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Peristrophe bicalyculata (Retz) Nees contains principles that are cytotoxic to cancer cells and induce caspase-mediated, intrinsic apoptotic death through oxidative stress, mitochondrial depolarisation and DNA damage

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ABSTRACT

The plant *Peristrophe bicalyculata* (Retz) Nees is used for the treatment of cancer. While its leaf extracts have been shown to inhibit the growth of some cancer cells, there is little information supporting the constituents' anti-tumour potential. This study, therefore, investigated the effects of the plant's leaf extracts on cancer cells and the associated cellular/molecular mechanisms. Extracts were prepared using hexane (PBH), chloroform (PBC), ethyl acetate (PBE) and methanol (PBM) and constituents were identified by Liquid Chromatography-Mass Spectrometry (LC-MS). Their cytotoxic effects on human cervical (HeLa) and lung cancer (MRC5-SV2) cells were assessed using the MTT and LDH release assays. Reactive oxygen species (ROS) production was assessed using 2′,7′-dichlorofluorescein diacetate (DCFDA) and mitochondrial membrane potential by staining with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide). Caspase activation was determined using a Caspase-Glo-3/7 assay, and DNA damage by the Comet assay. Changes to mRNA expression were assessed using Quantitative Real-Time PCR. PBC, PBE and PBM reduced cell viability and induced LDH release, with IC_{50} values (48 h, MTT, in μg/ml), respectively, of 6.21 ± 0.70, 23.39 ± 3.92, and 22.43 ± 3.58 (HeLa); and 1.98 ± 0.33, 8.57 ± 1.91 and 28.24 ± 5.57 (MRC5-SV2). PBC induced ROS, while PBC, PBE and PBM impaired mitochondrial membrane potential and induced caspase 3/7 activation. PBC and PBE induced DNA damage, and PBE induced *caspase-3* mRNA expression. Constituents of the extracts included derivatives of gallic acid, dipeptides, diterpenoids and flavones. We conclude that *P. bicalyculata* contains cytotoxic principles that could be potential leads for developing novel anti-cancer agents.

1. Introduction

Globally, cancer is the second leading cause of mortality, with an estimated 9.6 million deaths in 2018 [1]. In less developed countries, the incidence of the disease is on the increase, due to population growth, ageing and lifestyle changes incorporating more Western diets [2]. Cervical cancer is currently the fourth most common cancer in women worldwide, with approximately 570,000 new cases and 311,000 deaths in 2018, over 85% of which occurred in low- and middle-income countries (LMICs) [1]. In Nigeria, it is the second most common after

Abbreviations: DCFDA, 2′,7′ -dichlorofluorescein diacetate; DMEM, Dulbecco's Modified Eagle Medium; DOX, doxorubicin; ECACC, European Collection of Authenticated Cell Cultures; FBS, Foetal Bovine Serum; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; LC-MS, Liquid Chromatography-Mass Spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; ROS, Reactive oxygen species; UV/DAD, Ultraviolet Diode-Array Detector; LDH, Lactate Dehydrogenase; LMICs, Low- and middle-income countries.

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breast cancer [3], with 53.1 million women at risk, 14,943 cases and 10, 403 deaths annually [4]. These are very high figures compared to 13, 170 new cases and 4250 deaths in the more populous United States [5]. Increased use of pap smear to screen for cervical cancer and availability in recent years of vaccines against the human papilloma virus (HPV), which is linked to several types of cervical cancer, have decreased the incidence and the mortality of the disease in developed countries [1]. Unfortunately, however, cervical cancer continues to be a major public health problem in LMICs, as most cases are diagnosed late, due to a dearth or unavailability of diagnostic cancer screening programmes, lack of awareness of such screening opportunities where they exist, and limited access to oncology and radiation therapy services [6-8].

According to the World Health Organization (WHO), a government-led survey of national capacity for cancer control programmes in 2001 revealed that anticancer drugs were only available in 22% and affordable in 11% of the 39 African countries that participated in the survey, including Nigeria [9]. A majority of those who cannot afford these drugs depend on herbal medicines, an alternative to Western medicine that has been used for centuries, based on the ability of plants or their extracts to treat diseases and promote health and well-being [7]. Most of these plants have been shown to contain bioactive compounds with medicinal properties, such as antioxidant, anti-inflammatory, antibacterial, anti-mutagenic, antidiabetic and anticarcinogenic activities. However, only a small percentage have been sufficiently evaluated to date for their potential as therapeutic agents [10]. Yet, knowledge of the biodiversity and traditional medical uses of these herbal medicines has contributed to the development of almost 70% of the conventional drugs currently in use. In fact, at least 700 natural products, natural products-derived or natural products-inspired New Chemical Entities (NCEs) were approved between 1981 and 2019, aside from natural product mimics [11]. Natural products have, therefore, been an invaluable source of drug leads for many years as a result of their chemical diversity [12].

Peristrophe bicalyculata (Retz.) Nees (Acanthaceae), which we investigated as reported in this work, is considered one of the plants with potential anti-cancer efficacy. It is an erect shrub native to the tropical parts of Africa, from Mauritania to Niger and Nigeria, and also to Asia, being found in India, Burma and Thailand. It is commonly called "Goddess of Mercy" [13]. *P. bicalyculata* is used by traditional healers to treat skin-related ailments, fever, cough and colds, and the deployment of the plant for such conditions is likely related to its anti-inflammatory, anti-oxidant and antimicrobial properties [14-16]. Extracts from the leaf of the plant have been shown to inhibit the growth of human oral epidermal carcinoma (KB) cells and Ehrlich ascites carcinoma (EAC) cells [17-19]. Also, oils extracted from the leaf have been reported to inhibit human breast cancer cells (MCF-7 and MDA-MB-468) [13]. However, there is paucity of information on the wider potential anti-tumour effects of the leaf extracts of *P. bicalyculata,* especially in the context of cervical cancer. This work, therefore, explored the ability of the leaf extracts to induce cytotoxicity and cell death in cervical cancer cells, as well as some of the cellular and molecular mechanisms involved. The study demonstrates *P. bicalyculata* as a promising source of novel anticancer remedies or drugs to treat cervical cancer, and even other cancers, such as lung cancer.

2. Materials and methods

2.1. P. bicalyculata extracts preparation and standard drug

Fresh leaves of *P. bicalyculata* were obtained from a farm in Ibadan, Oyo State, Nigeria, in June, 2017. The plant was identified and authenticated by the botanist at the herbarium of the Department of Biological Science, Ahmadu Bello University (ABU), Zaria, Nigeria, with voucher number 2863. The leaves were air-dried in the laboratory and ground into powder. The powdered material was extracted with hexane, chloroform, ethyl acetate and methanol, each separately, using a Soxhlet apparatus. Each extract was then concentrated under reduced pressure using a rotary evaporator (Buchi, Switzerland) and lyophilized to obtain hexane, chloroform, ethyl acetate and methanol extracts. Doxorubicin, an anti-cancer drug, was used as a positive control (standard). The extracts and standard drug were stored at − 20° C until required. Each extract was dissolved in DMSO to a final stock concentration of 100 mg/ml.

2.2. Cell lines and cell culture reagents and materials

The human cervical adenocarcinoma cell line (HeLa) and the human foetal lung cancer cell line MRC5-SV2 used in the study were originally purchased from the European Collection of Authenticated Cell Cultures (ECACC). Each cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mM) and 1% penicillin-streptomycin antibiotic combination (anti-anti) at 37 °C in a humidified atmosphere of 5% CO_2. Cells growing in 75 cm^2 (T75) tissue culture flasks were harvested at 70–80% confluency using TrypLE (recombinant trypsin). All cell culture reagents were obtained from Thermo Fisher Scientific UK, apart from FBS and the standard drug (doxorubicin), which were purchased from Sigma-Aldrich, UK. Black 96-well plates were obtained from Greiner Bio-One, UK.

2.3. Cell culture

Adherent monolayer cultures were prepared as previously reported [20]. Briefly, the growth medium on cultured cells in a T75 flask was decanted, the culture was rinsed with phosphate-buffered saline (PBS), PBS was removed, culture was exposed to TrypLE for about 1 min, TrypLE was removed, and the flask was incubated for up to 5 min to allow cells to completely detach. Cells were then flooded with growth medium and triturated into a suspension of single cells. The density of the suspension was determined with a haemocytometer and adjusted to the desired density (as indicated in the appropriate section for each assay) before it was seeded into a 96-well plate at 100 μL/well. Cells were allowed to adhere overnight before they were used for experiments.

2.4. Assessment of cytotoxicity

The starting density was 5×10^3 cells/well in 100 μL of growth medium. The cells were treated 24 h after plating. The cytotoxicity screening was done in two phases. In the first phase, the cells were treated with the hexane, chloroform, ethyl acetate and methanol extracts at 50 and 200 μg/ml for 48 h and the most potent extracts were progressed to the second phase. In the second phase, cells were treated for 24 and 48 h with the chloroform extract of the plant at 0.5, 1, 5, 10, 25 and 50 μg/ml, and the ethyl acetate and methanol extracts at 5, 10, 50, 100 and 200 μg/ml. The anti-cancer drug, doxorubicin, was used as positive control. The DMSO concentration to which cells were exposed (negative control) was not more than 0.2% v/v (corresponding to the % v/v DMSO concentration in the highest extract concentration tested) and this DMSO concentration was confirmed to have no effect on cell viability. Each treatment was done in triplicate and blank controls were included. After treatment, the cytotoxic effects of the extracts were determined by assessing resultant cell viability using the 3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyl tetrazolium bromide (MTT) assay as earlier described [20]. 10% v/v (10 μL) of warm MTT (5 mg/ml, prepared in PBS) was added to each well and the plate was incubated for 2.5 h. The content of each well was aspirated and 100 μL of DMSO was added to dissolve the insoluble purple formazan formed. The plate was placed on an orbital shaker for 5 min to completely solubilise the formazan crystals. Absorbance was read at 570 nm using a microplate reader (CLARIOstar, BMG LABTECH, Germany). To analyse the data, an average of each triplicate treatment was taken. The average for the negative control was then set to 100% and the average of each other

treatment was normalised to it. The cytotoxic potency of each extract was then determined by calculating from the MTT assay data the IC_{50} value using the "log concentration-response (inhibition)" equation (GraphPad Prism).

2.5. Bright-field imaging to assess morphological damage

To assess changes to the morphology of the cells induced by treatments, bright-field images were acquired on an Olympus CKX41 microscope fitted with an Olympus DP71 U-TVIX-2 camera, using the Olympus cellSens entry software.

