Cyathula prostrata ethanol extract activates the extrinsic pathway of apoptosis in HeLa and U937 cell lines

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1. Introduction

Normal cells follow an orderly pattern of cell growth, division and death while cancer cells do not. Cancer is described as a class of disease in which cells display uncontrolled and accelerated proliferation and are unable to die, leading to their accumulation and expansion (Kasibhatla and Tseng, 2003). Defective apoptosis has been shown to be a major factor in the development and progression of cancer as tumour cells have the ability to evade apoptosis (Schafer, 1998) and thus represents a potential therapeutic target.

Apoptosis is initiated by extracellular or intracellular signals which lead to the activation of a cascade of events leading to the degradation of the cell and is characterised by morphological changes such as membrane blebbing, cytoplasmic condensation and apoptotic body formation. Biochemical changes within the cell are also evident including caspase activation, DNA fragmentation and phosphatidylserine translocation (Elmore, 2007). Two major pathways of apoptosis have been described. Death-receptor mediated apoptosis, also known as the extrinsic pathway, is mediated by one of several death receptors binding to an appropriate ligand on the cell surface (Kasibhatla and Tseng, 2003). This leads to the activation of cysteine-dependent aspartate-directed proteases (caspases) in the cytosol of the cell (Vermeulen et al., 2005).

MITochondria-mediated apoptosis, also referred to as the intrinsic pathway, is characterised by the release of cytochrome c and other pro-apoptotic molecules from the mitochondrial intermembrane space into the cytosol of the cell which, in turn, activates caspases. The release of cytochrome c and the balance of activity of pro- and anti-apoptotic members of the bcl-2 superfamily of proteins are associated with the loss of the outer mitochondrial membrane potential (Vermeulen et al., 2005) and the state of the mitochondrial membrane may serve as a marker for the onset of the intrinsic pathway of apoptosis.

Plants have played an important role in the discovery of anti-cancer agents. Over 60% of anti-cancer agents have been derived from natural sources (Cragg and Newman, 2005). Cyathula prostrata (L.) Blume is an annual, branched herb/shrub reaching a length of approximately 1 m or more. C. prostrata is widely distributed and can be found in tropical Africa and America and in Asia as well as in Australia. It has many medicinal uses. The leaves are typically used for treatment against rheumatic fever, dysentery, wounds and eye trouble. The sap is traditionally used as an eardrop to treat otitis and is also applied to skin sores and burns (Burkill, 1995). It has been reported that an ethanolic extract of C. prostrata exhibits cytotoxic effects against cervical and breast cancer cells (Sowemimo et al., 2009).

Although there appears to be no documentation that C. prostrata is traditionally used as an anti-cancer therapy, the report of cytotoxic activity has led to our investigation of the mechanism of cell death caused by the ethanolic extract of C. prostrata.
2. Methods and materials

2.1. Materials, reagents and chemicals

Cervical (HeLa) cancer and human promonocytic leukaemia U937 cells were purchased from Highveld Biological, South Africa. Cleaved caspase 8 (Asp391), p21 Waf1/Cip1 (12D1) rabbit and cytomegle-c (136F3) rabbit antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The Coulter® DNA Prep™ reagent kit, goat anti-rabbit IgG (H + L chain specific) and rabbit IgG isotype, both labelled with fluorescein (FITC) conjugate, and IsoFlow™ EPICS™ sheath fluid were purchased from Beckman Coulter (CA, USA). IntraPrep™ permeabilising reagent was purchased from Immunotech (Marseille, France). Annexin V–FITC/PI kit was purchased from MACS Miltenyi Biotec (Auburn, USA). CellTiter-Blue® reagent was purchased from Promega (Madison, WI, USA). RPMI 1640 cell culture medium containing 25 mM Hepes, 2 mM glutamine and foetal bovine serum was purchased from Thermo Scientific (St. Louis, MO, USA).

