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Research Article

Studies on *Desmodium velutinum* (Willd.) DC. Leaf Extract's Polyphenol Profile, *in-vitro* Antioxidant Capacity, and Anti-Proliferative Activity of A549 Cell Lines

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ABSTRACT

In African and Asian ethnomedicinal studies, *Desmodium velutinum* (Willd) has been used in the treatment of tooth and head aches, diarrhoea, cancer, and haematuria and as repellent and aphrodisiac. The study is aimed at determining polyphenol profile and antioxidant activities of *D. velutinum* methanol leaf extract and its ethyl acetate and butanol fraction. The study also investigated the effect of this plant on lung adenocarcinoma (A549) cell line. The polyphenol constituents in *D. velutinum* were evaluated with the U-HPLC technique. While, the antioxidant activities of the methanol plant extract and its fractions were assessed with the DPPH, metal chelating, reducing power and DNA nicking assays. MTT assay was employed to analyse the effect of the plant on A549 cell line. Kaempferol is the principal polyphenol in the leaf extract/fraction of *D. velutinum*. Conversely, its ethyl acetate fraction possessed a higher concentration of catechin than kaempferol. Total phenolic content (TPC) result was directly proportional to the antioxidant activities of extract and fractions, a higher TPC value was obtained in ethyl-acetate fraction (787.1 ± 21.4 mg GAE/g) than the crude methanol extract (710.9 ± 24.5 mg GAE/g) and its n-butanol fraction (759.2 ± 1.3 mg GAE/g), thus, ethyl acetate fraction performed best in the antioxidant assays. *D. velutinum* also inhibited oxidative damage to supercoiled plasmid DNA and suppressed the proliferation of A549 cells in a dose-dependent manner. This study shows that *D. velutinum* does have potentials to protect against many diseases linked with reactive oxygen species.

Keywords: *Desmodium velutinum, U-HPLC, Kaempferol, Reactive oxygen species*

INTRODUCTION

The culture of using plants as remedies for diseases is as ancient as the existence of man itself. According to the World Health Organisation (2005), 90% of Africans, 70% of Indians, and 40% of the Chinese population rely on traditional medicine to help meet their health care needs. This is in spite of the great advances in modern scientific medicine, which have led to the development and mass production of chemically synthesised drugs in most parts of the world. *Desmodium velutinum* (Willd) DC is one of the plants employed to meet the health care needs in some African countries and in India. *D. velutinum*, an erect or semi-erect perennial shrub, belongs to the family Fabaceae (Leguminosae) and can grow up to 3 metres high. It has a velutinous, short, hooked-hairy branch and a 1-foliate leaf about 4–20 cm long with a densely velutinous undersurface.

It has 2–5 purple–pink flowers at each node of its inflorescence (Akinola and Afolayan, 1991). Some of its ethnomedicinal uses include pain relief, aphrodisiac, antipyretic, antimalarial, and anticancer agents, enamel against haematuria, and treatment of diarrhoea (Abubakar *et al.*, 2007; Ibrahim *et al*., 2010; Kanthale and Biradar, 2012; Sorgho *et al*., 2013; Eze-Steven *et al*., 2014; Ezike *et al*., 2014; Isah *et al*., 2016). While some of these claims have been proven scientifically, there is a dearth of knowledge as to the compounds contained in the leaves of this plant that are responsible for their actions. Polyphenols are one of the active constituents of plants that have generated a lot of recent research attention (Sajid *et al*., 2019). Polyphenols, broadly divided into eight categories of phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids, are widely known for their varied biological activities (Mutha *et al*., 2022). Not only are they widely known as natural antioxidants, but there are reports on their anticancer properties. In this research paper, some of the polyphenolic compounds contained in the leaves of *D. velutinum* were investigated using the Ultra High-Performance Liquid Chromatography (U-HPLC) provided with a diode array detector. This research also evaluated the anti-oxidant activities of *D. velutinum* methanol leaf extract and fractions and investigated their actions on the lung cancer cell line A549.

MATERIALS AND METHODS

Reagents

The reagents used in this research includes rutin hydrate, Gallic acid, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine 123 (Rh-123), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and Dapi (4', 6-diamidine-2'-phenylindole) dyes which were purchased from Sigma Chemical Co. St. Louis, MO, USA. Also, DPPH, Methanol (HPLC grade and analytical grade) were purchased from Himedia Laboratory Pvt Ltd. Other reagents used include Dimethyl sulfoxide obtained from Merck specialities Private Limited Mumbai India, Ammonium acetate (Thermo Fisher scientific media India Pvt Ltd.), cupric chloride and Neocuproine from LOBA chemie Pvt Ltd Mumbai India.

Collection of plant materials

Fresh samples of *D. velutinum* were collected from farm land in Zaria, Kaduna state, Nigeria. The plant was identified by Alhaji Namadi Sanusi of the Herbarium section, Department of Biological Sciences, Ahmadu Bello University (ABU) Zaria, Kaduna State, Nigeria. The issued voucher number is 636. The sample was washed and dried under ambient conditions.

Extraction of plant material

The powdered leaf of *D. velutinum* was extracted with 80% methanol by employing maceration method. The 80% methanol extract (M.E) so obtained was further fractionated using different solvents viz. ethyl acetate and n-butanol to obtain ethyl acetate fraction (E.F) and n-butanol fraction (B.F) respectively. The solvents of different polarities used, partitioned and separated the various metabolites of methanol extract as per their solubility. The supernatant obtained was pooled and filtered after extracting in respective solvents, three times, using Whatman no. 1 sheet and concentrated by vacuum rotary evaporator (Strike 202, Stereo glass, Italy) according to the protocol described by Makanjuola *et al*., (2023).

Total phenolic content

The Total phenolic content of the methanol extract of *D. velutinum* (M.E) and its fractions, E.F and B.F was assessed by the Folin-Ciocalteu method previously describe by Ainsworth and Gillespie (2007) with slight modifications. The absorbance of the reaction mixture (10% Folin-Ciocalteu, 7.5% Na_2CO_3 and extract/fraction) was recorded at 765 nm by multi detection microplate reader (Synergy HT Biotek). TPC of the extract and fractions was calculated from a standard curve for Gallic acid (G.A), the value obtained was expressed as mg Gallic acid equivalent per g of the extract.

Total antioxidant capacity (molybdate ion reduction assay)

This assay is based on the principle of diminution of Mo (VI) to Mo (V), this reduction leads to the appearance of green coloured phosphate/Mo (V) complex (Prieto *et al*., 1999). The intensity of this coloration was measured at 695 nm by spectrophotometry. The total antioxidant capacity (TAC) of the extract and each fraction of *D. velutinum* was established through separate reactions (at 1000 g/mL) with a reagent mixture composed of hydrogen sulphate (0.6 M), disodium sulphate (28 mM), and ammonium molybdate (4 mM). The TAC was derived using the linear regression equation of a standard curve obtained from the spectrophotometric measurement of the colour intensity of the reaction of ascorbic acid with the reagent mixture at various concentrations. Therefore, the TAC of Extract/fractions of *D. velutinum* was stated as mg Ascorbic Acid Equivalents (AAE)/g dry weight of extract or fraction. Prior to spectrophotometric analysis, each reaction was left to cool at room temperature after 60 minutes of incubation at 95°C.

Ultra high-performance chromatography profile

To determine the polyphenolic constituents in the *D. velutinum* leaf, samples of the M.E and its fractions (E.F and

B.F) were analysed using a Shimadzu UHPLC Nexera system (Shimadzu, MA, USA) provided with diode array detector, using C18 column (150 mm x 4.6 mm, i.d 5 µm). The temperature of the column was maintained at 25 °C. The gradient mobile phase utilised in the experiment consisted of 0.1% acetic acid aqueous as solution A and Methanol as solution B. The gradient elusion was set at: 0 – 1 min: 35% B; 1-10 min: 65% B; 10-14 min: 80% B; 14-16 min: 80% A; 16-17 min: 40% B; 17-20 min: 35% B; 20-21 min: 30% B. The flow rate was set at 1 mL/min and the injection volume was 5 µL. The peaks were quantified using the software included with the Shimadzu UHPLC Nexera system. Eleven natural polyphenols were tested using the provided standard. They were mixed in different concentrations (2000, 4000, 6000, and 8000 ppm) to quantify these eleven natural polyphenols in the plant extract/fractions, and the calibration curves obtained were used to determine the various concentrations in the extract/fractions.

Diphenylpicrylhydrazyl (DPPH) assay

The free radical scavenging ability of *D. velutinum* M.E and its fractions, E.F and B.F was tested with DPPH assay. The experiment was executed by adding varying concentrations of the extract/fractions dissolved in methanol to the DPPH solution as described by BLOIS (1958). The DPPH solution and methanol mixture only served as a negative control; the resulting negative control mixture produced a purple coloration (maximum absorbance) after shaking and standing for 30 minutes. The decrease in the purple coloration in presence of the extract or fraction signifies the conversion of the free radical DPPH to its non-radical form. An increase in this discoloration (reduction in absorbance) at 517 nm indicates higher hydrogen donating potential or free radical scavenging ability.

