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Caspase Dependent Inhibitory Effect of the Growth of HeLa Cells by *Desmodium velutinum* (Willd.) DC. Ethanol Leaf Extract

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ABSTRACT

Cervical cancer is the most common cancer in women in sub-Saharan Africa and the fourth leading cause of cancer deaths in women. Adverse side effects associated with orthodox therapies have given rise to increase in natural therapies. Natural products from organisms such as plants and microbes are a rich source of therapeutic lead compounds. *Desmodium velutinum* (Willd.) DC. (Papilionaceae) is a perennial shrub which has been reported to be used in folkloric medicine for the treatment and management of tumours and abscesses. The anti-proliferative activity of the ethanol leaf extract of *D. velutinum* was assessed using MTT assay on HeLa cells. The mechanism of anti-proliferative activity was studied using cell cycle analysis, Annexin V-FITC/PI, mitochondrial membrane depolarisation, caspase 3 and 8 activation assays. *D. velutinum* ethanol leaf extract had an IC_{50} value of 53.27µg/mL on HeLa cells and caused accumulation of cells in the G0/G1 and early M phase. The extract also induced necrosis and led to the depolarisation of the mitochondrial membrane and activation of both caspase 3 and 8. The extract exhibited its anti-proliferative potential by mediating cell cycle arrest at the G1-S phase and cell death due to necrosis while activating the caspase system.

Keywords: *Desmodium velutinum*, Caspase, Necrosis, Cell cycle arrest.

Introduction

Cancer is a disease of the cells and is caused by mutation of genes responsible for cell proliferation, metabolism and death.[1] It is a major health challenge in Africa due to the various socio-economic factors and it is estimated that the number of cancer cases in Africa would double from 2018-2040.[2] Cervical cancer is the fourth most commonly diagnosed cancer in women worldwide. In 2018, there was an estimated 570,000 new cervical cancer occurrences with 311,000 deaths worldwide, with low- and middle-income countries especially sub-Saharan Africa accounting for 90% of the deaths.[3-5]
Natural products from plants and microbes are a rich source of therapeutic lead compounds in cancer therapy; and the high rate of adverse side effects and low accessibility to current orthodox cancer therapies in sub-Saharan Africa has given rise to increased interest in natural remedies for the management of cancers.[6,7]
Desmodium velutinum (Willd.) DC. (Papilionaceae) is a perennial shrub which is erect or semi-erect and grows to about 3 m. The leaves are simple and ovate with branches that are dark red to yellow-brown when young. Traditionally, the plant is used in Edo State as a blood purifier, while the root is used to treat malignant ulcers. The leaf, bark and root are used in treatment of syphilitic chancres, pain management and in the treatment of tumours.[8]

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Previous pharmacological studies have shown the hepatoprotective activity of the ethanol stem bark extract; anti-lipidemic activity of the aqueous extract of the leaves; anti-microbial activity of the leaves; gastroprotective and antibacterial properties of the dichloromethane:methanol leaf extract and its anti-pyretic activities.[9-12] Although the plant has been traditionally used in the treatment of tumours, no scientific work has been carried out to validate the claimed property hence the aim of this study was to determine the anti-proliferative activity of *D. velutinum* leaf extract as well as to elucidate the mechanism by which it exerts its anti-proliferative activity.

Materials and Methods

Plant collection and extraction
D. velutinum leaves were collected in September 2014 from Shere hills, Gwash District, Plateau State, Jos, Plateau State, Nigeria by Mr. J.J Azila of the Federal College of Forestry Jos. Voucher specimen number FHJ 203 was obtained and deposited in the herbarium of Department of Pharmacognosy, University of Lagos. The leaves of *D. velutinum* were air-dried and pulverised. The pulverised leaves (500 g) were macerated in 5 L of absolute ethanol for 72 hours. The resulting extract was then filtered and concentrated in *vacuo* using a rotary evaporator at 40 °C to give a yield of 6.8%.

Brine shrimp lethality test
Assessment of toxicity was carried out using the brine shrimp lethality test.[13] Briefly, stock solution of the plant extract was prepared and serial dilutions of 10, 100 and 1000 µg/ml were prepared in 5ml filtered seawater in test tubes. For each test tube, ten brine shrimp larvae were added and the samples incubated at room temperature for 24 hours. The experiment was done in triplicate. After 24 hours, the surviving larvae were counted and recorded. Probit method was used for the LD_{50} calculation for the extract.[14]

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Cell lines
Cervical cancer (HeLa) and Vero (African Green monkey kidney) cells were obtained from Cellonex and breast cancer (MCF-7) cells from Highveld Biological, South Africa. Cells were maintained in RPMI 1640 (HeLa cells) or DMEM (MCF-7 and Vero cells) culture medium with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and streptomycin) at 37 ℃ in a humified 5% CO_2 incubator.

