



## Caspase-dependent apoptosis is induced by *Artemisia afra* Jacq. ex Willd in a mitochondria-dependent manner after G2/M arrest

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### ABSTRACT

*Artemisia afra* is one of the oldest, most well known and widely used traditional medicinal plants in South Africa. It is used to treat many different medical conditions, particularly respiratory and inflammatory ailments (Liu et al., 2009). There is no reported evidence of its use for the treatment of cancer but due to its reported cytotoxicity (Fouche et al., 2008; Mativandlela et al., 2008), we investigated the mode of cell death induced by an ethanolic *A. afra* extract by using two cancer cell lines. IC<sub>50</sub> values of 18.21 and 31.88 µg/mL of ethanol extracts were determined against U937 and HeLa cancer cells, respectively. An IC<sub>50</sub> value of the aqueous extract was greater than 250 µg/mL. The effect of the cytotoxic ethanolic *A. afra* extract on U937 and HeLa cells and their progression through the cell cycle, apoptosis and mitochondrial membrane potential were investigated. Melphalan was used as a positive control. After 12 h of treatment with *A. afra* a delay in G2/M phase of the cell cycle was evident. Apoptosis was confirmed by using the TUNEL assay for DNA fragmentation, as well as fluorescent staining with annexin V-FITC. Apoptosis was evident with the positive control and *A. afra* treatment at 24 and 48 h. JC-1 staining showed a decrease in mitochondrial membrane potential at 24 h. The results obtained suggest that *A. afra* potentially has medicinal anticancer properties.

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### 1. Introduction

A large percentage of the African population depends on medicinal plants for health care. Africa is one of the continents with the richest biodiversity in the world, however little effort has been devoted to development of chemotherapeutic agents from medicinal plants. It is estimated that only 15% of the world's plants have been screened for their therapeutic values (Louw et al., 2002; McGaw and Eloff, 2008).

The World Health Organization (WHO) has recognized that 80% of the African population makes use of traditional medicine (Gurib-Fakim, 2006) and in sub-Saharan Africa, the ratio of traditional healers to the rest of the population is 1:500, compared to the ratio of medical doctors to the rest of the population, which is 1:40,000 (Abdool Karim et al., 1994). Thus, it is clear that traditional healers play an important role in the lives of African people and have the potential to serve as crucial components of a comprehensive health care strategy.

Cancer is a class of disease in which a group of cells grow and divide uncontrollably and do not die. Normal cells in the body follow an orderly pattern of cell division, growth and death, known as the

cell cycle. The event of programmed cell death, known as apoptosis, does not occur in cancer cells and thus the cells continue to divide and grow and cancer begins to form (Schafer, 1998).

Apoptosis is initiated by extracellular or intracellular signals in which a complex machinery is activated to start a cascade of events ultimately leading to the degradation of nuclear DNA and dismantling of the cell. The event of cell suicide needs to be regulated as too little apoptosis may result in cancer, autoimmune or chronic inflammatory diseases; too much apoptosis may lead to stroke-induced neuronal damage and neurodegenerative disorders (Zangemeister-Wittke and Simon, 2001).

Apoptosis is characterized by morphological changes such as membrane blebbing, cytoplasmic and chromatin condensation as well as apoptotic body formation. It is also characterized by biochemical changes including cysteine-dependent aspartate-directed proteases (caspase) activation, DNA fragmentation and phosphatidyl serine translocation (Elmore, 2007). Caspases are responsible for the proteolytic cleavage of cellular proteins leading to the characteristic apoptotic features (Vermeulen et al., 2005).

Plants have played an important role as a source of anticancer agents and it is reported that over 60% of anticancer agents are derived from natural products such as plants, marine organisms and microorganisms (Cragg and Newman, 2005). *Artemisia afra* is one of the oldest, most well known and widely used traditional medicinal plants in South Africa. It is commonly referred to as the African wormwood and it is used to

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treat many different medical conditions, particularly respiratory and inflammatory ailments. The plant is prepared in many different ways, traditionally, for specific uses. An infusion of leaves and roots are used for the treatment of diabetes in the Eastern Cape region of South Africa. The leaves are boiled and the vapors are inhaled to treat respiratory conditions. For the treatment of colic, a tincture of leaves wetted with brandy is used. Leaves are also added to boiling water and allowed to draw for 10 min and this infusion is strained and drunk for relief of flu type conditions (Liu et al., 2009). Mativandela et al. (2008) showed cytotoxicity of *A. afra* in Vero cells and Fouche et al. (2008) reported that a dichloromethane:methanol (1:1) extract of *A. afra* induced cytotoxicity against renal and breast cancer cells as well as melanoma cells. Thus we investigated the mode of cell death caused by an ethanolic extract of *A. afra* on HeLa cervical cancer cells and a promonocytic leukemia cell line, U937.

