Cytotoxicity of Syringin and 4-Methoxycinnamyl alcohol isolated from *Foeniculum vulgare* on selected human cell lines

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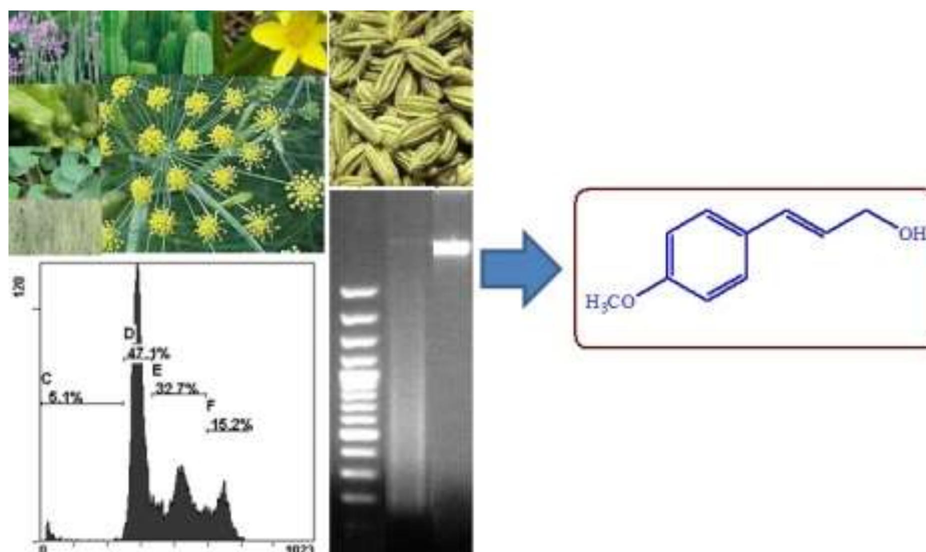
Graphical abstract

Cytotoxicity of isolated compounds from *Foeniculum vulgare* on various human cancer cell lines.



Abstract

The present study was carried out to determine the cytotoxic effect of seven plant extracts and the isolated compounds; syringin and 4-methoxycinnamyl alcohol on cancerous and non-cancerous cells. The ethanol extract of *F. vulgare* was found to exhibit the most significant toxicity with an IC_{50} value of 19.97 µg/mL on HeLa cells. Bioassay guided fractionation lead to the isolation of two compounds, syringin (1) and 4-methoxycinnamyl alcohol (2). Both compounds showed toxicity against MCF-7, HeLa, and DU145 cancer cell line. The results showed that compound 2 showed high toxicity against all the cancer cell lines with IC_{50} values of 14.24, 7.82 and 22.10 µg/mL, respectively. 4-Methoxycinnamyl alcohol also showed no apoptotic effect in cell cycle analysis after 48 hours at a concentration of 10 µg/mL. However DNA fragmentation study revealed that, necrosis took place at a concentration of 10 µg/mL after 48 h exposure.

Keywords: *Foeniculum vulgare*, cytotoxicity, 4-methoxycinnamyl alcohol, cell cycle analysis, DNA fragmentation

1. Introduction

Cancer is the second leading cause of death worldwide despite intensive research that is being conducted for new anticancer agents. Plants have been used for the treatment of cancer for a long time as a valuable source of anticancer constituents with minimal side-effects. It has been estimated that, 50% of breast cancer and 37% of prostate cancer patients have used plant products as a method of treatment (Nisa et al. 2011) and majority of South African people especially in rural communities still depend, to a large extent, on medicinal plants to treat various diseases (Van Wyk et al. 2008). The plants *Artemisia afra*, *Centella asiatica*, *Tulbaghia violacea*, *Euclea natalensis*, *Euphorbia ingens*, *Foeniculum vulgare* and *Hypoxis hemerocallidea* are used traditionally for the treatment of cancer in South Africa (Van Wyk et al. 1997).