2.6. Lactate dehydrogenase (LDH) release (cell death/cytotoxicity) assay

LDH release as a surrogate of cytotoxicity and cell death was assayed using Pierce LDH Cytotoxicity Assay Kit (Cat. No. 88953, Thermo Fisher Scientific, UK) according to the manufacturer's instruction. Briefly, cultures were prepared as described in the 'Cell Culture' section, seeded into a 96-well plate at 5×10^3 cells/well in 100 μL growth medium, and incubated overnight at 37 °C and 5% CO_2. They were then treated for 24 h with various concentrations of the extracts and the standard drug, as earlier described. A blank control (medium only, no cells) was included on the plate and two sets of triplicate wells (with cells) were included, for the measurement of spontaneous LDH activity and maximum LDH activity, respectively. After the 24 h treatment, 10 μL water and 10 μL 10x lysis buffer (supplied in the kit) were added to the sets of triplicate wells for spontaneous LDH activity and maximum LDH activity, respectively, and the plate was incubated for 45 min. This was followed by transferring 50 μL of the medium in each of the wells (for negative control, treatments, positive control, assay controls) to another (virgin) 96-well plate. The reaction mixture was prepared by dissolving one vial of lyophilised substrate mix in 11.4 ml of ultra-pure water, followed by addition of 0.6 ml of the assay buffer and mixing. The reaction mixture was then added to each well of the virgin plate (50 μL per well) and gently mixed with a multi-channel pipette. The plate was incubated at room temperature for 30 min, protected from light, after which 50 μL of stop solution was added and well content mixed by gentle tapping. The process was carefully done to avoid bubbles and any bubbles generated were burst using pipette tips or a needle. Absorbance was then measured at 490 nm and 680 nm using a microplate reader (CLARIOstar, BMG LABTECH, Germany). Absorbance at 680 nm (background) was subtracted from absorbance at 490 nm and, using the resultant data, the average of each control or treatment set of wells was determined and used to calculate % cytotoxicity (indicative of cell death) thus:

$$\%\,\text{Cytotoxicity} = \frac{(\text{Extract treatment LDH Activity} - \text{Spontaneous LDH Acivity})}{(\text{Maximum LDH Activity} - \text{Spontaneous LDH Activity})} \times 100$$

2.7. DCFDA cellular reactive oxygen species (ROS) assay

To investigate the potential of each extract of *P. bicalyculata* to alter intracellular ROS level, with a view to exploring whether induction of ROS was involved in the cytotoxic effects of the extracts, ROS generation following treatment with each extract was monitored over 24 h using 2′, 7′- dichlorofluorescein diacetate (DCFDA) (Abcam, UK – Cat. No. ab113851), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activities within the cell. DCFDA diffuses into the cells and is deacetylated enzymatically by cell esterases to a non-fluorescent compound, which upon oxidation by ROS yields a highly fluorescent compound, 2′,7′ dichlorofluorescein (DCF). It was used according to the manufacturer's instructions and as previously described [21]. Briefly, HeLa cells (2.5×10^4 cells/well) were plated in a black, clear bottom 96-well plate and allowed to adhere overnight. The media was aspirated and cells washed with buffer (supplied), before staining (after buffer was removed) with 25 μL DCFDA (100 μL/well, prepared in buffer) and incubating the plate for 45 min at 37 °C in the dark (incubator). Blank wells (no cells, medium only) and non-stained control wells (cells but no stain) were included. After incubation, DCFDA was aspirated and the cells were washed with buffer before they were treated with the extracts and standard at various concentrations prepared using growth medium without phenol red. The plate was put back in the incubator and fluorescence read at 1, 3, 6 and 24 h afterwards at Ex/Em = 485/535 nm on a plate reader (CLARIOstar). The average fluorescence of the negative control (treatment with vehicle only) was set to '1′ (unity) and the fluorescence of each treatment was computed as fold change compared to the negative control.

2.8. Assessment of mitochondrial membrane potential, Δψm (mitochondrial integrity), by JC-1 staining

Mitochondrial membrane integrity following treatment was assessed with a JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) mitochondrial membrane potential assay kit (Fisher Scientific, UK – Cat. No. 15540597), following instructions from the manufacturer. HeLa cells were seeded at 1.5×10^5 cells/ml into a black, clear bottom 96-well microplate and allowed to attach overnight. Blank controls were included. After 18–24 h cells were washed with 100 μL PBS, 100 μL of the working JC-1 solution (1.5 μM) was added to each well, and the plate was incubated for 15 min at 37 °C in the dark. Cells were then washed with PBS and treated with the extracts and standard for 24 h at 37 °C. Mitochondrial membrane depolarisation was examined at 1, 3, 6 and 24 h post-treatment by measuring the fluorescence emission shift (red to green) of the dye (Ex/Em of 550 ± 15 nm and 600 ± 20 nm for the red; Ex/Em of 485 ± 15 nm and 535 ± 20 nm for the green), using a microplate reader (CLARIOstar). To analyse the data, values were blanked, the average fluorescence of each treatment was determined, and the ratio of red average fluorescence to green average fluorescence was computed. A significant decrease in that ratio was considered an indicator of loss of mitochondrial integrity, and thus of mitochondria-mediated apoptosis.

2.9. Assessment of caspase-mediated apoptosis using caspase 3/7 assay

The activity of caspase 3/7 in treated HeLa cells was determined using a homogeneous, luminescent, Caspase-Glo-3/7 Assay kit (assay kit from Promega, UK – Cat. No G8091). Briefly, HeLa cells were plated at 5×10^4 cells/ml in a white, microclear 96-well plate and allowed to adhere overnight. The cells were then treated with the extracts and standard drug, with the inclusion of a blank control, and the plate was transferred back into the incubator at 37 °C for 24 h. The caspase reagent, constituted by mixing the substrate with the buffer, was added to the growth medium in the wells in a 1:1 vol ratio, the content was mixed gently by placing the plate on a shaker for 30 s, and the plate was incubated at room temperature for 30 min, 1 h or 1.5 h before luminescence was read on a microplate reader (CLARIOstar). Fold change in caspase activity was obtained by first subtracting the luminescence value for the blank well from every other value and then expressing the average luminescence of each treatment as a fold change with respect to the average luminescence of the negative control.

2.10. Assessment of DNA damage using the comet assay

DNA damage was analysed using a comet assay kit (Abcam, UK - Cat. No. ab238544), according to the manufacturer's instruction. Briefly, HeLa cells were plated at 3×10^5 cells/well in a 6-well plate and allowed to adhere overnight before treating them with the extracts and standard drug. After 24 h treatment, the medium was removed and cells harvested after trypsinisation. The number of cells in each well was then counted and cells were resuspended at 1×10^5 cells/ml in cold PBS. This was used for alkaline, single-cell gel electrophoresis performed in three

steps. Comet agarose was added to each well on the comet slide (three slides per sample) and solidified at 4 °C for 15 min. Each sample was mixed with the agarose gel at a 1:10 ratio (10:90 μL) - layered on top of the comet agarose base - and transferred to 4 °C refrigerator for 15 min. The slide was then immersed in a pre-chilled lysis buffer for 30 min at 4 °C in the dark, before replacing the lysis buffer with cold alkaline solution at 4 °C too. While still in the horizontal position, the slide was taken to a horizontal electrophoresis chamber, filled with alkaline electrophoresis solution and run at 15 V for 30 min. Still maintaining its horizontal position, the slide was immersed in cold, distilled water for 2 min (repeated twice), before placing it in cold ethanol (70%) for 5 min and allowing it to air-dry. It was then stained with diluted Vista Green DNA Dye (1:10000 in TE buffer), incubated at room temperature for 15 min, and viewed with a fluorescence microscope (Leica Microsystems CMS GmbH, Germany, with 510–560 nm excitation and 590 nm emission filters). Images of 50–75 cells were captured for each treatment and the comet tail DNA (%) and DNA moment were measured using Comet Score^{pro} Automatic Assay Software (Tritek CometScore 2.0.0.38, Tritek Corporation, DE, USA).

2.11. Assessment of caspase-3 and BAX gene expression changes using quantitative Real-Time PCR

Changes to the expression of *caspase-3* and *BAX* genes in HeLa cells treated with the extracts were assessed by quantitative RT-PCR. Briefly, HeLa cells plated at 1×10^5 cells/well in 6-well plates overnight were treated with the extracts and standard drug at different concentrations. Total RNA was isolated from the cells 24 h after treatment, using ReliaPrep™ miRNA cell and tissue miniprep kit (Promega, UK), following the manufacturer's protocol. The RNA concentration was quantified with a Nanodrop spectrophotometer (NanoDrop 1000, Thermo Scientific, MA, USA) and 200 ng used in cDNA synthesis with miRScript reagents that target mRNA (Qiagen, UK). The cDNA was diluted with 200 μL nuclease-free water and 2 μL used in PCR reactions with SYBR Green reagents (Novacyt, UK) and RT^2 qPCR Primer Assays (Qiagen, UK) for *CASP3* (catalogue ID PPH00107C), *BAX* (PPH00078B) and *GAPDH* (PPH00150F). Levels of *caspase-3* and *BAX* were normalised to *GAPDH*. Relative quantification was performed using the comparative ΔΔCt method. The experiment was carried out in duplicate.

2.12. Liquid chromatography-mass spectrometry (LC-MS-MS) analysis to identify components of extracts

To identify the compounds present in the extracts, the samples were analysed using liquid chromatography (LC)-tandem mass spectrometry (MS) (LC Waters e2695 Separation Module with W2998 PDA and coupled to ACQ-QDA MS), as previously described [22], but with some modifications. The extracted samples were reconstituted in methanol and filtered through polytetrafluoroethylene (PTFE) membrane filter with 0.45 μm size. The filtrate (10.0 μL) was injected into the LC system and allowed to separate on a Sunfire C18 5.0 μm, 4.6 mm × 150 mm column. The run was carried out at a flow rate of 1.0 ml/min, with sample and column temperature at 25 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), with a gradient as follows (A:B), in %v/v: The starting ratio was 95:5, which was maintained for a further 1 min; then 5:95–13 min and 15 min; then 95:5–17 min, 19 min and finally 20 min. The PDA detector was set at 210–400 nm with a resolution of 1.2 nm and sampling rate of 10 points/sec. The mass spectra were acquired with a scan range from *m/z* 100 – 1250, after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8 kv (positive) and 0.8 kv (negative); probe temperature 600 °C; flow rate 10 ml/min; nebulizer gas, 45 psi; MS set in automatic mode applying fragmentation voltage of 125 V. Data was processed with Empower 3. The compounds were identified on the basis of the following information: elution order, retention time (tR), fragmentation pattern, and base *m/z*.

