CHAPTER 4

Cytotoxicity of 7-methyljuglone and its derivatives on cancer cells and on non cancerous cells

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4.1 Introduction

An essential route to new pharmaceuticals emerged with the modern medicine and single pure drugs era, which were greatly provided by plant-derived active principles and their semi-synthetic and synthetic analogs (Lee, 1999). During the rational drug design modification and development stage of new anticancer drugs, the aim is to create new synthetic analogs with better activity, enhanced pharmacological profiles and reduced toxicity (Lee, 1999).

In the US bioactivity directed fractionation and isolation led to the acceptance of nine plant-derived compounds (vinblastine (Velban), vincristine (Oncovin), etoposide (VP-16), teniposide (VM-26), taxol (Paclitaxel), navelbine (Vinorelbine) for use as anticancer drugs since 1961. Newer accepted (in 1996) anticancer drugs include taxotere (docetaxel), topotecan (Hycamtin), and irinotecan (Camptosar) (Lee, 1999). In China, 11 other anticancer agents (d-tetrandrine, colchicinamide, 10-hydroxycamptothecin, homoharringtonine, monocrotaline, gossypol, (-)-sophocarpine, curcumol, lycobetaine, curdione and indirubin) are currently also used.

4.1.1 Quinonoids

Quinoid compounds are widely distributed in nature and they are found in all respiring animal and plant cells as well as in fungi and bacteria, primarily as secondary metabolites (Chakrabarty, 2002). Naturally occurring quinones are divided in three main groups: benzoquinones, naphthoquinones and anthraquinones, in order of increasing size (Powis, 1987). A number of natural quinoids such as anthracyclines, mitoxantrones and saintopin as well as many other quinoids and their synthetic analogues (simple and more complex) have been found to possess significant antitumour activity, selected throughout the random screening of an enormous number of compounds (Powis, 1987), by virtue of their facile redox capacity (Chakrabarty, 2002; Chung *et al.*, 2004; Kim *et al.*, 2006). By the year 1974 the National cancer Institute's Drug Research and Development Programme in the USA tested more than 1500 quinones. Quinonoids were initially studied in the late 1950s in soil fungi, i.e. *Streptomyces* strains, formed as pigmented antibiotics (O'Brien, 1991). In 1963 the anthraquinones glycosidic antibiotic (daunorubicin) was the first to be isolated and its antileukemic activity was revealed. The first synthetic anticancer drugs, rationally designed and synthesized in 1954, which were able to cross the blood brain barrier was the aziridinylbenzoquinones (O'Brien, 1991).

The quinoids comprise also the second largest class of antitumour agents currently in use. Many traditional oriental herbal preparations include several commonly used medicinal plants from which numerous quinonoid compounds were isolated recently, which are currently being studied for their anticancer properties and mechanisms of action associated with these properties (Hazra *et al.*, 2005). Most antitumour quinoid compounds have complex structures. The chemical reactivity contributions are therefore often tricky to separate from the different metabolic pathways and from the overall biological activity (Powis, 1987). Antitumour activity flaunted by many naphthoquinone derivatives (e.g. menadione) has been used as antitumour drugs (Chen *et al.*, 2004). However, the prospective anticancer activity of quinonoid compounds and their derivatives has remained unexplored at large. Furthermore studies confirmed the powerful pharmacologic effects of several 1,4-naphthoquinone derivatives and also their connection with distinct antimicrobial and antitumour activities (Chung *et al.*, 2004; Kim *et al.*, 2006). Naphthoquinones are capable of eliciting and inducing both apoptotic as well as necrotic cell death (Montoya *et*

al., 2005; Stasiauskaite *et al*., 2006). Strong corresponding relationships occur primarily between apoptosis frequency and growth inhibition potency (Stasiauskaite *et al*., 2006). Semiquinone radicals' formation can also be induced by quinone analogues. Superoxide is formed by a process catalyzed by flavoenzymes (e.g. nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome-P-450 reductase) as soon as semiquinone radicals are able to transfer an electron to oxygen (Powis, 1987; Kim *et al*., 2006). The semiquinone radical anions as well as the superoxide of the naphthoquinone analogues can produce the hydroxyl radical which can cause DNA strands to break (Kim *et al*., 2006). It was recently confirmed that a number of 1,4-naphthoquinones were capable of inhibiting Cdc25, including vitamin K_3 (Ham & Lee, 2004).

Studies conducted by Orrenius and his co-workers with 2-methyl-1,4-naphthoquinone known as menadione and isolated hepatocytes provided significant insights into the manner in which simple quinones metabolism could possibly lead to damage and death of cells (Powis, 1987). Depletion of the protein thiol groups and intracellular glutathione can take place with no a modification in the redox state of cellular pyridine nucleotides and lead to toxicity, as observed with menadione-induced hepatocyte toxicity (Powis, 1987). It has been established that the alkylation of vital protein thiol or amine groups and/or the oxidation of necessary protein thiols by triggered oxygen species and/or glutathione (oxidized form) (GSSG) in resting or non-dividing cells is the molecular basis used for launching the quinone cytotoxicity (O'Brien, 1991). When reductases reduce a quinone to a semiquinone radical, oxidative stress occurs. Eventually the quinone is reformed after superoxide radicals were produced once the semiquinone radical reduces oxygen. Cytotoxic levels of hydrogen peroxide and GSSG is retained by the cell due to this futile redox cycling and oxygen activation and furthermore causes cytotoxic mixed protein disulfide formation (O'Brien, 1991). Glutathione (reduced form) GSH conjugates are formed by most quinones. These GSH conjugates also go through futile redox cycling and oxygen activation (O'Brien, 1991). Naphthoquinones and benzoquinones are very cytotoxic since they have higher electrophilicity and thiol reactivity and also because these quinines or GSH conjugates are easily reduced to semiquinones which will in turn activate oxygen (O'Brien, 1991). A wide range of toxic effects are exerted by quinonoids and when administered cause side effects such as: nausea, vomiting, hair loss and tissue necrosis (Young *et a*l., 1981).

4.1.2 Bioactivity, cytotoxicity of 7-methyljuglone

The hydroxynaphthoquinonoids are known to have antiprotozoal activities since the 1930's and have attracted renewed attention during the past three decades, when some synthetic compounds of this class were identified as potential drugs against several protozoan parasitic diseases (Croft *et al.*, 1992). Marked improvement were showed from *in vitro* studies in the antileishmanial, antitrypanosomal activities of diospyrin and its derivatives which was attributable to minor changes in the functional groups of the natural product (Yardley *et al.*, 1996). Similar observations were recorded in the studies on antitumour and antiplasmodial effects of these compounds (Hazra *et al.*, 1994, 1995).

Plant extracts should have the ability to trigger/induce a cleavable complex with purified mammalian topoisomerases in order for them to be recognizing new plant metabolites with antitumour activity (Fujii *et al.*, 1992). It was then found by Fujii and co-workers, in 1992, that 'plumbagin' and 'shikonin', two plant naphthoquinones, are effective inducers, *in vitro*, of the cleavable complex formation by means of topoisomerase II. Therefore, these naphthoquinones induced topoisomerases II-mediated DNA cleavage by creating a cleavable complex. This is also seen with antitumour agents such as 'Lawson', 'demethylepipodophyllotoxin', ethylidene-β-glucoside' and 'lapacol'. These antitumour plant metabolites are structurally related to naphthoquinone moieties, but plumbagin and shikonin proved to have a parallel DNA cleavage pattern with topoisomerase II which was nothing like the cleavage patterns induced by other prominent topoisomerase II-active drugs (Fujii *et al.*, 1992).

Diospyrin derivatives were synthesized with the objective of improving the therapeutic effects, and reduce toxicity towards normal cell lines as well as opting to enhancing their efficacy to tumour cells. Diospyrin and its synthetic derivatives have been shown to induce apoptosis in different human tumour cells (Chakrabarty *et al.*, 2002). Tazi *et al.* (2005) found that even though diospyrin and its derivatives inhibit kinase activity of topoisomerase I and interfere with camptothecin-independent topoisomerase I mediated DNA cleavage, on the other hand, on DNA no reactions of topoisomerase I were block. Diospyrin derivatives therefore mediate the topoisomerase I conformational modification. They also

showed that diospyrin derivatives change several steps, either the first or the second catalytic step, of splicing *in vitro*, but not spliceosome assembly and that they are able to stall the assembly (dynamic) of the spliceosome (Tazi *et al.*, 2005). Full spliceosome formation was however prevented by diospyrin. The antitumour activity of diospyrin was enhanced through liposomal encapsulation, *in vivo* (Hazra *et al.*, 2005). Ting *et al.* (2003) found that isodiospyrin showed cytotoxicity against colon carcinoma (COLO-205) as well as lymphocytic leukaemia (P-388) and also a novel human DNA topoisomerase I inhibitor. Isodiospyrin has been examined for its inhibition of human DNA topoisomerase I, antibacterial and anti-inflammatory activities (Gafner *et al.*, 1987).

7-Methyljuglone, a monomer of diospyrin has been proved to have potent anti-tuberculosis and anticancer activity. It has been reported to possess among other activities antibacterial and cytotoxic properties against human colon carcinoma cells. There are several reports on the anticancer activity of naphthoquinones on a panel of cancer cell lines. 7-Methyljuglone, isolated from *E. divinorum,* showed marked cytotoxicity against murine lymphocytic leukaemia (P-388) and human prostate cancer (LNCaP) (Mebe *et al.*, 1998). Considering reports of previous researchers on various naphthoquinones, including 7-methyljuglone, it was decided to investigate the anticancer/cytotoxic activity of the synthetic derivatives on various cancer cell lines.

4.2 Materials and Methods

4.2.1 Synthesis of 7-methyljuglone and its derivatives

The parent compound 7-methyljuglone and its derivatives were successfully synthesized by Dr A. Mahapatra (Mahapatra, *et al*, 2007) (Table 4.1, Figure 4.1).

Table 4.1 List of naphthoquinones studied for anticancer activity modified from (Mahapatra, *et al*, 2007).

Naphthoquinones	X	R1	R2	R3
1) 8-Chloro-5-hydroxy-7-methyl-1, 4-naphthoquinone	Cl	Me	H	OH
2) 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone	Br	Me	H	OH
3) 8-Fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone	F	Me	H	OH
4) 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone	Cl	H	Me	OH
5) 5-Hydroxy-6-methyl-1,4-naphthoquinone	H	H	Me	OH
6) 5-Hydroxy-7-methyl-1,4-naphthoquinone (7-MJ)	H	Me	H	OH
7) 8-Chloro-5-methoxy-7-methyl-1,4-naphthoquinone	Cl	Me	H	OMe
8) 5-Ethoxy-8-chloro-7-methyl-1,4-naphthoquinone	Cl	Me	H	OEt
9) 5-Methoxy-7-methyl-1,4-naphthoquinone	H	Me	H	OMe
10) 5-Ethoxy-7-methyl-1,4-naphthoquinone	H	Me	H	OEt
11) 8-Chloro-5-methoxy-6-methyl-1,4-naphthoquinone	Cl	H	Me	OMe
12) 5-Ethoxy-8-chloro-6-methyl-1,4-naphthoquinone	Cl	H	Me	OEt
13) 5-Acetoxy-8-chloro-7-methyl-1,4-naphthoquinone	Cl	Me	H	OAc
14) 5-Acetoxy-7-methyl-1,4-naphthoquinone	H	Me	H	OAc
15) 5-Acetoxy-8-chloro-6-methyl-1,4-naphthoquinone	Cl	H	Me	OAc
16) 8-Chloro-7-methylnaphthalene-1,2,4,5-tetra-O-acetate	Cl	Me	H	
17) 7-Methylnaphthalene-1,2,4,5-tetra-O-acetate	H	Me	H	
18) 8-Chloro-6-methylnaphthalene-1,2,4,5-tetra-O-acetate	Cl	H	Me	
19) 2,5-dihydroxy-7-methyl-1,4-naphthoquinone		CH_3		OH

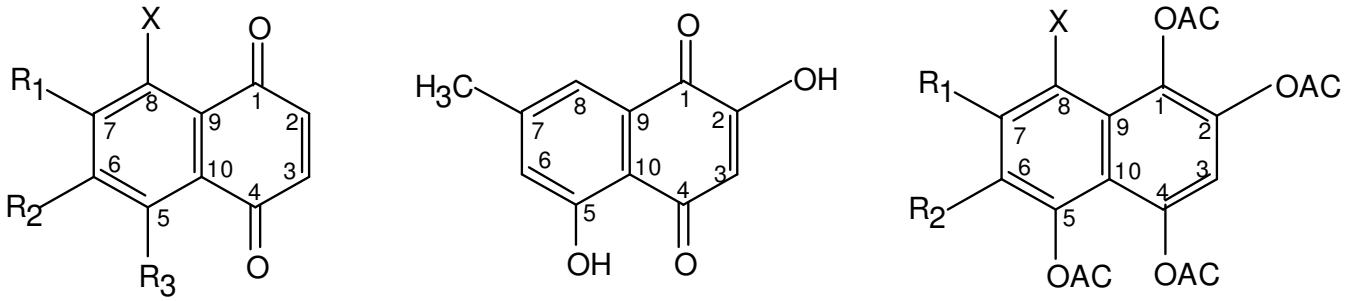

Figure 4.1 (A) Compounds 1-15 (B) 19 (C) 16-18.

4.2.2Culture of cancer cells

U937 cells were maintained in culture flasks in complete RPMI1640 medium (Sigma, Germany) supplemented with 10% fetal bovine serum (Delta Bioproducts, SA), containing 25mM HEPES and 2mM glutamine, in a humidified 5% CO_2 incubator at 37℃.

All the cell culture procedures were the same, as for the extracts (see chapter 3) the naphthoquinone derivatives were tested on the same cancer cell lines and the same concentrations (0.781 µg/ml – 100 µg/ml) as for the extracts.

4.2.3Cytotoxicity 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. Blood was drawn into the tubes and immediately the tubes were inverted 10 times to mix the anticoagulant additive with the blood. The tubes were centrifuged within 30 minutes after the blood collection at room temperature for 30 minutes at 1800xg. After centrifugation different layers were observed in the BD Vacutainer™ CPT™ cell preparation tubes. The plasma, mononuclear cells and platelets were transferred to other sterile 15 ml tubes and were inverted gently 10 times. The tubes were centrifuged for 15 minutes at 300xg at room temperature, the supernatants were aspirated and the pellets were resuspended in complete RPMI1640 medium (Figure 4.2). The centrifugation step was repeated, the supernatants were aspirated and this time the pellets were resuspended in complete RPMI1640 medium, after which the PBMCs were counted with trypan blue and a hemacytometer. Thereafter, the PBMCs were quite densely resuspended, $2x10^6$ cells per millilitre and were seeded in 96-well plates at 200 µl/well (or $4x10^5$ cells per well). The plates were incubated at 37℃ in a humidified atmosphere until they were used.