2.2. Methods

2.2.1. Cell culture conditions

Cells were cultured in a humidified atmosphere containing 5% CO2 in air at 37 °C. The adherent cancer cell line, HeLa, and the suspension U937 cells were used for the experimental procedures. The cells were routinely maintained in 10-cm culture dishes and 25 cm2 flasks, respectively, without antibiotics in RPMI 1640 cell culture medium supplemented with 10% foetal bovine serum. Trypan blue was used to determine cell viability and cell number.

2.2.2. Plant extraction

C. prostrata (whole plant) was collected from the Olokemeji Forest Reserve and from the Campus of Obafemi Awolowo University, Ile-Ife in Nigeria in July 2006. The plant was authenticated by comparison with corresponding herbarium specimens at the Forestry Research Institute, Ibadan, Nigeria (FRIN) where voucher specimens (FHI 107232) were also deposited. The plant was air dried for two days followed by drying in a hot air oven at 40 °C. Thereafter, the dried plant material was ground to powder and stored in amber coloured bottles. Powdered plant material (100 g) was macerated with 80% ethanol at room temperature. Extract was prepared from the plant material (100 g) was macerated with 80% ethanol at room temperature (Sowemimo et al., 2009). Stocks of the extract were made fresh on the day of experiment at 100 mg/mL by dissolving 1 mg extract in 10 μL dimethyl sulfoxide (DMSO).

2.2.3. Cytotoxicity

Cytotoxicity assays were performed using CellTiter-Blue. HeLa and U937 cells were seeded in 200 μL and 100 μL aliquots, respectively, at 3 × 10^5 cells/mL in 96 well plates and HeLa cells were left overnight to attach. U937 cells was also incubated overnight at 37 °C before treatment. The medium was replaced with fresh medium containing varying concentrations (4 to 500 μg/mL) of ethanolic C. prostrata extract for treatment of HeLa cells. One hundred microlitre aliquots of fresh medium containing double the appropriate extract concentration were added to the respective wells for treatment of U937 cells. Both cell lines were incubated at 37 °C in a humidified 5% CO2 incubator for 48 h. cisplatin (1.5 μM–100 μM) was used as a positive control and DMSO (0.05–0.25% v/v) as a vehicle control. Following incubation, 40 μL of CellTiter-Blue reagent was added to each well and cells were incubated for 2 h at 37 °C, in the dark. Thereafter, 200 μL of supernatant was transferred from each well to a black 96 well microtitre plate. Fluorescence was read at 544/590 nm by using a Fluoroskan Ascent FL Fluorometer (Thermo Labsystems, Finland).

Cytotoxicity of an ethanolic extract of C. prostrata was also tested against peripheral blood mononuclear cells (PBMCs). Blood was collected from a healthy donor and within 30 min after collection, PBMCs were isolated. Briefly, blood was centrifuged at 1800 × g for 30 min at 20 °C. The plasma containing PBMCs was transferred to sterile 15 mL polypropylene centrifugation tubes and centrifuged at 300 × g for 15 min. The cell pellet was resuspended gently and washed with 10 mL growth medium and centrifuged. Washing of the cell pellet was repeated twice more. The final cell pellet was resuspended in growth medium and cells were seeded in 200 μL aliquots at 1 × 10^5 cells/mL in a 96 well microtitre plate. Cells were treated with varying concentrations of C. prostrata (16–250 μg/mL), cisplatin (12.5 and 50 μg/mL) and DMSO (0.05%–0.5% v/v). Treatments were performed in quadruplicate. A viability assay using CellTiter-Blue was performed after 48 h of treatment as described above.

2.2.4. Cell cycle analysis

HeLa cells were seeded in 10 cm culture dishes at 1.2 × 10^6 cells/10 mL and allowed to attach for 6 h. After attachment, medium was aspirated and cells were treated with 125 μg/mL of C. prostrata extract and incubated for 24 and 48 h. U937 cells were seeded at a concentration of 1 × 10^5 cells/mL and treated and incubated as for HeLa cells. Following incubation of 24 and 48 h, all cells were harvested and collected in polypropylene flow cytometer tubes (Beckman Coulter). Adherent HeLa cells were trypsinised and re-suspended in 1 mL phosphate buffered saline (PBS). U937 cells did not require trypsinisation. Cells were washed by centrifugation at 500 × g for 5 min and resuspended in 1 mL PBS. This washing step was repeated twice. The DNA Prep kit from Beckman Coulter was used for DNA cell cycle analysis and the assay was performed as per manufacturer’s recommendations with modifications. Briefly, 100 μL of lysis buffer was added, tubes very gently vortexed and incubated for 5 min at room temperature. Thereafter, 500 μL propidium iodide (50 μg/mL) was added and allowed to incubate for 15 min at 37 °C in the dark. Samples were then analysed immediately on a Beckman Coulter FC 500 flow cytometer.

