that the acetone extract of *D. alata* contains potent antiproliferative properties. Based on the study performed in the two cancer cell lines, DaJa-3 displayed anticancer properties through G_1 blockage which resulted in the initiation of apoptosis. This study also revealed that *D. alata* may serve as a rich source for isolation of anticancer compounds. Further study is currently being done to identify the compounds present in the DaJa-3 extract as well as to elucidate the *in vivo* efficacy of DaJa-3 to further strengthen its potential use in anticancer treatment.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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