The test compounds were weighed and dissolved in DMSO to give a 40 µg/µl stock solution. The stock solution of each of the compounds was diluted with RPMI1640 complete medium to give the different final concentrations tested (1, 10 and 100 µg/ml)

which were added to the 96-well plate. Control wells were prepared in which only complete RPMI1640 medium was added. The 96-well plates were then incubated for 48 hours at 37℃ in a humidified atmosphere with 5% CO_2.

After 48 hours, 40 µl of the signal reagent CellTiter-Blue® (Promega) was added, to all the wells. The plates were incubated at 37℃ in a humidified atmosphere with 5 % CO_2 in aluminium foil for four hours. After the incubation period, 200 µl from each well was transferred to a black 96-well plate (Nunc). Data were recorded using a ThermoLabsystem Fluoroskan AscentFL fluorescence microplate reader at excitation and emission wavelengths of 560 and 590 nm, respectively.

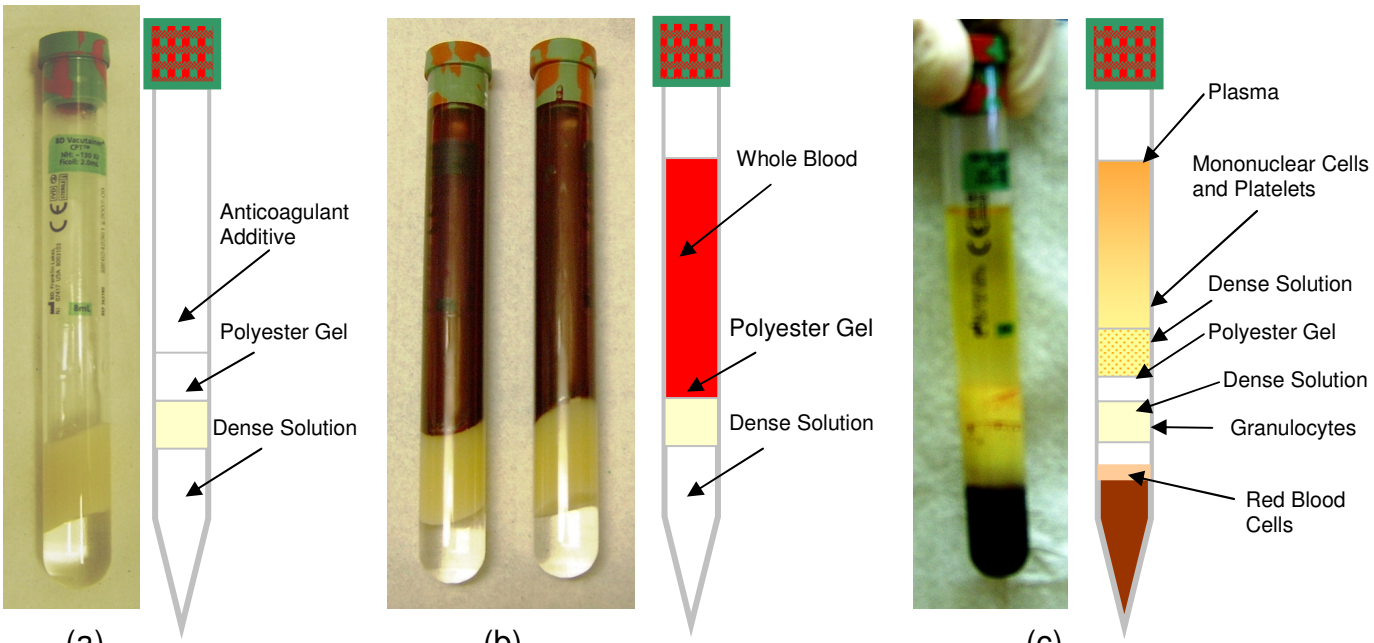

Figure 4.2 (a) A tube before use. (b) After blood collection. (c) After centrifugation

4.3 Results

4.3.1 Cytotoxicity on four human cancer cell lines

The growth inhibition results on the four adherent cancer cell lines (Figure 4.3) are examples of the dose response curves (Table 4.2) showed that all the naphthoquinone derivatives showed activity against these cell lines. With GraphPad Prism 4 a sigmoidal

dose-response (variable slope) curve fit was done and the IC_{50} values of the extracts were determined from the concentration-effect relationship (Figure 4.4). However, the positive control doxorubicin was more effective, by at least an order of magnitude, than any of the synthesized derivatives. It is clearly evident, however, that most of these display considerable cytotoxicity against the cancer cell lines.

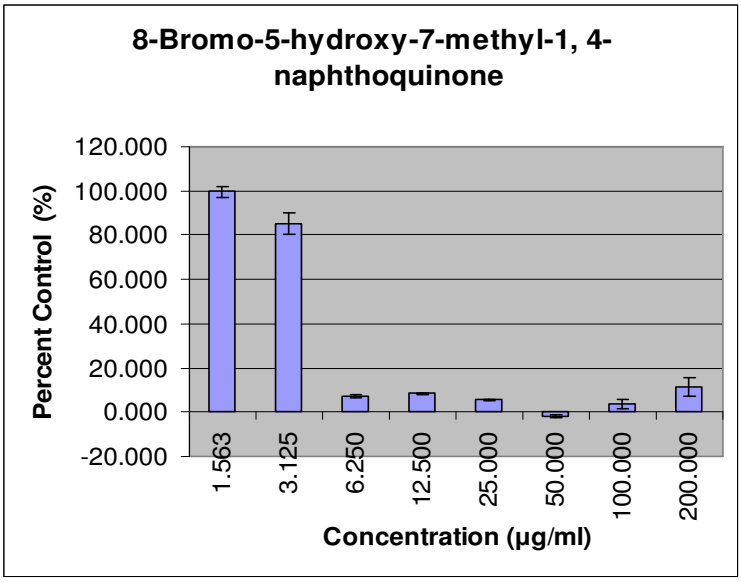

Figure 4.3 Dose response of 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone on MCF-7 cell viability.

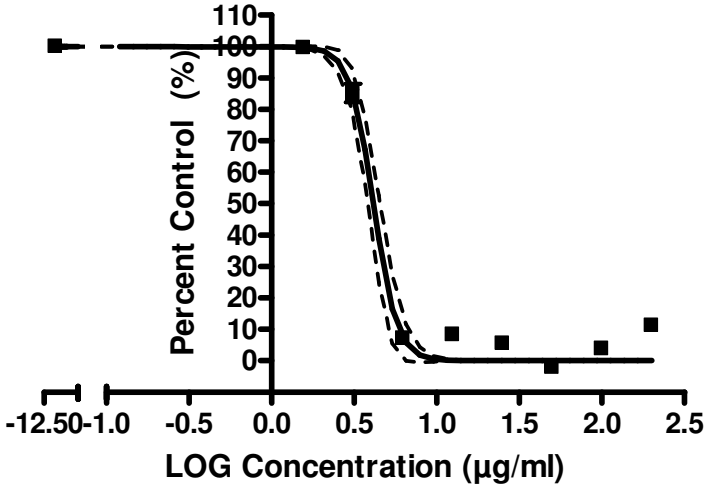

Figure 4.4 Dose response curve fit of 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone on MCF-7 cells.

Table 4.2 Summary of all the IC_{50} results on all four the human cancer cell lines of all the derivatives of 7-methyljuglone and the positive controls.

Compound	**MCF-7**	**HeLa**	**SNO**	**DU145**
	IC_{50} (µg/ml) ± SD	**IC_{50} (µg/ml) ± SD**	**IC_{50} (µg/ml) ± SD**	**IC_{50} (µg/ml) ± SD**
1. 8-Chloro-5-hydroxy-7-methyl-1, 4-naphthoquinone	3.50 ± 0.16	3.02 ± 0.87	3.85 ± 2.01	6.57 ± 0.88
2. 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone	4.15 ± 0.28	3.04 ± 0.92	3.98 ± 1.10	12.37 ± 1.25
3. 8-Fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone	4.19 ± 0.35	2.11 ± 0.67	3.91 ± 2.20	3.18 ± 1.91
4. 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone	2.23 ± 0.18	2.24 ± 0.90	2.11 ± 0.23	2.07 ± 2.11
5. 5-Hydroxy-6-methyl-1,4-naphthoquinone	2.89 ± 0.21	3.09 ± 1.24	3.59 ± 2.17	2.91 ± 2.14
6. 5-Hydroxy-7-methyl-1,4-naphthoquinone (7-MJ)	5.11 ± 0.62	12. 54 ± 2.45	15.33 ± 4.32	2.24 ± 0.27
7. 8-Chloro-5-methoxy-7-methyl-1,4-naphthoquinone	4.88 ± 0.20	4.54 ± 1.03	5.83 ± 1.60	6.31 ± 2.35
8. 5-Ethoxy-8-chloro-7-methyl-1,4-naphthoquinone	10.05 ± 1.54	4.78 ± 1.81	11.10 ± 2.00	6.37 ± 1.43
9. 5-Methoxy-7-methyl-1,4-naphthoquinone	3.10 ± 0.76	4.27 ± 0.82	4.56 ± 1.56	6.22 ± 1.53
10. 5-Ethoxy-7-methyl-1,4-naphthoquinone	5.14 ± 1.13	6.43 ± 2.69	5.48 ± 1.63	3.25 ± 1.24

Compound	MCF-7	HeLa	SNO	DU145
	IC_{50} (µg/ml) ± SD	IC_{50} (µg/ml) ± SD	IC_{50} (µg/ml) ± SD	IC_{50} (µg/ml) ± SD
11. 8-Chloro-5-methoxy-6-methyl-1,4-naphthoquinone	3.84 ± 0.65	9.83 ± 3.41	10.63 ± 1.56	8.66 ± 0.96
12. 5-Ethoxy-8-chloro-6-methyl-1,4-naphthoquinone	8.09 ± 1.81	8.33 ± 7.68	9.37 ± 1.83	6.59 ± 1.31
13. 5-Acetoxy-8-chloro-7-methyl-1,4-naphthoquinone	10.04 ± 1.71	6.96 ± 1.32	7.64 ± 1.35	6.76 ± 1.73
14. 5-Acetoxy-7-methyl-1,4-naphthoquinone	3.39 ± 0.60	3.98 ± 3.76	6.49 ± 1.62	5.57 ± 1.66
15. 5-Acetoxy-8-chloro-6-methyl-1,4-naphthoquinone	4.95 ± 1.01	5.77 ± 1.57	7.58 ± 1.63	3.32 ± 2.17
16. 8-Chloro-7-methylnaphthalene-1,2,4,5-tetra-O-acetate	20.90 ± 3.08	21.64 ± 5.75	23.29 ± 0.03	24.37 ± 1.37
17. 7-Methylnaphthalene-1,2,4,5-tetra-O-acetate	30.70 ± 4.86	8.39 ± 4.51	9.39 ± 3.52	24.21 ± 1.38
18. 8-Chloro-6-methylnaphthalene-1,2,4,5-tetra-O-acetate	23.52 ± 4.22	11.42 ± 1.80	21.91 ± 1.41	33.97 ± 1.31
19. 2,5-dihydroxy-7-methyl-1,4-naphthoquinone	2.99 ± 0.52	1.09 ± 0.22	4.74 ± 1.35	1.39 ± 0.75
20. Doxorubicin	0.36 ± 0.18	0.0086 ± 0.0011	0.0085 ± 0.0045	0.0088
21. Zearalenone	2.62 ± 1.15	1.63 ± 0.30	1.68 ± 0.10	2.43 ± 0.23

4.3.2 Cytotoxicity on U937 cells

The comparative growth inhibition results on U937 cells (Figure 4.5) suggest that compounds 1-4 were the least cytotoxic, whilst the other compounds showed much greater cytotoxicity. Compound 5 had the highest cytotoxicity followed by compound 6 and 19.

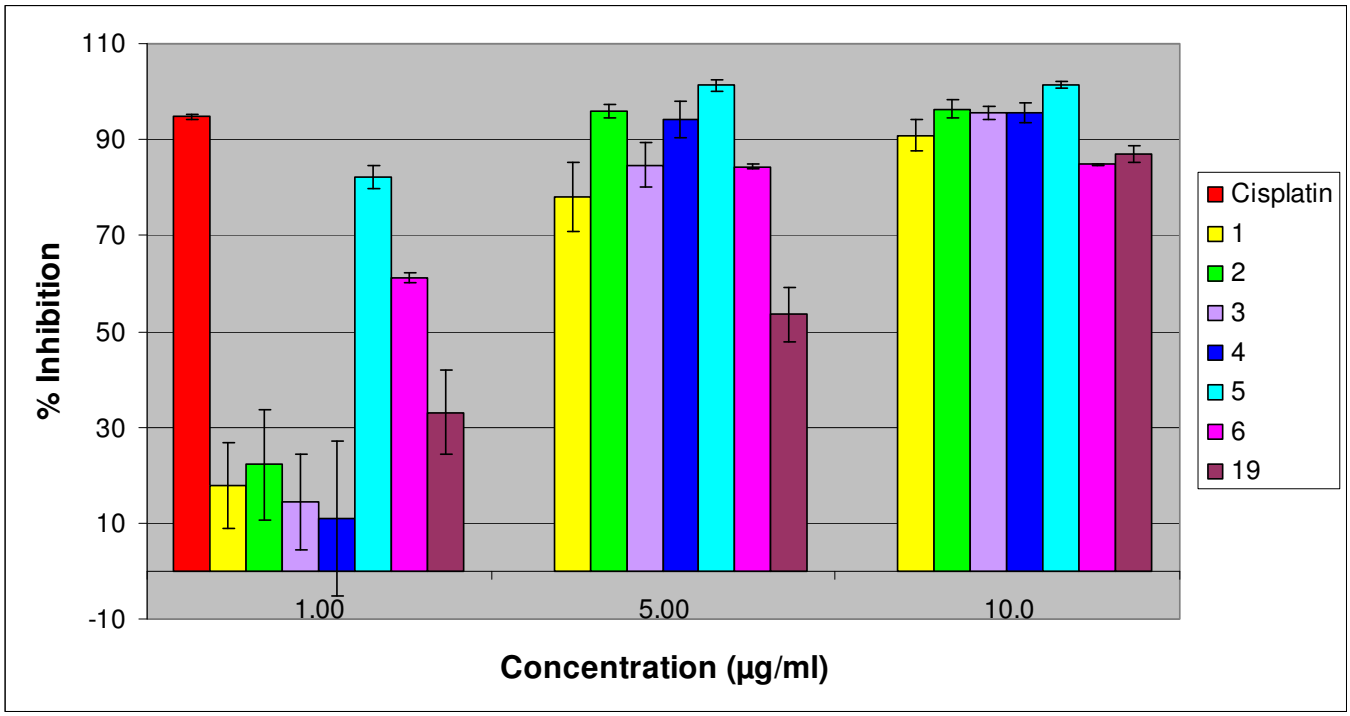

Figure 4.5 Percentage inhibition of selected compounds on U937 cells. Bars and error bars indicate mean ± SD of quadruplicates U937

4.3.3 Cytotoxicity on PBMCs

The comparative growth inhibition results on PBMCs (Figure 4.6) suggest that compounds 3 and 19 were the least cytotoxic, whilst the other compounds showed much greater cytotoxicity at the lower concentrations tested (Table 4.3).