Foeniculum vulgare is a medicinal and aromatic herb found in many parts of the world. Previous reports have revealed that the fennel extract exhibited antifungal, antioxidant, antithrombotic, anti-inflammatory, antibacterial, oestrogenic, hepato-protective and antidiabetic activity (Ibrahim and El-Khateeb 2013). Previously several volatile compounds, phenols and phenolic glycosides have been isolated from this plant (Diaaz-Maroto et al. 2006; Faudale et al. 2008). Therefore, the present paper describes the anticancer activity of seven ethanolic plant extracts (*Artemisia afra*, *Centella asiatica*, *Tulbaghia violacea*, *Euclea natalensis*, *Euphorbia ingens*, *Foeniculum vulgare* and *Hypoxis hemerocallidea*) on MCF-7, HeLa, SNO and DU145 human cancer cell lines. The cytoxicity of the isolated compounds; syringing and 4-methoxycinnamyl alcohol, was also determined after which the mechanistic studies of 4-methoxycinnamyl was evaluated.

2. Result and discussion

2.1 *Cytotoxicity of crude ethanol extracts*

From the study it is clear that *F. vulgare* extract showed significant cytotoxicity ($P<0.05$) towards the HeLa cells and little cytotoxicity towards the monkey Vero cells, with IC_{50} values of 19.97μg/ml and >100μg/ml respectively (Table 1). *Foeniculum vulgare* seeds were similar to those obtained in a study conducted by Berrington & Lall (2012) where an acetone extract of *Foeniculum vulgare* leaves showed an IC_{50} of 38.36±0.76μg/ml on HeLa cells. Furthermore, *A. afra* was significantly the least toxic

Table 1: Activity of crude extracts against cancer cell lines and Vero cells (IC_{50} in µg/mL)

Treatments	**Human cancers cell lines**				**Monkey Vero cells**
Ethanol crude extract	**MCF-7**	**HeLa**	**SNO**	**DU145**	
Artemisia afra	64.59±3.55	22.12±1.55	29.95±0.04	20.90±0.11	14.49±0.12
Centella asiatica	>100	83.24±3.10	>100	66.58±0.16	13.55±0.19
Euphorbia ingens	>100	>100	>100	85.31±0.05	14.45±0.18
Euclea natalensis	25.27±1.40	29.49±0.34	*	6.82±0.39	35.16±0.18
Foeniculum vulgare	>100	**19.97±0.048**	>100	56.41±0.28	>100
Hypoxis hemerocallidea	>100	52.63±2.02	>100	>100	27.89±0.09
Tulbaghia violacea	30.83±2.71	20.35±0.39	>100	22.29±1.35	70.28±0.06

*Not tested

($P<0.05$) to the MCF-7 cells than to the other cancerous cell lines. However, the toxicity towards monkey Vero cells was higher than the toxicity towards any of the cancerous cell lines. In a similar study an aqueous extract of *A. afra* was found to be cytotoxic at higher concentrations towards HeLa, Vero, Jurkat E 6.1, AA-Z and CEM-SS cells (Scott et al., 2004). These results were similar to those found in the present study on HeLa and monkey Vero cells.*T. violacea* showed higher cytotoxicity ($P<0.05$) than *A. afra* with regards to MCF-7 and HeLa cells. In a study conducted by Bungu et al., (2006) a methanolic extract from the leaves and bulbs of *T violacea* inhibited the growth of MCF-7, WHCO3, HT29 and HeLa cell lines. *C. asiatica* and *H. hemerocallidae* showed very low cytotoxicity towards the cancerous cell lines and high cytotoxicity ($P<0.05$) towards the monkey Vero cells. In an earlier study it was found that an aqueous extract of *C asiatica* stimulated the growth of DU-145, MDA-MB-231 and MCF-7 cells and the aqueous extract of *H. hemerocallidae* showed an increased growth of DU-145 and inhibited the growth of MCF-7 cells (Steenkamp & Gouws, 2006). *E. natalensis* showed relatively good cytotoxicity ($P<0.05$) towards all the cancerous cell lines with an IC_{50} value below 30µg/ml. *E. ingens* was relatively non-toxic ($P<0.05$) towards the cancerous cell lines but was considerably toxic towards the monkey Vero cells.