## 2. Materials and methods

### 2.1. Materials, chemicals and reagents

Cervical (HeLa) cancer and human promonocytic leukemia U937 cells were purchased from Highveld Biological, South Africa. Cleaved caspase-3 (Asp175) and caspase 8 (Asp391) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The Coulter® DNA Prep™ reagents kit, goat anti-rabbit IgG (H + L chain specific) and rabbit IgG isotype, both labeled with fluorescein (FITC) conjugate and IsoFlow™ EPICS™ sheath fluid, were purchased from Beckman Coulter (CA, USA). MEBSTAIN apoptosis kit direct and IntraPrep™ permeabilizing reagent were purchased from Immunotech (Marseille, France). Annexin V-FITC/PI kit was purchased from MACS Miltenyi Biotec (Auburn, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1), trypan blue and melphalan were purchased from Sigma (St. Louis, MO, USA). CellTiter-Blue® reagent was purchased from Promega (Madison, WI, USA). RPMI 1640 cell culture medium containing 25 mM Hepes, 2 mM glutamine and fetal bovine serum was purchased from ThermoScientific (Logan, Utah, USA).

### 2.2. Methods

#### 2.2.1. Extraction

Herbarium specimen voucher number *A. afra* 15487 was deposited in the Nelson Mandela Metropolitan University Herbarium, Botany Department, South Campus. Leaves of the plant were dried at 80 °C and thereafter ground by using a blender. Aqueous and ethanolic extracts were prepared by submerging the ground leaves in deionized water and 80% ethanol, respectively. The submerged leaves were incubated in the dark at room temperature overnight. After incubation, the liquid was filtered through Whatman filter paper (1.1 µm). The aqueous extract was freeze dried and the ethanol extract concentrated to dryness by evaporating ethanol by using a rotary evaporator (Buchi) and further freeze dried. The extracts were stored separately in a desiccator at 4 °C in the dark.

#### 2.2.2. Cell culture conditions

The adherent cancer cell line, HeLa cells, and the suspension U937 cells were used for the experimental procedures. The cells were routinely maintained in 10-cm culture dishes and flasks, respectively, without antibiotics in RPMI 1640 cell culture medium containing 25 mM Hepes, 2 mM glutamine, supplemented with 10% fetal bovine serum and incubated in a humidified 5% CO<sub>2</sub> incubator at 37 °C. Trypan blue was used to determine cell viability and cell number. Counting of cells was done by using an improved Neubauer hemocytometer and cell densities (number of cells per mL) were altered according to the number of cells required for each experiment.

#### 2.2.3. Cytotoxicity

HeLa and U937 cells were seeded in 200 µL and 100 µL aliquots, respectively, at  $3 \times 10^4$  cells/mL in 96 well plates and HeLa cells were left overnight to attach. U937 cell were also incubated overnight at 37 °C before treatment. The medium was replaced with fresh medium containing varying concentrations (1.25–250 µg/mL) of aqueous and ethanolic extracts. One hundred microliter aliquots of fresh medium containing double the appropriate extract concentration was added to the respective wells for treatment of U937 cells. Both cell lines were incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 48 h. The medium containing *A. afra* extract was removed prior to addition of MTT to HeLa cell. Cytotoxicity assays were performed by using CellTiter-Blue® and MTT (5 mg/mL) for U937 and HeLa respectively. Melphalan (40 µM) was used as a positive control for all experiments. Absorbance was read at 540 nm by using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) for HeLa cells and fluorescence was read at 544<sub>Ex</sub>/590<sub>Em</sub> by using a Fluoroskan Ascent FL fluorometer (ThermoLabsystems, Finland) for U937 cells.

#### 2.2.4. Cell cycle analysis

U937 cells were seeded at  $1 \times 10^5$  cells/mL in culture flasks and HeLa cells were seeded at  $5 \times 10^4$  cells/mL in 10 mL aliquots in 10 cm culture dishes and treated with 20 µg/mL and 30 µg/mL of ethanolic *A. afra* extract, respectively. Cells were incubated for 12, 24 and 48 h. After the appropriate incubation period, HeLa cells were trypsinized for 10 min, resuspended in phosphate buffered saline (PBS) and transferred to polypropylene tubes. U937 cells did not require trypsinization. The Coulter® DNA Prep™ reagents kit was used for DNA cell cycle analysis, as per manufacturer's instructions. Briefly, 100 µL lysis reagent was added to each tube and incubated for 5 min at room temperature. Thereafter 500 µL propidium iodide (50 µg/mL) was added and tubes were incubated for 15 min at 37 °C. Flow cytometric analysis was performed directly after incubation. A Beckman Coulter Cytomics FC500 was used for all flow cytometry analysis.