2.2.5. Phosphatidyserine translocation

The Annexin V–FITC/PI staining kit (Miltenyi) was used to analyse phosphatidyserine translocation, a biochemical change evident at a late stage of apoptosis. Cells were seeded and treated for both 24 and 48 h studies. U937 cells were seeded in 24 well culture plates at a cell density of 1 × 10^5 cells/mL. Following incubation with treatments, cells were transferred to polypropylene tubes and centrifuged at 500 × g for 5 min. The supernatant was discarded and the pellet washed in 1 mL binding buffer. Washed cells were centrifuged at 300 × g for 10 min, supernatant discarded and the washing step repeated. Cells were re-suspended in 100 μL 1 × binding buffer, followed by staining. Annexin V–FITC (1 μL; 25 μg/mL) was added to each tube, vortexed briefly and incubated in the dark. Cells were then washed as described. Thereafter, cells were re-suspended in 500 μL (1 ×) binding buffer followed by the addition of propidium iodide (5 μL; 250 μg/mL). Samples were immediately analysed by flow cytometry.

2.2.6. Measurement of intracellular p21 levels

HeLa and U937 cells were seeded at 4 × 10^4 cells/mL and 1 × 10^5 cells/mL, respectively. HeLa cells were left to attach for 6 h. Cells were treated with C. prostrata (125 μg/mL) and cisplatin (50 μM) separately. Cells were collected and washed using the centrifugation method as previously described. Cells were fixed and permeabilised using the IntraPrep permeabilisation kit as per manufacturer’s instructions. p21 Waf1/Cip1 (12D1) rabbit monoclonal antibody was used to determine p21 levels in the cells. Cells were first blocked by using PBS supplemented with 0.5% BSA and thereafter incubated with p21 antibody (1:50). Cells were then stained with goat anti-rabbit IgG (H + L) mouse/human ads-FITC secondary antibody and incubated
for 30 min at room temperature in the dark. p21 levels were analysed using flow cytometry. An isotype control of rabbit IgG-FITC was also prepared and analysed.

2.2.7. Measurement of activated caspase 8

Cells were seeded, treated, fixed and permeabilised as described in Section 2.2.6. Cleaved caspase 8 (Asp391 18C8) rabbit monoclonal antibody was used to determine activation of caspase 8. Secondary antibody staining was performed as described for p21 level analysis.

2.2.8. Mitochondrial membrane potential ($\psi_m$)

To determine a change in the mitochondrial membrane potential ($\psi_m$), Rhodamine 123 (R123) and nonyl-acridine orange (NAO) were used. HeLa and U937 cells were seeded and treated as described in Section 2.2.6 and collected as described in Section 2.2.4. Cell pellets were resuspended in 0.5 mL R123 or NAO (5 μg/mL) and left to incubate at 37 °C for 10 min. Cells were then centrifuged at 500 × g for 5 min and the supernatant discarded. Cells were washed twice using 1 mL PBS. The cell pellet was resuspended in 500 μL PBS and analysed by flow cytometry.