2.13. Statistical analysis

Results are expressed as the mean ± SEM (standard error of the mean) for at least three independent experiments (unless indicated otherwise). Data were analysed using GraphPad Prism v8.0 software (GraphPad Software Inc., CA, USA). Statistical significance of differences of means across different treatment groups was determined by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxic activities of P. bicalyculata extracts

In this study, the cytotoxic activities of hexane (PBH), chloroform (PBC), ethyl acetate (PBE) and methanol (PBM) extracts of *P. bicalyculata* were investigated in human cervical adenocarcinoma (HeLa) and human foetal lung cancer (MRC5-SV2) cell lines using the MTT reduction assay, which measures viability of cells based on reducing equivalents generated in metabolically active cells [23]. The MRC5-SV2 cell line was included as a second cancer cell line to investigate whether the plant could also be used to treat other cancers apart from cervical cancer. As shown in Fig. 1, treatment of HeLa and MRC5-SV2 cancer cells with each of doxorubicin, PBH, PBC, PBE and PBM for 48 h significantly ($P < 0.0001$) reduced cell viability compared to the negative control, and the reductions in cell viability were concentration-dependent, especially for the HeLa cells, as viability values for the extracts at 200 μg/ml were significantly lower than at 50 μg/ml. PBH was the least toxic of the extracts against both cell lines.

The three extracts that showed more significant cytotoxic effects, namely PBC, PBE and PBM, were then further tested at a wider range of concentrations, up to 200 μg/ml, for both 24 h and 48 h, in order to assess both the concentration- and exposure time-dependence of their effects. There were significant decreases in the viability of HeLa and MRC5-SV2 cells with increasing concentrations of each extract, although most effects peaked at the penultimate concentration (Figs. 2 and 3A). Most of the effects, especially against the HeLa cells, also appeared time-dependent. For example, the viability of HeLa cells treated with PBC at 50 μg/ml reduced from 25.94 ± 7.09% after 24 h to 14.57 ± 3.06% after 48 h, while PBE and PBM at 200 μg/ml reduced cell viability from 28.79 ± 4.42% at 24 h to 16.13 ± 4.97% at 48 h, and from 37.64 ± 1.04% at 24 h to 18.05 ± 5.38% at 48 h, respectively (Fig. 2). The viability of MRC5-SV2 cells treated with PBC decreased significantly ($p < 0.05$) from 99.47 ± 1.89% at 0.5 μg/ml to 26.26 ± 2.98% at 50 μg/ml after 24 h. Also, after 24 h treatment, MRC5-SV2 cell viability decreased from 72.76 ± 4.12% at 5 μg/ml PBE to 27.50 ± 2.96% at 200 μg/ml PBE. PBM at its higher concentrations decreased MRC5-SV2 viability to levels similar to those induced by the same concentrations of PBE, but the effects of both PBM and PBE did not appear significantly time-dependent at concentrations beyond 50 μg/ml (Fig. 3).

The cytotoxic effects of the extracts as depicted by reductions in cell viability were also revealed through detrimental changes to morphology as demonstrated by photomicrographs. For example, following 48 h treatments of the MRC5-SV2 cell line, the negative control cells appeared confluent, with a fibroblast-like, elongated shape, whereas images of extract-treated cells showed loss of cells and additionally, in the case of PBC, a shrunken appearance (Fig. 3B). The manner of morphological damage in PBC suggests that its cytotoxic mechanisms might be different to those of PBE or PBM.

The IC_{50} values for the cytotoxic effects are shown in Table 1. They demonstrate that the HeLa cell was generally more sensitive to the cytotoxic effects of doxorubicin but less sensitive to the effects of the

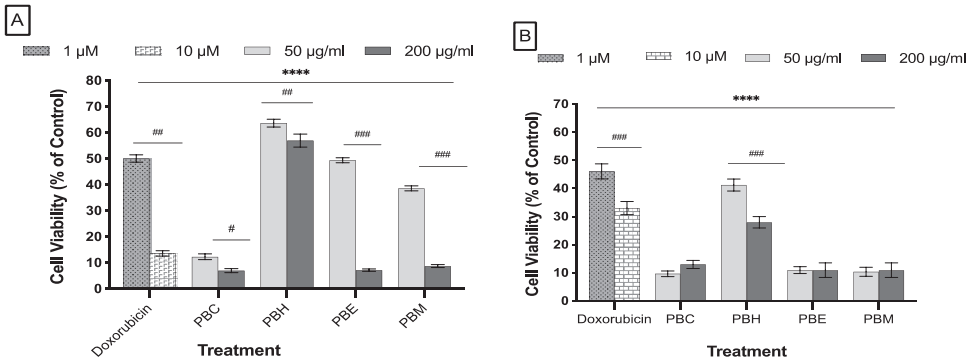

Fig. 1. Effects of doxorubicin and *P. bicalyculata* extracts (48 h treatment) on the viability of (A) HeLa and (B) MRC5-SV2 cancer cell lines. Each value represents the Mean ± SEM of three independent experiments. * ** *P < 0.0001 compared to negative control; #P < 0.05, ##P < 0.01, ###P < 0.001 for comparison of the different concentrations of the same treatment.

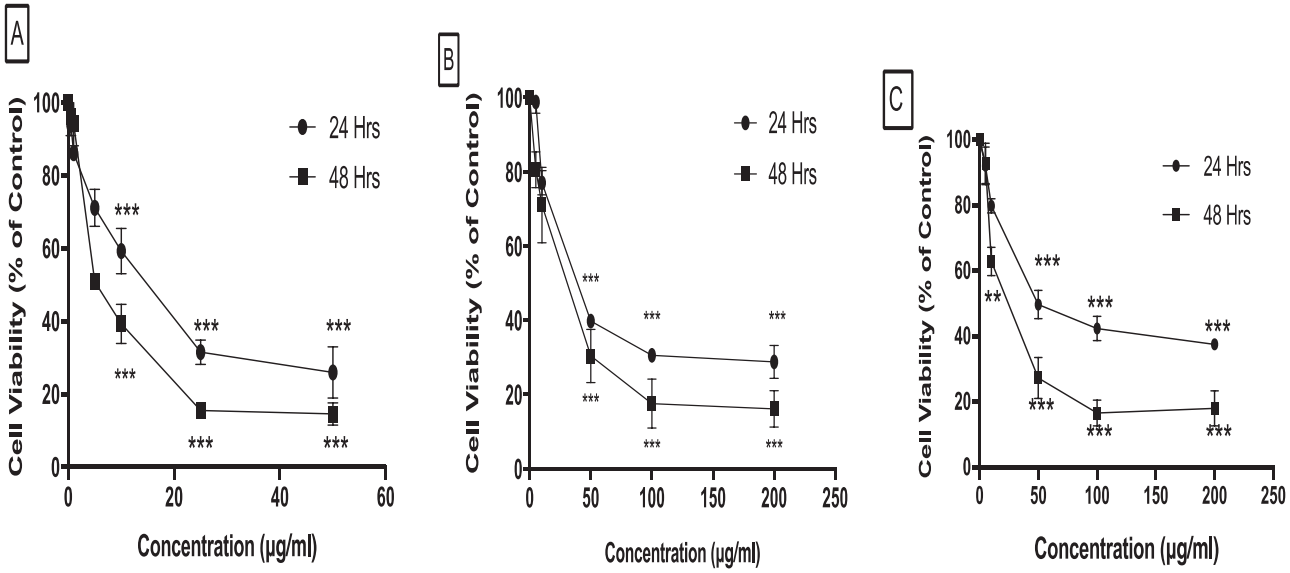

Fig. 2. Cytotoxic effects of (A) PBC (B) PBE and (C) PBM on HeLa cells after 24 h and 48 h treatments with the extracts. Each value represents the Mean ± SEM of three independent experiments. *P < 0.05, * *P < 0.01 and * **P < 0.001 compared to the negative control.

extracts than the MRC5-SV2 cell. In most cases, for both cell lines, the IC_{50} values for doxorubicin and the extracts were significantly higher at the 24 h time point than at the 48 h time point, implying a time-dependent effect. The IC_{50} values for PBC against HeLa cell at 24 h and 48 h were lower than the respective values for PBE and PBM, and a similar trend was found for the MRC5-SV2 cells. Overall, the IC_{50} values revealed that, of the three extracts, PBC was the most potent, while PBM was the least potent.

In order to examine whether these extracts could target cancer cells more selectively and, as a result, relatively spare normal cells, we compared the sensitivities of the normal MRC5 cell line (the parental cell line from which the cancer cell line MRC5-SV2 was derived) and the MRC5-SV2 cancer cell line. We then calculated the IC_{50} values to determine the Selectivity Index (SI), a parameter that indicates how (much) less sensitive to the toxic agent the normal cells are compared to the cancer cells (SI was calculated by dividing the IC_{50} of the normal cell line by the IC_{50} of the cancer cell line). A SI value greater than unity (1) implies that the test agent targets cancer cells more selectively and is thus more likely to kill them than it is likely to kill the normal cells. The SI values for doxorubicin, PBC, PBE and PBM (48 h treatment), respectively, were 21.5, 15.3, 11.5 and 5.6.

3.2. Extracts of P. bicalyculata induced lactate dehydrogenase (LDH) release

The LDH release assay assesses the level of cell death induced, especially by necrosis, as the cell membrane becomes compromised in necrosis or late-phase apoptosis, allowing the release of some intracellular contents such as the cytosolic enzyme LDH. The measurement of LDH release in the growth medium in which cells have been treated serves as a surrogate for the extent of cell death characterised by membrane permeability. We, therefore, employed the assay to assess necrotic or late-phase apoptotic cell death induced by the extracts. Treatment of HeLa cells for 24 h with the different concentrations of the standard drug doxorubicin and of PBC, PBE and PBM caused a marked and significant increase ($P < 0.001$ or $P < 0.0001$) in LDH release compared to the negative control with a value of 9.93 ± 3.35% (Fig. 4). Doxorubicin at its highest tested concentration (10 µg/ml) induced 59.38 ± 12.00% of maximum LDH release. For each of PBC, PBE, and PBM, the highest LDH release was induced by the highest concentration tested, corresponding to 55.83 ± 9.21%, 59.88 ± 6.04% and 47.77 ± 7.58% of the maximum LDH release, respectively, with the effects of PBE being clearly concentration-dependent (Fig. 4).