#### 2.2.5. Phosphatidylserine translocation

U937 cells were seeded at  $1 \times 10^5$  cells/mL in culture flasks and HeLa cells were seeded at  $5 \times 10^4$  cells/mL in 6 well culture plates, by using 3 mL per well, and treated with 20 µg/mL and 30 µg/mL of ethanolic *A. afra* extract, respectively. Cells were incubated for 24 h at 37 °C. After treatment and incubation, HeLa cells were washed with ice-cold Dulbecco's Modified Eagle's Medium (DMEM). Accutase was used to detach the HeLa cells from the culture plate. Cells were incubated for 10 min with accutase and then resuspended in PBS. Cells were transferred to polypropylene tubes and centrifuged at 500 ×g for 5 min at room temperature and washed with PBS to remove accutase. U937 cells did not require the detachment step. Cells were then stained according to Annexin V-FITC/PI kit protocol (MACS Miltenyi Biotec). In brief, after centrifugation, the supernatant was discarded and cell pellets resuspended in ice-cold 1 × binding buffer. Annexin V-FITC (1 µL; 25 µg/mL) and PI (5 µL; 250 µg/mL) were added to each tube. Compensation control tubes contained cells with Annexin V-FITC only, PI only and a combination of Annexin V-FITC and PI. Tubes were gently mixed and incubated on ice for 15 min in the dark. Samples were read within 30 min on a Beckman Coulter Cytomics FC500.

#### 2.2.6. Mitochondrial membrane potential (MMP) analysis

U937 and HeLa cells were seeded and treated as described for phosphatidylserine translocation analysis. Cells were incubated for 24 and 48 h. After the incubation periods, cells were transferred to polypropylene tubes and cells collected by centrifuging at 500 ×g for 5 min at room temperature. After treatment and incubation, HeLa cells were washed with Ca<sup>2+</sup> and Mg<sup>2+</sup> free PBS, trypsinized for 10 min and resuspended in PBS. Cells were centrifuged at 500 ×g for 5 min at room temperature and washed with PBS to remove trypsin. U937 cells

did not require the trypsinization step. Thereafter, a lipophilic cation dye, JC-1, was added to a final concentration of 2  $\mu\text{g}/\text{mL}$ . JC-1 was used to determine a change in the mitochondrial membrane potential. Cells were incubated for 10 min at room temperature in the dark. The cells were washed by using 500  $\mu\text{L}$  PBS and centrifuged at 500  $\times g$  for 5 min. The wash step was repeated three times before the flow cytometric analysis.

### 2.2.7. Caspase 8 and 3 activation

Cells were seeded and treated as described for MMP analysis. Cells were fixed and permeabilized by using the IntraPrep kit (Beckman Coulter). Cleaved caspase 8 (Asp 391) and cleaved caspase 3 (Asp 175) monoclonal antibodies (Cell Signaling) were used to determine the activation of caspase 8 and caspase 3 respectively. Cells were first blocked by using PBS containing 0.5% BSA and thereafter incubated with the antibodies separately (1:100 for caspase 8 and 1:50 for caspase 3) for 1 h at 37 °C. Cells were washed and incubated with the conjugated secondary antibody (1:1000) for 30 min at 37 °C in the dark. This step was not required for caspase 3 analysis as the antibody contains a conjugated FITC fluorophore. Cells were then analyzed by using flow cytometry.

### 2.2.8. DNA fragmentation

Cells were seeded, treated, fixed and permeabilized as described for caspase analysis. The MEBSTAIN apoptosis kit (Immunotech) was then used as per the manufacturer's instructions to determine the effect of *A. afra* on DNA fragmentation.

### 2.2.9. Statistical analysis

$\text{IC}_{50}$  values of *A. afra* extracts were determined by using the GraphPad Prism Version 4.0 (GraphPad Software, San Diego, USA). Statistical significance was determined by using the two-tailed Student's *t*-test and  $p < 0.05$  was considered significant. For flow cytometry, a minimum of 10,000 events were recorded for each sample. Cell cycle analysis results were analyzed by using a Multicycle version 4.0 software.

## 3. Results

### 3.1. Cytotoxicity

The cytotoxic effects of the aqueous and ethanol *A. afra* extracts were determined by using the MTT assay and Cell Titre Blue assay for HeLa and U937 cancer cells respectively. The aqueous extract yielded  $\text{IC}_{50}$  values of greater than 250  $\mu\text{g}/\text{mL}$  and thus was considered non-toxic (results not shown). Cytotoxicity was also determined by using confluent Chang liver cells and an  $\text{IC}_{50}$  value greater than 250  $\mu\text{g}/\text{mL}$  was achieved for both extracts. The  $\text{IC}_{50}$  values obtained for the ethanolic extract against HeLa and U937 cancer cell lines were  $31.88 \pm 1.09 \mu\text{g}/\text{mL}$  and  $18.21 \pm 0.9 \mu\text{g}/\text{mL}$ , respectively (Fig. 1). From these results, the concentration of the ethanolic extract to be used for further experiments was fixed at 30  $\mu\text{g}/\text{mL}$  for HeLa and 20  $\mu\text{g}/\text{mL}$  for U937 cells.