2.2.9. Measurement of cytochrome c release

Cytochrome c release from the mitochondria was measured according to the method of Waterhouse and Trapani (2003) with modifications. Briefly, cells were seeded in 24 well culture plates at 1 × 10⁵ and 1 × 10⁶ cells/mL for HeLa and U937, respectively. Cells were treated with C. prostrata and incubated for 24 and 48 h. Following incubation, the cells were harvested and treated with 100 μL digitonin (50 μg/mL in Dulbecco’s PBS with 100 mM KCl) for 5 min on ice (until ~95% of the cells were permeabilised as assessed by trypan blue exclusion). Cells were fixed in 4% v/v paraformaldehyde in DPBS for 20 min at room temperature. The cell pellets were resuspended in 1 mL PBS and incubated in blocking buffer (3% BSA, 0.05% saponin in DPBS) for 1 h. Cells were incubated overnight at 4 °C with 1:100 cytochrome-c (136F3) rabbit mAb in blocking buffer, washed three times and incubated with 1:200 goat anti-rabbit IgG (H + L), mouse/human ads-FITC secondary antibody in blocking buffer for 1 h at room temperature. Cells were then analysed by flow cytometry. An isotype control of rabbit IgG-FITC was also used.

2.2.10. Statistical analysis

IC₅₀ values were calculated using GraphPad Prism Version 4.1 (GraphPad software, San Diego, USA). Flow cytometry was performed using the Beckman Coulter FC500 and a minimum of 10,000 gated events were recorded for each sample unless stated otherwise. Where appropriate, data are expressed as mean ± SD in triplicate or quadruplicate for each experimental point. The statistical differences between treated groups and control groups were determined by two-tailed Student’s t-test, and the significance threshold was set to p < 0.05. Histograms obtained from cell cycle analysis were analysed with MultiCycle software version 4.0 to calculate the percentage cells in each of the cell cycle phases.

3. Results

3.1. Cytotoxicity

CellTiter-Blue assay was performed to determine the cytotoxic effect of an ethanolic extract of C. prostrata on HeLa and U937 cancer cells. The extract proved to be cytotoxic against both HeLa and U937 cells, yielding IC₅₀ values of 100.8 μg/mL and 64.43 μg/mL, respectively. A fixed extract concentration of 125 μg/mL was used to treat both cell lines for further experiments. IC₅₀ values of 12.5 and 8 μM were obtained for cisplatin on HeLa and U937 cells, respectively. Fifty μM of cisplatin was used as a positive control for all further experiments.

PBMCs were isolated from blood donated by a healthy individual to test the cytotoxicity of an ethanolic extract of C. prostrata against normal cells. After a 48 hour exposure of PBMCs to cisplatin, an IC₅₀ of 50 μM was obtained. The percentage of viable cells after 24 h of exposure to 125 μg/mL extract, a concentration similar to that of the IC₅₀ value obtained against HeLa cells, was 106.56 ± 4.92%. When PBMCs were exposed to 500 μg/mL extract for 24 h, a percentage cell viability of 101.57 ± 7.40% was obtained. Only after 48 h of exposure to 500 μg/mL extract was there a decrease of approximately 50% in the number of viable PBMCs (data not shown).

3.2. Cell cycle analysis

Cell cycle analysis indicates that HeLa cells treated with the ethanolic extract of C. prostrata arrest in the G1/G0 phase of the cell cycle after 24 and 48 h of exposure (Table 1). U937 cells responded more slowly and the same phase arrest as for HeLa cells was evident after 48 h of exposure. Accumulation of cells in the S phase was evident after treatment with cisplatin.

3.3. Phosphatidyserine translocation

Dual staining with Annexin V–FITC and PI was used to establish the integrity of the cell membrane. After 24 h of exposure to C. prostrata, a significant increase in the percentage of apoptotic cells from 4.9% in the vehicle control to 48.1% was evident. An increase after treatment with cisplatin was also evident (Fig. 1).

3.4. Measurement of intracellular p21 levels

Levels of the cyclin–CDK inhibitor p21, a gene product of the Cip/Kip family of genes, are increased by p53 which is triggered by DNA damage. p21 halts the cell cycle in the G1 phase by binding to, and inactivating, cyclin–CDK complexes (Flores-Rozas et al., 1994). Following the observation of G1/G0 arrest in C. prostrata treated cells, measurements of p21 levels were performed to see whether G1 arrest was due to an increase in p21 levels. Immune labelling of control and treated cells revealed no significant increase in p21 levels upon C. prostrata treatment for 24 and 48 h (Fig. 2) and thus it is deduced that G1/G0 arrest is not induced by this pathway.