The result of extract/fraction in this experiment was compared with Rutin and Gallic acid. The percentage DPPH scavenging ability was calculated as:

$$\% \text{ Inhibition of DPPH} = \left(\frac{\text{TD control} - \text{TD sample}}{\text{TD control}}\right) 100$$

Where, TD control = Absorbance (Transmission Density) of negative control (Blank without extract/fraction or reference standard compound) and TD sample = Absorbance of the extract/fraction/standard drug at a given concentration

Metal chelating activity

The ability of the M.E of *D. velutinum* and its fractions to chelate ferrous ions was evaluated by the method described by Dinis *et al.* (1994) with slight modification. In brief, 100 µL of each concentration (i.e. 6.25, 12.5, 25, 50, 100, 500, 1000 µg/ml) of extract/fraction was added to methanol (370 µL) and 2 mM of $FeCl_2$ (10 µL). To initiate a reaction, 20 µL of 5 mM of Ferrozine was added to the mix. This reaction mixture was allowed to stand at room temperature for 10 mins. Absorbance was determined at 562 nm against blank (i.e. reaction mixture without the extract/fraction). EDTA was used as positive control. The percentage inhibition of ferrozine - Fe^{2+} complex formation was calculated as

$$\% \text{ Inhibition} = \left(\frac{\text{TD control} - \text{TD sample}}{\text{TD control}}\right) 100$$

Where, TD control = Absorbance of negative control (Blank without extract/fraction or reference standard compound) and TD sample = Absorbance of the extract/fraction/standard drug at a given concentration

Reducing power assay

The procedure outlined by Oyaizu (1986) and Romero *et al.* (2014) with slight variation was used to examine the electron-donating potential of the M.E. and fractions of *D. velutinum*. In brief, the mixture containing 100 µL of extract/fraction in methanol and 250 µL of Phosphate buffer solution (PBS) was incubated with 250 µL of 1% potassium ferricyanide for 20 minutes at 50 °C. Following the incubation period, 250 µL of 10% TCA and distilled water was added to mixture. Finally, the mix was supplemented with freshly prepared 0.1% $FeCl_3$ (50 µL). Absorbance of the reaction was measured at 700 nm. The extract's ability to donate electrons was expressed as a percentage of gallic acid at the highest concentration tested, with gallic acid serving as the benchmark.

Cupric ion reduction (CUPRAC) assay

CUPRAC assay was performed as described by Apak *et al.* (2004) with minor modifications. The assay was conducted by adding 100 µL of 10 mM Copper (II) Chloride to 100 µL of 7.5 mM Neocuproine. To this reaction 100 µL of Ammonium acetate was added to buffer the liberated protons. After which 100 µL of extract/fraction was added and the total volume was brought up to 2.5 mL by adding 800 µL of distilled water. The final reaction mixture was incubated at room temperature for 30 minutes and absorbance was measured at 450 nm. Cupric ion reduction of extract/fraction was calculated as:

$$\left(1 - \left(\text{TDr} - \frac{\text{TDs}}{\text{TDr}}\right)\right) * 100$$

Where T Dr is the absorbance of reference compound at the maximum concentration and T Ds is the absorbance of extract/fraction at a given concentration.

DNA nicking assay

The ability of *D. velutinum* plant to prevent oxidative damage of supercoiled pBR 322 plasmid DNA was assessed

by DNA nicking assay. The experiment was performed with agarose gel electrophoresis as described by Lee *et al.* (2002) and Robin *et al.* (2015). Oxidative damage was induced by fenton reaction which liberates hydroxyl radical that causes breakage of the supercoiled DNA. The negative control was in the absence of fenton reagent, while the positive control was done in the presence of fenton reagent only. Experimental groups contained varying concentration of extract/fractions in the presence of fenton reagent and the outcome was compared to the standard antioxidant compound, rutin. Analysis of the activity of the extract/fraction in DNA nicking assay was done by Gel documentation system (Gel Doc XR, Bio-Rad USA) and densitometric analysis was performed by using AlphaEaseFC 4.0 software.

Cell culture

Non-small cell lung cancer cell line A549 was procured from the National Centre for Cell Science Pune, Mumbai. A549 cells were routinely cultured in DMEM (Dulbecco's Modification of Eagle's Medium) supplemented with 10% FBS, penicillin, 100 µg/mL streptomycin (complete medium) in a humidified incubator maintaining 5% CO_2 at 37 °C. For experimental purposes, A549 cells were harvested by centrifugation for 5 min at 1500 rpm.

MTT colorimetric assay (Anti-proliferative Assay)

The potential anti-proliferative activity of M.E of *D. velutinum* and its fractions on A549 cells was determined by MTT assay described by Fischer *et al*. (2003) and Vinjamuri *et al*. (2015). A549 cells were seeded on 96-well plates with 5 × 104 cells in 200 µL medium per well. Then, the plates were incubated at 37 °C for 24 h under a 5% CO_2 atmosphere and humid chamber till the cells adhered to the surface of the plates. Then, different concentrations of extract/fraction (12.5, 25, 50, 100, 500 and 1000 µg/mL) in culture medium were added to the wells, and the culture was further incubated for another 24 hours. Culture medium without extract was used as a negative control, while camptothecin (10 mM) was used as positive control. After this treatment, 200 µL of MTT was added (5mg in 10 ml of DMEM medium without FBS) in the 96-well plates. Afterwards, the plates were incubated for another 4 h at 37 °C and centrifuged. Subsequently, the culture medium was removed carefully, and the plates were washed carefully twice with PBS buffer. Then, aliquots of 150 µL of DMSO were added into each well and oscillated until the formazan crystals were dissolved completely. The mixture was measured at 540 nm using a BioTek synergy HT Elisa microplate reader. Percentage inhibition of proliferation by extract fraction was calculated as follows:

$$\%\text{Inhibition} = \left(1 - \frac{\text{A}}{\text{B}}\right)100$$

Where A is the average optical density of the extract/fraction-treated cells and B is the average optical density of the negative control wells (culture medium without extract with cells).

Dapi DAPI (4', 6-diamidine-2'-phenylindole) staining

To determine the morphological changes in A549 cancer cells after treatment with *D. velutinum*, DAPI staining technique was employed as described by Alam *et al.* (2017) and Kaur *et al.* (2019) with minor modifications. In brief, A549 cancer cells at concentration of 5 x 10^5 cells/well were seeded in 6 well plate containing a slide coverslip per well. These cells were allowed to adhere to the cover slips for 24 hours, after which the cells were treated with E.F of *D. velutinum* for 24 hours. Cells were subsequently washed with PBS (1 x) and fixed with 4% PFA for 30 minutes. After fixation, the cells were stained with DAPI (concentration of 10 µg/mL), and then washed in PBS twice after which the coverslips were picked up from the wells using a forceps and mounted on a slide with a fluoromount aqueous medium (Sigma-Aldrich). Slides were allowed to dry and thereafter taken for imaging using Nikon laser scanning confocal microscope (eclipse Ti), A1R model with resonate scanner (Nikon Corp., Japan). Fluorescence was observed with a long-pass 488 emission filter.

Measurement of mitochondrial membrane potential

The effect of *D. velutinum* leaves on mitochondrial membrane potential of A549 cells was evaluated using the ethyl-acetate fraction (E.F) with the aid of rhodamine-123, a fluorescent dye which binds to mitochondria membrane (Deng *et al.*, 2013). The A549 cells were treated with E.F for 24 hours in a 24 well plate with various concentrations (representing the IC_{30}, IC_{50} and IC_{70} values obtained in the MTT assay). Thereafter, cells were incubated with rhodamine 123 (1 µM) for 45 minutes. Cells were washed three times with PBS (1x). Rhodamine 123 fluorescence was evaluated using ELISA plate reader (BioTek Multi-Mode Microplate Reader) with a laser excitation of 488/20 nm and 528/20 nm emission filter.

Measurement of intracellular reactive oxygen species

This is a study conducted to determine the mechanism in which E.F induces cell death in A549 cells. This experiment was conducted as described by Zhu *et al*. (2012) with slight modification, A549 cells were treated with various concentrations of E.F of *D. velutinum*. The concentrations used in this experiment represent the IC_{30}, IC_{50} and IC_{70} values obtained in the MTT assay. After 24 hours of treatment with E.F, the cells were incubated with 10 µg/mL of the fluorescent probe DCFH-DA (2'-7'-dichloro dihydro fluorescein) for 30 min at 37 °C. Thereafter, the cells were

washed with phosphate buffer solution (1xPBS) for three times to remove the extracellular DCFH-DA. Intra-cellular ROS levels in the A549 cells with or without extract/fraction treatment were detected through determining the fluorescence intensity of DCF using fluorescence spectrophotometer at an excitation wavelength of 488/20 nm and emission wavelength of 528/20 nm using the Synergy HT BioTek Microplate reader.