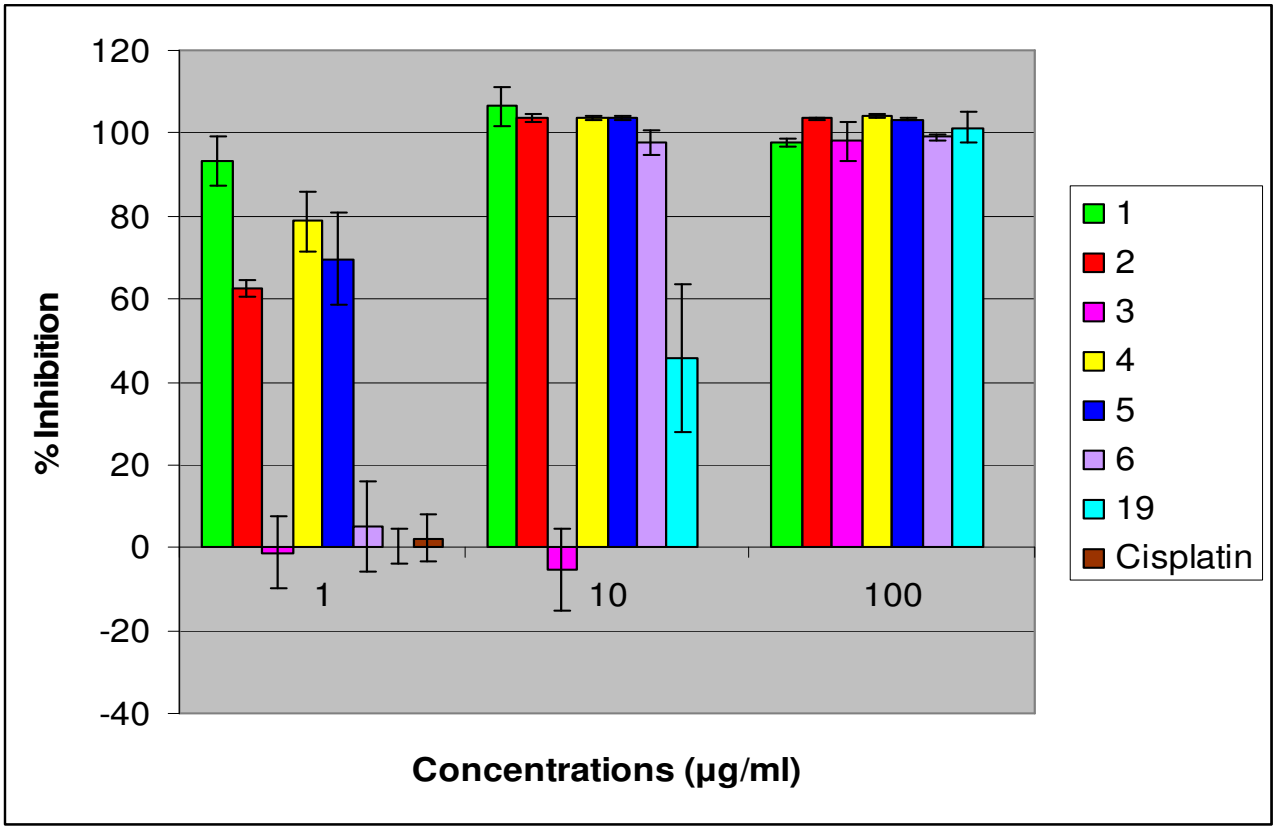

Figure 4.6 Percentage inhibition of selected compounds on peripheral blood mononuclear cells. Bars and error bars indicate mean ± SD of quadruplicates.

Table 4.3 Cytotoxic activity of 8-fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone, 5-hydroxy-7-methyl-1,4-naphthoquinone (7-MJ) and 2,5-dihydroxy-7-methyl-1,4-naphthoquinone on peripheral blood mononuclear cells.

Treatment	**(Peripheral blood mononuclear cells) Lymphocytes IC_{50} (µM)**
Compound 3	188.70
Compound 6	18.40
Compound 19	53.97

4.4 Discussion

4.4.1 Cytotoxicity on four human cancer cell lines

From the synthesized compounds the 1,2,4,5-tetra-*O*-acetate derivatives: compounds 17, 16 and 18 gave the least anticancer or cytotoxic activity, mostly with activity above 20 µg/ml, of all the synthesized compounds on all the cancer cell lines except compound 17 (without the Cl at the 8 position) which had higher cytotoxic activity on the HeLa and SNO cancer cell lines, but was also the least cytotoxic on the MCF-7, IC_{50} = 30.70 ± 4.86 µg/ml. Of the 1,2,4,5-tetra-*O*-acetate derivatives it is also has the best cytotoxic activity and specificity towards the DU145 cells with an IC_{50} = 24.21 ± 1.38 µg/ml. Therefore, substituting compound 6 (the parent) with 1,2,4,5-tetra-*O*-acetate groups did give less cytotoxicity, but also the least for anticancer activity and were not worth further investigating. The 5-ethoxy derivatives, compounds 8, 12 and 10 had anticancer activity of 11.10 ± 2.00 µg/ml and less on all four cancer cell lines, with compound 10 (without the Cl at the 8 position) the best anticancer activity of the 5-ethoxy derivatives with a value as low as 3.25 ± 1.24 µg/ml on DU145 cells. Substituting with 5-ethoxy led to enhanced cytotoxic activity than the 1,2,4,5-tetra-*O*-acetate derivatives, and as a result thereof to greater anticancer activity. The novel compound 11 and the other two 5-methoxy derivatives, compounds 9 and 7, had activity of 10.63 ± 1.56 µg/ml and less on the four cell lines. Compound 9 had the best cytotoxic activity of the on MCF-7 cells with an IC_{50} = 3.84 ± 0.65 µg/ml. But of all the 5-methoxy derivatives, compound 9 (without the Cl at the 8 position) had the least cytotoxic activity of IC_{50} = 3.10 ± 0.76 µg/ml on MCF-7 cells. Subsequently it were concluded that compounds without a Cl functional group at the 8 position had less cytotoxic activity and consequently more anticancer activity.

Compound 3 had the best cytotoxic activity on all the cell lines with an IC_{50} value on HeLa cells IC_{50} =2.11 ± 0.67 µg/ml and had activity of 4.19 ± 0.35 µg/ml on MCF-7 cells and less than that on all four the other cell lines. The introduction of a fluorine atom into antibiotic quinolones has been shown to enhance their activity (Domagala *et al.*, 1986; Miyamoto *et al.*, 1990), and now the same was found with its cytotoxic activity. The other 5-hydroxy (8-Halogen substituted) derivatives, compounds 1, 4 and 2 also had cytotoxic activity on all

the cell lines with 6.57 ± 0.88 µg/ml and less on all four the tested cell lines, except compound 2 which had less cytotoxic activity on DU145 cells with an IC_{50} value of 12.37 ± 1.25 µg/ml.

Previous studies have shown that mono- or dihydroxy substitution (at C5 or C5 & C8 positions in the aromatic ring) of naphthoquinones results in higher toxicity as compared to the parent 1,4-naphthoquinone due to increased efficiency of redox cycling (Ollinger & Brunmark, 1991). The 2,5-dihydroxy substitution of compound 19 consequently led to the greatest toxicity of all the derivatives on HeLa cells with an IC_{50}= 1.09 ± 0.22 µg/ml (utmost cytotoxic) while the parent compound 6 was less cytotoxic than several of these derivatives. Compound 6 had its highest cytotoxic activity and specificity towards DU145 cells, IC_{50}= 2.24 ± 0.27 µg/ml, but it ranged between 2.24 ± 0.27 and 15.33 ± 4.32 µg/ml, on the four cell lines. In this study higher cytotoxicity were found in most of the substituted compounds, except compounds 16-18 and others on some of the selected cell lines, than was found in compound 6 (the parent) except on DU145 cells where compound 6 had an IC_{50} = 2.243 ± 0.271µg/ml. However, the positive controls: doxorubicin was much more effective than any of the synthesized derivatives with its IC_{50} values between 0.0085 ± 0.0045 and 0.36 ± 0.18 µg/ml. Zearalenone had less cytotoxic activity than doxorubicin, IC_{50} values ranging between 2.62 ± 1.15 and 1.63 ± 0.30 µg/ml on MCF-7 and HeLa respectively.

Some naphthoquinones and naphthoquinone derivatives that were proved to have antitumour activity, by one mechanism of action, is the stabilization of the cleavable complexes which were formed by topoisomerase II (topo II) along with DNA leading to apoptosis (Chen *et al.*, 2004). Therefore several compounds with the lowest IC_{50} values were selected for further investigation for the mechanism of action used by these compounds.

4.4.2 Cytotoxicity on U937 cells

Compounds 1-6 and 19 were selected based on their toxicity, and were tested on U937 cells (Figure 4.5). All these compounds were toxic at 5 and 10 µg/ml. Cisplatin, the

positive control, had the highest toxicity/percentage of inhibition even at 1 µg/ml. Compound 5 was the most toxic of all the selected compounds, and the other compounds had IC_{50} values between 1 and 5 µg/ml and were therefore less toxic. Compound 4 were the least toxic of the selected compounds.

4.3.3 Cytotoxicity on PBMCs

Compounds 3, 6 and 19 were the least cytotoxic on the PBMCs, whilst the other compounds showed much greater cytotoxicity. Compound 6 had the lowest IC_{50} at 18.40 µM and was followed by compound 19 IC_{50} at 53.97 µM and these compounds (3 and 6) was therefore more toxic towards PBMCs than compound 3 which had an IC_{50} of 188.70 µM (Table 4.3). The IC_{50} values of the other compounds (compounds 1, 2, 4 and 5) were less than 1 µM, the lowest concentration tested, and therefore very cytotoxic towards PBMCs (Figure 4.6).

4.5 References

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CHAPTER 5

Isolation of the bioactive compounds from *Foeniculum vulgare*

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Isolation of the bioactive compounds from *Foeniculum vulgare*

5.1 Introduction

The skilful application of fractionation methods exploits the fact that an extract can be separated into groups of compounds sharing similar physico-chemical characteristics and this process is called fractionation and can be carried out in various ways, each, of which group compounds according to one or more particular feature such as solubility, size, shape, electrical charge and several other features which may influence grouping (Houghton & Raman, 1998). By the application of fractionation methods many different fractions of a single extract can be obtained which will contain different components.

5.1.1 Chromatography

Chromatography is one of the most widely used techniques in the fractioning of techniques. The major uses of chromatography are: as an analytical method to quantify a certain compound, and in the isolation of compounds for identifying. All chromatographic techniques are based on a method that uses stationary and mobile phase. The compound has different properties for each of them making the separation of compounds with different

properties easy. Compounds that move slowly through the column have a high affinity for the stationary phase, and a compound that moves fast or at a rapid pace have high affinity for the mobile phase. There are quit a few principles involved in the separation of compounds from each other in a mixture. These are adsorption, partitioning, reversed-phase partitioning, ion exchange and affinity.

5.1.4 Steps for isolation

There are sequences of events that need to be followed to get a usable drug from any sample. According to Houghton & Raman (1998) the steps involved are as follows:

- Step 1 Preparation of sample in form suitable for tests.
- Step 2 Testing of sample, preferably in a range of conditions.
- Step 3 If sample gives a positive reaction to test, fractionation of the sample is carried out.
- Step 4 Testing of fractions.
- Step 5 Fractions showing activity are further fractionated, usually by a different method than that first employed.
- Step 6 Testing of each new fraction.
  (NB steps 5 and 6 may be repeated several times until one or more pure compounds are obtained which display activity.)
- Step 7 Elucidation of the molecule structure of the active molecules.
- Step 8 Determination of the concentration of the active compound necessary for a defined biological activity to be observed.
- Step 9 Determination of the amount of active compound present in an active amount of the original extract and comparison with the value found in step 8.
- Step 10 Isolation, synthesis and testing of related compounds.
- Step 11 Investigation of mode of action and metabolism of the active component.

The time-tested method, bioassay-guided fractionation, has enhanced success in the natural product discovery field. It however remains subjective even though it is the 'best' bioassay system for examining cancer chemoprevention (Park & Pezzuto, 2002). A suitable number of lead starting materials can be identified by means of *in vitro* assays and

it also enable the procurement of active agents through bioassay directed isolation in a fixed period of time from a realistic number of sources (Park & Pezzuto, 2002). Numerous classes of cytotoxic compounds with novel antitumour activities were isolated from medicinal herbs/plants via bioactivity-directed fractionation; these include polyphenolic compounds, sesquiterpene lactones, ligans, triterpene glucosides, flavonoids, colchicines derivatives, and quinine derivatives (Lee, 1999).

5.3 Materials and Methods

5.3.1 Collection of plant material

Fennel seeds were bought from a local shop, and were identified at the H.G.W.J. Schwelckerdt Herbarium (PRU) of the University of Pretoria.

5.3.2 Isolation of bioactive compounds

5.3.2.1 Preparation of extract

Two kilogram (kg) of *Foeniculum vulgare* seeds were ground with a kitchen grinder until and was extracted with 1 litre (l) of ethanol, then left for 24 hours at room temperature while constantly stirring. The extracts were then filtered after which the rest of the plant material was extracted with equal amount of fresh solvent. This procedure was repeated three times. The filtered extracts were collected after filtration and concentrated under reduced pressure to yield 70 g of total extract.

5.3.2.2 Column chromatography

The total extract of 70 g was applied to silica gel column chromatography using hexane and ethyl acetate in different ratios ((9:1); (8:2), (7.3), (0.100)) as eluent. Similar fractions were colleted together according to the thin layer chromatography (TLC) profiles developed with 0.34% vanillin in 3.5% sulphuric acid in methanol.

Twelve final fractions from column were obtained and tested for their anticancer activity on HeLa cells. Based on the results fraction eight (443 mg) which exhibited the highest cytotoxicity was fractionated on silica gel column with hexane: ethyl acetate at ratios of (8:2), (7.3) and (0.100) again as eluent (Figure 5.1). A series of columns silica gel (1 column) with hexane: ethyl acetate as eluent and shephadex columns (2 columns) with 100% ethanol as eluent.