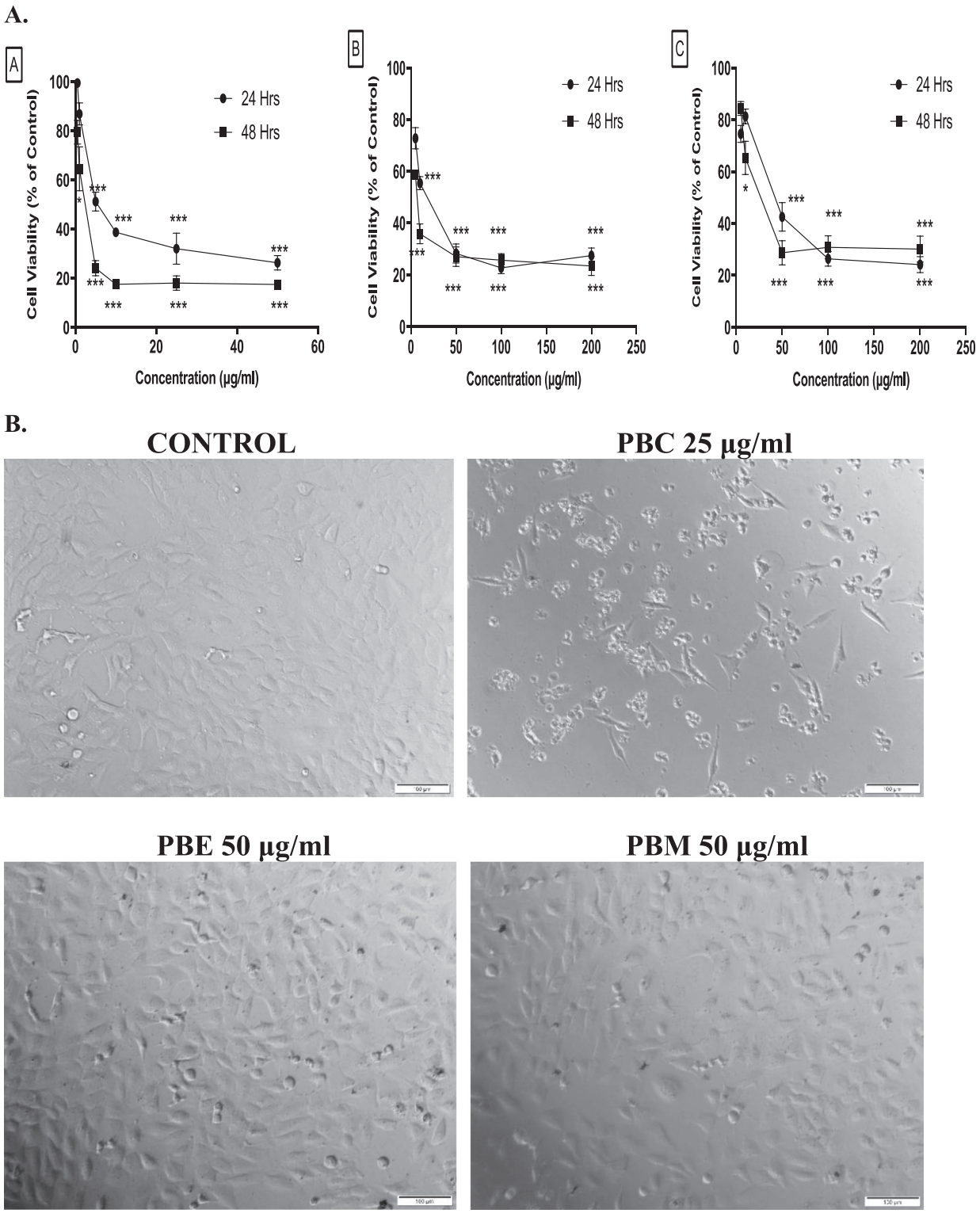

Fig. 3. Cytotoxic effects on the (A) viability (24 h and 48 h treatments) and (B) morphology (48 h treatment) of the MRC5-SV2 cells following their exposure to the extracts (A) PBC (B) PBE and (C) PBM. Each value in Panel (A) represents the Mean ± SEM of three independent experiments. *P < 0.05, * *P < 0.01 and * **P < 0.001 compared to the negative control. Scale bar (for Panel B) = 100 µm.

3.3. Extracts increased levels of intracellular reactive oxygen species (ROS)

Having established the ability of the extracts to induce cytotoxicity and cell death, we sought to identify potential mechanisms that might underlie such effects. As oxidative stress induced by reactive oxygen species (ROS) is a major cause of cytotoxicity, we investigated the potential of each extract of *P. bicalyculata* to alter intracellular ROS levels. HeLa cells were treated with doxorubicin or each extract and ROS levels, indicated by 2′,7′ dichlorofluorescein (DCF) fluorescence, were assessed from 1 h up to 24 h post-treatment. Fig. 5 clearly demonstrates that doxorubicin and PBC generated ROS production in HeLa cells. The ROS

Table 1
IC_{50} values for the cytotoxicity of the chloroform (PBC), ethyl acetate (PBE) and methanol (PBM) extracts of *P. bicalyculata* against HeLa and MRC5-SV2 cell lines after 24 and 48 h treatments. Values are expressed as mean ± SEM of three independent experiments. $^{###}$ $P < 0.001$ significantly different from doxorubicin within the same column; *** $P < 0.001$ and ** $P < 0.01$ significantly different from PBC within the same column; ^{yy} $P < 0.01$ significantly different from PBE within the same column; ^{a,b} significantly different at $P < 0.05$ when compared at 24 and 48 h.

Extracts	IC_{50} (μg/ml)			
	HeLa		MRC5-SV2	
	24 h	48 h	24 h	48 h
Doxorubicin	2.97 ± 0.84	0.40 ± 0.08	15.05 ± 2.63^{a}	0.78 ± 0.24^{b}
PBC	13.16 ± 1.44	6.21 ± 0.70$^{###}$	7.59 ± 1.01	1.98 ± 0.33
PBE	43.97 ± 5.70$^{###, **}$	23.29 ± 3.92$^{###}$	17.70 ± 2.96	8.57 ± 1.91
PBM	67.97 ± 9.47$^{###,***,a}$	22.43 ± 3.58$^{###,b}$	37.01 ± 4.79***	28.24 ± 5.57$^{###, ***, yy}$

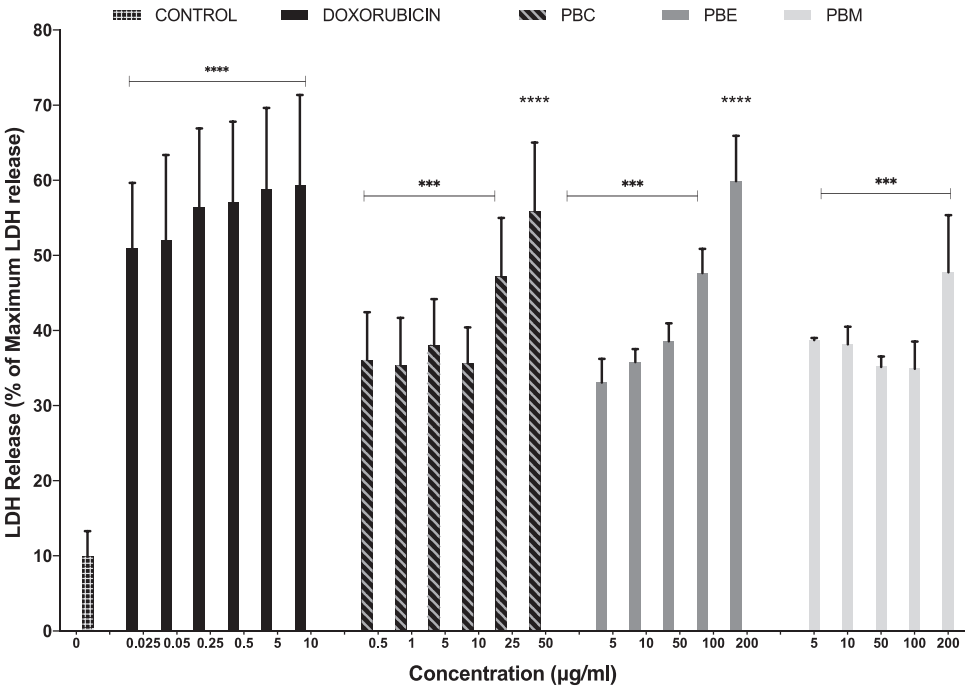

Fig. 4. Effects of 24 h treatment with doxorubicin and *P. bicalyculata* extracts on Lactate Dehydrogenase (LDH) release in HeLa cells. Each value represents the Mean ± SEM of three independent experiments. * **P < 0.001 and * *** *P < 0.0001 compared to negative control (CONTROL). Where the statistical symbol * ** is indicated on a horizontal line above a group of bars, the indicated significance applies independently to each of the bars compared to the negative control.

level in cells treated with doxorubicin increased with increasing concentrations. Doxorubicin at 10 μg/ml induced a significant 5.96 ± 2.71 fold increase in ROS ($P < 0.001$) at the 1 h time point. The effect, which though progressively decreased up to the 24 h time point, remained significantly higher than the negative control at all time points, with a 1.96 ± 0.55 fold increase at the 24 h time point. Similarly, PBC at 50 μg/ml induced a significant increase in ROS ($P < 0.05$) up to 24 h, peaking at a 1.96 ± 0.94 fold increase at the 1 h time point, but the effect was significantly lower than that of doxorubicin. In contrast, PBE or PBM did not have a significant effect on ROS levels (Fig. 5).

3.4. Extracts induced mitochondrial depolarisation, compromising mitochondrial integrity

One of the mechanisms that could underlie cytotoxicity and eventual cell death is the impairment of mitochondrial health (integrity) as a result of the depolarisation of the mitochondria, linked to apoptotic cell death. Mitochondrial integrity could be assessed by determining the mitochondrial membrane potential (Δψm). We therefore assessed the potential effects of doxorubicin and the extracts on mitochondrial membrane potential, using the dye JC-1, a membrane-permeant, potential-dependent, cationic dye that accumulates in energised mitochondria. At low concentrations (as a result of low mitochondrial membrane potential), JC-1 is predominantly a monomer that yields green fluorescence, while at high concentrations (as a result of high mitochondrial membrane potential) it aggregates, yielding a red to orange emission. Therefore, JC-1 accumulates in aggregated form and emits orange/red fluorescence in polarised mitochondria, whereas it spreads into the cytoplasm and gives a monomeric green in depolarised mitochondria. A significant decrease in average red-to-green fluorescence ratio is considered an indicator of loss of mitochondrial integrity, and thus of mitochondrial-mediated apoptosis. This study demonstrates that doxorubicin, PBC, PBE and PBM each caused concentration-dependent and, in some cases, time-dependent loss in mitochondrial membrane potential of HeLa cells (Fig. 6). For doxorubicin or each extract, the red-green fluorescence ratio at each concentration tested or at each time point examined, up to 24 h, was significantly lower than that of the negative control. Over the 24 h time course of measurement, the initial decrease in red-green fluorescence ratio induced by PBE at 5 μg/ml appeared to lessen, rather than worsen, up to 24 h, although values at all time points were significantly lower than that of the negative control.