Statistical analysis

All experiments were performed at least three times. Numerical data were expressed as mean ± standard error (SE). The difference between means was analysed by using one-way ANOVA and Tukey Post-hoc test. All statistical analyses were performed by using SPSS 17.0 software (Chicago, IL, USA). Values of $p < 0.05$ and $p < 0.01$ were considered statistically significant.

RESULTS

Total phenolic contents (TPC) and total antioxidant capacity (TAC)

Results of TPC in *D. velutinum* M.E and its fractions, E.F and B.F as presented in Table 1 revealed that M.E possess lower amount of phenolic content (710.9 ± 24.5 mg GAE/g dry extract) compared with the fractions studied. The fraction, E.F displayed a higher TPC (787.1 ± 21.4 mg GAE/g dry extract) compared with B.F (759.2 ± 1.3 mg GAE/g dry extract). TPC was calculated using the equation, $Y = 0.2249\,in(x) - 0.5631$ with $R^2 = 0.97$ derived from a standard curve for Gallic acid.

Estimated by molybdate ion reduction assay, the total antioxidant capacity (TAC) of M.E of *D. velutinum* was 52.2 µg Ascorbic acid equivalent (AAE)/mg of dry extract, while TAC in the fractions, E.F and B.F was 87.6 µg (AAE)/mg of dry extract and 46.2 µg (AAE)/mg of dry extract respectively. This showed that TAC in E.F > M.E > B.F (Table 1).

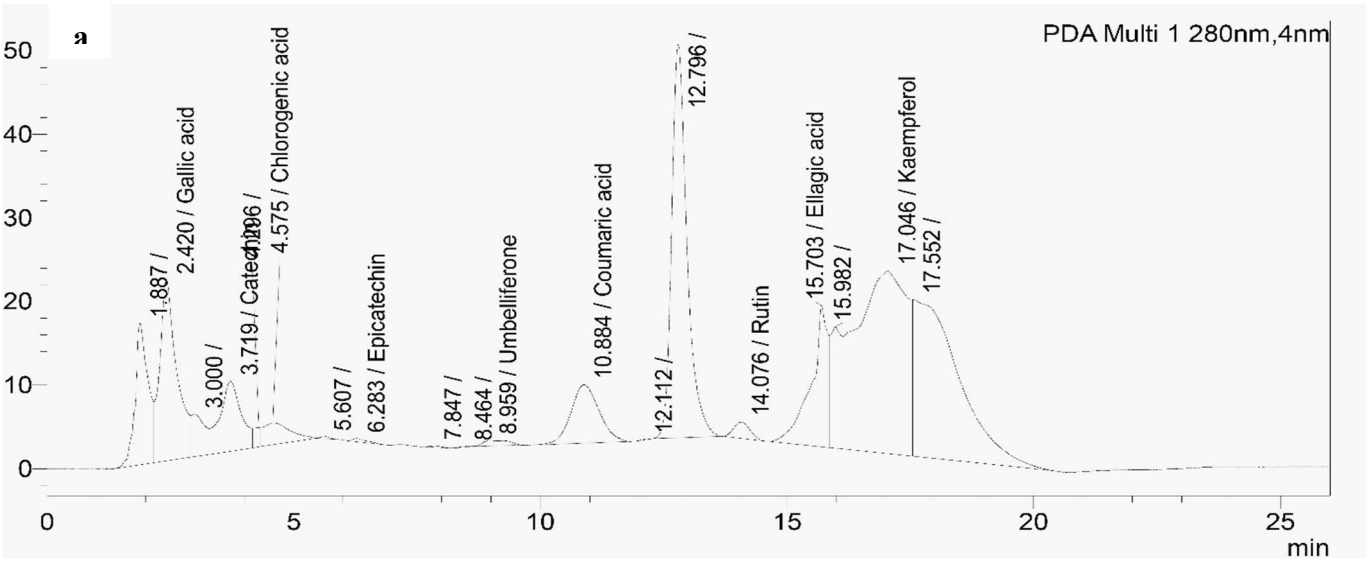
Table 1. Total Phenolic Contents (mg GAE/g dry extract) and Total Antioxidant Capacity (µg AAE/mg dry extract) of Methanol Extract *of D. velutitnum* and its Butanol and Aqueous Fractions

Extract/Fraction	TPC (µg GAE/mg dry extract)	TAC (µg AAE/mg dry extract)
Methanol Extract (M.E)	710.9 ± 24.5	52.2 ± 5.4
Ethyl-acetate Fraction (E.F)	**787.1 ± 21.4**	**87.6 ± 1.9** [a, b]
Butanol Fraction (B.F)	759.2 ± 1.3	46.2 ± 2.8

Mean ± SEM; [a, b] = significantly different from M.E and B.F respectively at $p < 0.05$ (one way ANOVA)

U-HPLC analysis

The chromatogram of U-HPLC analysis of *D. velutinum* leaves extract and fractions as displayed in Figure 1(a-c) revealed several peaks which were compared with pure polyphenol standards. However, four phenolic acids (gallic acid, chlorogenic acid, coumaric acid and ellagic acid); four flavonoids (catechin, epicatechin, rutin and kaempferol); and umbelliferone, a coumarin were identified and quantified in the crude M.E and B.F of the plant. In addition to these polyphenols, a phenolic acid (caffeic acid) and a flavonoid (quercetin) were detected in the E.F. Kaempferol was the predominant polyphenol identified in the crude extract (M.E) and the fraction, B.F. Conversely, the fraction, E.F exhibited an array of dominant polyphenol (Figure 2). The concentration of the array of dominant polyphenols in E.F ranked from the highest is catechin > kaempferol > ellagic acid > gallic acid > chlorogenic acid > epicatechin.



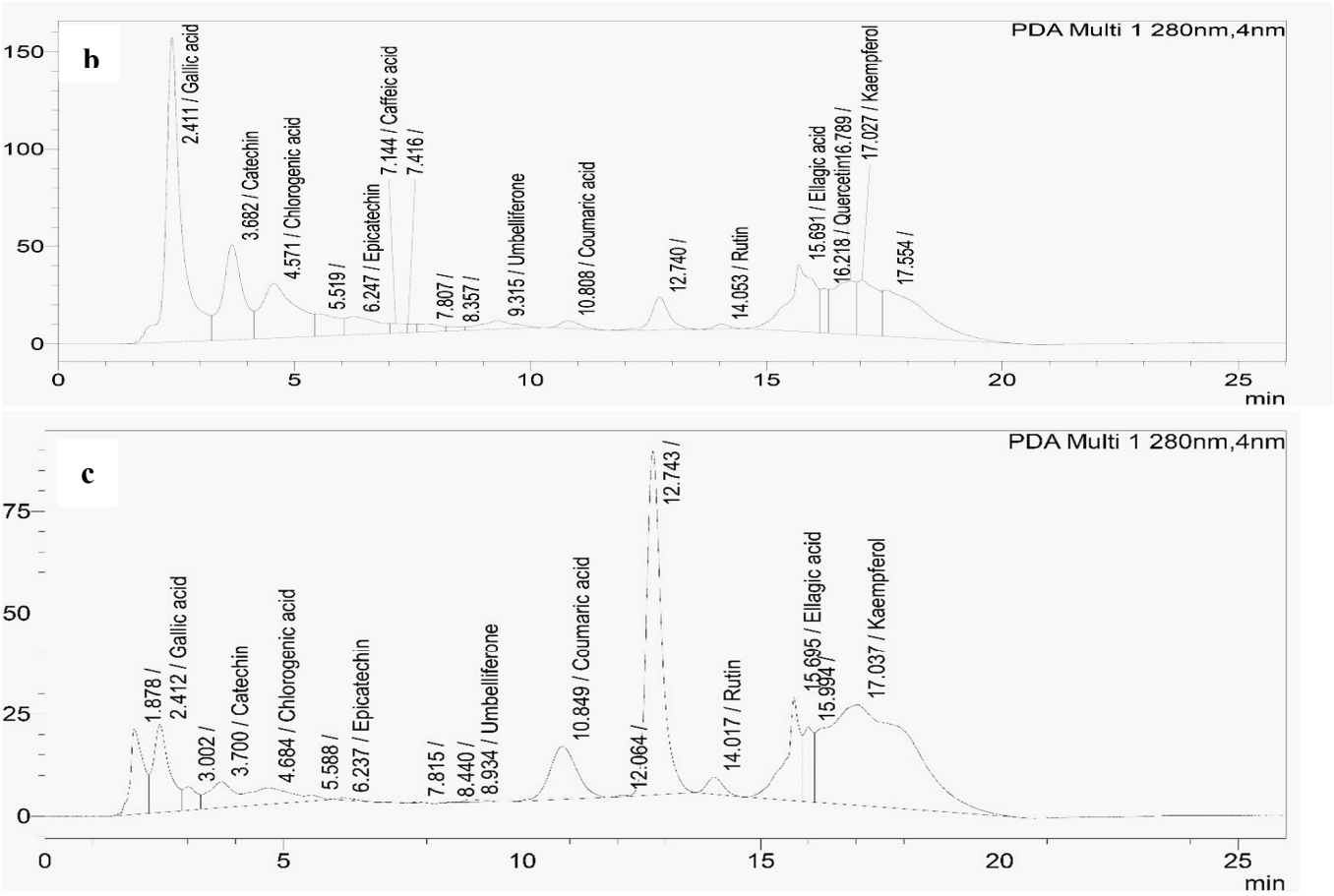


Figure 1. U-HPLC Chromatogram of Methanol Extract (a), Ethyl-acetate Fraction (b) and N-butanol Fraction (c) of *D. velutinum*