3.5. Measurement of activated caspase 8

Caspase 8 is an initiator caspase and forms an important part of the extrinsic pathway of apoptosis. Activated caspase 8 was measured using immune labelling and flow cytometry. After 24 h of exposure, no significant increase in activated caspase 8 was evident (data not shown).

Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Time (h)</th>
<th>Treatment</th>
<th>% cells in each phase (Ave ± SD; n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G1/G0</td>
</tr>
<tr>
<td>HeLa</td>
<td></td>
<td>Vehicle control</td>
<td>45.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>Cisplatin</td>
<td>14.9 ± 2.9**</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>C. prostrata</td>
<td>88.3 ± 1.2***</td>
</tr>
<tr>
<td>U937</td>
<td></td>
<td>Vehicle control</td>
<td>63.4 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>Cisplatin</td>
<td>3.1 ± 1.8***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. prostrata</td>
<td>80.1 ± 6.2*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>C. prostrata</td>
<td>782.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cisplatin</td>
<td>747.4 ± 0.7**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. prostrata</td>
<td>73.8 ± 0.8*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Cisplatin</td>
<td>579.5 ± 5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. prostrata</td>
<td>437.3 ± 0.3*</td>
</tr>
</tbody>
</table>

Significant differences compared to corresponding vehicle control cell values: *p < 0.05, **p < 0.01, and ***p < 0.001.
shown); however an increase in cleaved/activated caspase 8 after 48 h of exposure to *C. prostrata* in both HeLa and U937 cells is evident (Fig. 3).

### 3.6. Mitochondrial membrane potential ($\Psi_m$)

Mitochondria often play a central role in apoptosis. Measurement of the mitochondrial membrane potential ($\Delta\Psi_m$), using Rhodamine 123 (R123) was done in conjunction with nonyl-acridine orange (NAO). NAO results revealed that there was no fluctuation in the mitochondrial mass (results not shown) and therefore differences in R123 staining could be interpreted as changes in $\Psi_m$. Cells treated with *C. prostrata* appeared to have no change to the state of the mitochondria in both cell lines after 24 and 48 h of exposure as indicated in Fig. 4. In contrast, cisplatin treatment resulted in a time dependent decrease in the mitochondrial membrane potential.

### 3.7. Measurement of cytochrome c release

To confirm the lack of mitochondrial mediated apoptosis a method developed by Waterhouse and Trapani (2003) that utilises digitonin to selectively permeabilise the plasma membrane and keep the mitochondrial membrane intact was used to investigate cytochrome c release, the hallmark of the intrinsic apoptosis pathway. Selective membrane permeabilisation after treatment of the cells allows cytoplasmic cytochrome-c to leak out of the cells before fixation. Mitochondrial membrane permeabilisation with saponin after fixation provides

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Fig. 1. Dot plots showing Annexin V–FITC stained U937 cells after 24 h (A, B and C) exposure to 0.25% DMSO (A), 50 μM cisplatin (B) and 125 μg/ml C. prostrata (C). Four quadrants represent unstained/live cells (A3: Annexin V—negative; PI—negative), early apoptotic cells (A4: Annexin V—positive; PI—negative), late apoptotic cells (A2: Annexin V—positive; PI—positive) and necrotic cells (A1: Annexin V—negative; PI—positive). A minimum of 20,000 events were recorded for each sample.

Fig. 2. Histogram overlays of p21 immunostaining after treatment with *C. prostrata* (…), cisplatin (---) and vehicle control (___) for A; HeLa (24 h), B; U937 (24 h), C; HeLa (48 h) and D; U937 (48 h). One representative of 3 individual experiments. A minimum of 10,000 events were recorded for each sample.
entry for the cytochrome-c antibody into the mitochondrial membrane for immune staining and quantification of the amount of cytochrome-c remaining in the mitochondria. The release of cytochrome-c was not evident for both HeLa (data not shown) and U937 cells (Fig. 5Ba and E) after treatment with *C. prostrata* for 24 and 48 h, confirming the maintenance of the mitochondrial function. Treatment with cisplatin revealed a significant increase in cytochrome-c release which corroborates with the decreased mitochondrial membrane potential.