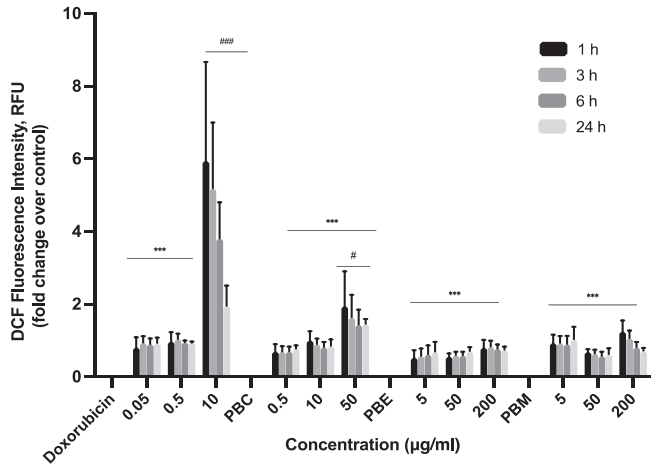

Fig. 5. Intracellular ROS generation in HeLa cells following treatment with doxorubicin or the *P. bicalyculata* extracts PBC, PBE and PBM. The fluorescence intensity (Relative Fluorescence Unit, RFU) of DCF was measured at 1, 3, 6 and 24 h post-treatment and expressed as fold change over negative control values. Each value represents the Mean ± SEM of three independent experiments. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared to negative control, * **P < 0.001 compared to 10 µg/ml doxorubicin at 3 h or beyond. The statistical significance symbol on each horizontal line on top of bar groups indicates the significance level for each bar within the group for the applicable comparison.

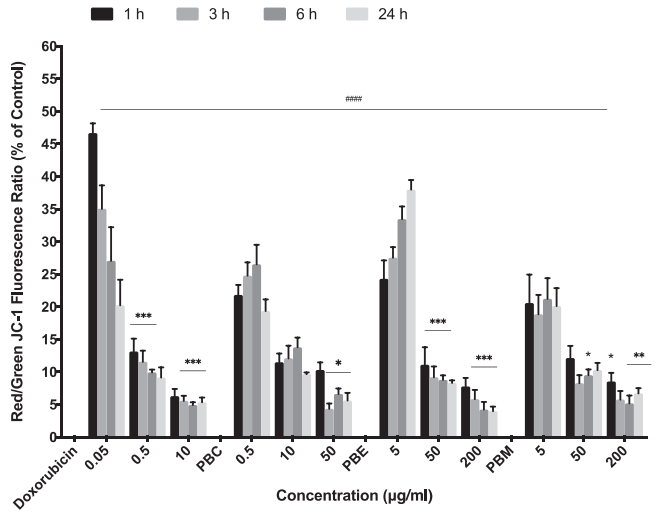

Fig. 6. Residual mitochondrial membrane potential (Δψm) of HeLa cells treated with doxorubicin or extracts of *P. bicalyculata* measured at 1, 3, 6 and 24 h post-treatment. Red and green fluorescence intensities were measured and red/green fluorescence ratios were determined. Each fluorescence ratio for the treatments was then expressed as a percentage of the fluorescence ratio of negative control that was taken as 100%. Each value represents the Mean ± SEM of three independent experiments. $^{\#\#\#\#}P < 0.0001$ for each bar compared to negative control; *P < 0.05, * *P < 0.01 and * **P < 0.001 for each extract or doxorubicin compared to the effect of its lowest concentration.

3.5. Extracts induced concentration-dependent increases in caspase 3/7 activity

Caspases mediate apoptosis, with caspases 3 and 7 representing major executioner caspases. Therefore, to confirm if an apoptotic phenotype was exhibited by HeLa cells after treatment with extracts of P. *bicalyculata*, changes to the activity of caspase 3/7 were assayed. As shown in Fig. 7, following treatment with the standard drug, doxorubicin, caspase 3/7 activity increased significantly in a concentration-dependent manner, with 10 µg/ml doxorubicin producing a 5-fold

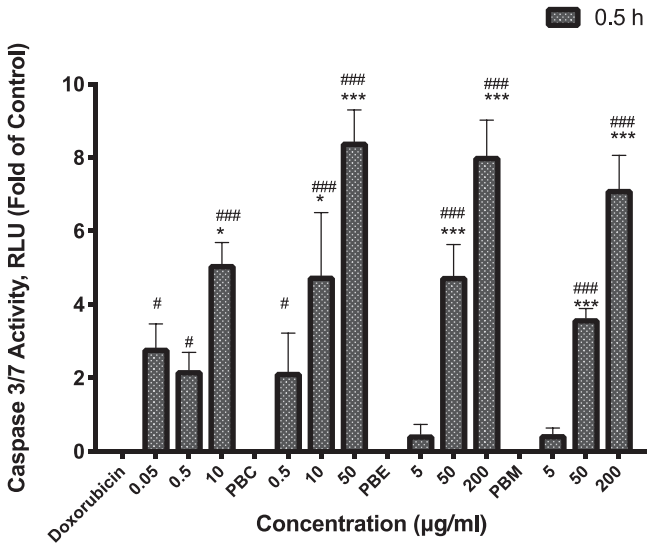

Fig. 7. Effects of doxorubicin and *P. bicalyculata* extracts on caspase 3/7 activity in HeLa cells following 24 h treatment. Values are fold changes in luminescence compared to the negative control, with the luminescence measured at 0.5 h (shown), 1 h or 1.5 h after adding the caspase reagent at the end of the 24 h treatment period. Each value represents the Mean ± SEM of three independent experiments. $^{\#\#\#}P < 0.001$ compared to negative control; *P < 0.05 and * **P < 0.001 compared to cells treated with the least concentration of the same extract. Results for the other two time points (1 h and 1.5 h) are not shown but are similar to those of the 0.5 h time point shown, for all treatments.

increase compared to the control. Similarly, each extract induced a significant concentration-dependent increase in caspase 3/7 activity, with PBC and PBE at their highest concentrations each inducing an 8-fold increase, while PBM at its highest concentration induced a 7-fold increase.

3.6. The comet assay revealed extracts caused DNA damage

To investigate the effects of the extracts on DNA, the Comet Assay, a rapid and sensitive method for assessing genotoxicity of drugs (Pak et al., 2016), was employed. Results from the single-cell comet assay indicated significant DNA damage in extract-treated HeLa cells compared to negative control cells, both in terms of tail moment and tail DNA (%), the results of which were similar to each other (Fig. 8A and B). Doxorubicin, PBC and PBE each produced a significant increase in tail DNA of 65.92 ± 7.68% ($P < 0.01$), 51.94 ± 11.89% ($P < 0.05$) and 66.35 ± 5.93% ($P < 0.01$), compared to the negative control at 3.95 ± 0.11%. A similar trend was obtained using the tail moment quantification. Fluorescence images revealed there were no comet tails (comet tails indicate DNA damage) in the negative control cells, while they were found in the extract-treated cells (Fig. 8C; arrows point to some comet tails).

3.7. Extracts increased mRNA levels of genes regulating apoptosis

To further confirm the molecular mechanisms underlying the cytotoxicity and cell death induced by the extracts, treatment-induced changes to mRNA expression levels of two apoptosis-regulatory genes, *BAX* and *caspase-3*, were determined. Doxorubicin at 10 µg/ml significantly increased mRNA expression levels of *BAX* ($P < 0.01$) and *caspase-3* ($P < 0.001$) two-fold and 6-fold, respectively (Fig. 9A and B). PBC at 50 µg/ml and PBE at 200 µg/ml showed a tendency to increase mRNA expression level of *BAX* (Fig. 9A), while PBE at 200 µg/ml increased *caspase-3* mRNA expression level nearly 11-fold ($P < 0.001$) (Fig. 9B).