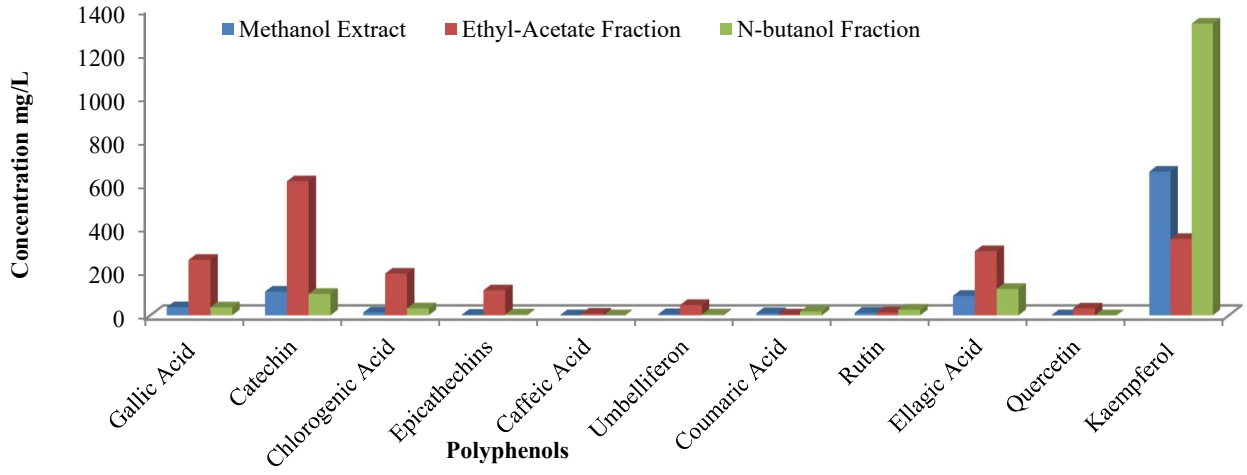


Figure 2. Polyphenol Constituents of Methanol Extract of *D. velutinum*, Ethyl-acetate and N-butanol Fraction

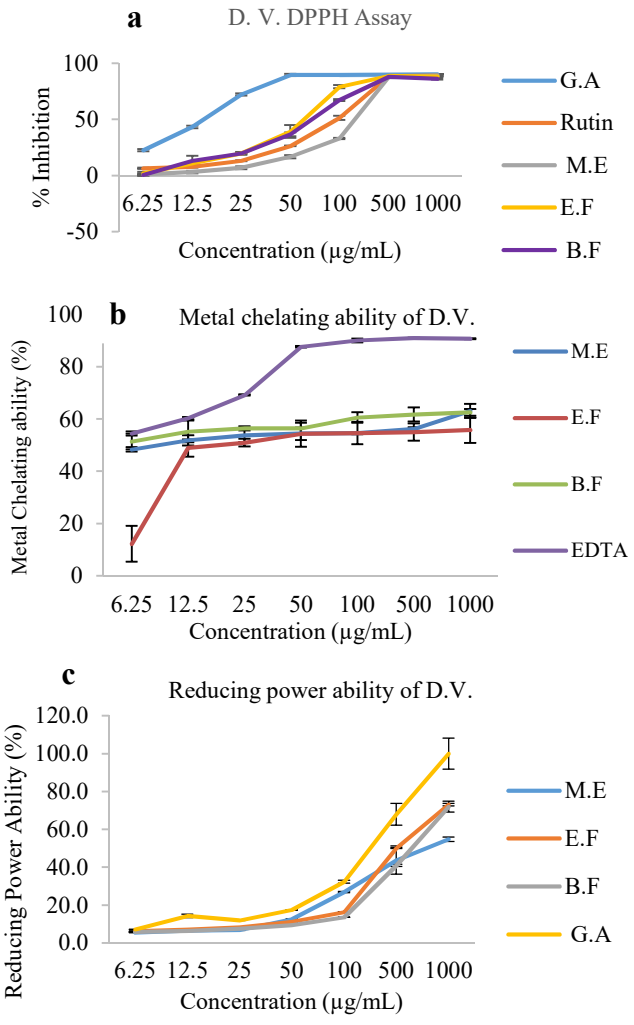
Antioxidant capacity of *D. veultinum* extract

Four antioxidant assays viz. DPPH, metal chelating, reducing power and CUPRAC were performed to evaluate and compare the antioxidant power of the crude methanol extract (M.E) of *D. velutinum* leaves and the studied fractions, E.F and B.F.

As shown in Figure 3(a), M.E, E.F and B.F exhibited dose dependent scavenging potentials against DPPH assay, with E.F demonstrating the greatest potential with an IC_{50} value of 76.2 µg/mL. B.F with an IC_{50} value of 88.3 µg/mL exhibited a greater scavenging potential than M.E (IC_{50} value of 145.6 µg/mL). Furthermore, E.F and B.F performed better than the pure antioxidant, rutin but not as good as the pure antioxidant gallic acid. The IC_{50} values of gallic acid and rutin in this study were 55.8 µg/mL and 102.1 µg/mL respectively.

The metal chelating activity of M.E and its fractions is displayed Figure 3b. The result showed that D.B showed a superior metal chelating power of 62.5% with IC_{50} value of 1.5 µg/mL compared to M.E. which exhibited a maximum chelating power of 63.1% and IC_{50} value of 8.1 µg/mL. E.F, in contrast, showed the least chelating power of 55.7% and IC_{50} value of 104.8 µg/mL. However, the standard chelating compound, EDTA, showed a superior chelating power of 91% and IC_{50} value of 1.5 µg/mL compared to *D. velutinum* extract and fractions.

E.F showed a greater antioxidant potential than M.E and B.F in the reducing and CUPPRAC assays as displayed in Figure 3(c-d). The electron donating capacity of the extract/fraction to reduce ferri-cyanide ion to ferro-cyanide ion was expressed as a percentage of the standard compound, Gallic acid at 1000 µg/mL. The maximum reducing capacity expressed by E.F (73.0 %) was greater than B.F (72.0 %), which in turn was greater than M.E (54.8 %). Similarly, the ability of the extract/fraction to reduce Cu^{2+} chelate to yellow orange coloured Cu^{1+} chelate was greater in E.F (81.6 %) than M.E (76.6 %) and B.F (69.8 %).



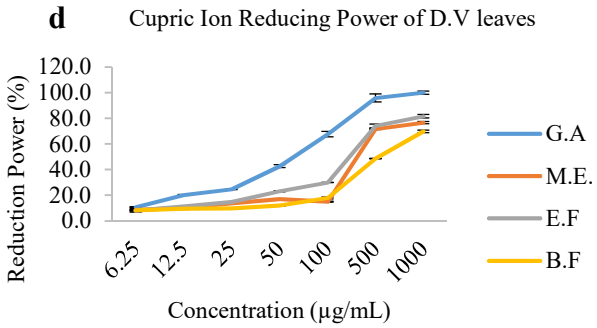


Figure 3. Antioxidant Activity of Different Concentrations of Extract/Fractions of *D. velutinum* Leaves Assessed by DPPH Radical Scavenging Assay (a), Metal chelating assay (b), Reducing power assay (c), Cupric-Ion-Reducing Antioxidant Capacity (CUPRAC) assay (d).
Data expressed as mean of % inhibition ± SE of triplicates

DNA nicking assay

For the reason that E.F of *D. velutinum* leaf extract emerged as the most potent antioxidant in majority of the bioassays, its antioxidant capacity was further evaluated in the DNA nicking assay. The outcome of the experiment showed that the ability of E.F to avert fenton induced oxidative damage of supercoiled plasmid DNA is dose dependent, protecting 60.7 % of the supercoiled plasmid DNA at 1000 µg/mL, the maximum dose tested (Figure 4 and 5). In the absence of the rutin or *D. velutinum*, fenton reagent induced oxidative damage on supercoiled plasmid DNA (form I) to produce open circular DNA (form II) or linear DNA (form III) which results from a single or double DNA strand break respectively.