2.2 *Cytotoxicity of major fractions and isolation of compounds from F. vulgare*

Twelve major fractions were collected during column chromatography of *F. vulgare* extract. All fractions displayed cytotoxic activities ranging between 56.04-20.98 µg/mL on HeLa cells. Fraction eight exhibited the highest cytotoxicity with an IC_{50} of 20.98 µg/mL and therefore, was chosen for further isolation. Two known compounds, syringin (**1**) and 4-methoxycinnamyl alcohol (**2**) were identified.

2.3 *Cytotoxicity of isolated compounds*

The isolated compounds were tested on the MCF-7, HeLa and DU145 cancer cell lines using the XTT assay. The cytotoxicity was also performed on PBMC and U937 cells. Syringin showed low cytotoxicity on the U937 and PBMC cells with IC_{50} of >100µg/ml and 91.14µg/mL respectively. 4-methoxycinnamyl alcohol showed low cytotoxicity on the PBMC cells with IC_{50} value >100µg/mL, however high cytotoxicity was observed on the U937 cell line with an IC_{50} value of 3.55µg/ml (Figure 1, Table 2). The highest cytotoxicity was seen for 4-methoxycinnamyl alcohol with an IC_{50} value of 7.82 µg/mL whereas syringin was slightly less cytotoxic with an IC_{50} of 10.26 µg/mL on the HeLa cell line. The positive control doxorubicin showed a significantly high toxicity towards the HeLa cell line with an IC_{50} of 0.009µg/ml (Table 2. The positive control Cisplatin exhibited slightly higher cytotoxicity value on the U937 cell line than 4-methoxycinnamyl alcohol with an IC_{50} value of 5.71µg/mL.

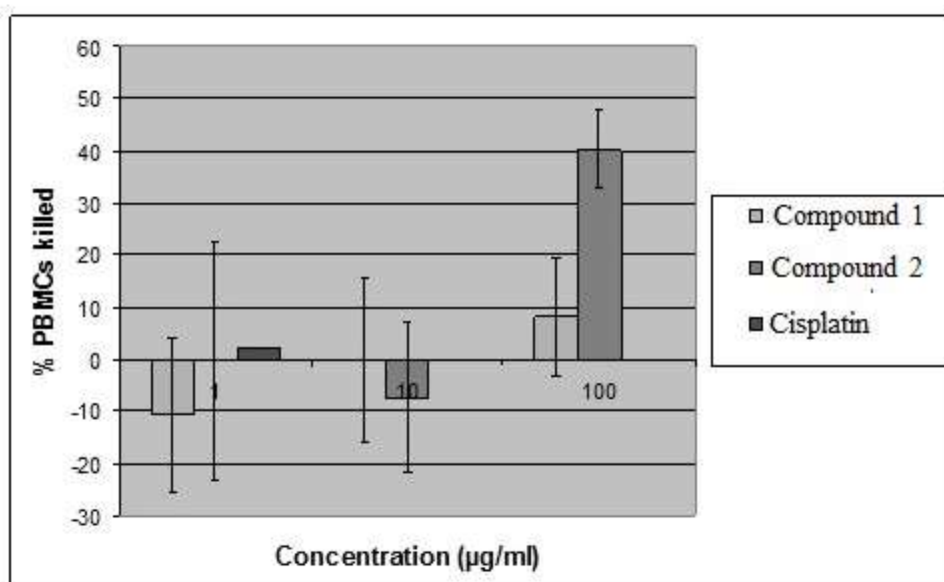


Figure 1: Cytotoxicity of isolated compounds on PBMCs. Percentage inhibition of isolated compounds on PBMCs and error bars indicate mean ± SD of quadruplicates.

Table 2: Cytotoxicity of syringin and 4-methoxycinnamyl alcohol on MCF-7, HeLa, DU145 and U937 cell lines

Treatment (IC_{50} in µg/ml)	**MCF-7**	**HeLa**	**DU145**	**U937**
Compound 1	21.88 ± 0.13	10.26 ± 0.18	>100	91.14 ± 0.63
Compound 2	14.21 ± 0.16	**7.82 ± 0.28**	22.10 ± 0.14	3.55 ± 0.18
Doxorubicin	0.36 ± 0.18	0.009 ± 0.001	0.009 ± 0.005	-
Cisplatin	-	-	-	5.71 ± 0.13