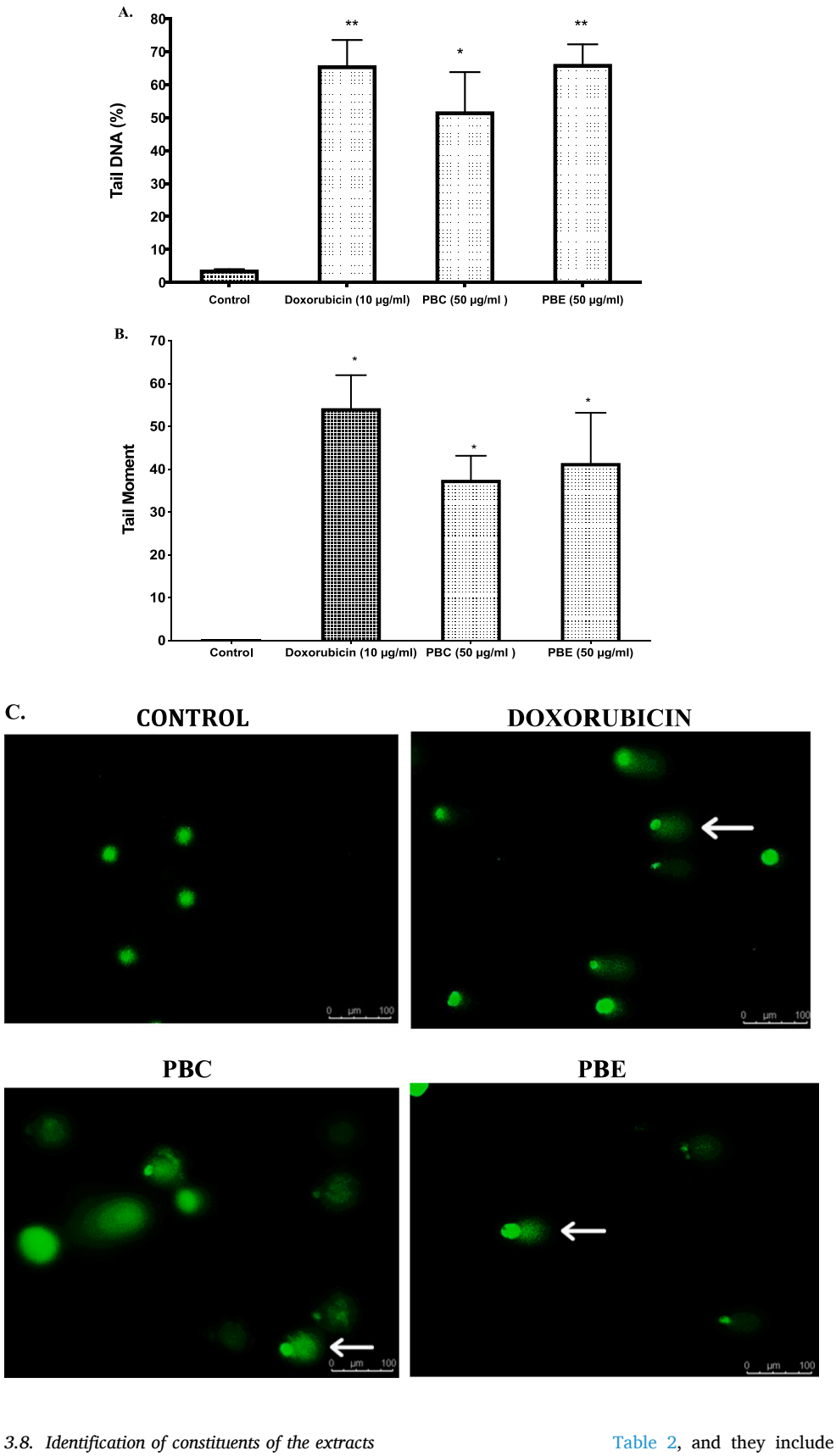

Fig. 8. The effects of doxorubicin and the most active *P. bicalyculata* extracts (PBC, PBE) on DNA damage as determined by the Comet Assay. (A) Effects on Tail DNA; (B) Effects on Tail Moment; and (C) Comet visualisation images (fluorescence microscopy) - comets are absent in negative control cells (Control) but present (arrows) in cells treated with doxorubicin (DOX) or extracts (PBC, PBE). Each experiment in A and B was repeated twice. *$P < 0.05$ and * *$P < 0.01$ compared to the negative control.

3.8. Identification of constituents of the extracts

The total ion chromatograms of the ethyl acetate and chloroform fractions of *P. bicalyculata* (which were the most active fractions) are presented in Fig. 10, and the constituent compounds are identified in Table 2, and they include a diterpenoid and a flavonoid from the chlorofom (PBC) fraction; and a gallic acid derivative, quassin and a dipeptide from the ethyl acetate (PBE) fraction.

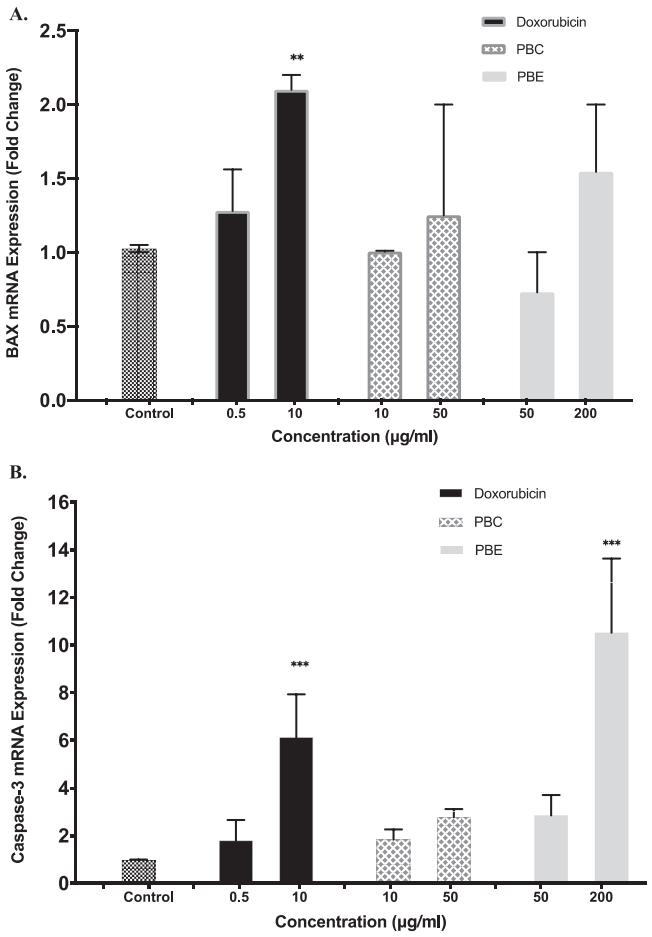

Fig. 9. The effects of doxorubicin and *P. bicalyculata* extracts (PBC, PBE) on (A) *BAX* mRNA expression and (B) *Caspase-3* mRNA in HeLa cells. Levels were normalised to GAPDH. Each experiment was repeated twice. * *P < 0.01 and * **P < 0.001 compared to the negative control.

4. Discussion

This paper reports the potential of the plant *P. bicalyculata* as a source of anti-cancer agents by investigating the cytotoxic potential of its extracts, the cellular and molecular mechanisms of the cytotoxic effects, and the constituent compounds that could be responsible for the cytotoxicity.

Despite current improvements in cancer prevention, diagnosis and treatment, mortality from the disease remains unacceptably high. Also, the side effects of standard chemotherapy sometimes compromise continued treatment, making affected patients in some parts of the world to consider alternative treatments, including herbal medicines [24]. Although currently there is insufficient scientific validation of efficacy of most herbal medicines for treating or preventing cancer, renewed and concerted efforts are now geared towards the discovery and development of safer and well-tolerated anticancer drugs from natural products, especially plants [2,25]. Such efforts include exploration of Nigerian medicinal plants, as Nigeria possesses diverse plant species whose traditional historical uses span many centuries. The Nigerian flora, therefore, has significant potential as a veritable source of novel pharmaceuticals and other therapeutic entities, but many of these plants are yet to receive enough research attention to make local sourcing of pharmaceutical products a reality [26]. As part of our efforts to address this challenge, we have identified *P. bicalyculata* as one of the plants that deserve attention. It is well established that plants are a source of dietary supplements and drugs for the management of several diseases [10]. These plants (natural alternatives or natural products) are now widely appreciated and accepted due to their contributions to health and international commerce [11]. With respect to cancer, a growing body of evidence has associated the ability of plants to inhibit cell growth and/or cause cell death with anticancer properties of their bioactive constituents [10]. Almost 60% of drugs currently used for the treatment of cancer were isolated from natural products, with plants contributing considerably [9,27]. However, only a small percentage of plants have been evaluated for their potential as sources of anticancer agents, thus creating research gaps and opportunities for developing new, potent, safer and, perhaps, more selective anticancer drugs.

In this study, four extracts of *P. bicalyculata* (hexane extract (PBH), ethyl acetate extract (PBE), chloroform extract (PBC) and methanolic extract (PBM)) were tested for their cytotoxic activities; and three of them, PBE, PBC and PBM were each found to cause significant cytotoxicity in HeLa and MRC5-SV2 cancer cells in a concentration- and time-dependent manner. The ability of the extracts to induce marked LDH release further demonstrated their cytotoxic activities, as well as the involvement of necrosis and/or late-phase apoptosis in the resultant cell death process. Cancer cells can produce and retain LDH for growth, and elevation of LDH levels in the growth medium occurs from dead cancer cells due to the breakdown of the integrity of their cell membranes [28]. The extracts were also able to target cancer cells more selectively compared to normal cells, thus showing potential to kill cancer cells and relatively spare normal cells, a property desirable in anti-cancer compounds. The cytotoxic activities of the extracts could be attributed to the nature and concentration of bioactive compounds in the extracts [29,30], as it is known that bioactive constituents of plants, including phytochemicals such as alkaloids, saponins, flavonoids, taxanes, vitamins, minerals, terpenes and lignans, exhibit anticancer potential of varying potencies due to their ability to inhibit enzymes and cancer-signalling pathways, activate DNA repair mechanisms, and stimulate formation of protective enzymes [29]. The significantly high cytotoxic activity of PBC indicates that the chloroform extract may contain more potent bioactive compounds or compounds capable of killing cancer cells than are contained in the methanolic (PBM) and ethyl acetate (PBE) extracts, although the methanolic extract of *P. bicalyculata* has been previously shown to be cytotoxic against human mouth epidermal carcinoma cells (KB) [17]. Oils from the plant were also found to be cytotoxic against the human MCF-7 and MDA-MB-468 breast tumour cells [13]. Our present findings establish that extracts of this plant contain compounds that may be beneficial for the treatment of not only human breast cancers but also human cervical and lung cancers. Interestingly, the constituents of the two most active (potent) fractions (PBC and PBE) were identified as vital secondary metabolites, including derivatives of gallic acid, dipeptides, diterpenoids and flavones. These compounds could act through a range of mechanisms, including enzyme inhibition and modulation of levels of reactive oxygen species (oxidative stress) and inflammation, thus tipping the balance against the survival of cancer cells [31].

To understand the mechanisms of cytotoxic action of the extracts, we investigated their apoptosis-inducing ability, as it is well established that cytotoxic anticancer drugs may exert their effects by inducing apoptosis, cell cycle arrest and DNA damage [32,33]. First, two early events associated with apoptosis, the production of ROS and changes to the mitochondrial membrane potential, were examined. ROS such as singlet oxygen ($^1\Delta_g$), nitrogen dioxide ($^{\bullet}NO_2$), hydroxyl radical ($^{\bullet}OH$), superoxide ($^{\bullet}O^{-}_2$), nitric oxide ($^{\bullet}NO$), and lipid peroxyl play significant roles in the development of several diseases, including cancer [34]. To maintain homeostasis, under normal conditions cellular ROS produced are kept in check by a ROS-scavenging system, failing which the accumulation of excessive ROS induces oxidative stress and causes DNA damage, thus increasing the risk of cancer [34]. Studies have demonstrated the ability of some chemopreventive drugs to scavenge ROS, thus acting as antioxidants [35,36], while many anticancer drugs generate high ROS levels to induce cancer cell death [34,37]. In this study, although all three extracts that induced cytotoxicity showed a tendency