Anti-proliferating activity of methanol extracts of *D. velutinum* leaf and the fractions

The anti-proliferation activity of *D. velutinum* leaves, M.E and the fractions, E.F and B.F was studied on A549 cell line in MTT assay. As shown in the Figure 6, the extract and fractions were able to inhibit the proliferation of A549 cells in a dose dependent pattern after 24 hours of incubation. The outcome showed cytotoxic potential against A549 cells when compared with the standard drug, camptothecin. However, ranked on the basis of their IC_{50} values, the anti-proliferating activity of E.F (IC_{50} = 274.1 µg/mL) < M.E (IC_{50} = 244.3 µg/mL) < B.F (IC_{50} = 131.5 µg/mL) as shown in Table 2.

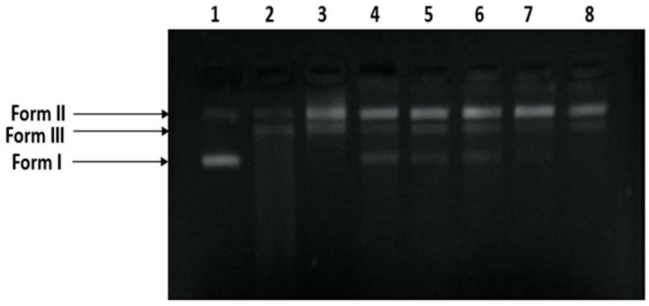
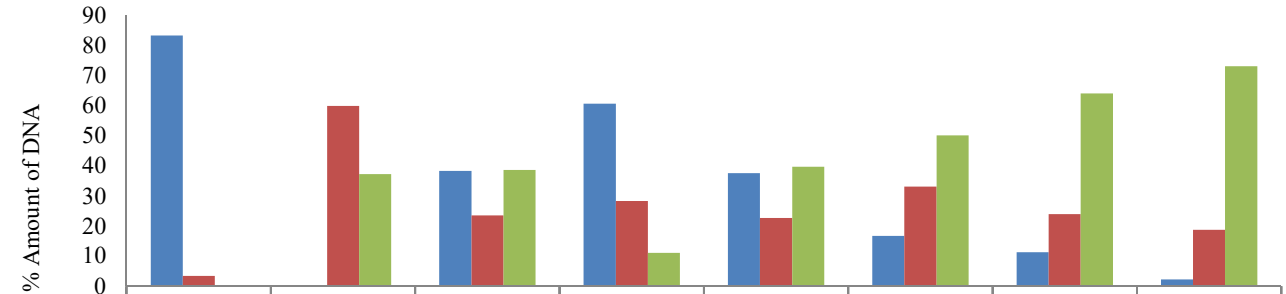


Figure 4. Outcome of Ethyl-Acetate Fraction (E.F) of *D. velutinum* in DNA Nicking Assay

Lane 1: Negative control (deionised water); lane 2: Fenton's reagent; lane 3: Positive control (Rutin); lane 4–8: Fenton's reagent + different concentrations of E.F of D. velutinum (1000, 500, 100, 50 and 25g/mL); Form I-supercoiled plasmid DNA; Form II- open circular; Form III- Linear DNA

Anti-proliferating activity of methanol extracts of *D. velutinum* leaf and the fractions

The anti-proliferation activity of *D. velutinum* leaves, M.E and the fractions, E.F and B.F was studied on A549 cell line in MTT assay. As shown in the Figure 6, the extract and fractions were able to inhibit the proliferation of A549 cells in a dose dependent pattern after 24 hours of incubation. The outcome showed cytotoxic potential against A549 cells when compared with the standard drug, camptothecin. However, ranked on the basis of their IC_{50} values, the anti-proliferating activity of E.F (IC_{50} = 274.1 µg/mL) < M.E (IC_{50} = 244.3 µg/mL) < B.F (IC_{50} = 131.5 µg/mL) as shown in Table 2.



	lane 1 control	Lane2 FR+DNA	lane3 FR+Rutin	lane4 Extract (1000 µg/ml	lane5 Extract (500 µg/ml	lane6 Extract (100 µg/ml	lane7 Extract (50 µg/ml	lane8 Extract (25 µg/ml
FORMI	83.42	0	38.3	60.66	37.56	16.69	11.23	2.17
FORMII	3.4	59.96	23.56	28.3	22.68	33.15	23.89	18.7
FORMIII	0	37.27	38.6	11	39.76	50.16	64.11	73.2

Figure 5. Densitometric Analysis of DNA in Plasmid Nicking Assay of Ethyl Acetate Fraction (E.F) of *D. velutinum*

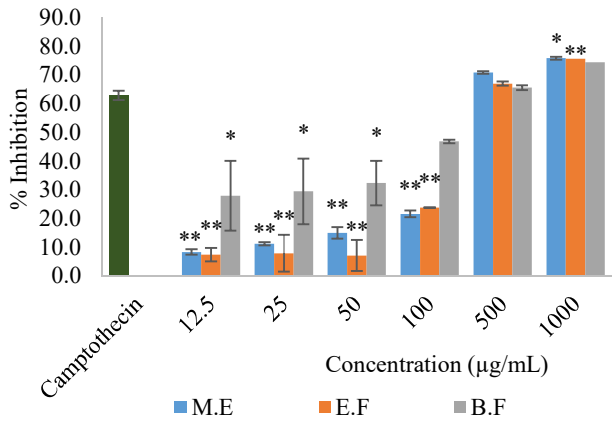


Figure 6. Inhibitory Effect of 10 µM of Camtothecin and Different Concentration of Methanol Extract of *D. velutinum*, Ethyl Acetate and Butanol Fraction on A549 cell Growth in MTT assay.

* Indicates significant difference from camptothecin at $p < 0.05$, ** indicates significance difference from camptothecin at $p < 0.01$ (one-way ANOVA; Tukey post-hoc test).

Table 2. Calculation of 50 Percent Inhibitory Concentration of A549 Cells by Methanol Extract *of D. velutinum* and its Ethyl Acetate and Butanol Fractions

S/N	Extract	IC_{50} (µg/mL)	Regression formula	R^2 Value
1	M.E. of *D. velutinum*	244.3	y = 17.369ln(x) - 45.503	0.9179
2	Ethyl-acetate fraction	274.1	y = 17.684ln(x) - 49.272	0.9148
3	Butanol fraction	131.5	y = 11.419ln(x) - 6.067	0.9644

Effect of treatment of *D. velutinum* leaves extract on A549 cell nucleus and DNA material

The A549 cells, with or without treatment, were visualised under the confocal microscope for morphological changes after being stained with DAPI. The outcome of the result was compared with A549 cells treated with camptothecin (a standard drug). It was observed that A549 cells in the negative experiment exhibited elongated to oval nuclei, while the A549 cells treated with either captothecin or E.F. exhibited spherical nuclei with chromatin condensation, blebbing, and apoptotic bodies (Figure 7).

Analysis of mitochondrial membrane potential (MMP) in A549 cells

To possibly elucidate the mechanism of by which E.F of *D. velutinum* induces apoptosis as observed under the confocal microscope, the mitochondrial membrane potential (MMP) in A549 cells was assessed. The MMP of A549 cells was evaluated by monitoring the intensity of fluorescence staining of rhodamine 123 (Figure 8). The result showed a dose dependent decrease in relative mean of fluorescence after treating with E.F. However, this decrease was significant ($p < 0.05$) from the control group at the 70% inhibitory concentration (850 µg/mL). Camptothecin (10 µM) was observed to cause significant decrease in MPP compared with control at $p < 0.01$).

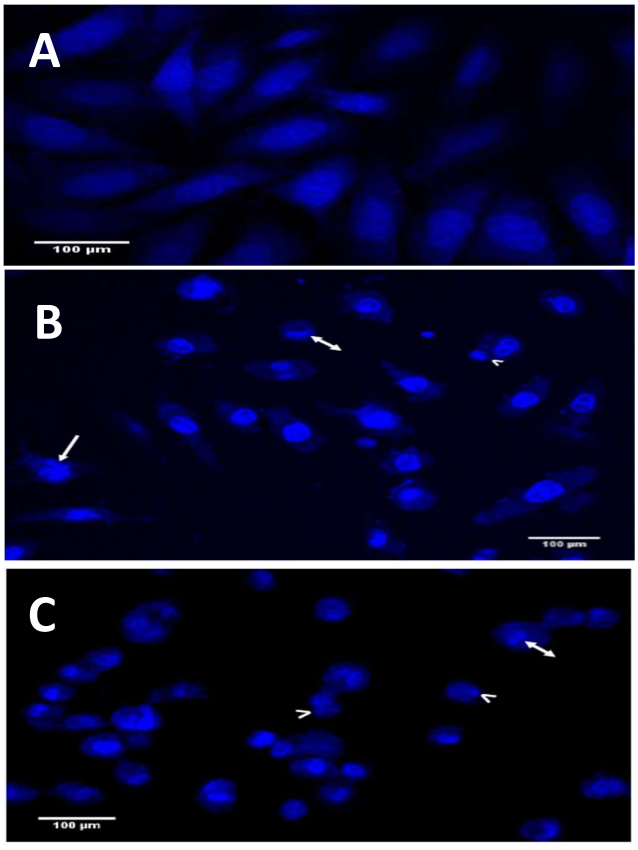


Figure 7. Effect of Ethyl-Acetate Fraction (E.F) of *D. velutinum* at 50% Inhibition Concentration on the Morphology of A549 cell line (DAPI stain).