2.4 *Cell cycle analysis*

4-Methoxycinnamyl alcohol was examined for its cell cycle regulatory activities using PI staining. The treatment of U937 cells with 10µg/mL of 4-methoxycinnamyl alcohol indicated similar results to that of the negative DMSO control (Figure S1). However, the positive control showed an increase in the proportion of the sub-G1 cells compared to the negative control, which was equal to 71.8% and 5.8% respectively. As the sub-G_1 events are indicative of apoptotic activity it was clear that cell death occurred through apoptosis when exposed to Cisplatin, however the cells were not arrested in sub-G1 phase when treated with 4-methoxycinnamyl alcohol, which further indicates the lack of any apoptotic induction property. This indicated that at 10µg/mL of 4-methoxycinnamyl alcohol, no toxicity was observed on the U937 cells, however significant toxicity was observed on the HeLa cells.

2.5 *Annexin V-FITC/PI on U937 cells*

During the cell cycle analysis, when the U937 cells were exposed to 4-methoxycinnamyl alcohol at 10 µg/mL, it was found that there was no significant change in cell cycle when compared to that of the negative control. However, to confirm these results Annexin V-FITC/PI staining was done on the U937 cells at the same concentration, 10µg/mL, as during cell cycle analysis. The flow cytometric data analysis revealed that after 48 hours of treatment with 4-methoxycinnamyl alcohol, 92.4% of the U937 cells were live which is comparable to the negative control which showed 88% of the cells. However, when analysing the positive control the majority of cells were found to be necrotic, indicating that apoptosis had already taken place (Figure S2). These results confirm the similar finding during cell cycle analysis where the U937 cells exposed to DMSO and 4-methoxycinnamyl alcohol.

2.6 *DNA fragmentation*

DNA fragmentation (Figure 2) was used to confirm that 4-methoxycinnamyl alcohol did not lead to the accumulation of fragments after U937 cells were exposed for 48 hours. In this study the exposure of U937 cells to 4-methoxycinnamyl alcohol over a 48h incubation period at a concentration of 10µg/mL showed no ladder formation, which is characteristic of apoptosis however, smeared DNA bands were observed which was characteristic of necrosis.

3. Experimental (see supplementary file)

4. Conclusion

It was evident from the cytotoxicity results that *Foeniculum vulgare* showed high potential as an anti-cancer agent, specifically against the HeLa cell line. Two compounds were isolated from the seeds of *F. vulgare*; Syringin and 4-methoxycinnamyl alcohol. Furthermore, the isolated compound, 4-methoxy-cinnamyl alcoholappeared to show signs of necrotic cell death.These observations raise the prospects of using *F. vulgare* for the treatment of cancer. Further experimental analysis would need to be performed to determine the mechanism of cell death on cancerous HeLa cells.

Acknowledgements

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Supplementary Material

3. Experimental

3.1 *General*

Column chromatography: silica gel 60 (70-230 mesh, Sigma-Aldrich). NMR spectra were recorded on a Varian Oxford AV-200 MHz spectrometer, using reference line as a standard. IR spectra were recorded on a Nexus 670 FT-IR instrument from KBr pellets. The XTT Cell proliferation Kit II was purchased from Roche diagnostics. The Cell Titre-Blue was obtained from Promega. The Coulter® DNA Prep™ Reagent Kit as well as the Annexin V-FITC/PI kit was purchased from Beckman Coulter and the FexiGene DNA Kit from Qiagen. All the chemicals were purchased from Sigma-Aldrich and Merck SA Pty Ltd.

3.2 *Plant material and preparation of extracts*

The plant material was collected from the botanical garden at the University of Pretoria, Pretoria. .The plants were identified and authenticated at the H.G.W.J Schwelckerdt Herbarium of the University of Pretoria and voucher specimens were submitted. All plant material was collected and shade dired. Thereafter 30 g of each plant material was extracted with 200 mL of ethanol for 24 h on a shaker (120 rpm). The filtrate of each plant was collected after filtration with a Buchner funnel (1000mL; pore size 3) and Whatman filter paper 3. The extracts were then evaporated under reduced pressure at 40 °C using a rotary evaporator.