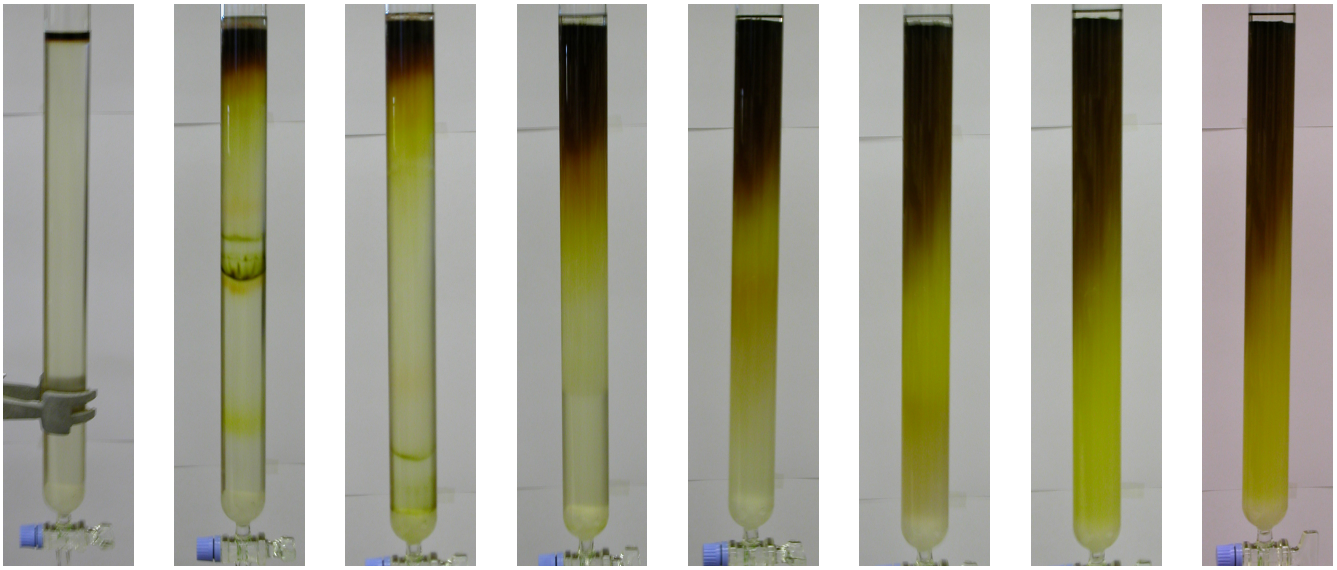

Figure 5.1 Silica gel column of the *F. vulgare* ethanol extract.

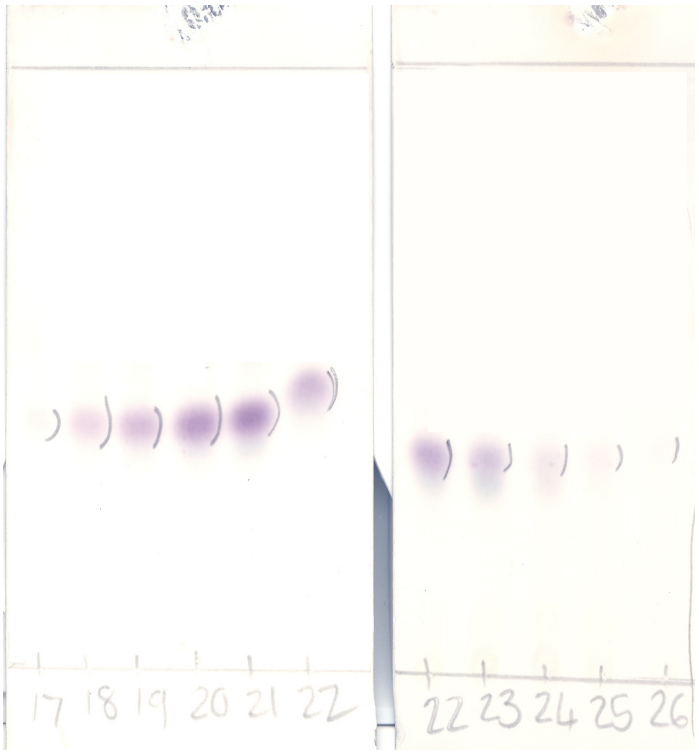

Figure 5.2 TLC plates (hexane: ethyl acetate (6:4) as eluent) after treatment with Vanillin in sulphuric acid (H_2SO_4),

The second total ethanol extract of 249.645g was also applied to silica gel column chromatography using hexane and ethyl acetate in different ratios ((9:1); (8:2), (7.3), (0.100)) as eluent. Twenty-one (1 litre) fractions were collected. All the fractions were spotted five times on separate TLC plates and developed with different solvents as mobile phase; hexane: ethyl acetate (7:3), (2:8) and (1:1) and dichloromethane: methanol (95:5) and (90:10). Fraction 18 was selected for further isolation and was subjected to a sephadex column (LH x 20, Sigma-Aldrich, South Africa) with 100% ethanol as eluent. Thereafter another two sephadex columns and a silica gel column followed after which the selected fraction was subjected to HPLC.

5.3.2.3 Identification of the isolated compounds

The first compound (syringin) was isolated as a amorphous powder, the ^{1}HNMR spectrum exhibited singlet integrated for 6H (2xOMe) at 3.85, multiple's signals at 3.00-3.50 (4H, H-2", 3", 4", 5"), two protons at 3.64, 3.77 (dd each H-6"), another two protons at 4.21 (*dd, J*, = 5.4, 1.2 Hz, H-1), H-1'' anomeric proton at 4.87, two proton signals at 6.30 (1H, *dt*, *J*, =

16.2, 5.0 Hz, H-2), 6.54 (1H, *d*, *J*, = 16.2 Hz, H-3) and 6.7s (2H, *s*, H-2', 6'). The ^{13}CNMR (50 MHz, MeOH-d_4): δ 56.1, 61.6 (2 x OMe), 61.6 (C-6"), 62.6 (C-3'), 70.4 (C-4"), 74.7 (C-3"), 76.8 (C-2"), 77.4 (C-5"), 104.39 (C-1"), 104.52 (C-2, C-6), 129.1 (C-2'), 130.0 (C-1'), 132.0 (C-1), 135.1 (C-4) and 153.4 (C-3, C-5) (Wazir *et al.*, 1995).

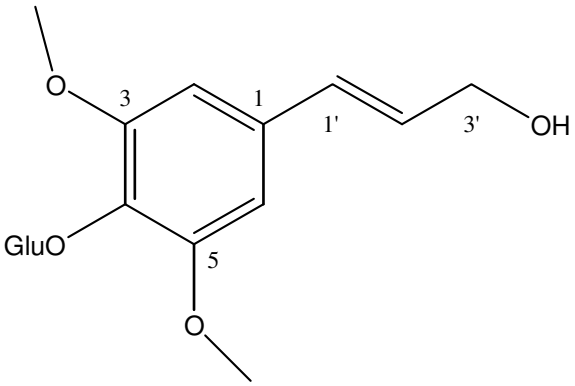

Figure 5.3 Syringin the first isolated compound.

The second compound was (4-methoxycinnamyl alcohol or 3-(4-methoxyphenyl)-2-propen-1-ol) was islolated as colorless crystals mp 130–132 ◦C; IR (KBr): 3345, 2924, 1604, 1243, 1018 cm−1; the ^{1}HNMR(CD_3OD) showed signals at *δ* 3.76 (3H, s, OMe), 4.18 (2H, d, *J* = 5.9 Hz, CH_2-1), 6.20 (1H, dt, *J* = 5.9, 16.0 Hz, CH-2), 6.48 (1H, d, *J* = 16.0 Hz, CH-3), 6.85 (2H, d, *J* = 8.8 Hz, CH-5,6), 7.31 (2H, d, *J* = 8.8 Hz, CH-7,8). According to the data demonstrated by the ^{1}H NMR we concluded that the compound 2 is 4-methoxycinnamyl alcohol (Akita *et al.*, 2006).

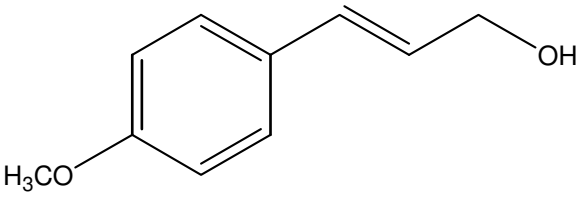

Figure 5.4 Second isolated compound 4-methoxycinnamyl alcohol.

5.3.2.4 Culture of cancer cells

The procedures are all the same as for the naphthoquinone derivatives that were tested on the same cancer cell lines, and the same concentrations were tested as for the naphthoquinone derivatives.

5.3.2.5 Cytotoxicity of various fractions from *F. vulgare*

The cytotoxic activity of the first 12 fractions of the ethanol extract was determined on HeLa cells (Table 5.1). Two fractions (1 and 2) had cytotoxic activity of larger than100 µg/ml. All the other fractions had various cytotoxic activities.

Table 5.1 The IC_{50} values of various fractions from column on the HeLa cell line.

Fractions of the ethanol extract of *F. vulgare*	**HeLa**
	IC_{50} (µg/ml) ± SD
1	>100
2	>100
3	38.55 ± 3.235
4	50.04 ± 2.646
5	52.43 ± 3.574
6	31.26 ± 3.965
7	31.13 ± 3.670
8	20.98 ± 4.480
9	36.91 ± 7.150
10	53.24 ± 7.955
11	56.04 ± 13.130
12	48.46 ± 9.500

5.3.2.6 Cytotoxicity of the compounds Isolated *F. vulgare*

The compounds which were isolated from the ethanol extract of *F. vulgare* were tested for their cytotoxicity on MCF-7, HeLa and DU145 (Table 5.2).

Table 5.2 The IC_{50} values of the compounds tested on the selected human cancer cell lines

Isolated compounds	**MCF-7**	**HeLa**	**DU145**
	IC50 (µg/ml) ± SD	**IC50 (µg/ml) ± SD**	**IC50 (µg/ml) ± SD**
Siringin	21.88 ± 0.13	10.26 ± 0.18	>100
4-methoxycinnamyl alcohol	14.21 ± 0.16	7.82 ± 0.28	22.10 ± 0.14

5.3.2.7 Cytotoxicity of the compounds Isolated *F. vulgare* on U937 cells

The compounds which were isolated from the ethanol extract of *F. vulgare* were tested for their cytotoxicity on U937 cells, and on PBMCs following the methods as described previously.

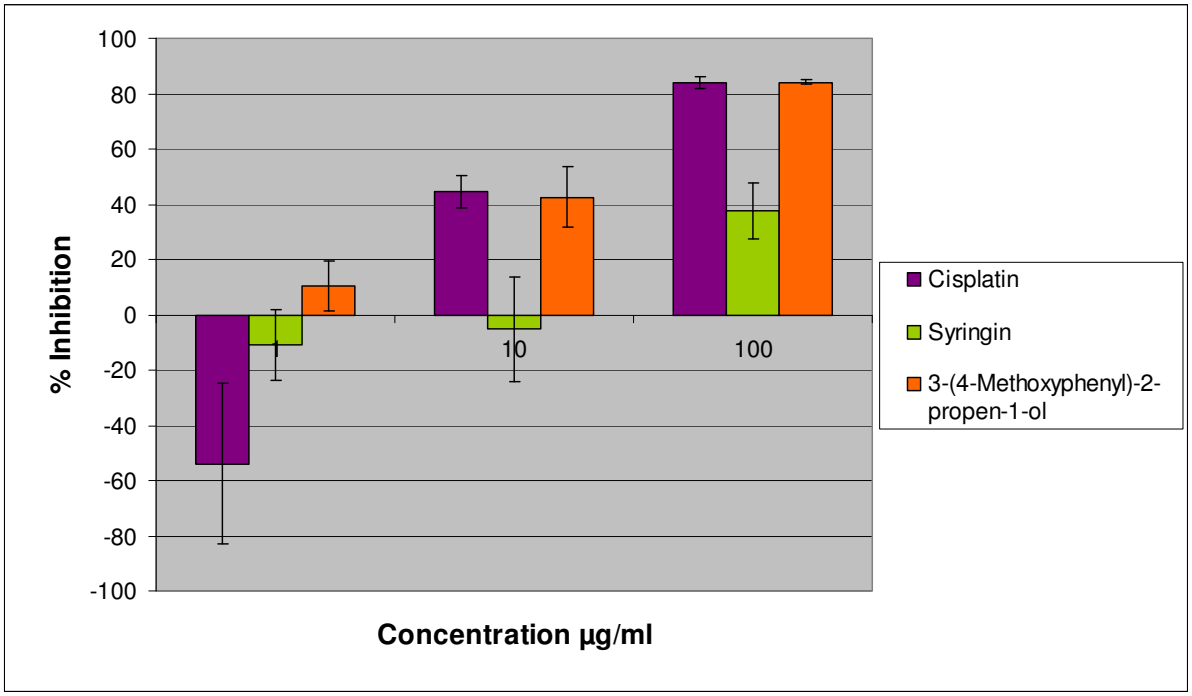

Figure 5.5 Percentage inhibition of selected compounds on U937 cells. Bars and error bars indicate mean ± SD of quadruplicates.

5.3.2.8 Cytotoxicity of the compounds Isolated *F. vulgare* on PBMCs

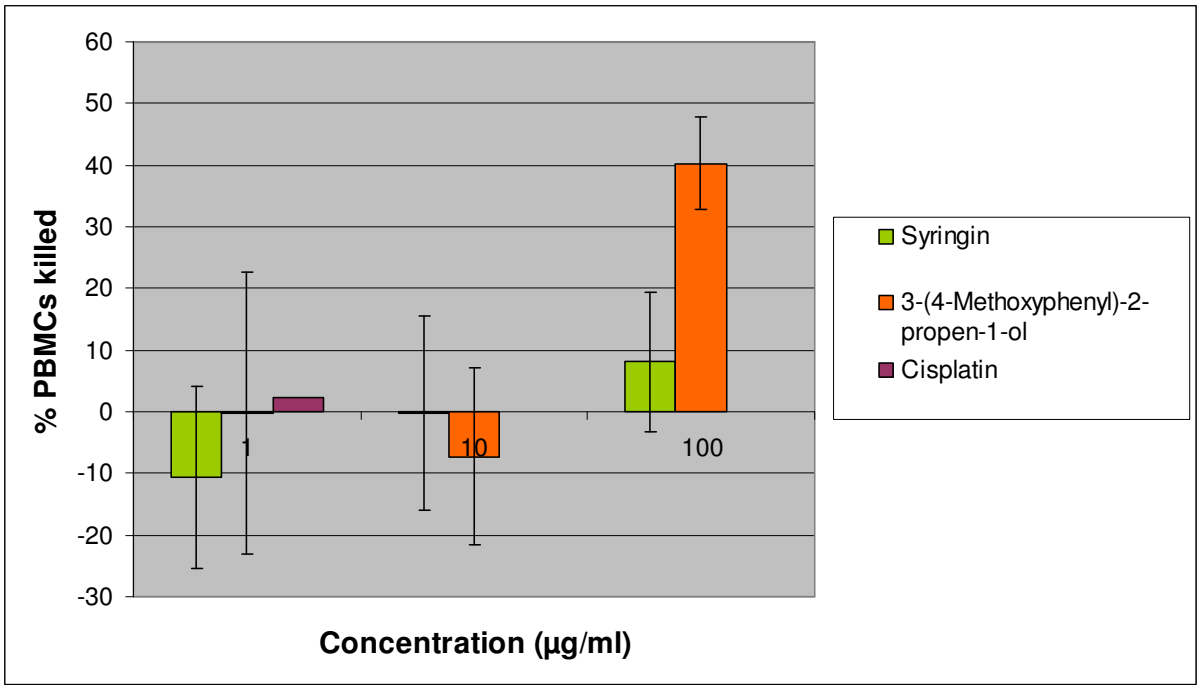

Figure 5.6 Percentage inhibition of selected compounds on peripheral blood mononuclear cells. Bars and error bars indicate mean ± SD of quadruplicates.