A – control experiment; B – Camptothecin group; C – E.F group. Double headed arrow shows chromatin condensation; Single headed arrow shows nuclear fragmentation and blebbing; arrow head shows apoptotic bodies.

Effect of *D. velutinum* leaves on intracellular reactive xxygen species in A549 cells

To further elucidate the mechanism of action of E.F against A549 cells, the experiment to assess the intracellular reactive oxygen species (ROS) was conducted. Figure 9 shows the result of the study after treatment with camptothecin (standard drug) and E.F. The intracellular ROS was estimated from expression of DCFH-DA fluorescence. It was observed that E.F induces generation of free radicals in A549 cells but this was not significant ($p < 0.05$) until the highest dose considered (850 µg/ml). The effect of E.F on the intracellular ROS of A549 is not as prominent as camptothecin.

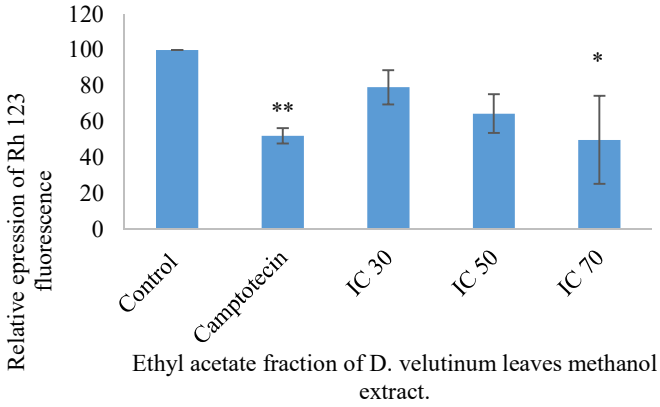


Figure 8. Changes of Mitochondria Transmembrane Potential in A549 Cells with or without (Control) Treatment with Ethyl-Acetate Extract of D.V.

Camtothecin was used as positive control. * Indicates significant difference from control (without treatment) at $P < 0.05$; ** indicates significant difference at $P < 0.01$ (one way ANOVA; Tukey Post-hoc test).

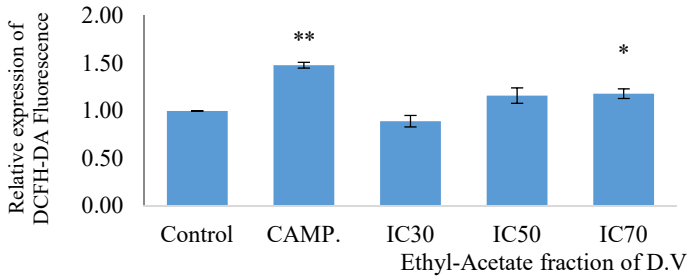


Figure 9. Level of ROS in A549 Cells with or without (Control) Treatment with Ethyl-Acetate Fraction of *D. velutinum*

Camtothecin was used as positive control. * Indicates significant difference from control (without treatment) at $P < 0.05$; ** indicates significant difference from control at $P < 0.01$ (one way ANOVA; Tukey Post-hoc test).

DISCUSSION

Researches over the years have shown the link between development of various diseases such as cancer, diabetes, neurodegenerative disease and oxidative stress (Wang *et al*., 2021). Therefore, the ability of plant extracts to mop up free radicals or prevent their generation in such a large amount that leads to oxidative stress is analysed to determine their potential health benefits. In spite of various uses of *D. vlutinum* leaves in folk medicine, there is a dearth of empirical data to support these claims. However, this research investigated the antioxidant activities, total phenolic content (TPC) and the polyphenol profile of methanol leaf extract (M.E) of *D. velutinum* and its fractions. Also, the anti- proliferative activities of this plant extracts was analysed.

Plant polyphenols have attracted a lot of study interest because of their antioxidant capability, and have been utilised in the prevention and/or control of numerous diseases linked to oxidative stress (Okafo *et al*., 2021). The UHPLC analysis of *D. velutinum* leaf extract demonstrated numerous peaks, some of which were not identified because of lack of suitable standards. However, the major polyphenol identified in M.E of *D. velutinum* and the B.F is kaempferol. To the best of our knowledge this is the first research to demonstrate kaempferol in any specie of *Desmodium*. Even though a significant amount of kaempferol was identified in the fraction, E.F, cathechin was predominant. Little amount of cathechin have been reported in water extract of *D. adscendes* leaves (Muanda *et al*., 2011), the disparity in concentration in the cathechin in these plants may be largely due to the chemical structure of the solvent used for extraction (Ezealigo *et al*., 2021).

From the analysis of the activities of *D. vulutinum* in four antioxidant assays (DPPH, metal chelating, CUPRAC and reducing power assays), it was discovered that the crude plant extract and its fractions exhibited a proficient antioxidant activity which corroborates previous reports by Tsai *et al.* (2011). In their study to examine the antioxidant activities and phenolic components of the crude extracts of ten *Desmodium* species from Taiwan, Tsai *et al.* (2011), concluded that most of the species studied exhibited strong antioxidant activities. Although their research did not report on *D. velutinum*, the crude extract (M.E) and the fractions of *D. velutinum* demonstrated a better DPPH free radical scavenging than the ten Taiwan species as shown by the lower IC_{50} value. IC_{50} is the amount of antioxidant containing plant material needed to scavenge 50% of the initial DPPH. The more effective the plant material is at scavenging DPPH and hence, the lower the IC_{50} value, the greater the antioxidant activity of the plant material (Olugbami *et al*., 2014). Conversely, lower IC_{50} values in DPPH assay have been reported by Chowdhury *et al*., (2013) in methanol extract and fractions of *D. motorium*.

Comparing the antioxidant potency of the crude extract, and its fractions, it was observed that ethyl-acetate fraction (E.F) of *D. vulutinum* demonstrated a better antioxidant activity than the crude extract, M.E and the n-butanol fraction, B.F in DPPH, CUPRAC and reducing power assays. Hence, E.F showed a significantly higher antioxidant potency ($p < 0.05$) as analysed by molybdate ion reduction assay. This result is consistent with previous research on *Desmodium triflorum* (Lai *et al*., 2010) and Desmodium *ramosissimumis* (Ezealigo *et al*., 2021). As reported by Lai *et al*. (2010) and Ezealigo *et al*. (2021), the higher antioxidant activity expressed in the ethyl-acetate fraction of the *Desmodium* plant is due to the higher total phenolic content. Similarly, in this study, E.F. presented with a higher total phenolic when compared with M.E and the fraction, B.F. However, the fraction, B.F showed a greater potency in metal chelation than the crude extract and E.F fraction. Although no significant difference ($p < 0.05$) was observed between the total phenolic contents of the fractions. Plant polyphenols have been reported to perform their antioxidant activities by scavenging radical nitrogen and radical oxygen species (RNS and ROS), blocking a number of enzymes, chelating trace metals involved in the creation of free radicals, and boosting the rate and protection of antioxidant defence (Okafo *et al*., 2021). Furthermore, the presence of different chemical constituents may account for the variation in antioxidant activity of the extract and its fractions. The antioxidant activities of the extract/fraction could be attributed to synergistic interactions between polyphenolic compounds and/or other compounds present in them (Kaur *et al*., 2016).

The outcome of the DNA nicking assay investigation shows that E.F. of *D. velutinum* effectively scavenged hydroxyl radical, thus preserving a significant amount of DNA from oxidative damage. In a biological reaction, superoxide radical is converted to hydrogen peroxide by superoxide dismutase, which can subsequently produce extremely reactive hydroxyl radicals in the presence of divalent metal ions like iron and copper. One of the primary targets of these radicals is DNA, damage to which leads to the development of various cancer types. Therefore, eliminating the hydroxyl radicals is undoubtedly one of the most valuable defences against numerous cancer types (Robin *et al*., 2015; Olajuyin *et al*., 2021).