4. Discussion

Plants have been a prime source of highly effective cancer drugs. Active compounds isolated from plants have led to the development of anti-cancer drugs (Cragg and Newman, 2005). *C. prostrata* grows naturally in Nigeria as well as in other countries such as China (Zheng and Xing, 2009), Congo (Mbatchi et al., 2006), Ivory Coast (Kamanzi Atindehou et al., 2004) and Australia (Burkill, 1995). It has been reported that a methanolic extract of *C. prostrata* exhibits anti-inflammatory and analgesic activities (Ibrahim et al., 2012) and antibacterial activity has also been reported (Oladimeji et al., 2005; Unni et al., 2009). *C. officinalis* is used in China as an herbal medicine for the treatment of cancer and five compounds isolated from this plant have shown to exhibit anti-cancer properties (Rong et al., 2004). *C. prostrata* was collected and included in a cytotoxicity screening experiment of Nigerian plant extracts by Sowemimo et al. (2009). *C. prostrata* showed to have cytotoxic effects against HeLa cells at a concentration of 250 μg/mL. A recent study by Priya et al. (2013) reported on the anticancer activity of a methanolic extract of *C. prostrata* leaves against Daltons ascites in Swiss albino mice. It was shown that treatment with this extract caused DNA fragmentation in the cancer cells, which is indicative of apoptosis induction. Thus the mode of reported cytotoxicity was investigated in the present study.

Dose–response assays were conducted on both adherent HeLa and suspension U937 cancer cell lines. Gertsch (2009) questioned the value of extracts being used at high concentrations of >200 μg/mL as such concentrations will likely display artificial results in in vitro assays. Previous studies have indicated that *C. prostrata* may possess potential anti-cancer properties, however the cytotoxic effect was only determined at a single concentration of 250 μg/mL (Sowemimo et al., 2009). A more comprehensive characterisation of the cytotoxicity yielded IC₅₀ values of 100.8 μg/mL and 64.4 μg/mL against HeLa and U937 cells, respectively, and thus may be considered to be biologically relevant. Furthermore, using peripheral blood mononuclear cells (PBMCs) as a model for cells that do not divide frequently in culture, the specificity of *C. prostrata* as an anti-proliferatory/cytotoxic agent could be established. PBMCs were relatively insensitive to the toxic effects of *C. prostrata* as judged from the IC₅₀ value of >500 μg/mL after 48 h of exposure.

DNA cell cycle analysis was performed to determine which phase of the cell cycle arrests in, if at all (Table 1). After 24 h of exposure to *C. prostrata*, an arrest in the G0/G1 phase of the cell cycle was evident for HeLa cells relative to the vehicle control. The same was evident after 48 h of exposure for U937 cells. Upon treatment of cells with cisplatin, cells arrest in the S phase of the cell cycle as would be expected from the known mechanism of DNA damage. The mechanism of G0/G1 arrest cannot be deduced by PI staining of DNA. In the G1 phase, DNA damage activates p53, which in turn, activates the expression of p21, a cyclin dependent kinase (Cdk) inhibitor. In the presence of p21, cyclin D is destabilised and this leads to redistribution of p21 from Cdk4 to Cdk2 and this also contributes to an arrest in G1 (Cheng et al., 1999; Huang et al., 2005). Due to the evident arrest of cells in G0/G1, p21 levels were investigated using flow cytometry. Histogram overlays in Fig. 2 show that no increase in p21 levels was evident after 24 and 48 h of exposure to *C. prostrata* when comparing the result to the vehicle control cells. Thus, it is deduced that G0/G1 arrest is not caused by an increase in p21 levels and it can therefore be assumed.
that *C. prostrata* extract does not induce DNA damage. In contrast to *C. prostrata*, cisplatin treatment, which is known to induce DNA damage, increased the levels of p21, however the effect was more pronounced in HeLa cells than in U937 cells.