### 3.2. Cell cycle analysis

DNA cell cycle analysis was performed on HeLa and U937 cancer cells after 12, 24 and 48 h of exposure to *A. afra* ethanol extract. After 12 h of exposure, an arrest of the cell cycle in the G2/M phase was noticed in HeLa cells (Fig. 2C) and U937 cancer cells (Fig. 2F). This was still evident after 24 and 48 h (data not shown).

### 3.3. Phosphatidylserine translocation

To determine the effect of *A. afra* exposure on the integrity of the cell membrane, Annexin-V FITC and PI staining was performed. HeLa and U937 cells were stained positive for Annexin-V and negative for

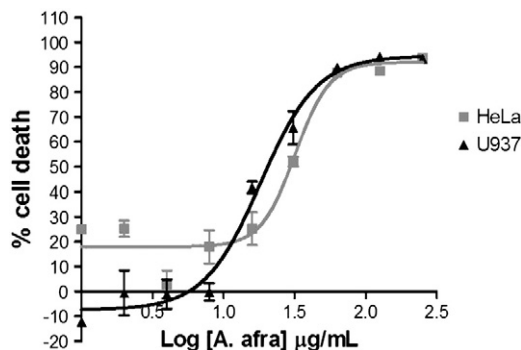


Fig. 1. Cytotoxic effect of *Artemisia afra* ethanol extract after 48 h of exposure. Cell viability was determined by using the MTT assay for HeLa and CellTitre Blue assay for U937 cells. Error bars indicate SD of four replicate values.

PI after 24 h of exposure (Fig. 3A–C and 5D–F, respectively). A significant increase in the percentage of cells undergoing apoptosis was evident after 24 h of exposure to *A. afra* ethanol extract in both HeLa and U937 cancer cells. An increase from  $5.83 \pm 1.33\%$  to  $37.53 \pm 2.68\%$  ( $p < 0.005$ ) of apoptotic cells was recorded in HeLa cells and an increase from  $4.73 \pm 0.38\%$  to  $15.23 \pm 0.57\%$  ( $p < 0.005$ ) was recorded in U937 cancer cells.

### 3.4. Mitochondrial membrane potential analysis

To determine the involvement of the mitochondria in the cell death pathway induced by *A. afra*, the mitochondrial potential was measured by using a lipophilic cationic dye, JC-1. This dye reversibly changes color from green to orange as the membrane potential increases. This property is due to the reversible formation of JC-1 aggregates upon membrane polarization. An increase in the mean green fluorescence intensity indicates a decrease in the mitochondrial membrane potential, suggesting that the mitochondria are involved in the onset of apoptosis (Table 1). Depolarization of the mitochondrial membrane is often associated with apoptosis by the release of pro-apoptotic proteins, such as cytochrome c, from the mitochondria and the formation of a pro-apoptotic complex (Adams and Cory, 1998; Vermeulen et al., 2005).

An increase in the mean green fluorescence intensity is noticed after 24 and 48 h of exposure to *A. afra*, thus the depolarization of the mitochondrial membrane. This suggests that the mitochondria are involved in the onset of apoptosis.