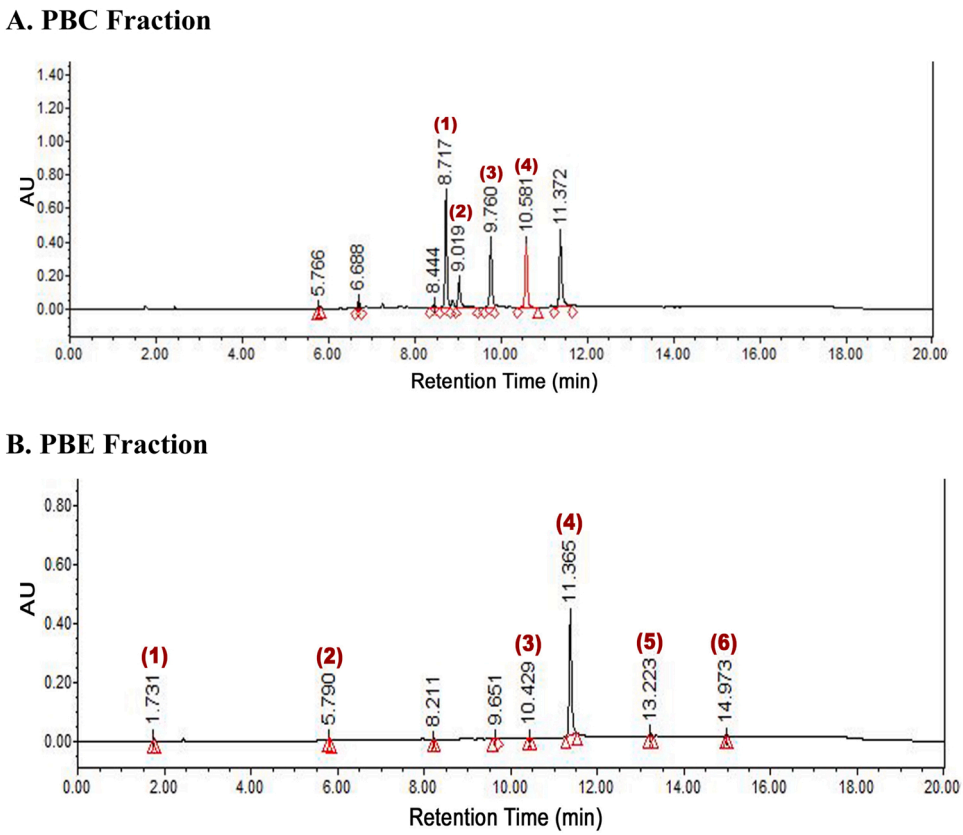

Fig. 10. Total ion chromatograms of the (A) chloroform fraction (PBC) and (B) ethyl acetate fraction (PBE) of *P. bicalyculata*. Numbers in red in brackets indicate peak numbers for constituents that were tentatively identified, as highlighted in Table 2.

Table 2
Compounds identified by LC-MS (positive mode) from the chloroform fraction (PBC) and ethyl acetate fraction (PBE) of *P. bicalyculata*.

Compound Peak	Retention Time (min)	Compound Mass (Da)	M+H (m/z)	Tentative Identification
CHLOROFORM FRACTION (PBC)				
1	8.717	314	315.34	7,8-Dihydropteroic acid
2	9.019	286	287.11	Kaempferol (flavonols)
3	9.760	270	271.15	Dehydroabietan (Diterpenoids)
4	10.581	296	297.21	2,5-dihydroflavone-5-acetate (flavones)
ETHYL ACETATE FRACTION (PBE)				
1	1.731	394	395.18	Tylactone
2	5.790	198	199.20	Syringic acid (Gallic acid)
3	10.429	416	417.61	Ramipril (Dipeptides)
4	11.365	390	391.68	Nutriacholic acid
5	13.223	276	277.35	[8]-Shogaol
6	14.973	388	389.42	Quassin (Quassinoids)

to increase ROS, only PBC increased it significantly ($P < 0.05$), clearly showing ROS as a mediator of PBC-induced cell death. The high ROS production by the anticancer drug doxorubicin used as a positive control agrees with studies demonstrating that the drug induces apoptosis in several cancer cell lines by generating free radicals [38,39]. These results are consistent with those from studies showing that free radical formation plays a significant role in the cytotoxic effects of some anticancer drugs [40].

Impairment of mitochondrial health is integral to intrinsic apoptosis, and as the induction of ROS is known to activate some enzymes of the apoptotic pathway [41] by causing damage to the mitochondrial membrane, the effects of the extracts on mitochondria membrane potential (Δψm) were examined. These results showed concentration- and time-dependent decreases in the mitochondria membrane potential (Δψm) of HeLa cells treated with the extracts. Thus, treatment of cells with the extracts resulted in oxidative stress due to accumulation of ROS, which disrupted the mitochondrial membrane potential, resulting in dysfunctional mitochondria [42], an early indicator of apoptosis [40,43]. We then confirmed through the comet assay experiment that the moderate-to-excessive levels of ROS generated by the extracts did not only affect membrane integrity, but also impaired genetic material, causing DNA damage.

Apoptosis is a homeostatic and defensive mechanism that eliminates unwanted cells via the intrinsic (mitochondrial) or extrinsic (death receptor) pathways, both of which converge at the level of proteases called caspases [44]. Activation of the initiator caspase 9 and 8 by the intrinsic and extrinsic pathways, respectively, triggers the activation of the executioner caspases 3, 6 and 7, causing the destruction of functional and structural proteins, chromatin condensation, DNA fragmentation, membrane blebbing, and other morphological changes associated with apoptosis [44]. Findings from this work showed that all the extracts elevated caspase 3/7 activities in HeLa cells. This increase in caspase-3 activity at the protein level was correlated with changes to gene expression, as caspase-3 mRNA levels also increased after treatment. Thus, it could be established that these extracts induced apoptosis via the mitochondria-mediated pathway. This can be attributed to the loss of $\Delta\Psi_m$, as we confirmed, which causes release of cytochrome *c* from the mitochondria to the cytosol. Cytosolic cytochrome *c* binds to apoptotic protease-activating factor 1 (Apaf-1) to generate the apoptosome, which then binds and activates caspase-9 via reciprocal cleavage. Effector caspases such as caspase-3 become activated by binding to the

apoptosome complex via caspase-9, eventually triggering apoptosis [41, 44]. Thus, the results of this study imply that the extracts triggered the upstream signal of caspase-9 in HeLa cells to activate caspase-3 and initiate cell apoptosis. This agrees with Kayacan et al. [45] and Li et al. [41] who reported that the induction of apoptosis in HeLa cells usually occurs via caspase-3. The role of BAX, a Bcl-2 family protein known to control intrinsic apoptosis events and play an important role in maintaining mitochondrial membrane integrity [46] was also studied. Our results show that, although PBC and PBE extracts tended to increase *BAX* mRNA level in HeLa cells, the effects were not statistically significant at the concentrations tested. This result is consistent with reports by Park et al. [47] who demonstrated that, to induce apoptosis, BAX levels may not necessarily increase, as its activity does not directly block apoptosis, but inhibits the anti-apoptotic role of Bcl-2 [48].

5. Conclusions

Taken together, the findings of this study demonstrate that *P. bicalyculata* extracts could induce cytotoxicity and cell death in cancer cells. This represents a unique evidence base justifying the use of *P. bicalyculata* by traditional medicine practitioners for the treatment of cancers. The molecular mechanisms by which preparations from the plant used in anticancer remedies exert their anti-cancer effects are suggested to include ROS induction, loss of mitochondrial integrity, DNA damage and activation of apoptotic signalling pathways. Our work suggests that *P. bicalyculata* contains a range of bioactive compounds that can be beneficially exploited in the development of novel anticancer therapies for a variety of human cancers, including cervical cancer.

CRediT authorship contribution statement

Mansurah A. Abdulazeez: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Hiba A. Jasim:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Musa Bashir:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Kehinde Ross:** Methodology, Resources, Supervision, Validation, Writing – review & editing. **Amos A. Fatokun**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Conflict of interest statement

Authors declare that there are no conflicts of interests.

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References

[1] World Health Organization. Human Papillomavirus (HPV) and Cervical Cancer. 2019. ⟨https://www.who.int/en/news-room/fact-sheets/detail/human-papillomavirus⟩⟨-(hpv)-and-cervical-cancer⟩. Available: 24 October 2019.

[2] I. Rady, M.B. Bloch, R.-C.N. Chamcheu, S. Banang Mbeumi, M.R. Anwar, H. Mohamed, A.S. Babatunde, J.-R. Kuiate, F.K. Noubissi, K.A. El Sayed, Anticancer properties of Graviola (Annona muricata): a comprehensive mechanistic review, Oxid. Med. Cell. Longev. 2018 (2018), 1826170, https://doi.org/10.1155/2018/1826170.

[3] A.I. Bisi-Onyemaechi, U.N. Chikani, O. Nduagubam, Reducing incidence of cervical cancer: knowledge and attitudes of caregivers in Nigerian city to human papilloma virus vaccination, Infect. Agent. Cancer 13 (2018) 1–6, https://doi.org/10.1186/s13027-018-0202-9.

[4] L. Bruni, G. Albero, B. Serrano, M. Mena, D. Gómez, J. Muñoz, F.X. Bosch, S. de Sanjosé, ICO/IARC information centre on HPV and cancer (HPV information centre). Hum. papillomavirus Relat. Dis. world. Summ. Rep. 17, Date Assess. 24th Oct. (2019) 2019.

[5] American Cancer Society, Cancer Facts & Figures 2019, American Cancer Society,, Atlanta, Ga, 2019. ⟨https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2019/cancer-facts-and-figures-2019.pdf⟩.

[6] A. Idowu, S.A. Olowookere, A.T. Fagbemi, O.A. Ogunlaja, Determinants of cervical cancer screening uptake among women in Ilorin, North Central Nigeria: a community-based study, J. Cancer Epidemiol. 2016 (2016), 6469240, https://doi.org/10.1155/2016/6469240.

[7] F. Ishola, O. Omole, A vision for improved cancer screening in Nigeria, Lancet Glob. Heal. 4 (2016) e359–e360, https://doi.org/10.1016/S2214-109X(16)30062-6.

[8] A.D. Shrestha, D. Neupane, P. Vedsted, P. Kallestrup, Cervical cancer prevalence, incidence and mortality in low and middle income countries: a systematic review, Asian Pac. J. Cancer Prev. APJCP 19 (2018) 319–324, https://doi:10.22034/APJCP.2018.19.2.319.

[9] V. Kuete, T. Efferth, African flora has the potential to fight multidrug resistance of cancer, Biomed. Res. Int (2015) 2015, https://doi.org/10.1155/2015/914813.

[10] B.J. Taiwo, G.O. Taiwo, O.O. Olubiyi, A.A. Fatokun, Polyphenolic compounds with anti-tumour potential from *Corchorus olitorius* (L.) Tiliaceae, a Nigerian leaf vegetable, Bioorg. Med. Chem. Lett. 26 (2016) 3404–3410.

[11] D.J. Newman, G.M. Cragg, Natural products as sources of new drugs from 01/1981 to 09/2019, J. Nat. Prod. 83 (2020) 770–803.

[12] S.M. Ogbourne, P.G. Parsons, The value of nature's natural product library for the discovery of new chemical entities: the discovery of ingenol mebutate, Fitoterapia 98 (2014) 36–44.

[13] I.A. Ogunwande, T.M. Walker, A. Bansal, W.N. Setzer, E.E. Essien, Essential oil constituents and biological activities of *Peristrophe bicalyculata* and *Borreria verticillata*, Nat. Prod. Commun. 5 (2010) 1815–1818, https://doi.org/10.1177/1934578×1000501125, 1934578×1000501125.