During apoptosis phosphatidylserine is translocated from the inner surface of the plasma membrane to the outer surface. Annexin-V has a high affinity for the negatively charged PS and thus it is used to determine the presence of PS on the outer surface of the cell (Van Engeland et al., 1998). U937 cells were treated with *C. prostrata* and cisplatin separately and analysed after 24 h of exposure. Fig. 1 shows the result of this assay. After 24 h of treatment, an increase in the percentage of apoptotic cells was evident through increased Annexin-V staining. No increase in PI positive cells was observed in this study, thus indicating that necrotic cell death is unlikely and cell death is caused by the onset of apoptosis as a result of exposure to *C. prostrata*. PS translocation analysis was not conducted on HeLa cells as trypsin damages the cell membrane, which is used to detach the cells from the culture plates. This damage leads to PS translocation and will therefore result in false positive data (Van Engeland et al., 1996).

Cellular changes during apoptosis occur after a cascade of cell signalling and caspase-mediated events triggered by one of or both apoptotic pathways, namely the death-receptor induced extrinsic pathway or the mitochondria–apoptosome intrinsic pathway. Both pathways lead to caspase activation (Hu and Kavanagh, 2003). Caspases are divided into 2 groups, the initiator and the executioner caspases. Caspase 8 is an initiator caspase that, once cleaved and activated, initiates apoptosis via a mitochondria-dependent or -independent pathway. In the mitochondria-dependent pathway, caspase 8 will activate the executioner caspase, i.e. caspase 3, without exerting an effect in the mitochondria. The mitochondria-dependent pathway induces apoptosis by depolarisation of the mitochondrial membrane and the release of pro-apoptotic proteins and cytochrome-c into the cytosol of the cells (Fulda and Debatin, 2006). The activation of caspase 8 after exposure to *C. prostrata* was investigated using immunocytochemistry and flow Fig. 4.
cytometry. After 48 h of treatment with the plant extract, an increase in activated caspase 8 was evident in both cell lines (Fig. 3). This suggested that the cells respond to the plant treatment via the death-receptor extrinsic apoptotic pathway.

Although activation of caspase 8 is evident in both cell lines upon treatment with C. prostrata which suggests the onset of a death-receptor mediated mechanism, mitochondrial depolarisation and cytochrome c release were investigated to confirm the absence of an intrinsic apoptosis pathway which is dependent in the mitochondrial involvement. Figs. 4A, D, G and J, R123 accumulated in the control cells of the mitochondria, yielding high fluorescence intensities as is evident from the peak. Cells with lower membrane potential were detected in the region marked “C” of each histogram. A decrease in \(\Psi_m\) will therefore be noted by any increase in the percentage cells in region “C” and compared to the vehicle control of DMSO. Cells treated with C. prostrata showed to have no significant increase in percentage cells in region “C”, i.e. depolarisation of the membrane is not evident after 24 and 48 h of treatment in both cell lines (Fig. 4C, F, I and L). The positive control, cisplatin, showed to have a significant effect in the mitochondrial membrane in accordance to the activation of the intrinsic apoptotic pathway. Depolarisation of the membrane is clearly evident by the increase in the percentage of cells in region “C” of the histograms (Fig. 4B, E, H and K). Pro-apoptotic proteins are normally released from the mitochondria if depolarisation of the membrane occurs. One such protein is cytochrome c. Because depolarisation was not evident after treatment with C. prostrata, no change in cytochrome c levels was expected (Fig. 5B and E). Cisplatin showed to have a decrease in cytochrome c associated with the mitochondrial membrane (Fig. 5C and F), as expected due to depolarisation of the mitochondrial membrane after treatment.

Taken together our results, for the first time, argue in favour of a cytotoxic mechanism involving the activation of the extrinsic pathway of apoptosis in HeLa and U937 cells treated with an ethanol extract of C. prostrata. The activation of caspase 8 and an increased translocation of PS to the cell surface without any significant PI staining indicate that the cytotoxicity involves apoptosis. Furthermore, the absence of the mitochondrial involvement in the cytotoxic mechanism suggests that C. prostrata causes apoptosis by activating the death-receptor extrinsic pathway only. Generally, plant extracts that induce apoptosis do so with involvement of both extrinsic and intrinsic pathways, making this a novel finding that requires further investigation.

References