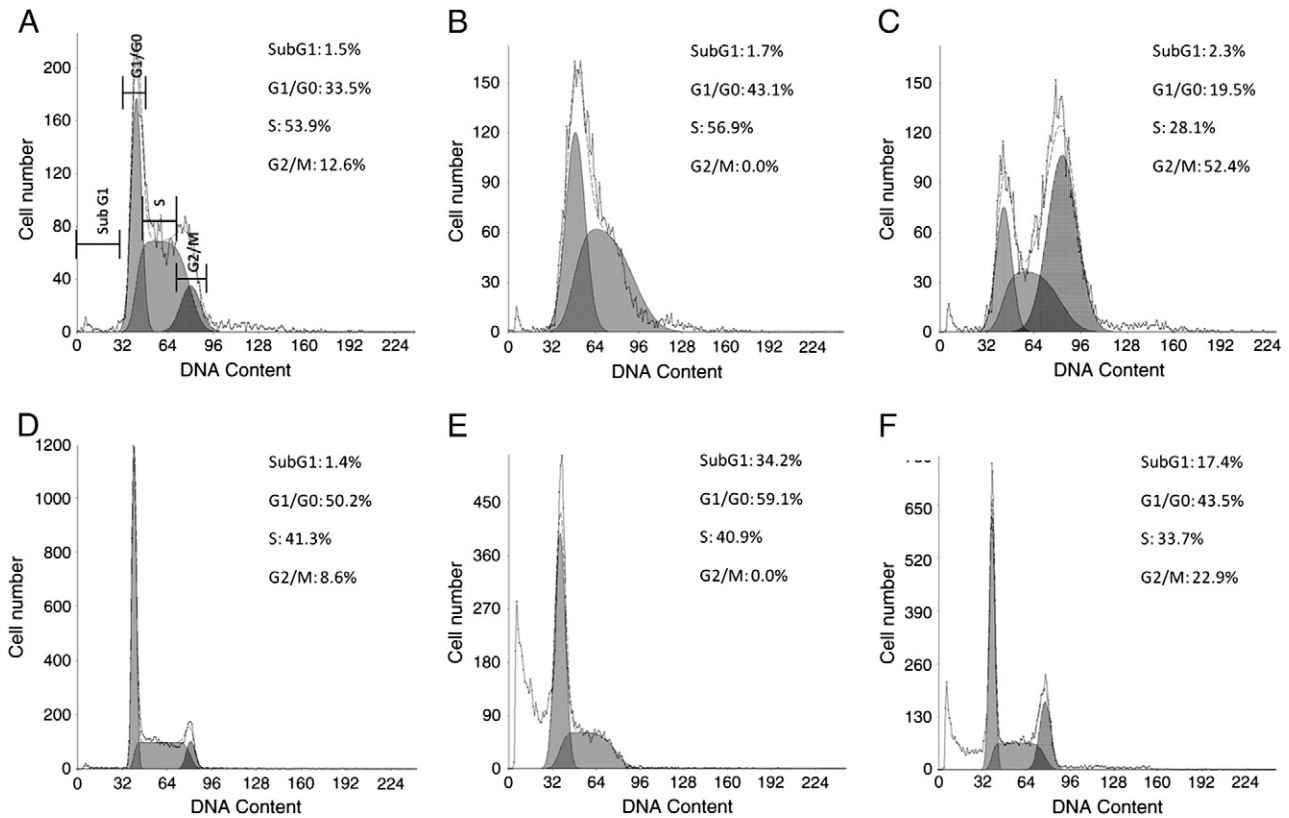
### 3.5. Caspase 8 and 3 activation

The caspase cascade plays a central role in the execution of apoptosis. Caspase 8 is an initiator caspase which forms part of the death inducing signaling complex (DISC) once a death signal is received by the cell. The formation of this complex allows for the autocatalytic cleavage of procaspases into active caspases. Activated caspases allow for the activation of the effector caspases, including caspase 3. Caspase 3 is known to be the main executioner of apoptosis by facilitating DNA fragmentation (Thornberry and Lazebnik, 1998; Vermeulen et al., 2005; Zangemeister-Wittke and Simon, 2001).

An increase in the log mean fluorescence value when compared to the untreated cell sample (control) indicates the presence of cleaved, or activated, caspase 8 or -3. A significant increase in both activated caspase 8 and -3 in HeLa and U937 (data not shown) cells was noticed after 24 h and 48 h of exposure to *A. afra*, respectively (Table 2).

### 3.6. DNA fragmentation

DNA fragmentation is characteristic of late apoptosis and was investigated in HeLa and U937 cancer cells (Fig. 4). Significant increases in



**Fig. 2.** Histograms representing DNA cell cycle analysis after 12 h of treatment in HeLa cells (A–C) and U937 cells (D–F). HeLa cells were treated with 40  $\mu$ M melphalan (B) and 30  $\mu$ g/mL *Artemisia afra* (C). U937 cells were treated with 40  $\mu$ M melphalan (E) and 20  $\mu$ g/mL *A. afra* (F). Control cell populations are represented by histograms A and D for HeLa and U937 cells respectively. Cell cycle analysis was done on a Beckman Coulter Cytomics FC500 after PI staining of DNA. Ten thousand events were recorded for each sample.

the amount of fragmented DNA were evident after 24 and 48 h in both cell lines.