5.4 Discussion

5.4.1 Cytotoxicity of the first fractions from *F. vulgare* (Bioassay guided isolation)

Fractions one and two didn't have any cytotoxic activity on HeLa cells at the highest concentration tested (100 µg/ml), and are in fact stimulating the growth of HeLa cells (Table 5.1). The other fraction 3 to 12 had cytotoxic activities ranging between 56.04 ± 13.130 and 20.98 ± 4.480 (Table 5.1). Fraction 11 had the second least cytotoxic activity of 56.04 ± 13.130. According to the guidelines from the American National Cancer institute the limit for crude extracts and fractions for anticancer potential are less than 30 µg/ml, after a 72 hour of exposure. Only fractions seven and eight had cytotoxic activity less than 30 µg/ml. Fraction eight had the best cytotoxic activity and was therefore selected for further investigation.

5.4.2 Cytotoxicity of the compounds isolated from *F. vulgare*

The American National Cancer Institute's guidelines set the limit of activity for pure compounds less than 10 µg/ml, after an exposure time of 72 hours. 4-Methoxycinnamyl alcohol had high cytotoxic activity on HeLa cells at 7.82 ± 0.28 µg/ml (Table 5.2). On the other two cell lines the results were less cytotoxic at 14.21 ± 0.16 on MCF-7 and 22.10 ± 0.14 on DU145 cells respectively.

According to a previous report no cytotoxic activity were found for syringin, against HeLa, A549, and HCT116 cancer cell lines at the highest concentration tested (30 µg/ml). There are however, patents existing on the anticancer activity of this compound on MCF-7 and SNO cells. In this study syringin had high cytotoxic activity on HeLa cells at 10.26 ± 0.18 µg/ml, and less cytotoxic activity on MCF-7 cells at 21.88 ± 0.13 µg/ml (Table 5.2). It is however not toxic towards the DU145 cells with and IC_{50} of larger than 100 µg/ml the highest concentration tested. Syringin was also previously tested *in vitro* for its antioxidant activity against DPPH and since it do not have any free phenolic groups showed an IC_{50} > 500 µM (Pauletti *et al.*, 2006). .

5.4.3 Cytotoxicity of the compounds Isolated *F. vulgare* on U937 cells

Cisplatin, the positive control, had high toxicity on the U937 cells at 5.71 ± 0.13 µg/ml. 4-Methoxycinnamyl alcohol had higher cytotoxic activity than cisplatin, with an IC_{50} value of 3.550 ± 0.18 µg/ml. At the concentrations of 10 and 100 µg/ml 4-methoxycinnamyl alcohol was comparable to that of cisplatin. Syringin however, had much lower cytotoxicity on the U937 cells than 4-methoxycinnamyl alcohol, at 91.14 ± 0.63 µg/ml.

5.4.4 Cytotoxicity of the compounds Isolated *F. vulgare* on PBMCs

Syringin also had the lowest cytotoxicity on the PBMCs, at all the concentration tested. Both syringin and 4-methoxycinnamyl alcohol were not cytotoxic at concentrations of 1 and

10 µg/ml on the PBMCs when compared to cisplatin. At 100 µg/ml, 4-methoxycinnamyl alcohol had the highest toxicity towards the PBMCs.

Based on all the results 3-(4-methoxyphenyl)-2-propen-1-ol was selected for further investigation to examine the mechanism involved.

5.5 References

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CHAPTER 6

Mechanistic studies of potent anticancer compounds

Chapter 6

Mechanistic studies of potent anticancer compounds

There are different methods to detect whether the compounds induce cell death (specifically apoptosis) and by which mechanism these compounds induce cell death:

6.1 Different methods to detect different types of cell death with the focus on apoptosis

6.1.1 Cell cycle analysis

A crucial part of a cells life cycle is death, together with growth and differentiation (Schwartzman & Cidlowski, 1993). Novel antitumour agents from natural sources were initially discovered by testing them for cytotoxic activity (an assays), *in vivo* (models) or *in vitro* (cell lines growing) until in the 1990s when it was found that these antitumour agents (vinblastine, vincristine, colchicine, combretastatins and maytansine) use also other mechanisms together with exerting their cytotoxic action via interaction with tubulin, which encourage tubili depolymerisation (Cragg & Newman., 2005). With the taxanes and the plant-derived chemotype such as the jatrophane esters, it was revealed that these agents exhibit a different method; microtubules are “bundled” due to stabilization against depolymerization. This further led to the finding of another mechanism exerted by the plant-derived compounds (camptothecin derivatives, topotecan and irinotecan, which exert their cytotoxic action via inhibition of topoisomerse I (Cragg & Newman, 2004).

Mechanism based receptor screens were developed through progress made in the molecular cancer biology. Now anticancer drug discovery basically take place by means of high throughput screening and enhanced molecular target (linked with particular cancers) development, which allows screening of many compounds against a wide variety of targets. These screens nowadays permit probing of interactions connecting large molecules and locating new small natural product molecules as potentially improved chemotherapy drug candidates (Mukherjee *et al.*, 2001). These techniques are greatly used for plant-based medicines which are widely studied. Another extensively studied area is the mechanism of action involved in many plant-based phytochemicals and cancer cells which play important roles in the treatment of cancer and the discovery of new and improved anticancer drugs (Mukherjee *et al.*, 2001). An essential role is played in the regulation of the cell cycle progression by the cyclin-dependant kinases together with their cyclin partners, since they are intimately involved in the majority of cell cycle stages (Cragg & Newman, 2005). The cell cycle progression is delayed or arrested at specific stage/s when their activity is inhibited and a series of interactions occur within the cell cycle which then induced the cell to undergo apoptosis (Newman *et al.*, 2002; Cragg & Newman, 2005).

The human cell life cycle consist of the following stages: (1) proliferation – is a period of normal growth, (2) differentiation – when the tissue matured more of the cells will differentiate into various specialized subsets that are required for the tissue to function and starts producing the chemicals that are required for maintenance or function of the organism; and (3) cell death.

In eukaryotes, cell cycle control is a vital process (Newman *et al.*, 2002). The cell reproductive life cycle has four phases, as taken from Mukherjee *et al.* (2001): (1) G0 phase – is a stage of quiescence, during which the cell carries out its ordinary role for the organism; when there is any proliferation, then purines and pyrimidines, the building blocks for DNA synthesis, must be produced then the cell enters the; (2) G1 phase - in which nucleotides and enzymes are synthesized; (3) the S phase – it is when DNA synthesis occurs; one enzyme responsible for replication of DNA for the new cell that seems to be particularly vulnerable to exogenous plant chemicals is topoisomerase; the next stage is the: (4) G2 phase – when the cell prepares other structures needed for mitosis, (5) the M

phase is mitosis itself, where two daughter cells are produced (Figure 6.1). During the S phase the cell first checks if the DNA was entirely replicated and without damage before it will enter to the M phase (mitosis). Arrest takes place at the checkpoint (mitotic spindle checkpoint) if the spindle wasn't correctly formed or when the chromosomes weren't properly attached. Aneuploidy and/or mutations will probable be introduced if these controls are not employed and the cell divide without it, but before a cell proceed into mitosis, the damaged DNA is repaired during a permitted pausing time at a checkpoint or replication is completed (Newman *et al.*, 2002). Apoptosis occur in mammalian cells which are incapable of repairing the damage, and these controls on the cell cycle might vanish during cancer (Newman *et al.*, 2002).

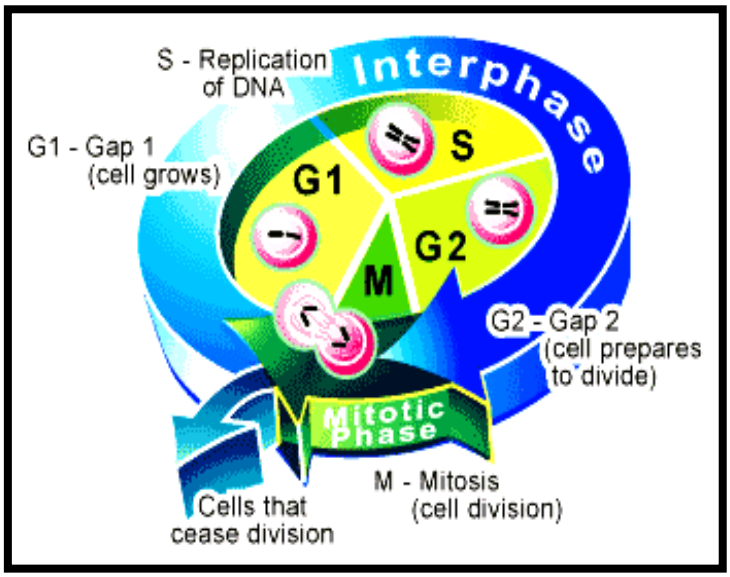


Figure 6.1 The different stages of the cell cycle G1 (cell grows), S (replication of DNA), G2-(cell prepare to divide) and M (cell division).

A cell cycle analysis assay using propidium iodide (PI) was applied for this project to give more detailed evidence for the features of apoptosis induced by the naphthoquinone derivatives. The fluorescent dye most widely-used for staining DNA in whole cells or isolated nuclei is propidium iodide (PI), since the intact plasma membrane of viable cells cannot be cross by it, although it can readily enter through damaged plasma membranes of cells in apoptosis (late stages) or dead cells (Fang, 2006). PI will intercalate into the DNA helix during the cell cycle analysis of fixed permeabilized cells.

6.1.2 Annexin V-FITC/PI

All the defining characteristic aspects of apoptosis, are a complete change in cellular morphology (Kerr, 1991). One of the earliest features of apoptosis is changes in the plasma membrane. Phosphatidylserine is normally located on the inner membrane surface of viable cells. Convincing proof exist that during apoptosis of cells (in many cells if not all) the phosphatidylserine (PS), a plasma membrane phospholipid, is translocated from the inner to the outer leaflet and externalized via an active process based on kinetics and active transport of phosphatidylcholine to the extra cellular environment (Zhu & Chun, 1998; Van Engeland *et al*., 1998).

Bohn and colleagues, was first to report on the protein Annexin V which was isolated from human placenta. They and termed it plavental protein 4 (PP4) (Van Engeland *et al*., 1998). Annexin V was at that time also isolated by Reutelingsperger *et* al., from the umbilical cord by its good quality of anticoagulant activity and identified it as vascular-anticoagulant-α (Van Engeland *et al*., 1998). It was later called Annexin because of the proteins homology with the family of annexin proteins. This family of structurally related proteins are called annexins. These annexins are also able to bind specifically to cellular membranes (Crutz, 1992). Annexin V (a phospholipids binding protein) has anticoagulant activity, it inhibit phospholipids-dependant reactions via high-affinity binding to phospholipids thus inhibiting their catalytic activity and is a monomer with a molecular weight of 35.8 kDa (Maurer-Fogy, 1989; Zhu & Chun, 1998) which can detect cell-surface PS. It has a very high affinity for membranes containing the negatively charged phospholipids such as PS (Darzynkiewicz, 1997a, Koopman *et al*., 1994), specific binding is rapid and contain high calcium-dependent affinities for aminophospholipids (Zhu & Chun, 1998). In the presence of PS liposomes inhibit the binding of annexin V to cell-surface PS. The binding of a annexin V was however found to be unaffected by liposomes having other phospholipids (like phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and sphingomyelin) (Zhu & Chun, 1998).

During apoptosis, Annexin V is used as a probe to monitor change in the distribution of PS in the plasmamembrane (Figure 6.2) and serves as a marker for apoptotic cells. It may be

conjugated to florochromes such as fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The translocation of PS to the exposed membrane surface or extra cellular environment is an early stage in apoptosis, where it serves as a signal for the attack of phagocytic cells. An earlier stage of apoptosis based on nuclear changes such as DNA fragmentation can be detected by FITC coupled to annexin V. Early apoptotic cells will stain annexin-V FITC positive and PI negative where dead cells (necrotic cells) or later stages of apoptosis will stain annexin-V FITC positive and PI positive. It was also found that all cells that show chromatin condensation and DNA fragmentation after initiation of apoptosis strongly stain with annexin V (annexin V-positive cells), while annexin V-negative cells are normal (Koopman *et al.*, 1994).

The Annexin-V FITC detection kit was designed to detect specific biochemical changes in the cell surface membrane which are signature events of early apoptosis and analysis can be done by flow cytometry or by fluorescence microscope. Propidium iodide (counterstain) was also given in the kit and it was used to distinguish apoptotic cells with intact membranes from lysed, necrotic cells. Therefore, staining cells with a combination of Annexin-V FITC and PI allow flow cytometry to detect nonapoptostic live cells (annexin V negative/PI negative), early apoptotic cells (annexin V positive/PI negative), late apoptotic cells (annexin V positive/PI positive) and necrotic cells (annexin V negative/PI positive) (Darzynkiewicz, 1997). The assay can however not differentiate between cells that have died as a result of the necrotic pathway from those that have undergone apoptotic death, since the combination of annexin V and PI will stain both dead cell types positive.

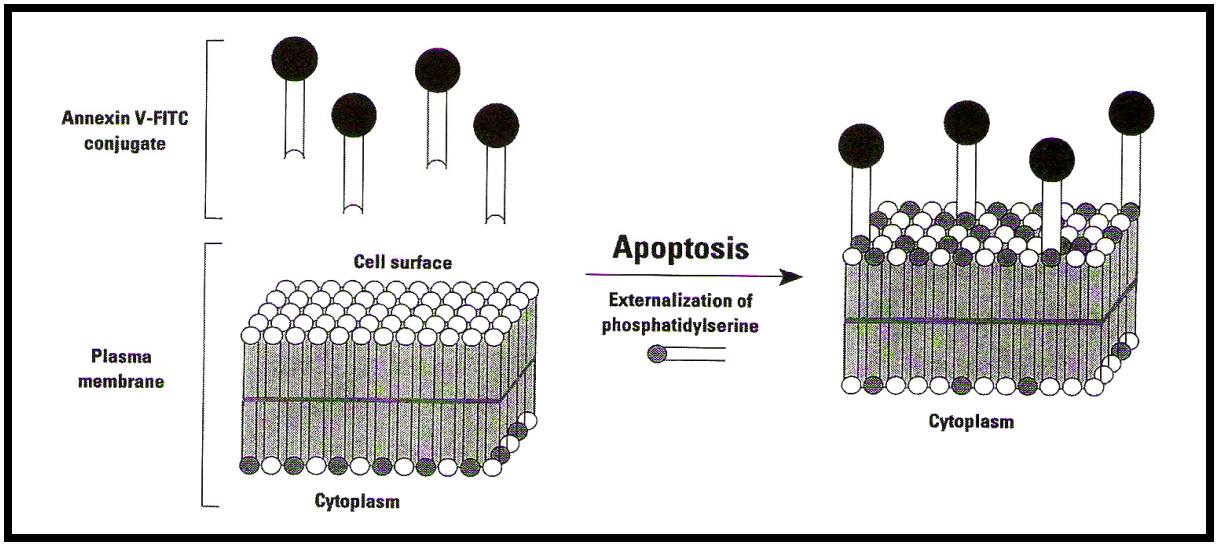


Figure 6.2 Biological basis of annexin V-FITC binding assay (Zhu & Chun, 1998).