Lung cancer is one of the leading causes of cancer deaths worldwide, with non-small cell lung cancer (NSCLC) accounting for nearly 85% of cases. Adenocarcinomas are the most prevalent subtype of NSCLC class (Li *et al*., 2020; Thai *et al*., 2021). Lung cancer is currently treated mostly with surgery, chemotherapy and radiotherapy. These treatments have negative side effects that can be very

uncomfortable. On the other hand, herbal and plant derived compounds have the advantages of being less toxic and having fewer side effects, which can enhance patients' quality of life and lessen the drawbacks of currently available therapeutic medications (Khan *et al*., 2019; Li *et al*., 2022). In this research, it was observed that introduction of *D. velutinum* crude extract and its fractions to human lung adenocarcinoma (A549) cell lines hindered proliferation in a dose dependent manner, with the n-butanol fraction showing a greater anti-proliferative potency. The pattern of potency appears to be directly proportional to the concentration of the kaempferol present in the extract/fraction of plant. Previous studies have demonstrated that kaempferol was able to inhibit proliferation of A549 cells and has been shown to be a useful and promising anticancer agent (Hang *et al*., 2015; Jo *et al*., 2015; Zhang *et al*., 2018; Muller *et al*., 2019).

Apoptosis (programmed cell death) is a necessary process for normal cell turnover, immune system development and function, embryonic development, and chemical-induced cell death. Therefore, disruptions of apoptosis are crucial for the development of cancer and many other diseases (Azzwali and Azab, 2019). One of the various mechanisms by which plant polyphenols exert their anticancer activity is by promoting apoptosis (Li *et al*., 2022). In the confocal microscopic study using DAPI stain, exposure of A549 cell lines to E.F of *D. velutinum* showed induction of apoptosis, which was evidenced by chromatin condensation, nuclear fragmentation and production of apoptotic bodies. In an attempt to gain an insight into the mechanism of induction of apoptosis by the plant, it was discovered that A549 cell lines in the presence of the extract using rhodamine 123 fluorescent dyes as a probe indicated loss of mitochondria membrane potential and in DCFH-DA assay exhibited generation of intracellular ROS in a dose dependent manner. Majority of anticancer agents instigates over production of ROS, which result in oxidative stress induced cell death. By causing damage to organelles, lipids, membranes, proteins, and nucleic acids, excessive intracellular ROS initiates the apoptosis process, which is arbitrated by mitochondria, death receptors, and the ER signaling pathway (Sajna *et al*., 2020). Hence, E.F of *D. velutinium* induces apoptosis via mitochondria membrane potential mediated apoptosis in A549 cells. The outcome of the result could be attributed to the polyphenol elements in this plant. Polyphenols have been reported to trigger apoptosis by pro-oxidative activity rather than anti-oxidative action, depending on concentration, target molecule(s), and environmental circumstances (Mojzer *et al*., 2016).

CONCLUSION

From this study, it was discovered that ethyl acetate fraction exhibited good antioxidant activities. These activities may be attributed to the fraction's high phenolic content and the synergistic interaction of its dominant polyphenols. The n-butanol fraction exhibited a better anti-proliferative action against A549 cell line. This useful anti-proliferative action may be attributed to the concentration of kaempferol, a flavonoid.

AUTHORS' CONTRIBUTIONS

Conceptualization- VOM, SA, FIOD, AAO.; Methodology- VOM, SA, FOD, AAO; Investigation and data acquisition- VOM, SA, OAA; Data interpretation- VOM, SA, FIOD, AAO.; Writing—original draft preparation- VOM, SA; Writing—review and editing- VOM, SA, FIOD.; Supervision, SA, FIOD, AAO.; Project administration, VOM, SA; Funding acquisition- VOM, SA**.**

FUNDING STATEMENT

None

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Abubakar, M. S., Musa, A. M., Ahmed, A. and Hussaini, I. M. (2007). The perception and practice of traditional medicine in the treatment of cancers and inflammations by the Hausa and Fulani tribes of Northern Nigeria. *Journal of Ethnopharmacology*, 111(3): 625–629. https://doi.org/10.1016/j.jep.2007.01.011

Ainsworth, E. A. and Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols*, *2*(4): 875–877. https://doi.org/10.1038/nprot.2007.102

Akinola, J. and Afolayan, R. (1991). Effects of storage, testa colour and scarification method on seed germination of *Desmodium velutinum* (Willd.) DC. *Seed Science and Technology*, 19(1): 159–166.

Alam, F., Najum us Saqib, Q. and Waheed, A. (2017). Cytotoxic activity of extracts and crude saponins from *Zanthoxylum armatum* DC. against human breast (MCF-7, MDA-MB-468) and colorectal (Caco-2) cancer cell lines. *BMC Complementary and Alternative Medicine*, 17(1): 1–9. https://doi.org/10.1186/s12906-017-1882-1

Apak, R., Güçlü, K., Özyürek, M. and Karademir, S. E. (2004). Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC Method. *Journal of Agricultural and Food Chemistry*, 52(26): 7970–7981. https://doi.org/10.1021/jf048741x

Azzwali, A. A. A. and Azab, A. E. (2019). Mechanisms of programmed cell death. *Journal of Applied Biotechnology and Bioengineering*, 6(4): 156-158.

BLOIS, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, 181(4617): 1199–1200. https://doi.org/10.1038/1811199a0

Chowdhury, A., Pal, T. K., Alam, M. A., Rahaman, M. S. and Rashid, M. A. (2013). Antioxidant, antimicrobial and cytotoxic activities of Desmodium motorium merr. *Journal of Scientific Research*, 5(1): 201-205.

Deng, S., Yuan, H., Yi, J., Lu, Y., Wei, Q., Guo, C., Wu, J., Yuan, L. and He, Z. (2013). Gossypol acetic acid induces apoptosis in RAW264.7 cells via a caspase-dependent mitochondrial signaling pathway. *Journal of Veterinary Science*, 14(3): 281–289. https://doi.org/10.4142/jvs.2013.14.3.281

Dinis, T. C. P., Madeira, V. M. C. and Almeida, L. M. (1994). Action of Phenolic Derivatives (Acetaminophen, Salicylate, and 5-Aminosalicylate) as Inhibitors of Membrane Lipid Peroxidation and as Peroxyl Radical Scavengers. *Archives of Biochemistry and Biophysics*, *315*(1): 161–169. https://doi.org/https://doi.org/10.1006/abbi.1994.1485

Eze-Steven, P. E., Udeozo, I. P., Emmanuel, O. and Farida, O. (2014). The effects of ethanol exract of Desmodium velutinum stem on liver markers of albino wistar rats fed with high fat diet. *World Applied Science Journal*, *31*(10): 1684–1688. https://doi.org/10.5829/idosi.aejsr.2014.9.2.83170

Ezealigo, U. S., Joshua, P. E., Ononiwu, C. P., Agbo, M. O., Asomadu, R. O. and Ogugua, V. N. (2021). Total phenolic and flavonoid content and in vitro antioxidant activity of methanol extract and solvent fractions of *Desmodium ramosissimum* G. Don. *Medical Science Forum,* 2: 15. https://doi.org/10.3390/CAHD2020-08594

Ezike, A. C., Akah, P. A., Okoli, C. O., Ufere, I. K., Ezeudu, E., Okoye, C. F., Ashara, C. and Igbokwe, I. N. (2014). Studies on gastrointestinal effects of *Desmodium velutinum*: A traditional remedy for diarrhea. *American Journal of Pharmacology and Toxicology*, 9(2): 114–124. https://doi.org/10.3844/ajptsp.2014.114.124

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J. and Kissel, T. (2003). *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials*, 24(7): 1121–1131. https://doi.org/https://doi.org/10.1016/S0142-9612(02)00445-3

Hang, M., Zhao, F., Chen, S., Sun, Q. and Zhang, C. (2015). Kaempferol modulates the metastasis of human non-small cell lung cancer cells by inhibiting epithelial-mesenchymal transition. *Bangladesh Journal of Pharmacology*, 10: 267–271.

Ibrahim, J. A., Muazzam, I., Jegede, I. A. and Kunle, O. F. (2010). Medicinal plants and animals sold by the "yan-shimfidas" of sabo wuse in Niger state, Nigeria. *African Journal of Pharmacy and Pharmacology*, 4(6): 386–394.

Isah, A. O., Agunu, A. and Danmalam, U. H. (2016). Evaluation of analgesic property of *Desmodium velutinum* (P.BEAUV.) DC (PAPILIONACEAE). *European Journal of Botany, Plant Sciences and Phytology*, 3(2): 30–34.

Jo, E., Park, S. J., Choi, Y. S., Jeon, W. K. and Kim, B.C. (2015). Kaempferol suppresses transforming growth factor-β1–induced epithelial-tomesenchymal transition and migration of A549 lung cancer cells by inhibiting Akt1-mediated phosphorylation of Smad3 at threonine-179. *Neoplasia*, 17: 525–37.

Kanthale, P. R. and Biradar, S. D. (2012). Ethnomedicinal plants and their utilization by tribals of mahur range forest of nanded district of Maharashtra, India. *Indian Journal of Natural Products and Resources*, 3(4): 578–581.