#### 4. Discussion

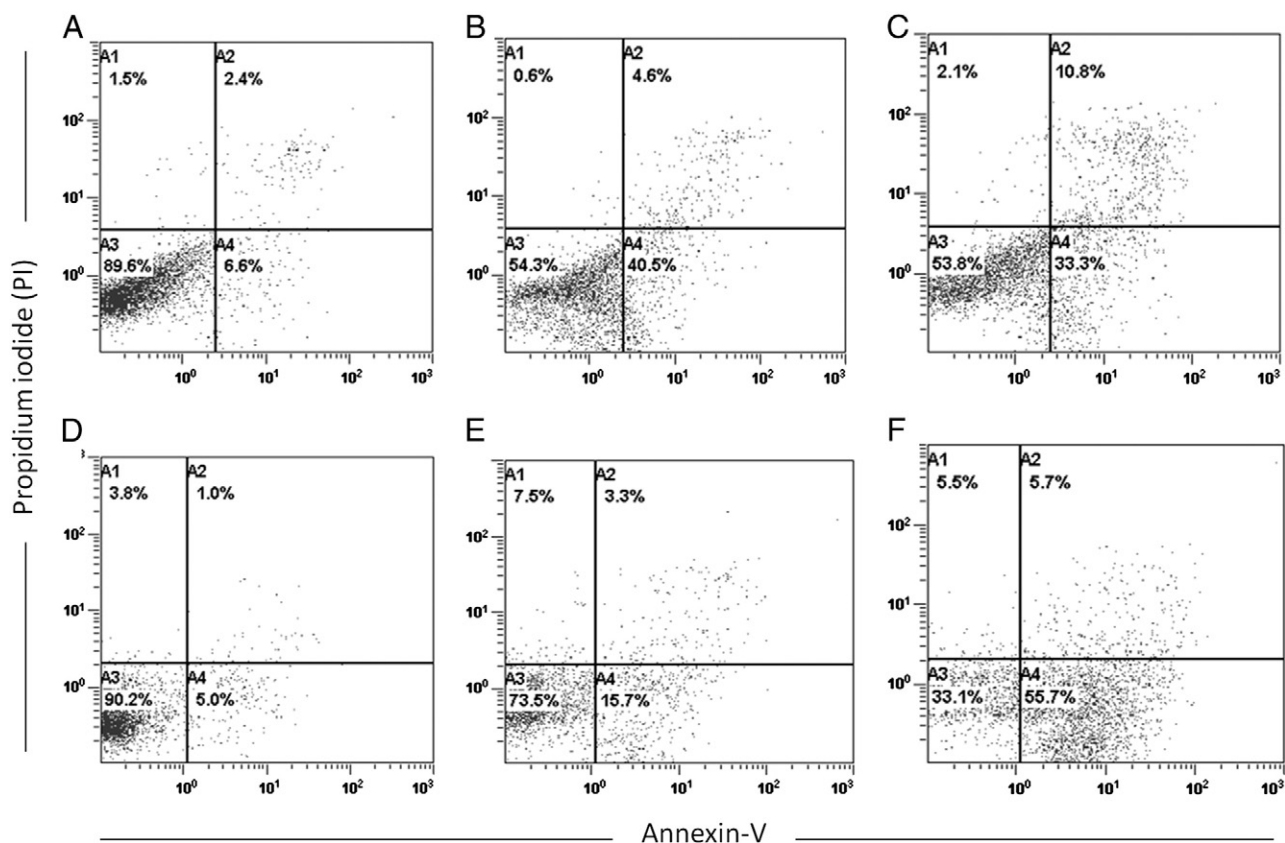
*A. afra* is known to be used to treat many different ailments and possesses antiviral, antibacterial and anti-inflammatory activities (Liu et al., 2009). Many different preparations of the plant including infusions, decoctions, brewing a tea and preparing a tincture by using the leaves and brandy are used to treat various symptoms. There is no reported evidence of its use for the treatment of cancer, but there has been reported evidence of its cytotoxicity (Fouche et al., 2008; Mativandlela et al., 2008).

Dose–response assays were performed by using the MTT assay for HeLa cells and the CellTitre Blue assay for U937 cells. An  $IC_{50}$  value of  $113.0 \pm 2.05$   $\mu$ g/mL against Vero cells was previously reported (Mativandlela et al., 2008). Vero cells are a continuous and non-tumor cell line and are used as a model for non-cancerous cells. The  $IC_{50}$  values achieved for both U937 and HeLa cells are promising as they are 3 to 5 times lower than that of the reported  $IC_{50}$  value obtained by using Vero cells for the ethanolic extract of *A. afra*. The  $IC_{50}$  value reported here for the Chang liver cells is  $>250$   $\mu$ g/mL, indicating that the ethanol extract is not toxic to this non-tumor cell line. Gertsch (2009) stated that extracts used at concentrations  $>200$   $\mu$ g/mL are likely to display artificial results in in vitro assays, despite their being reproducible and questions whether extracts used at concentrations  $>50$   $\mu$ g/mL are physiologically meaningful. Here, we achieved  $IC_{50}$  values below 50  $\mu$ g/mL and displayed reproducible bioassay results.