Table S1: Summary of selected plant material and voucher specimen numbers

S.N.	Plant species	Plant part used	Specimen number
1.	*Artemisia afra*	Leaves	PRU 112085
2.	*Centella asiatica*	Leaves	PRU 112086
3.	*Tulbaghia violacea*	Leaves	PRU 095452
4.	*Euclea natalensis*	Roots	NL 22
5.	*Euphorbia ingens*	Stem	PRU 112087
6.	*Foeniculum vulgare*	Seeds	PRU 112089
7.	*Hypoxis hemerocallidea*	Corms	PRU 112088

3.3 *Cell lines*

The adherent breast adenocarcinoma (MCF-7), cervical epithelial carcinoma (HeLa), oesophageal carcinoma (SNO), prostate epithelial carcinoma (DU145), andAfrican green monkey kidney (Vero) cell lines were maintained in culture flasks in complete Eagle's Minimum Essential Medium (EMEM), supplemented with 10% foetal bovine serum (Highveld Biological, SA) and 1% antibiotics (100 U/ml penicillin, 100µg/ml streptomycin and 250µg/ml fungizone).The histiocytic lymphoma (U937) cell line was maintained in complete RPMI1640 medium. The cells were grown in a humidified 5% CO_2 incubator at 37 ºC. Cells were sub-cultured after a confluent monolayer had formed. Adherent cells were detached from the culture flask using trypsin (0.25% trypsin containing 0.01% EDTA) for 10min at 37 ºC and then adding complete medium to inhibit the reaction. The U937 suspension cells were used in the cell cycle analysis studies as adherent cell lines can produce false positive results when adherent cell lines are detached for staining and analysis.

3.4 *Cytotoxicity assay*

Cytotoxicity of the samples was measured by the XTT (Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate) colorimetric assay using the Cell Proliferation Kit II (Roche Diagnostics) as described by Kishore et al. (2014). The cells were seeded (100 µL) at 1×10^5 cells/well in a 96-well microtitre plate and incubated for 24 h to allow for adherence. Serial dilutions were prepared from the extracts, isolated compounds and the positive controls (0.1-100 µg/mL), and were added to the microtitre plate and incubated for a further 48 h. The XTT reagent was added to a final concentration of 0.3 mg/mL and incubated for 2 h.

The absorbance of the colour complex was quantified at 490 nm using a BIO-TEK Power-Wave XS multi-plate reader with a reference wavelength set at 690 nm. The assay was performed in triplicate to calculate the fifty per cent inhibitory concentration (IC_{50}). The results were statistically analysed using Graph Pad Prism 4 (Version 4 Graph Pad Software, San Diego, Ca, USA) statistical program.

3.5 *Isolation and identification of bioactive compounds*

The shade dried and powdered seeds (2.0 kg) of *F. vulgare* were extracted with 3L of ethanol and then left for 24 h on a shaker (120 rpm). The procedure was repeated three times. The filtrates were collected and concentrated under reduced pressure by using rotary evaporator at 40 °C to produce 70 g of crude ethanol extract. Approximately 65

g of the extract was subjected to silica gel column chromatography (70 cm×120 cm) with hexane : ethyl acetate mixtures of increasing polarity (100:0 to 0:100) followed by 100% methanol (MeOH) as eluent. A total of 78 fractions were collected and similar fractions were combined according to thin-layer (TLC) profile, which resulted into 12 major fractions (MF).

The major fractions were tested for cytotoxicity against HeLa cells. Fraction 8 showed (Table S2) the highest toxicity and hence was subjected to column chromatography to isolate the bioactive compounds. MF 8 (443 mg) was separated on a silica gel column eluted with Hexane: ethyl acetate mixtures of increasing polarity which yielded syringin (1) (17 mg) and 4-methoxycinnamyl alcohol (2) (13 mg).

Table S2: Cytotoxicity of major fractions of *F. vulgare* on HeLa cells

Fractions	IC_{50} on HeLa cells (µg/mL)
1	>100
2	>100
3	38.55 ± 3.24
4	50.04 ± 2.65
5	52.43 ± 3.57
6	31.26 ± 3.97
7	27.13 ± 3.67
8	**20.98 ± 4.48**
9	36.91 ± 7.15
10	53.24 ± 7.96
11	56.04 ± 13.13
12	48.46 ± 9.50

Structural assessment of isolated compounds was done by Mass, ^{1}H and ^{13}C NMR spectroscopic data. Assignment of signals was facilitated by COSY, HSQC and HMBC experiments.