[14] N. Janakiraman, M. Johnson, S.S. Sahaya, GC–MS analysis of bioactive constituents of *Peristrophe bicalyculata* (Retz.) Nees.(Acanthaceae), Asian Pac. J. Trop. Biomed. 2 (2012) S46–S49.

[15] O. Nabere, G. Samson, H. Adama, C. Moussa, S.P. Eric, N.P. Aminata, M.-R. Jeanne, N.O. Germaine, Antioxidant and anticancer activities of polyphenolic compounds from three Acanthaceae medicinal species from Burkina Faso, Int. J. Phytomed. 4 (2012) 552–557.

[16] G. Rashmi, P. Jaya, P. Hardik, M. Bhumi, A. Shivani, Peristrophe bicalyculata-a review, Pharmacogn. J. 2 (2010) 39–45.

[17] M.A. Abdulazeez, I. Sani, D.A. Ameh, J.O. Ayo, L.J.C. Carvalho, Anticancer activities of extracts of *Peristrophe bicalyculata* (RETZ) Nees, Rom. Biotechnol. Lett. 18 (2013) 7995–8006.

[18] K. Ashokan, M.S. Muthuraman, Anticancer studies on *Orthosiphon pallidus* Royle and *Peristrophe bicalyculata* Nees, J. Pharm. Res. 4 (2011) 2654–2656.

[19] S.S. Tanavade, N.S. Naikwade, D.D. Chougule, In vitro anticancer activity of ethanolic extracts of *Peristrophe bicaliculata* Nees, Int. J. Chem. Sci. 10 (2012) 317–323.

[20] P.A. Ajibade, A.A. Fatokun, F.P. Andrew, Synthesis, characterization and anticancer studies of Mn (II), Cu (II), Zn (II) and Pt (II) dithiocarbamate complexes-crystal structures of the Cu (II) and Pt (II) complexes, Inorg. Chim. Acta 504 (2020), 119431, https://doi.org/10.1016/j.ica.2020.119431.

[21] R.O. Omondi, R. Bellam, S.O. Ojwach, D. Jaganyi, A.A. Fatokun, Palladium (II) complexes of tridentate bis (benzazole) ligands: Structural, substitution kinetics, DNA interactions and cytotoxicity studies, J. Inorg. Biochem. 210 (2020), 111156, https://doi.org/10.1016/j.jinorgbio.2020.111156.

[22] A. Piovesana, E. Rodrigues, C.P.Z. Norena, Composition analysis of carotenoids and phenolic compounds and antioxidant activity from Hibiscus calyces (Hibiscus sabdariffa L) by HPLC-DAD-MS/MS, Phytochem. Anal. 30 (2) (2019) 208–217, https://doi.org/10.1002/pca.2806.

[23] Z.D. Lv, X.P. Liu, W.J. Zhao, Q. Dong, F.N. Li, H.B. Wang, B. Kong, Curcumin induces apoptosis in breast cancer cells and inhibits tumor growth in vitro and in vivo, Int. J. Clin. Exp. Pathol. 7 (6) (2014) 2818–2824.

[24] A.J. Akindele, Z. Wani, G. Mahajan, S. Sharma, F.R. Aigbe, N. Satti, O.O. Adeyemi, D.M. Mondhe, Anticancer activity of *Aristolochia ringens* Vahl.(Aristolochiaceae), J. Tradit. Complement. Med. 5 (2015) 35–41.

[25] S.Z. Moghadamtousi, B.H. Goh, C.K. Chan, T. Shabab, H.A. Kadir, Biological activities and phytochemicals of Swietenia macrophylla King, Molecules 18 (2013) 10465–10483, https://doi.org/10.3390/molecules180910465.

[26] A. Sofowora, Recent trends in research into African medicinal plants, J. Ethnopharmacol. 38 (1993) 197–208.

[27] A.B. Da Rocha, R.M. Lopes, G. Schwartsmann, Natural products in anticancer therapy, Curr. Opin. Pharm. 1 (2001) 364–369.

[28] Y.-Q. Tang, I.B. Jaganath, S.D. Sekaran, Phyllanthus spp. induces selective growth inhibition of PC-3 and MeWo human cancer cells through modulation of cell cycle and induction of apoptosis, PLoS One 5 (2010), e12644, https://doi.org/10.1371/journal.pone.0012644.

[29] T.N. Aung, Z. Qu, R.D. Kortschak, D.L. Adelson, Understanding the effectiveness of natural compound mixtures in cancer through their molecular mode of action, Int. J. Mol. Sci. 18 (2017) 656, https://doi.org/10.3390/ijms18030656.

[30] J. Iqbal, B.A. Abbasi, T. Mahmood, S. Kanwal, B. Ali, S.A. Shah, A.T. Khalil, Plant-derived anticancer agents: a green anticancer approach, Asian Pac. J. Trop. Biomed. 7 (2017) 1129–1150.
[31] B. Scaria, S. Sood, C. Raad, J. Khanafer, R. Jayachandiran, A. Pupulin, S. Grewal, M. Okoko, M. Arora, L. Miles, S. Pandey, Natural health products (NHP's) and natural compounds as therapeutic agents for the treatment of cancer; mechanisms of anti-cancer activity of natural compounds and overall trends, Int. J. Mol. Sci. 21 (2020) 8480.
[32] A. Dwivedi, S.F. Mujtaba, H.N. Kushwaha, D. Ali, N. Yadav, S.K. Singh, R.S. Ray, Photosensitizing mechanism and identification of levofloxacin photoproducts at ambient UV radiation, Photochem. Photobio. 88 (2012) 344–355.
[33] K. Griffiths, B.B. Aggarwal, R.B. Singh, H.S. Buttar, D. Wilson, F. De Meester, Food antioxidants and their anti-inflammatory properties: a potential role in cardiovascular diseases and cancer prevention, Diseases 4 (2016) 28, https://doi.org/10.3390/diseases4030028.
[34] L. Dini, S. Mariano, E. Panzarini, Herbal Extracts from Carica papaya and Azadirachta indica: What Role for ROS in Cancer Cell Lines? in: Ferid Murad, Atta-ur-Rahman, Ka Bian (Eds.), Herbal Medicine: Back to the Future Cancer Therapy 3 Bentham Science, Singapore, 2019, pp. 1–45.
[35] A.R. Ndhlala, B. Ncube, H.A. Abdelgadir, C.P. Du Plooy, J. Van Staden, Antioxidant potential of African medicinal plants, in: K. Al-Gubory, I. Laher (Eds.), Nutritional Antioxidant Therapies: Treatments and Perspectives, Springer, Cham, 2017, pp. 65–88, https://doi.org/10.1007/978-3-319-67625-8_3.
[36] K.V. Ramana, A. Reddy, N.V. Majeti, S.S. Singhal, Therapeutic potential of natural antioxidants, Oxid. Med. Cell. Longev. 2018 (2018), 9471051, https://doi.org/10.1155/2018/9471051.
[37] E. Gansukh, K.K. Mya, M. Jung, Y.-S. Keum, D.H. Kim, R.K. Saini, Lutein derived from marigold (Tagetes erecta) petals triggers ROS generation and activates Bax and caspase-3 mediated apoptosis of human cervical carcinoma (HeLa) cells, Food Chem. Toxicol. 127 (2019) 11–18.
[38] A. Das, D. Durrant, C. Mitchell, E. Mayton, N.N. Hoke, F.N. Salloum, M.A. Park, I. Qureshi, R. Lee, P. Dent, Sildenafil increases chemotherapeutic efficacy of doxorubicin in prostate cancer and ameliorates cardiac dysfunction, Proc. Natl. Acad. Sci. 107 (2010) 18202–18207.
[39] A. Rogalska, A. Koceva-Chyła, Z. Jóźwiak, Aclarubicin-induced ROS generation and collapse of mitochondrial membrane potential in human cancer cell lines, Chem. Biol. Interact. 176 (2008) 58–70.
[40] A. Rogalska, A. Gajek, M. Szwed, Z. Jóźwiak, A. Marczak, The role of reactive oxygen species in WP 631-induced death of human ovarian cancer cells: a comparison with the effect of doxorubicin, Toxicol. Vitr 25 (2011) 1712–1720.
[41] X. Li, X. Luo, J. He, N. Wang, H. Zhou, P. Yang, T. Zhang, Induction of apoptosis in human cervical carcinoma HeLa cells by active compounds from *Hypericum ascyron* L, Oncol. Lett. 15 (2018) 3944–3950.
[42] W. Ahmad, B. Ijaz, K. Shabbiri, F. Ahmed, S. Rehman, Oxidative toxicity in diabetes and Alzheimer's disease: mechanisms behind ROS/RNS generation, J. Biomed. Sci. 24 (2017) 1–10, https://doi.org/10.1186/s12929-017-0379-z.
[43] Z. Liu, Y. Jiao, H. Lu, X. Shu, Q. Chen, Chemical characterization, antioxidant properties and anticancer activity of exopolysaccharides from Floccularia luteovirens, Carbohydr. Polym. 229 (2020), 115432, https://doi.org/10.1016/j.carbpol.2019.115432.
[44] Y. Kiraz, A. Adan, M.K. Yandim, Y. Baran, Major apoptotic mechanisms and genes involved in apoptosis, Tumor Biol. 37 (2016) 8471–8486.
[45] S. Kayacan, L.T. Sener, G. Melikoglu, S. Kultur, I. Albeniz, M. Ozturk, Induction of apoptosis by *Centaurea nerimaniae* extract in HeLa and MDA-MB-231 cells by a caspase-3 pathway, Biotech. Histochem. 93 (2018) 311–319.
[46] Q. Zhang, F. Zhang, K. Thakur, J. Wang, H. Wang, F. Hu, J.G. Zhang, Z.J. Wei, Molecular mechanism of anti-cancerous potential of Morin extracted from mulberry in Hela cells, Food Chem. Toxicol. 112 (2018) 466–475.
[47] K.I. Park, H.S. Park, A. Nagappan, G.E. Hong, D.H. Lee, S.R. Kang, J.A. Kim, J. Zhang, E.H. Kim, W.S. Lee, S.C. Shin, Induction of the cell cycle arrest and apoptosis by flavonoids isolated from Korean Citrus aurantium L. in non-small-cell lung cancer cells, Food Chem. 135 (4) (2012) 2728–2735.
[48] M. Ji, L. Yuan, X. Lv, W. Dong, X. Peng, EBP50 regulates the apoptosis of pancreatic cancer cells by decreasing the expression levels of Bcl-2, Exp. Ther. Med. 8 (2014) 919–924.