A DNA cell cycle analysis was performed in order to determine if and which phase of the cell cycle the cells arrest in. Fig. 2C shows a cell cycle arrest in the G2/M phase of HeLa and U937 cells after 12 h of exposure

to *A. afra*. Upon treatment of the cells with melphalan (40  $\mu$ M), cells arrest in the S phase of the cell cycle (Fig. 2B). The mechanism of this G2/M arrest cannot be deduced from the PI cell cycle analysis and more than one possibility exists. The phosphatases Cdc25B and Cdc25C are regulators of the progression of the cell cycle from the G2 phase through the M phase. These proteins regulate the progression by its activity on Cdc2/cyclinA and Cdc2/cyclinB complexes (Busino et al., 2004). DNA damage causes G2/M arrest by the inhibition of the activation of Cdc2 (Hwang and Muschell, 1998). Active Cdc2 complexed to cyclin B1 is required for the progression from the G2 phase to the M phase of the cell cycle. Cdc25C dephosphorylates the active site of Wee1, which increases the activity of Cdc2. When DNA damage occurs, Chk1 is activated and deactivates Cdc25C. This results in the phosphorylation and inactivity of Cdc2/cyclinB complex and the cell arrests in the G2/M phase. G2/M arrest can also be associated with problems in the formation of the mitotic spindle resulting in mitotic catastrophe. G2/M arrest is an early event as it is apparent after 12 h of exposure to *A. afra*. Other apoptotic markers investigated here were evident later. This suggests that G2/M arrest may be the primary event occurring upon treatment with *A. afra* and apoptosis results in response to the mitotic catastrophe (Vakifahmetoglu et al., 2008).

The negatively charged phospholipid phosphatidylserine (PS) is located on the inner side of the plasma membrane lipid bilayer in most normal cells. During apoptosis, PS is translocated from the inner surface to the outer surface of the plasma membrane. Annexin V has a high affinity for PS and thus is used to determine the presence of PS on the surface of the cells. After 24 h of treatment with *A. afra*, a significant increase in the percentage of cells stained positive for Annexin-V was evident, confirming the occurrence of apoptosis. Propidium iodide was also used in the staining procedure to detect necrotic and/or late apoptotic cells (the latter also referred to as



**Fig. 3.** Dot plots of Annexin V-FITC stained HeLa cells (A, B and C) and U937 cells (D, E and F) after 24 h exposure to medium only (A, D), 30 µg/mL and 20 µg/mL *Artemisia afra* (B, E; respectively) and 40 µM melphalan (C, F). 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 read ( $n = 3$ ).

“secondary necrosis”). During early apoptosis the plasma membrane is still intact, but becomes leaky with the onset of late apoptosis or necrosis and an increase in PI positive cells will be evident (Koopman et al., 1994). No increase was observed in PI positive cells in this study, confirming that cell death was not due to necrosis.

In the intrinsic pathway of apoptosis, caspase activation is closely linked to the permeabilization of the outer mitochondrial membrane by pro-apoptotic proteins. Cytotoxic stimuli may induce outer membrane permeabilization resulting in depolarization of the mitochondria. A set of proteins normally found between the inner and outer membranes is released including cytochrome *c*, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G. Once released in the cytosol, these proteins promote caspase activation or may act as caspase-independent death effectors (Saelens et al., 2004). As mentioned, the lipophilic dye, JC-1 was used to determine the state of the mitochondrial membrane after the treatment of cancer cells with *A. afra* extract. A significant increase in the mean green fluorescence intensity was observed, suggesting

the depolarization of the mitochondrial membrane. Thus it is deduced that the intrinsic pathway plays a role in the execution of apoptosis induced by *A. afra*. The intrinsic pathway can be activated in the presence of intracellular signals or in response to the activation of death receptors on the plasma membrane. Caspase 8 activation occurs when the death inducing signaling complex (DISC) is formed in response to an extracellular signal. Thus, the activation of caspase 8 in response to extract treatment would also suggest a cellular response to an external signal for cell death. Hence, activated caspase 8 can be used as a convenient indicator of involvement of the extrinsic death receptor pathway of apoptosis (Fulda and Debatin, 2006).

Caspase activation was determined by using immunocytochemistry. Antibodies against activated caspase 8 and caspase 3 were used. Caspase 8 is an initiator caspase which is first activated through a death signal, suggesting a cellular response to an external signal for cell death.

**Table 1**

Summary of results obtained for analysis of mitochondrial membrane potential after treatment with melphalan (40 µM) and *A. afra* ethanol extract.

Treatment	Mean green fluorescence intensity <sup>a</sup> for HeLa cells	Mean green fluorescence intensity <sup>a</sup> for U937 cells
24 h Control	19.03 ± 3.46	19.25 ± 3.46
Melphalan	41.93 ± 0.21**	85.75 ± 0.21**
<i>A. afra</i>	70.40 ± 0.92**	69.45 ± 0.92**
48 h Control	9.99 ± 2.81	24.93 ± 1.44
Melphalan	105.77 ± 9.62**	257.71 ± 16.40**
<i>A. afra</i>	34.83 ± 0.97**	144.25 ± 4.29**

<sup>a</sup> Mean of green fluorescence intensity. Depolarization is accompanied by an increase in green and a decrease in red fluorescence.