Syringin (1), white amorphous powder; $C_{17}H_{24}O_9$, m.p. 191-193 °C; ^{1}H NMR (200 MHz, CD_3OD): δ 6.70s (2H, s, H-2', 6'), 6.54 (1H, d, *J* = 16.2 Hz, H-3), 6.30 (1H, dt, *J* = 16.2, 5.0 Hz, H-2), 4.87 (1H, s, H-1″), 4.21 (2H, dd, *J* = 5.4, 1.2 Hz, H-1), 3.85 (6H, s, 2 x $-OCH_3$), 3.64-3.77 (2H, m, H-6″), 3.00-3.50 (4H, m, H-2″, 3″, 4″, 5″). ^{13}C NMR (50 MHz, CD_3OD): δ 153.4 (C-3', C-5'), 135.1 (C-4'), 132.0 (C-1'), 130.0 (C-1), 129.1 (C-2), 104.5 (C-2', C-6'), 104.3 (C-1″), 77.4 (C-5″), 76.8 (C-2″), 74.7 (C-3″), 70.4 (C-4″), 62.6 (C-3), 61.6 (C-6″), 56.1 (2 x $-OCH_3$).

4-Methoxycinnamyl alcohol (2), colourless crystals; m.p. 130-132 °C; ^{1}H NMR (200 MHz, CD_3OD): δ 7.31 (2H, m, CH-2', 8') 6.85 (2H, m, CH-5', 6'), 6.48 (1H, d, J = 16.0 Hz, CH-3), 6.20 (1H, dt, J = 5.9, 16.0 Hz, CH-2), 4.18 (2H, d, J = 5.9 Hz, CH_2-1), 3.76 (3H, s, -O CH_3). ^{13}C NMR (50 MHz, CD_3OD): δ 159.5 (C-4'), 131.2 (C-3), 129.4 (C-2), 127.7 (C-1'), 126.7 (C-2', C-6'), 114.1 (C-3', C-5'), 63.8 (C-1), 56.3 (-OCH_3).

3.6 *Cytotoxicity in peripheral blood mononuclear cells (PBMCs)*

Blood was obtained from healthy adult volunteers. PBMCs were separated with BD Vacutainer™ CPT™ cell preparation tubes containing sodium heparin. The plasma, mononuclear cells and platelets were transferred to sterile 15ml tubes and centrifuged for 15 minutes at 300 × g. The supernatant was discarded and the pellet was re-suspended in incomplete RPMI1640 medium. The centrifugation step was repeated and the pellet was re-suspended in complete RPMI1640 medium, after which the PBMCs were counted using trypan blue and a hemacytometer. The PBMCs were seeded in 96-well plates at 200 µL per well at a concentration of 4×10^5 cells/well and incubated at 37 ºC and 5% CO_2.

The isolated compounds were dissolved in DMSO to give a stock concentration of 400 µg/mL. Serial dilutions of thecompounds were prepared to give final concentrations of 1, 10 and 100 µg/mL which were added to the 96-well plate. Control wells included complete RPMI1640 medium and a vehicle control (2% DMSO).. The 96-well plates were incubated for 48 h at 37 ºC in a humidified atmosphere with 5% CO_2. Thereafter, 40 µL of the signal reagent CellTitre-Blue® (Promega) was added to the wells and incubated for 4 h. After the incubation period 200 µL from each well was transferred to a black 96-well plate. Data was recorded using ThermolabSystem Fluoroskan AscentFL fluorescence micro-plate reader at excitation and emission wavelengths of 560 nm and 590 nm respectively.