Cell populations that were detected were as follows: the viable cells were; the cells that were in the metabolically active stages of apoptosis will stain with Annexin V-FITC and not with the PI. Cells that were with compromised membranes were stained with both Annexin V-FITC and PI. Apoptotic bodies in the late-stages may enter secondary necrosis if they were not detected through phagocytosis, especially in suspension cultures and therefore stain with PI positive and not with annexin V-FITC.

For detecting apoptosis in this project the plasma membrane alteration assay with annexin-V FITC, and the cell viability assay with PI staining have been adopted. The optical properties of the fluorescent probes are given in Table 6.1.

Table 6.1 Optical properties for the fluorescent probes

Agent	Excitation	Emission
FITC	488nm	525nm
PI	536nm	620nm

6.1.3 Caspase 3 and 7 activity

Apoptosis or programmed cell death proceeds through an ordered series of steps (Slee *et al.*, 1999). A lot of work has been done on identifying endonucleases capable of cleaving DNA at internucleosomal sites (Martin & Green, 1995). Given that, in most forms of apoptosis (but not all) DNA degradation is a fixed trait (Martin & Green, 1995). It consists of the following different phases (Slee *et al.*, 1999; Huppertz *et al.*, 1999):

1. Initiation phase - the time during which the cell obtains the initial cell death activation signal and include the induction of the cascade which leads to the first proteolytic events.
2. Commitment phase - in which the cell becomes committed to apoptosis and these death signals become irreversible since they degrade several protein.
3. Amplification phase - which involves the multiple caspase activation, it involves the activation of execution caspases, recruited to assist in the destruction of the cell.

4. Demolition phase - consists of direct activation of caspase-mediated destruction or via activation of other enzymes (CAD/DFF45) which leads to dismantling of the cellular structures and in the end the collapse of the nucleus and the cell itself.

Protease activation is able to initiate activation and processing of other similar molecules or other proteases (such as calpain) which result in an amplified and escalating protease cascade that is vital for the processes of clotting and complement activation (Martin & Green, 1995). Besides it is a familiar topic in protease function. Apoptosis can be activated by means of a range of interactions and stimuli, but the pathway ultimately lead to the activation of the caspases. The caspase-family can be divided into three subfamilies based on their phylogenetic comparisons of structural homology and sequence similarities (Kidd 1998):

1. ICE-like caspases (includes caspases 1, 4, and 5)
2. CPP32-like caspases (includes caspases 3, 6, 7, 8, 9, and 10)
3. ICH-1 subfamily (caspase2).

The CPP32-like caspases are further classified as execution or initiator caspases due to the role that they play in the apoptosis cascade (Huppertz *et al.*, 1999).

During the apoptotic cell death pathway a family of enzymes, the caspases (Cysteinyl Aspartate-Specific Proteases) play a crucial role and mediate important key proteolytic events (Saleh *et al.*, 2004). As signified by caspase, a conserved cysteine residue functions in catalysis and substrate cleavage occurs on the carboxyl-side of an aspartate residue (Biomol AK-118, 2002). All the caspases are synthesized as single polypeptide zymogens which are triggered through proteolytic activation which arise during autocatalysis or by cleavage via an additional or active caspase or by cleavage with several serine proteases such as granzyme B and cathepsin –B. The caspases are now active caspases that were formed from tetramers. These tetramers consist of two large subunits, usually 20kDa, that were formed from the reminder of the zymogen, frequently minus the N-terminal prodomain and two small 10kDa subunits derived from the C-terminal part of the zymogen polypeptide, by cleavage at one site, or two closely spaced sites, C-terminal to the active-site cystein (Biomol AK-118, 2002). The substrate binding site is formed through residues contributed by both large and small subunits. Two binding sites

per tetramer are formed, except caspase 9 since the active mature enzyme, *in vivo,* hold on to the N-terminal prodomain as part of the large subunit which is 35 or 37 kDa.

Maintenance of the functional and structural integrity of the organs and tissues arise from the apoptosis cascade which are a tightly-regulated and controlled homeostatic mechanisms essentially required to manage the balance between proliferative activity and apoptotic activity (Erickson, 1997). Two distinct subfamilies of human caspases exist and they are functionally divided into: those involved in cytokine maturation (Caspase-1,-4 and -5) and those involved in cellular apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10) (Saleh *et al.*, 2004). The highly regulated process of apoptosis can be induced, stimulated and inhibited at different stages as taken from Huppertz *et al*. (1999) follows (Figure 6.3):

(1) Apoptosis can be induced by specific ligand-receptor interactions and these include TNFα and its p55-receptor (TNF-R1), Fas-ligand (FasL) and its Fas-receptor;

(2) Activation of these receptors leads to the formation of a signalling complex that subsequently activates a family of so-called initiator caspases, e.g. caspase 8;

(3) Initiator caspases (Figure 6.4) are responsible for the first proteolytic events e.g. cleavage of cytoskeletal and related proteins including vimentin, and fodrin (a membrane-associated cytoskeletal protein, and these early apoptotic events are thought to be responsible for the characteristic blebbing of the cell surface;

(4) Cleavage of translocase (flippase) and/or activation of scramblase (floppase) leads to a subsequent flip of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which is known to be a very early event during the initiation stages of apoptosis, externalization of phosphatidylserine is used by the cells as a signal for events such as the attraction of macrophages, induction of the coagulation cascade or induction of syncytial fusion;

(5) Initiator caspases (Figure 6.4) are positioned at the top of the caspase hierarchy and subsequently they cleave and thus activate a second subpopulation of caspases known as the execution caspases and irreversible progression of the apoptosis cascade commences with activation of the latter;

(6) Execution caspase (Figure 6.4) activation is tightly regulated by the bcl-2 family of mitochondrial proteins, the members of this family promote (bad, bak, bax, bcl-x_s, bik, hrk), or inhibit (A1, bcl-2, bcl-w, bcl-x_L, bfl-1, brag-1, mcl-1, NR13) cleavage of execution caspases;

(7) Irreversible progression of apoptosis by activated execution caspases (e.g. caspase 3) requires that these enzymes remain active for critical minimum period of time which may vary from minutes to hours depending on size, type and functional state of the cell;
(8) Once activated, the execution caspases, either directly or by means of other proteases, cleave a broad array of proteins critical for cell survival which include intermediate filament proteins (such as cytokeratin 18), nuclear envelope proteins (such as lamins A and B), proteins involved in DNA maintenance and repair (such as poly-(ADP-ribose), polymerase (PARP), enzymes involved in relaxation of the DNA-helix and separation of chromosomes during mitosis (topoisomerase IIα), the catalytic subunit of DNA-dependant protein kinase (DNA-PK_{CS});
(9) Execution caspases activate DFF (DNA fragmentation factor) and other endonucleases resulting in specific fragmentation of DNA;
(10) Finally, execution caspases cause characteristic intracellular shifts and activation of proteins such as of: TIAR (T-cell-restricted intracellular antigen-related protein, possibly involved in cytoplasmic DNA cleavage), which is shifted from a nuclear to cytoplasmic localization, transglutaminase II, which protects the endangered plasmalemma, by cross-linking proteins and forming large sub-plasmalemmal protein scaffolds that is shifted from a more diffuse cytoplasmic localization to directly underneath the plasma membrane.

Many plants and their bioactive compounds possess chemopreventative properties that are able to be used therapeutically and their antiproliferative activity is thought to be related via modulation of cell cycle progression and apoptosis induction which have effects on the cellular signaling processes (Kuo *et al.*, 2005).

Within whole living cells the substrate-based $CR(DEVD)_2$ Biomol CV-Caspase 3 and 7 Detection Kit can detect caspase activity. This kit was used since it is a user friendly kit which allows rapid visualization of the interacellular caspase activity in the particular cell line used. For detection of the caspase 3 and 7 activity the kit make use of the flourophore, cresyl violet coupled to the C-terminus of the optimal tetrapeptide recognition sequence DEVD ($CR(DEVD)_2$) (Biomol AK-118, 2002). Caspase 3 and 7 cleave the DEVD groups which are then detached which then lead to excitement of the mono- and un-substituted cresyl violet fluorophores fluoresce at 550-590nm.

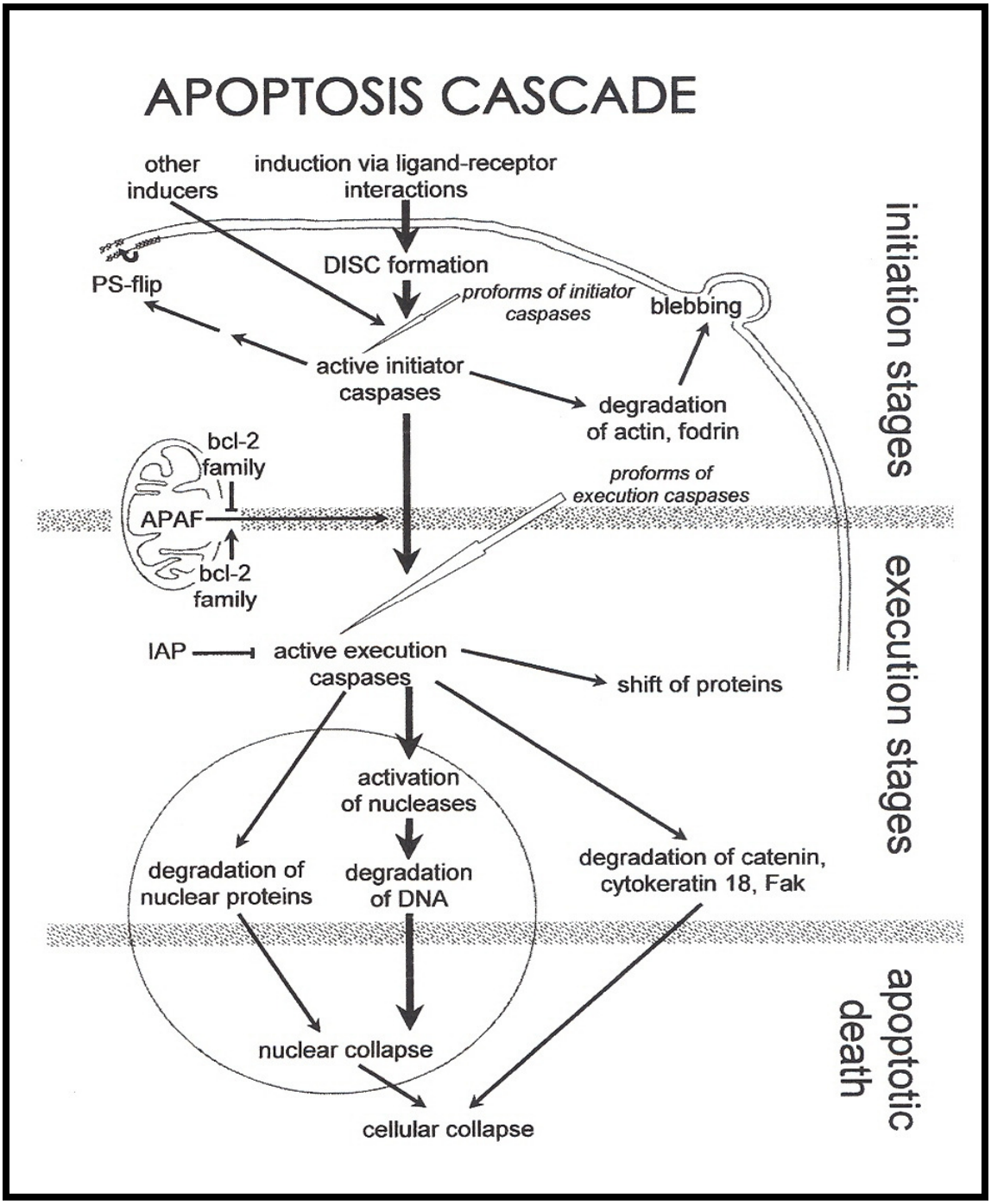


Figure 6.3 The three sequential stages of the apoptosis cascade (Huppertz *et al.*, 1999).

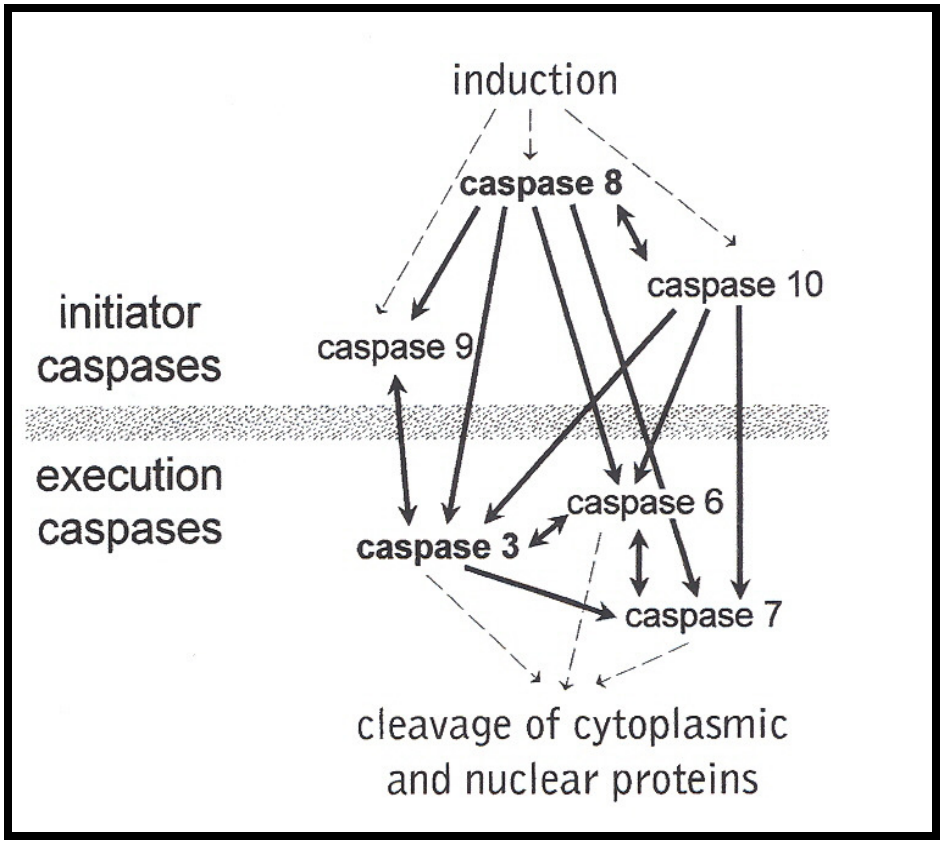


Figure 6.4 Induction of the initiator caspases and activation of the execution caspases which finally leads to apoptotic death as a result of the very complex cascade of events (Huppertz *et al*., 1999).