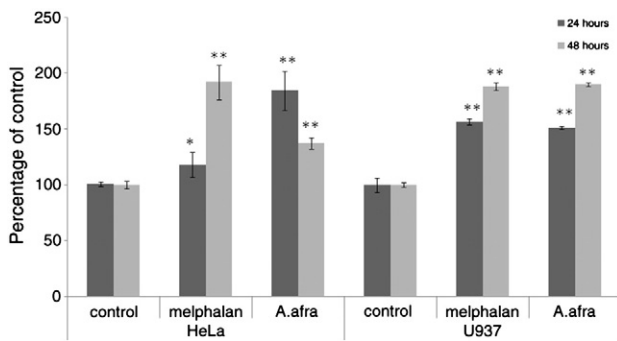
\*\* Significantly higher than control;  $p < 0.005$ : significance was determined by using the two-tailed Student's *t*-test.

**Table 2**

Summary of results obtained for analysis of caspase 8 and caspase 3 activation in HeLa cells after treatment with melphalan (40 µM) and *A. afra*.

Treatment	Percentage (%) of control	
	Cleaved caspase 8	Cleaved caspase 3
12 h Control	100.00 ± 5.324	100.00 ± 7.46
Melphalan	127.79 ± 13.43	92.50 ± 13.72
<i>A. afra</i>	105.36 ± 15.89	115.12 ± 22.99
24 h Control	100.00 ± 4.76	100.00 ± 8.67
Melphalan	157.47 ± 34.25**	161.80 ± 19.51**
<i>A. afra</i>	189.28 ± 44.82**	153.38 ± 19.61**
48 h Control	100.00 ± 5.83	100.00 ± 5.75
Melphalan	288.89 ± 39.49**	331.77 ± 39.30**
<i>A. afra</i>	143.07 ± 23.16*	285.15 ± 48.95**

Significance was determined by using the two-tailed Student's *t*-test: \* $p < 0.05$ ; \*\* $p < 0.005$  compared to control.



**Fig. 4.** DNA fragmentation in HeLa and U937 cells after 24 and 48 h of exposure to treatments. Cells were treated with 40  $\mu$ M melphalan, 20  $\mu$ g/mL *Artemisia afra* ethanolic extract (U937) or 30  $\mu$ g/mL *A. afra* ethanolic extract (HeLa). Bar graph represents the average of two individual experiments each performed in triplicate. 10,000 events were recorded per sample. Significance was determined by using the two-tailed Student's *t*-test: \* $p < 0.05$ ; \*\* $p < 0.005$  compared to control.

Executioner caspases, including caspase 3, are in turn activated by the initiator caspases. The activation of both caspase-8 and caspase-3 is evident for both HeLa (Table 2) and U937 cells although the data for U937 cells are not shown. It is thus deduced that the cell death pathway induced by *A. afra* is caspase dependent and involves both the extrinsic and intrinsic pathways.

One of the later steps in apoptosis is the degradation or fragmentation of DNA by activated endonucleases and/or activated executioner caspases. Caspase-3 and caspase-7 are executioner caspases responsible for the degradation of the chromatin structure into fragments of ~300 kb and then into smaller fragments of ~50 kb. In order to detect the DNA fragments, the TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) method was used. FITC-labeled dUTP DNA fragments were detected by using flow cytometry and a significant increase in the amount of fragmented DNA was noticed after 48 h of exposure to *A. afra* in both HeLa and U937 cell lines confirming the involvement of apoptosis in the cytotoxic mechanism of *A. afra* (Fig. 4).

Taken together our results demonstrate for the first time that *A. afra* has potential anti-cancer properties despite the absence of ethnobotanical reports. However, *A. afra* is used to treat inflammation associated with various diseases (Liu et al., 2009). This study has shown that upon treatment of HeLa and U937 cells with an ethanol extract of *A. afra*, apoptosis is induced. The mechanism of induction of cell death upon treatment with an ethanolic extract of *A. afra* is accompanied by G2/M cell cycle arrest and depolarization of the mitochondrial membrane. Caspase-8 and caspase-3 are both activated resulting in the fragmentation of the DNA, a characteristic of late apoptosis. Since these studies were conducted on a crude extract, we cannot rule out the

possibility that different primary cell death mechanisms were activated due to the multicomponent nature of the extract. Jennet-Siems et al. (2002) identified sesquiterpene lactones as the cytotoxic compounds of *A. afra*. Studies are currently underway to identify/confirm the active component(s) and to characterize the precise mechanism of action of cytotoxicity induced by *A. afra*.

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