Cytotoxicity assay
The anti-proliferative activity of *D. velutinum* ethanol leaf extract on HeLa, MCF-7 and Vero cells was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[15,16] Briefly, cells were seeded at 4 $\times 10^4$ cells/ml in 96 well plates and incubated overnight at 37℃, 5% CO_2, and 100% relative humidity for 24 hours to allow for cell attachment prior to addition of extracts. Cells were then treated with 200 µL of fresh medium containing the extracts at varying concentrations (9.4-300 µg/mL). After 48 hours, MTT (5mg/mL) was added to each well followed by a 3-hour incubation. The medium containing MTT was then removed and dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystals. Melphalan (50µM) was used as positive control. Absorbance was read using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). Dose-response curves were constructed using GraphPad prism and used to determine the IC_{50} values.

Cell cycle analysis
HeLa cells were seeded and treated with 53.27 µg/ml extract (IC_{50}) or 50 µM melphalan for 48 hours as described in the cytotoxicity assay. Cells were stained according to Annexin V-FITC/PI (Annexin V-fluorescein isothiocyanate/Propidium iodide) Kit protocol (MACS MiltenyiBiotec). However, experimental changes were made to include the nuclear dye, Hoechst 33342 and PI was not used. The binding buffer of the kit was prepared by performing a 1:20 dilution as recommended using PBS (Phosphate buffered saline, $+Ca^{2+}$ and Mg^{2+}). Annexin V-FITC and Hoechst were diluted in binding buffer to a final concentration of 2 µg/mL and 5 µg/mL, respectively. Treatment medium was aspirated from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) and Annexin V-FITC and incubated for 30 minutes at 37 ºC. The plate was acquired on the ImageXpress Micro XLS Widefield microscope using the DAPI and FITC (4',6-diamidino-2-phenylindole and fluorescein isothiocyanate) filters and 10 x Plan Fluor objectives (Molecular Devices).

Caspase- 3 and -8 activation assay
HeLa cells were seeded and treated with 53.27 µg/ml extract (IC_{50}) or 50 µM melphalan for 48 hours as previously described. The cells were first fixed and permeabilised using the IntraPrep kit as per manufacturer's instructions (Beckman Coulter). Cleaved caspase -8 (Asp 391) and cleaved caspase -3 (Asp 175) monoclonal antibodies (Cell Signaling Technology) were used to determine the presence of activated caspase enzymes. Cells were blocked using phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and thereafter incubated with the antibodies separately (1:200 for cleaved caspase -8 and 1:100 for cleaved caspase -3) for 1 hour at 37°C. The cells were washed and incubated with the Alexa 647 conjugated secondary antibody (1:1000) for 30 minutes at 37°C in the dark. Hoechst 33342 (5 µg/mL) was added to each well as a nuclear counterstain. Cells were then imaged using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). A 10x Plan Fluor objective was used with the DAPI and Cy5 (Sulfo-Cyanine5) filters for Hoechst 33342 and Alexa 647, respectively.

Mitochondrial membrane depolarisation
Positively charged, red orange dye tetramethylrhodamine, ethyl ester (TMRE) was used to quantify changes in the mitochondrial membrane potential using quantitative fluorescence microscopy. TMRE labels active mitochondria and readily accumulates due to the relative negative charge inside the matrix.
HeLa cells were seeded and treated for 24 hours as previously described. Staining solution containing 1 µM TMRE and 5 µg/mL Hoechst 33342 was prepared in 10 mL RPMI:PBS (1:1). Treatments were aspirated, 100 µL staining solution added to each well and cells incubated for 15 min at 37 ℃. Cells were then imaged using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) with a 10x Plan Fluor objective and DAPI and TRITC filter cubes.

Phosphatidylserine (PS) translocation
HeLa cells were seeded and treated for 24 hours as previously described. Cells were stained according to Annexin V-FITC/PI Kit protocol (MACS Miltenyi Biotec) with the addition of Hoechst 33342. The binding buffer of the kit was prepared by performing a 1:20 dilution as recommended using PBS ($+Ca^{2+}$ and Mg^{2+}). Annexin V-FITC and Hoechst were diluted in binding buffer to a final concentration of 2 µg/mL and 5 µg/mL, respectively. The treatments were aspirated from all wells and 100 µL of the prepared dyes was added to each well and incubated for 20 minutes at room temperature. Propidium iodide (PI) prepared in binding buffer to a final concentration of 100 µg/mL and 10 µL was added to each well. Cells were imaged immediately after addition of PI using the ImageXpress Microscope using the DAPI, FITC and Texas Red filters and 10x Plan Fluor objective (Molecular Devices).