In the non-fluorescent state this fluorogenic substrate easily enters the cell through effortless infiltration of the cell membrane and the membranes of the internal cellular organelles. The four amino acids (DEVD) caspase are the targeted sequences which are cleaved off in the presence of caspase 3 and 7 enzymes and consequently yield a red fluorescent product (Biomol AK-118, 2002). According to Huppertz *et al*. (1999) caspase 3 was found to degrade a variety of cytoplasmic and nuclear proteins and activate nucleases-thereby inducing degradation of DNA. The active form is located in the nucleus and cytoplasm and the inactive form is localized in the mitochondria and cytoplasm. Caspase 7 was found downstream of the initiator caspases and act as an execution caspase. DEVDase-mediated production of the red fluorophore signals apoptotic activity within that particular cell (Biomol AK-118, 2002). The red fluorescent signal, which have an optimal excitation and emission wavelength pairing of 592 nm and 628 nm, could be detected by use of fluorescence microscopy or 96-well microtiter plate fluorometry.

For this experiment the 96-well microtiter plate fluorometry method were used. The induction of apoptosis could be quantitated as the amount of red fluorescence generated in the induced versus non-induced cell populations (Biomol AK-118, 2002). A higher RFU intensity was generated in cell populations which were in the more advanced stages of apoptosis. It was also important to note that a variable base-level of DEVDase activity was present in a varying quantity in all cell lines, which were seen in the non-apoptotic cell populations and may possibly have been the effect of constitutively synthesized serine proteases.

6.1.4 Acridine orange and ethidium bromide nuclear staining

Cell death is not constantly accompanied by the typical characteristics of either apoptosis or necrosis (Darzynkiewicz *et al.*, 1997a). Previous cell death experiments have explained patterns of morphological and/or biochemical changes which differed from the classical features of neither apoptosis nor necrosis (Darzynkiewicz *et al.*, 1997a). Often cell death revealed features of both apoptosis and necrosis. Hematopoietic lineage cell types have typical features of apoptosis at cell death and are most usually “primed” to apoptosis whereas epithelial type cells death are more complex and occasionally even tricky to classify (Darzynkiewicz *et al.*, 1997a). The pattern of cell death may be especially confusing since several drugs cause apoptosis as a result of the drugs-induced secondary effects on the cell. All of this makes the identification of the characteristic features of apoptosis and necrosis extremely difficult.

The frequency of apoptotic induction of THP-1 and U937 cells following exposure to the selected naphthoquinone derivatives was determined by fluorescence microscopy of nuclear stains, acridine orange and ethidium bromide (Coligan, 1995). The cells were identified as viable, apoptotic, and necrotic as described previously by Stasiauskaite *et al.* (2006).

6.1.5 DNA fragmentation

DNA fragmentation plays a major part in the cell death mechanism since it take place very early (primarily appearing several hours before cell viability starts to decrease) in the

apoptotic process. According to Bortner *et al.* (1995) three types of DNA fragmentation can be distinguished which occur throughout apoptosis: internucleosomal DNA cleavage, fragmentation into large (500-300kbp) lengths and single-strand cleavage events. It is also thought that there is a near-universal occurrence of internucleosomal cleavage in apoptostic cells and therefore it is proposed by Schwartzman and Cidlowski, (1993) that in different cell types there possibly exist a frequent mechanism by which apoptosis occur. In several cell types the presence of zinc (Zn^{2+}) inhibits apoptosis and DNA degradation. DNA degradation in isolated nuclei is also blocked because of Zn^{2+}, which suggests that it inhibits the endogenous endonuclease further highlightening the significance of DNA degradation in apoptotic cell death (Schwartzman & Cidlowski, 1993). Proteases are also capable of contributing to DNA fragmentation through the activation of endonucleases (Martin & Green, 1995). Typical DNA ladders were induced in isolated nuclei in the presence of an additional partially characterized 24 kDa protease (Martin & Green, 1995). On the other hand, no nuclease activity is displayed by protease itself. DNA fragmentation is used as a diagnostic for the occurrence of apoptosis since it can detect internucleosomal chromatin degradation in apoptotic cells which is an identifiable biochemical parameter for the start of apoptosis. Caspase activated DNase (CAD) and DNA fragmentation factor (DFF 40) are the main enzymes responsible for fragmentation during apoptosis and are selectively activated upon cleavage by caspase 3.

Agarose gel ethidium bromide staining of extracted DNA reveals the appearance of nucleosomal DNA which is believed to be the hallmark of apoptosis. Wyllie was first to observed the quantitative measure of apoptosis, internucleosomal DNA fragmentation in apoptotic cells, in 1980 (Martin *et* al., 1994). But it is clear that apoptosis is far more than the degradation of nuclear DNA during apoptosis and is a more complex nuclease-mediated process that proceeds through a well-organized multi-step fashion and not all need to be finished for cell death to occur (Warrington *et al*., 2003). The degradation of DNA start with the generation of high molecular weight fragments of about 300 kb, which are fragmented more to about 50 kb fragments and more fragmentation takes place and in the end giving rise to 10 to 40 kb fragments which in the end give rise to the oligosomal DNA fragments (Warrington *et al*., 2003; Kerr *et al*., 1994). These oligosomal DNA fragments (Warrington *et al*., 2003) and double stranded DNA cleavage observed at linker regions between nuclosomes (180-200 base pair fragments) (Gerschenson & Rotello,

1992) are detectable on conventional agarose gels (gel electrophoresis) as distinctive "DNA ladders", when extracted from apoptotic cells (Figure 6.4). Random cleavage of DNA and degradation of histone occur generally in necrosis. Alternatively to apoptotic cells, DNA extracted from necrotic cell develops as a diffuse smear on DNA electrophoresis (Figure 6.4). Substantial variation exist in the aspect of apoptotic DNA degradation and these factors include things such as the cell type used, the toxic agent involved and even the function of the dose of a given agent in the same cells. According to Warrington and co-workers (2003) variation in the extent and characteristic features of apoptosis may also arise from the fact that cancer cell lines are studied, in which diverse perturbations of apoptotic pathway responses may be a prominent factor not only in the events leading to the generation of such cells, but also their subsequent apoptotic behaviour. Engulfment is also a factor that must be taken in account for because it happens *in vivo* and is excluded when experiments are done *in vitro* and therefore, a different picture can possibly be revealed during *in vitro* studies than what ensues in situ.

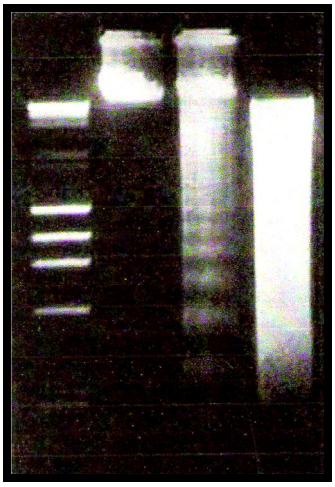


Figure 6.5 Agarose gel electrophoresis of DNA extracted from cultures of P-815 cells. Ethidium bromide stain photographed in ultraviolet light. Lane 1: DRIgest III molecular weight markers; lane 2: control culture; lane 3: culture showing extensive apoptosis induced by heating; lane 4: culture showing massive necrosis 72 hours after repeated freezing and thawing. (Kerr *et al.*, 1994).

The purpose of DNA fragmentation during apoptosis according to Martin *et al.* (1994):

1. may be to facilitate breakdown of the DNA upon uptake of apoptotic cells by phagocytes;
2. alternatively it may serve to destroy the information content of the cell and thus act as an

irreversible step in the process;

3. or it may simply be a by-product of ion redistributions that occur during apoptosis

6.2 Materials and Methods

6.2.1 Cell cycle analysis U937 cells

Compounds having obtained good anticancer activity were selected to further investigate the mechanism of action. U937 cells were grown in 25 cm^2 culture flasks (Nunc) at a density of $1x10^6$ cells/ml in 5 ml RPMI1640 complete medium. All the seeded cells were drawn with serological pipettes from the flasks and transferred to sterile 15 ml tubes. By centrifugation of the tubes at 500 x g for 5 minutes the cells were pelleted. The supernatant was removed with serological pipettes and the pellet loosened by gently tapping the tube. Thereafter, the cells were resuspended in one millilitre complete RPMI1640 medium after which the cell viability and number were determined when they were counted with trypan blue and a hemacytometer. The cells were seeded in complete RPMI1640 medium at a density of $2x10^6$ cells per millilitre in flasks. After a recovery period of 24h, the selected derivatives of 7-methyljuglone, compounds 1-5 and 19 together with 4-methoxycinnamyl alcohol were dissolved in DMSO and compounds 1-4, and 19 were added to the cells to a final concentration of 2 µg/ml and compound 5 and 4-methoxycinnamyl alcohol to a final concentrations of 0.5 µg/ml and 10 µg/ml, respectively. Cisplatin was added as a positive control to give a final concentration of 10µM, and DMSO was used as a negative control. The flasks were further incubated for 24 hours.

The Coulter® DNA Prep™ Reagents Kit (Beckman Coulter) was brought to room temperature before use. Two millilitres were drawn from the treated flasks and centrifuged for 5 minutes at 500 x g at 4°C. The supernatant was discarded and the pellet loosened in 500 µl sheath fluid (Beckman Coulter) and 500 µl lysis buffer added to all the tubes. The tubes were incubated at room temperature for 5 minutes after which 1 ml of PI was added, covered in foil and incubated for 5 minutes at 37°C. The cells were analysed on a Beckman-Coulter FC500 Flow Cytometer. A minimum of 10 000 events were acquired for each sample.

6.2.2 Cell cycle analysis MCF-7 cells

Basically the same method was followed as for the U937 cell cycle analysis, except that only the following derivatives of 7-methyljuglone 2, 3 and 5 were selected based on their activity (IC_{50}) and their quantity for testing on the MCF-7 cells. Cells were plated at 60-70 % confluency in T-25 flasks (Triple Red). After 24 hours one flask were treated with 500 nM of each compound (positive control 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), Taxol and 2-MeOE2) for 48 hours. Control cells were untreated and another was treated with THF vehicle only. To harvest cells for flow cytometric DNA analysis, cells were washed with phosphate buffer saline (PBS) before being trypsinised (0.25 % trypsin 0.05 % EDTA). Medium containing non-adherent cells was also collected and pooled with the trypsinised cells. The cells and PBS washings were pelleted by centrifugation at 500 x g for 5 minutes. The same Coulter® DNA Prep™ Reagents Kit (Beckman Coulter) (prepared as per kit instructions) was used as for the U937 cells and the cells were analysed using a flow cytometer (FACScan, Becton Dickinson).

6.2.3 Annexin V-FITC/PI U937 cells

The same cell suspensions prepared for the cell cycle analysis were used for the annexin V-FITC/PI staining. Reagents of the Annexin V-FITC Kit (Beckman Coulter) were prepared as per kit instructions and kept on ice until further use. Two millilitres were drawn from the treated flasks and centrifuged for 5 minutes at 500 x g at 4°C. The supernatant was discarded and the pellet loosened and resuspended in 1 x binding buffer after which all the tubes were kept on ice. To 100 µl of the cell suspension, 1 µl of annexin V-FITC solution and 5 µl of the dissolved PI were added and vortexed lightly. All the tubes were incubated on ice for 15 minutes in the dark. Thereafter 400 µl of the 1x buffer were added. The samples were analysed on a Beckman Coulter FC500 flow cytometer with a minimum of 10 000 events that were acquired per sample.

6.2.4 Annexin V-FITC/PI MCF-7 cells

The cells were prepared with the same method as for the MCF-7 cell cycle analysis, but using Annexin V-FITC/PI Kit (Beckman Coulter). The flasks were then treated with 500 nM of the positive control 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140) and the selected concentrations for each compound were the same as for the U937 cells. For this experiment only derivatives 2, 3 and 5 of 7-methyljuglone were selected based on their activity (IC_{50}) on the MCF-7 cells. A flask with control cells were also left untreated (only medium) and another were treated with the THF vehicle only. The cells were harvested with the same method as for the MCF-7 cell cycle analysis and were analysed using a flow cytometer (FACScan, Becton Dickinson).

6.2.5 Caspase 3/7 activity U937 cells

The Caspase 3/7 detection kit (Biomol) was used and the reagents were prepared as described in the directions of the kit.

The U937 cells and compounds were prepared the same as for the cell cycle analysis and annexin V-FITC. The compounds were added to the cells and controls prepared and incubated for 24 hours. The flasks were mixed gently by hand so that the cells mix well because they tend to settle while standing. Two millilitres of RPMI 1640 medium containing cells and compounds, positive control, and negative control were removed from each flask and transferred into separate 2 ml Eppendorf tubes. The eppendorf tubes were centrifuged and 290 µl of RPMI 1640 medium were added and the cell suspension were transferred to a black microtiter plate. At least $2x10^5$ cells per 100 µl aliquot was added per well.

Ten microlitres of the 30x $CR(DEVD)_2$ working solution were added to the 290 µl treated cells and mixed gently by aspiring and expelling the cells with a pipette. The black microtiter plate was wrapped in foil and incubated for 120 minutes at 37°C under 5% CO_2. The cells tend to settle and were gently resuspended every 20 minutes to ensure the $CR(DEVD)_2$ is evenly dispersed among all the cells. The plates were read with the ThermoLabsystem Fluoroskan AscentFL fluorescence microplate reader at excitation and emission wavelengths of 544:620 nm and 544:590 nm.

6.2.6 Acridine orange and ethidium bromide nuclear staining U937 and THP-1 cells

The frequency of apoptotic induction of THP-1 and U937 cells following exposure to the selected naphthoquinone derivatives was determined with nuclear stains, acridine orange and ethidium bromide, by fluorescent microscopy (Gu & Belury, 2005). The U937 cells and THP-1 cells were maintained the same as in section 6.2.1. Morphological changes of the U937 and THP-1 cells photographed, fluorescent microscope (Olympus BX40), following exposure to compounds1 to 4, 6 and 19 at final concentration of 2 µg/ml and compound 5 at a final concentration of 0.5 µg/ml, for 24 hours. Equal quantities of cell suspensions (25 µl) were mixed with a mixture of acridine orange and ethidium bromide (100 µg/ml). On microscope slides 15 µl of the mixtures were viewed under a fluorescent microscope. Each microscope slide was viewed immediately after preparation. Two hundred cells per replicate were counted and each compound was done in triplicate. During this study the cells were scored as viable, apoptotic or necrotic as judged by nuclear morphology and membrane integrity (Gu & Belury, 2005). Viable normal cells were easily distinguished with their bright green nuclei and intact structure where the viable apoptotic cells were bright green with their highly condensed or fragmented nuclei. Non-viable normal cells were identified with their bright orange chromatin with organized structure where as the non-viable cells with apoptotic nuclei were with bright orange chromatin which is highly condensed or fragmented (Gu & Belury, 2005).