Kaur, D., Kaur, A. and Arora, S. (2016). Delineation of attenuation of oxidative stress and mutagenic stress by Murraya exotica L. leaves. *Springer Plus,* 5: 1037. DOI: 10.1186/s40064-016-2709-0

Kaur, P., Robin, Mehta, R. G., Singh, B. and Arora, S. (2019). Development of aqueous-based multi-herbal combination using principal component analysis and its functional significance in HepG2 cells. *BMC Complementary and Alternative Medicine*, 19(1): 1–17. https://doi.org/10.1186/s12906-019-2432-9

Khan, T., Ali, M., Khan, A., Nisar, P., Jan, S. A., Afridi, S. and Shinwari, Z. K. (2019). Anticancer plants: a review of the active phytochemicals, applications in animal models, and regulatory aspects. *Biomolecules*, 10: 47.

Lai, S. C., HO, Y. L., Huang, S. C., Huang, T. H., Lai, Z. R., Wu, C. R., Lian, K.Y. and Chang, Y. S. (2010) Antioxidant and antiproliferative activities of *Desmodium triflorum* (L.) DC. *The American Journal of Chinese Medicine*, 38(2): 329–342.

Lee, J.-C., Kim, H.-R., Kim, J. and Jang, Y.-S. (2002). Antioxidant property of an ethanol extract of the stem of *Opuntia ficus-indica* var. Saboten. *Journal of Agricultural and Food Chemistry*, 50(22): 6490–6496. https://doi.org/10.1021/jf020388c

Li, J., Song, Y., Yu, B. and Yu, Y. (2020). TNFAIP2 promotes non-small cell lung cancer cells and targeted by miR-145-5p. *DNA and Cell Biology*, 00 (00): 1–8. DOI: 10.1089/dna.2020.5415

Li, J., Zhong, X., Zhao, Y., Shen, J., Pilapong, C. and Xiao, Z. (2022). Polyphenols as lung cancer chemopreventive agents by targeting microRNAs. *Molecules*, 27: 5903. https://doi.org/10.3390/molecules27185903

Makanjuola, V. O., Robin, R., Kaur, P., Arora, S., Duru, F. I., Osinubi, A. A., Okoli, B., Kumar, A., Haque, S. and Tuli, H. S. (2023). *Trema oreintalis* (Linn) Blume stem bark: polyphenol profile, *in-vitro* antioxidant and anti-proliferative activites on the A549 cell line. *Minerva Biotechnology and Biomolecular Research,* 35(3):161-172.

Mojzer, E. V., Hrncic, M. K., Skerget, M., Kenez, Z. and Bren, U. (2016). Polyphenols: Extraction methods, antioxidative action, bioavailabilty and anticancer effects. *Molecules*, 21: 901; doi:10.3390/molecules21070901

Muanda, F. N., Bouayed, J., Djilani, A., Yao, C., Soulimani, R. and Dicko, A. (2011). Chemical composition and, cellular evaluation of the antioxidant activity of *Desmodium adscendens* leaves. *Hindawi Publishing Corporation Evidence-Based Complementary and Alternative Medicine,* 2011, Article ID 620862, doi:10.1155/2011/620862

Muller, A. G., Sarker, S. D., Saleem, I. Y. and Hutcheon, G. A. (2019). Delivery of natural phenolic compounds for the potential treatment of lung cancer. *DARU Journal of Pharmaceutical Sciences*, 27: 433–449

Mutha, R. E., Tatiya, A. U. and Surana, S. J. (2022). Flavonoids as natural phenolic compounds and their role in therapeutics: an overview. *Future Journal of Pharmaceutical Sciences,* 7: 25

Okafor, J. N. C., Rautenbauch, F., Meyer, M., Le Roes-Hill, M., Harris, T. and Jideani, V. A. (2021). Phenolic content, antioxidant, cytotoxic and antiproliferative effects of fractions of *Vigna subterraenea* (L.) verdc from Mpumalanga, South Africa. *Heliyon*, 7: e08397 https://doi.org/10.1016/j.heliyon.2021.e08397

Olajuyin, A. M., Olajuyin , A. K., Wang, Z., Zhao, X. Xu, Z., Zhang, Q. and Zhang, X. (2021). Anti-proliferative, antioxidant effects of methanol extract of *Calotropis procera* leaf on lung cancer cells (H1299) and its ameliorative effect on expression of CD146 on blood cells. *Clinical Phytoscience*, 7: 51. https://doi.org/10.1186/s40816-021-00289-x

Olugbami, J.O., Gbadegesin, M.A. and Odunola, O.A. (2014). *In vitro* evaluation of the antioxidant potential, phenolic and flavonoid contents of the stem bark ethanol extract of *Anogeissus leiocarpus. African Journal of Medicine and Medical Sciences, 43*(Suppl 1):101-109

Oyaizu, M. (1986). Studies on products of browning reactions: antioxidative activities of product of browning reaction prepared from glucosamine. *Japan Journal of Nutrition*, 44: 307–315.

Prieto, P., Pineda, M. and Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical Biochemistry*, 269(2): 337–341.

Robin, Arora, S. and Vig, A. P. (2015). Inhibition of DNA oxidative damage and antimutagenic activity by dichloromethane extract of *Brassica rapa var. rapa* L. seeds. *Industrial Crops and Products*,74(2015):585–591.

Romero, M. C., Garro, O. A., Romero, A. M., Michaluk, A. G., Doval, M. M. and Judis, M. A. (2014). Assessment of the quality and shelf-life in enriched n3 PUFA raw beef patties using dry soybean sprouts as antioxidant. *Food and Nutrition Sciences*, 05(07): 658–670

Sajid, M., Yan, C., Li, D., Merugu, S. B., Negi, H. and Khan, M. R. (2019). Potent anti-cancer activity of Alnus nitida against lung cancer cells; *in-vitro* and *in-vivo* studies. *Biomedicine and Pharmacotherapy,* 110:254–264.

Sajna, K. V., Kamat, S. and Jayabaskaran, C. (2020). Antiproliferative role of secondary metabolites from *Aspergillus unguis* ag 1.1 (g) isolated from marine Macroalgae *Enteromorpha sp*. by inducing intracellular ROS production and mitochondrial membrane potential loss leading to apoptosis. *Frontiers in Marine Science*, 7: 543523. doi: 10.3389/fmars.2020.543523

Sorgho, H., Koog, C., Kazienga, A., Dabiré, K. R., Gouagna, C., Dakuyo, P. Z., Ouédraogo, J. B., Guissou, I. P., Guiguemdé, T. R., Bernard, U. C., Lyon, I., Pharmacie, F. De, & Faso, B. (2013). Antiplasmodial and repellent activity of indigenous plants used against malaria. *Journal of Medicinal Plants Research*, 7(42): 3105–3111. https://doi.org/10.5897/JMPR12.998

Thai, A. A., Solomon, B. J., Sequist, L. V., Gainor, J. F. and Heist, R. S. (2021). Seminar: Lung cancer. *Lancet*, 398: 535–54. https://doi.org/10.1016/ S0140-6736(21)00312-3

Tsai, J. C., Huang, G. J., Chiu, T. H., Huang, S. S., Huang, S. C., Huang, T. H., Lai, S. C. and Lee, C. Y. (2011). Antioxidant activities of phenolic components from various plants of Desmodium species. *African Journal of Pharmacy and Pharmacology,* 5(4): 468-476.

Vinjamuri, S., Shanker, D., Ramesh, R. and Nagarajan, S. (2015). *In vitro* evaluation of hemolytic activity and cell viability assay of hexanoic extracts of *Bridelia ferruginea* e. *World Journal of Pharmacy and Pharmaceutical Sciences*, 4(7): 1263–1268.

Wang, Z., Tu, Z., Xie, X., Cui, H., Kong, K.W. and Zhang, L. (2021). *Perilla frutescens* leaf extract and fractions: polyphenol composition, antioxidant, enzymes (α-glucosidase, acetylcholinesterase, and tyrosinase) inhibitory, anticancer, and antidiabetic activities. *Foods,* 10: 315. https://doi.org/10.3390/foods 10020315

Zhang, J., Shi, X., Meng, W., Ma, F., Zhao, L. and Zhao, J. (2018). Kaempferol inhibits invasion and migration of non-small cell lung cancer A549 cells by down regulating ERRα expression. *Chinese Journal of Cancer Biotherapy*, 6: 1230-1236.

Zhu, L., Yuan, H., Guo, C., Lu, Y., Deng, S., Yang, Y., Wei, Q., Wen, L. and He, Z. (2012). Zearalenone induces apoptosis and necrosis in porcine granulosa cells via a caspase-3- and caspase-9-dependent mitochondrial signaling pathway. *Journal of Cellular Physiology*, 227(5): 1814–1820. https://doi.org/10.1002/jcp.22906