3.7 *Cell cycle analysis*

The compound, 4-methoxycinnamyl alcohol, with the lowest IC_{50} value was selected to further investigate the mechanism of action. U937 cells were grown in 25 cm^2 culture flasks at a density of 1×10^6 cells/mL in 5ml of RPMI1640 complete medium. The cells were transferred to 15 mL tubes and centrifuged at 500 ×g for 5 min. Thereafter the cells were re-suspended in 1ml complete RPMI1640 medium after which the viability and number were determined using trypan blue. The cells were seeded in complete RPMI1640 medium at a density of 2×10^6 cells per mL in a culture flask. After a recovery period of 24h, the selected compound were dissolved in DMSO and added to

the cells to a final concentration of 10 µg/mL. Cisplatin was added as a positive control to give a final concentration of 10µM, and DMSO was added as the negative control. The flasks were further incubated for 48 h.

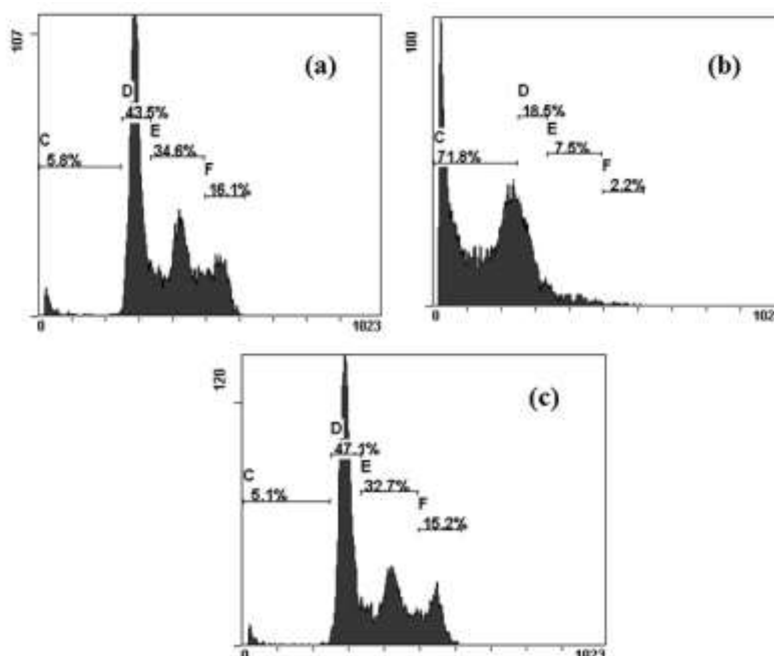

Figure S1: Cell cycle analysis of 4-methoxycinnamyl alcohol. Histograms representing DNA cell cycle arrest in U937 cells after 48 hours treatment with 0.25% DMSO (a), 10 µg/mL Cisplatin (b) and 10 µg/mL compound 2 (c). A minimum of 10,000 events were read. One representative of three experiments is shown.

The Coulter® DNA Prep™ Reagents Kit (Beckman Coulter) was left to reach room temperature before use. From the treated flasks, 2mL of medium was drawn and centrifuged for 5 minutes at 500 ×g at 4 ºC. The pellet was then loosened in 500 µL sheath fluid and thereafter 500 µL lysis buffer was added to all the tubes. The tubes were incubated at room temperature for 5 minutes after which 1mL of propidium iodide (PI) was added. The tubes were covered with foil and incubated for a further 5 minutes at 37 ºC. The cells were analysed on a Beckman-Coulter FC500 Flow Cytometer (Miami, FL, USA). A minimum of 10,000 events were acquired for each sample.

3.8 *Annexin V-FITC/PI on U937 cells*

The same cell suspensions prepared for the cell cycle analysis were used for the Annexin V-FITC/PI staining. Annexin V-FITC/PI Kit (Beckman Coulter) reagents were prepared as per kit instructions and kept on ice. From each treated flask, 2mL of medium was drawn and centrifuged for 5 minutes at 500 x g at 4 ºC. The pellet was re-suspended in 1 × binding buffer after which all the falcon tubes were kept on ice. To

100 μL of cell suspension, 1 μL of Annexin V-FITC solution and 5 μL of the dissolved PI was added and vortexed. The falcon tubes were then incubated on ice in the dark for 15 minutes. After incubation, 400 μL of the 1 × buffer solution was added and the samples were analysed on a Beckman Coulter FC500 flow cytometer. A minimum of 10,000 events per sample were acquired.