Statistical analysis
Image acquisition was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) and acquired images analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module and the Cell Cycle Module. Nine image sites were acquired in each well (covering 70% of the total well area). Acquired data was transferred to an EXCEL spreadsheet and data was analysed and processed using graph pad prism. The two-tailed Student's t-test was used to compare effects of treatments to control values.

Results and Discussion

Cytotoxicity assay
The anti-proliferative effect of *D. velutinum* ethanol leaf extract was investigated using brine shrimp larvae, HeLa and MCF-7 cells. After 24-hour exposure of the brine shrimp larvae to the ethanol extract of *D. velutinum*, an LD_{50} of 199.5 µg/mL was observed. The IC_{50} value on HeLa cells was 53.27µg/mL while that of MCF-7 was > 200 µg/mL. The IC_{50} values of the extract on MC-7 cells was > 200 µg/mL and previous studies have shown that IC_{50} values should be less than 100 µg/mL to avoid having false positive results for *in vitro* studies.[17] The extract exhibited selective toxicity, causing substantial inhibition of growth in HeLa cells when compared to MCF-7. For this reason, further studies were done on HeLa cells only. The extract showed no toxicity against Vero cells up to the highest tested concentration of 200 µg/mL. The relatively high LD_{50} of 199.5 µg/mL after a 24 h period provides further evidence of the selective toxicity of the extract.

Cell cycle analysis
A major hallmark in carcinogenesis is cell proliferation, which is associated with deregulation of cell cycle progression.[18,19] The dysregulation of the cell cycle check points and over expression of growth promoting cell cycle factors are associated with tumorigenesis.[20] The cell cycle has four sequential phases, with the two most important being the S phase, where DNA replication occurs and M phase, where the cell divides into two daughter cells.[21] After treatment of HeLa cells with *D. velutinum* ethanol leaf extract for 48 hours, there was a significant increase in the percentage of cells in the G0/G1 phase ($p<0.0001$) and the early M phase ($p<0.001$) when compared with the untreated control. The G1-S and G2-M check points are involved in detecting and correcting DNA damage.[22] Large percentage of cells found in these phases could suggest that cell cycle arrest by *D. velutinum* occurs at the G1-S check point or that the extract excited the cell cycle into the G0 phase. The mechanism involved cannot be determined by cell cycle analysis alone as further studies need to be carried out to determine the exact mechanism of cellular arrest.

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Phosphatidylserine (PS) translocation

Programmed cell death (PCD) can occur either via the apoptotic (PCD I), autophagic (PCD II) or programmed necrotic (PCD III) pathway.[23,24] Although, the apoptotic death pathway has been studied greatly in connection to programmed cell death, many death regulatory genes have been found to be common to more than one pathway, therefore programmed cell death can be regarded as a network of interconnected pathways comprising of the three main death modes.[24] Annexin V-FITC/PI in addition to Hoechst dye was used to analyse the mechanism of cell death induced by the plant extract. Cells stained with Annexin V are indicative of apoptotic cells, while those with PI staining are indicative of necrosis and cells stained with both means late apoptosis or necrosis. After treatment of HeLa cells with *D. velutinum* leaf extract for 24 hours, a greater percentage of the cells were found to be necrotic, this suggests that the anti-proliferative potential of *D. velutinum* leaves is not via the apoptotic pathway but can be attributed to programmed necrosis (Table 2). This data is supported by the cell cycle analysis where Annexin V staining was not observed. Necrosis in time past has been characterised as an unregulated or uncontrolled form of cell death. However, recent research findings suggest that necrosis can also be as regulated as apoptosis.[25,26]

Caspase- 3 and -8 activation assay

Caspases play an important role in apoptosis and are responsible for many of the morphological and biochemical features of apoptosis. Apoptosis occurs through two major pathways; the intrinsic (mitochondrial) and extrinsic (death receptor) pathways; both pathways are triggered by activated caspases. Caspase 8 is an initiator caspase of the extrinsic pathway and once activated triggers the activation of the effector caspase 3 and 7 which inevitably leads to apoptosis.[27,28] After treatment of HeLa cells with *D. velutinum* for 48 hours, there was an increase in the activation of cleaved caspase -8 and -3, confirming that the cytotoxic potential of *D. velutinum* is caspase dependent and that the extract activates the extrinsic pathway of apoptosis (Table 1). Caspase activation has been implicated in a number of cell death models, the most frequent being apoptosis.[29,30] However, because caspases have other functions, the activation of caspase is not a sufficient indicator of apoptosis. Caspases have been less implicated in the necrotic pathway of cell death, although reports have also shown that necrosis can also occur in the presence of activated caspases.[31] This is in contrast to the norm where necrosis is stimulated when caspases are inactivated in cells and indicates that cells can die via the necrotic pathway, despite the presence of active caspases.