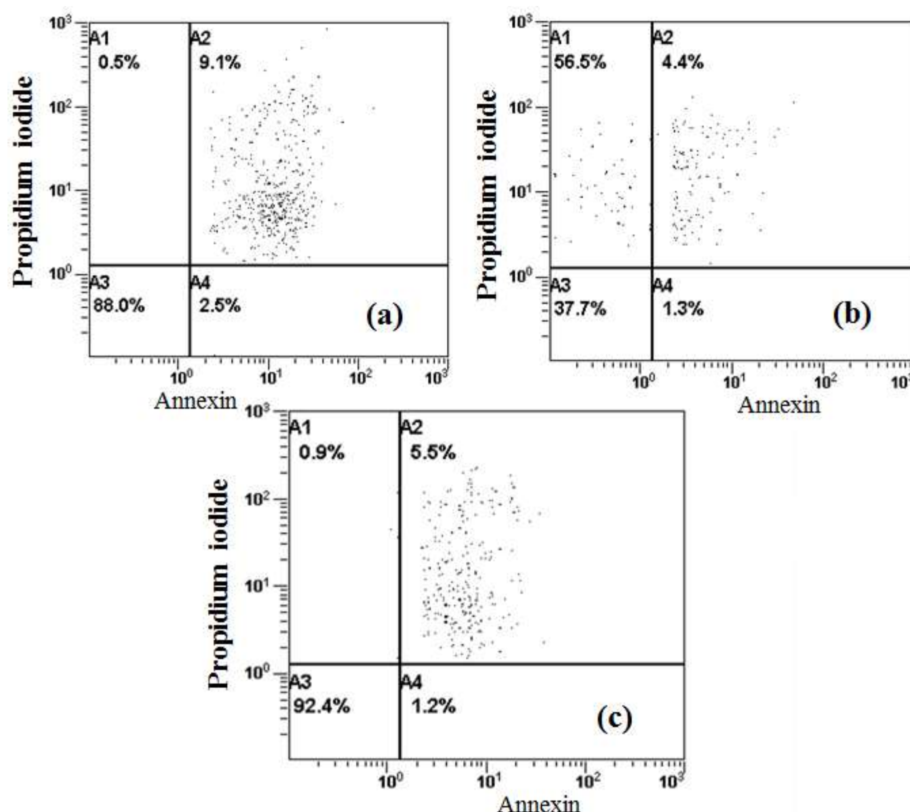

Figure S2: Annexin V-FITC analysis of 4-methoxycinnamyl alcohol. Dot plots of Annexin V-FITC-stained U937 cells after 48 hours exposure to 0.25% DMSO (A) 10μg/mL Cisplatin (B) and 10μg/ml 4-methoxycinnamyl alcohol (C). A minimum of 20,000 events were read. One representative of three experiments is shown.

3.9 *DNA fragmentation*

DNA fragmentation was performed to determine whether 4-methoxycinnamyl alcohol was inducing apoptosis or necrosis in U937 cells. U937 cells were treated with 4-methoxycinnamyl alcohol for 48 h, at a concentration of 10 μg/mL, after which the modulations of DNA fragmentation was detected using a FexiGene DNA kit (QIAGEN). Untreated cells were used as a control and prepared in the same manner. After the incubation period the DNA was extracted following the manufacturer's instructions. The DNA was resolved on a 1.5 and 2% TAE (tris/acetate/EDTA) electrophoresis agarose gel (White Sci).

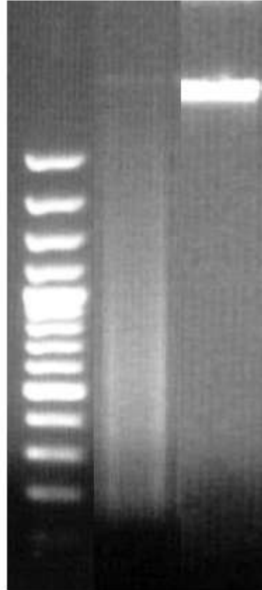

Figure S3: DNA fragmentation of 4-methoxycinnamyl alcohol on U937 cells. Gel electrophoresis of (1) DNA ladder, (2) 4-methoxycinnamyl alcohol with characteristic necrotic smear and (3) control cells without treatment.