Mitochondrial membrane depolarisation

Depolarisation of the mitochondrial membrane is a well-known feature of mitochondria-dependent apoptosis.[32] Mitochondrial membrane depolarisation was measured using tetramethylrhodamine, ethyl ester (TMRE) dye. *D. velutinum* caused a significant ($p<0.0001$) decrease in the mitochondrial membrane potential in HeLa cells compared to control cells after 24 hours (Table 1).

Necroptosis, a type of programmed necrotic pathway, shares several signalling pathways with apoptosis. Necroptosis is initiated by death receptors through a series of cell signalling cascades leading to membrane damage and mitochondrial fragmentation.[25] Programmed necrosis has been reported to lead to the depolarisation of the mitochondrial membrane as the pro-necrotic complex RIP 1-RIP3 interacts with some metabolic enzymes including glutamate ammonia ligase and glutamate dehydrogenase 1 causing increased presence of reactive oxygen species.[24] The reactive oxygen species produced lead to the depolarisation of the mitochondrial membrane. Therefore, the depolarisation of the mitochondrial membrane by *D. velutinum* can be said to be a result of programmed necrosis.

The results from this study show that *D. velutinum* exhibited its anti-proliferative potential by mediating cell cycle arrest at the G1-S phase and cell death due to necrosis while activating the caspase system and inducing mitochondrial membrane depolarisation.

Table 1: Fold changes in levels of active caspase -3 and -8 and mitochondrial membrane potential in HeLa cells after treatment with *D. velutinum* and melphalan.

	Untreated control	*D. velutinum* (53.27 µg/mL)	Melphalan (50µM)
Mitochondrial membrane potential	1	0.36 ± 0.10****	0.56 ± 0.06****
Cleaved caspase -8	1	11.89 ± 0.54****	1.86 ± 0.06***
Cleaved caspase -3	1	12.80 ± 0.88****	1.94 ± 0.08*

Caspase activation was monitored after 48 hours and mitochondrial membrane potential after 24 hours. Values are expressed as fold increase in comparison to an untreated control, represented as mean ± standard deviation. Asterisks indicate significant difference compared to control *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.

Table 2: Percentage live, apoptotic, necrotic and late apoptotic HeLa cells.

	Untreated	*D. velutinum* (53.27µg/mL)	Melphalan (50 µM)
Live	96.82 ± 0.87	76.75**** ± 4.75	79.36**** ± 2.28
Apoptotic	0.92 ± 0.14	0.11**** ± 0.04	2.20**** ± 0.24
Necrotic	1.38 ± 0.87	23.06**** ±4.79	10.99**** ± 1.80
Late apoptotic	0.88 ± 0.16	0.08** ± 0.04	7.46**** ± 0.80

Values are expressed as percentage mean ± standard deviation. Asterisks indicate significant difference compared to control *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.

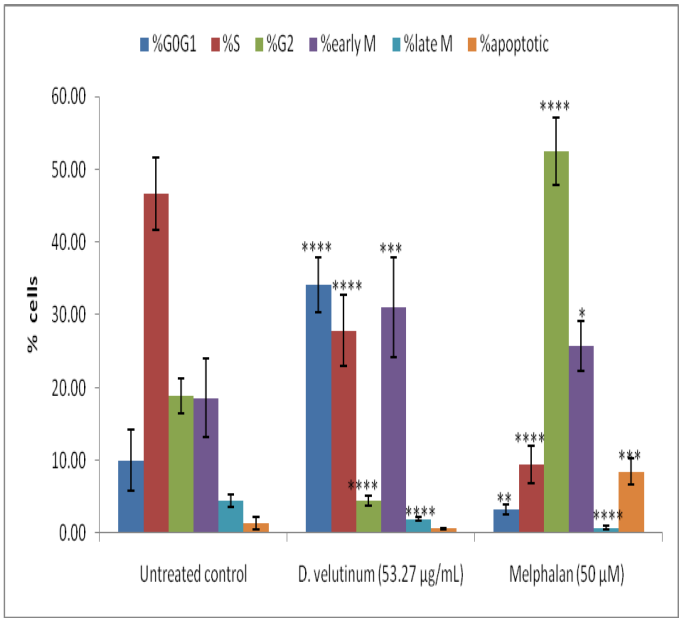

Figure 1: DNA cell cycle analysis using HeLa cells after 48 hours of exposure to *D. velutinum*.

Melphalan was used as a positive control. Bar graph represents the average of quadruplicate determinations. SD is represented as error bars. Asterisks indicate significant difference compared to control *p < 0.05, ** p < 0.01, ***p < 0.001, ****p < 0.0001.

Conclusion

In time past, necrosis has been associated with unregulated and uncontrolled cell death but recent finding point to the fact that necrosis can indeed be a regulated process involving the caspase system. The

anti-proliferative activity of *D. velutinum* via the necrotic pathway of cell death and the activation of the caspase system can be further explored to understand the exact pathway of necrotic cell death.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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