6.2.7 DNA fragmentation U937 cells

To determine whether the selected naphthoquinone compounds induced U937 cells death was caused by apoptosis, we examined the DNA fragmentation. U937 cells were treated with 4-methoxycinnamyl alcohol at a concentration of 10 µg/ml for 12, 24, 48, 72 and 96 hours after which the modulations of DNA fragmentation by these compounds were detected by using a FexiGene DNA kit (QIAGEN). Untreated cells was incubated at the same experimental conditions and times to serve as a control. At the end of each time of treatment the DNA was extracted following the manufacturer's instructions. The DNA was resolved on a 1 and 2% TAE (tris/acetate/EDTA) electrophoresis agarose gel (White Sci).

6.3 Results

6.3.1 Cell cycle analysis

6.3.1.1 Cell cycle analysis U937 cells

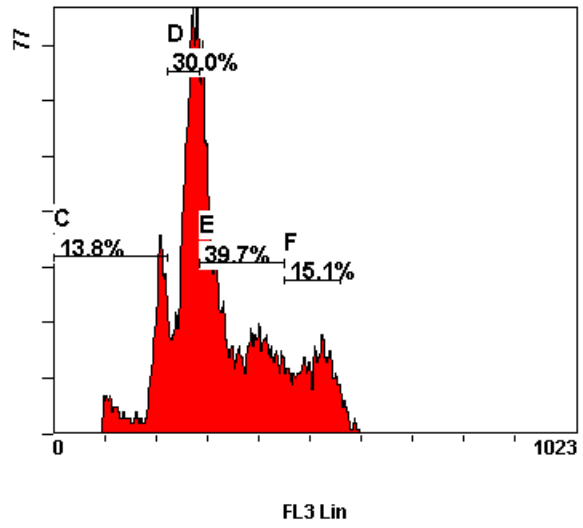
To establish whether the compounds inhibited cell growth by blocking cells in a certain phase of the cell cycle and/or inducing apoptosis, cellular DNA was stained with PI and cells analyzed using flow cytometry after 24 hours (Figure 6.6 and Table 6.2) and 48 hours (Figure 6.6 and Table 6.2). The sub-G1 phase of the cell cycle was used to calculate the percentage of apoptotic cells (Chang *et al.*, 1998).

Table 6.2 shows that, at 24 hours, cisplatin, when compared with the control at 24 hours, did not increase the number of apoptotic cells (Sub G1). There was, however, an increase in percentage at G0/G1, which was expected. At 48 hours cisplatin did give an increase in the percentage of apoptotic cells from 1.53 % to 13.59 %, when compared to the control as well as in the G0/G1 phase.

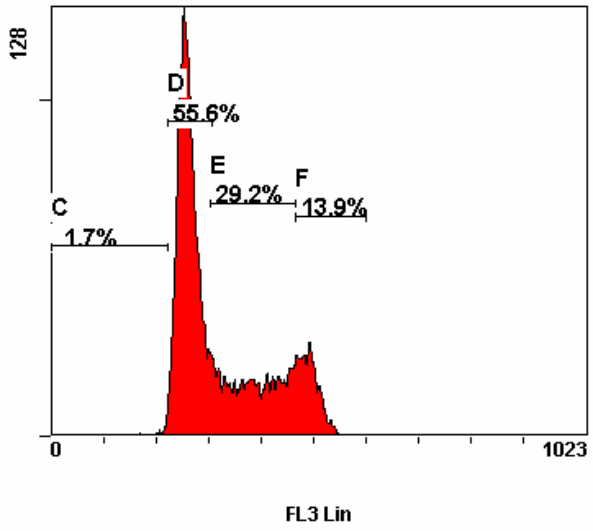
Compounds 1 and 4 did show a small increase in the percentage of apoptotic cells at 24 hours, and a higher percentage than the control at 48 hours. There were increases in the percentage apoptotic cells for both 1 and 4 from 1.53 % to 11.63 % and 1.53 % to 10.94 %, respectively. Compounds 2 and 3 did not show any increase in the percentage of apoptotic cells at 24 hours when they were compared with the control, but showed higher percentages than the control at 48 hours. At 48 hours compounds 2 and 3 both showed increase in the percentage of SubG1, from 1.53 % to 17.78 % and 1.53 % to 15.76 %, respectively.

Compound 5 showed, at 24 hours, a good increase in the percentage of apoptotic cells, from 15.03 % to 34.45 %, and at 48 hours there was an increase from 1.53% to 22.12%. With compound 19, a much lower number of apoptotic cells were observed, from 15.03 %

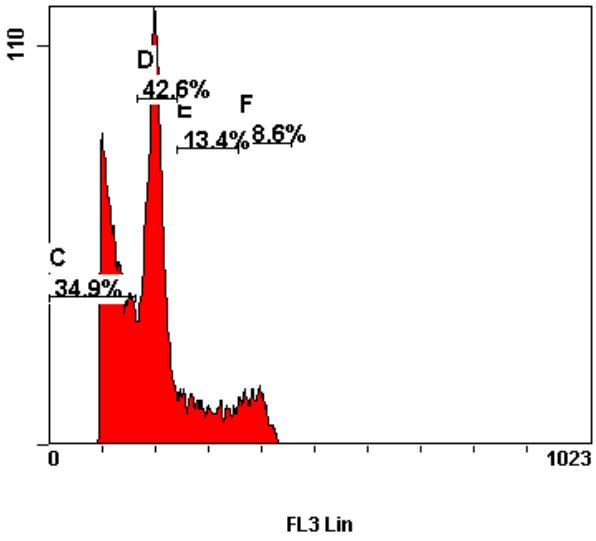
to 3.15 %, at 24 hours, and at 48 hours almost the same number as for the control were found.



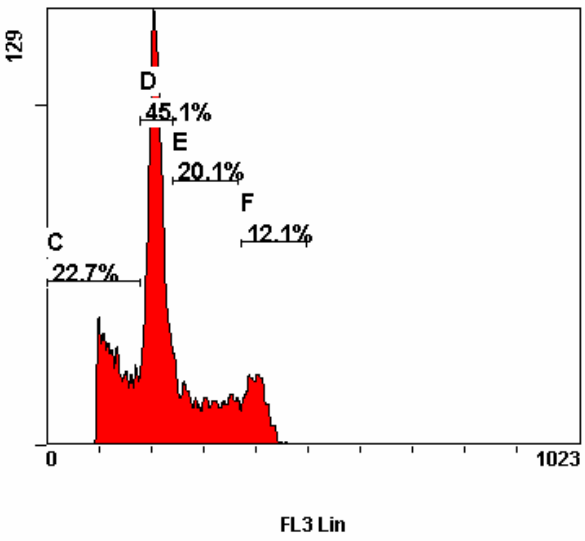
(a)



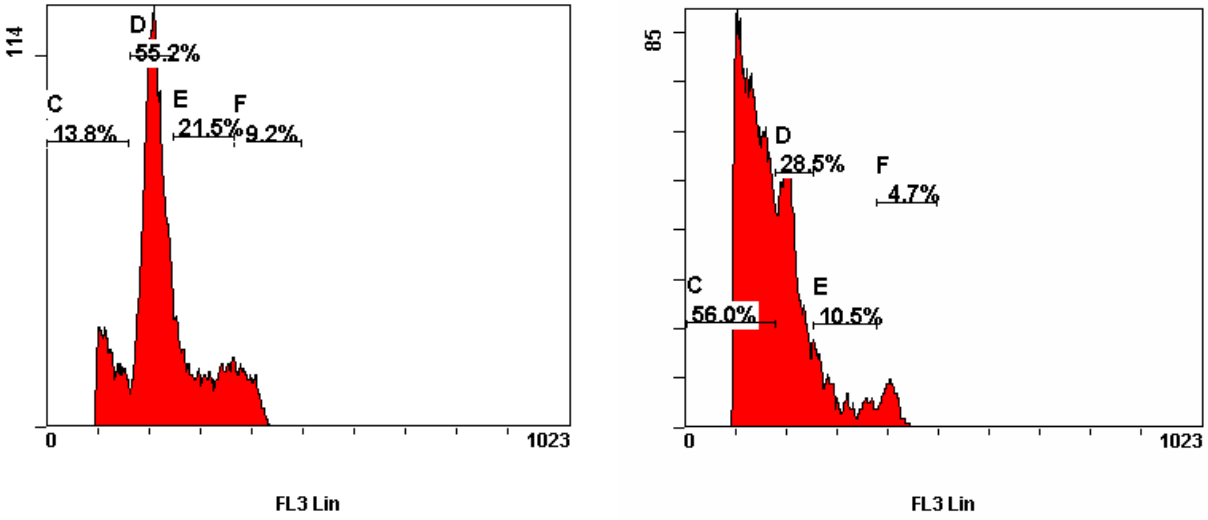
(b)



(c)



(d)



(e) (f)

Figure 6.6 DNA content histograms of U937 cell cycle analysis (a) Control after 24 hours (b) Control after 48 hours (c) Compound 5 after 24 hours (d) Compound 5 after 48 hours (e) Cisplatin after 24 hours (f) Cisplatin after 48 hours (Keyes: C=sub-G1 peak; D=G_0/G_1 peak; E=S peak; F=G_2/M peak).

Table 6.2 Results of cell cycle analysis using nuclear PI staining. U937 cells were exposed to the compounds at the indicated concentrations for 24 hours and 48 hours before cell cycle analysis was performed.

Cell Cycle analysis 24 hours				
Treatment	**Sub G1 (%)**	**G0/G1 (%)**	**S (%)**	**G2/M (%)**
Control	15.03	38.20	33.84	12.93
Cisplatin (10μM)	13.51	56.02	21.14	9.33
Compound 1 (2μg/ml)	17.15	61.03	16.27	5.55
Compound 2 (2μg/ml)	14.44	63.98	16.68	4.90
Compound 3 (2μg/ml)	8.57	69.40	17.33	4.70
Compound 4 (2μg/ml)	18.26	65.79	11.45	4.50
Compound 5 (0.5μg/ml)	34.45	42.53	13.93	9.09
Compound 19 (2μg/ml)	3.15	53.17	27.59	16.09

Cell cycle analysis 48 hours				
Treatment	**Sub G1 (%)**	**G0/G1 (%)**	**S (%)**	**G2/M (%)**
Control	1.53	53.24	29.46	15.77
Cisplatin (10µM)	13.59	56.06	21.13	9.22
Compound 1 (2µg/ml)	11.63	65.34	15.76	7.27
Compound 2 (2µg/ml)	17.78	62.45	13.94	5.83
Compound 3 (2µg/ml)	15.76	63.47	14.21	6.56
Compound 4 (2µg/ml)	10.94	65.35	15.78	7.93
Compound 5 (0.5µg/ml)	22.12	45.83	19.99	12.06
Compound 19 (2µg/ml)	1.22	55.56	25.60	17.62

Table 6.3 shows that most cells were in the Sub G1, or apoptotic, peak after cisplatin treatment for 48 hours, compared to only 5.80% of the control cells. 4-Methoxycinnamyl alcohol gave similar results to the control.

Table 6.3 Results of cell cycle analysis using nuclear PI staining. U937 cells were exposed to 4-methoxycinnamyl alcohol at the indicated concentration (10 µg/ml) for 48 hours before cell cycle analysis was performed.

Cell Cycle analysis 48 hours				
Treatment	**Sub G1 (%)**	**G0/G1 (%)**	**S (%)**	**G2/M (%)**
Control	5.80	43.51	34.63	16.06
Cisplatin (10 µM)	71.82	18.53	7.52	2.13
4-methoxycinnamyl alcohol (10 µg/ml)	5.10	47.12	32.73	15.05

6.3.1.2 Cell cycle analysis MCF-7 cells

For the MCF-7 cell cycle analysis, at 24 hours and 48 hours (Table 6.4), the positive control, 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), showed an increase in the number of apoptotic cells from 2.34 % to 13.57 % and 2.48 % to 30.22 %, respectively,

and in the G2/M phase from 22.50 % to 72.81 % at 24 hours and from 12.69 % to 44.26 % at 48 hours.

Again compounds 2 and 3 did not show any increase in the percentage of apoptotic cells at 24 hours. At 48 hours, both compounds showed increases in the percentage of SubG1, from 2.48 % to 4.30 % and 2.48 % to 4.54 %, respectively.

Even from 24 hours compound 5 appeared to be the best, it showed a good increase in the percentage of apoptotic cells, from 2.34 % to 35.54 %.

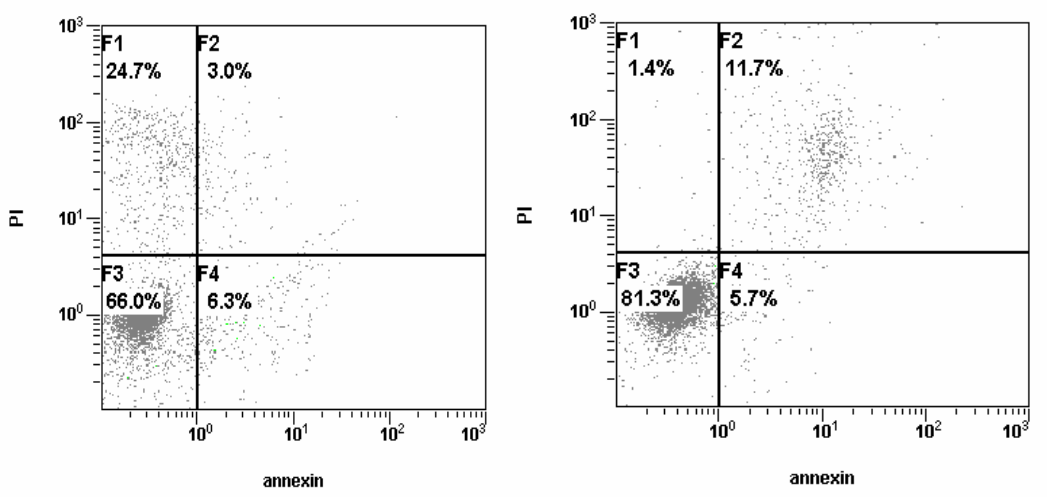
Table 6.4 Results of cell cycle analysis using nuclear PI staining. MCF-7 Cells were exposed to the compounds at the indicated concentrations for 24 and 48 hours before cell cycle analysis was performed.

Cell Cycle analysis 24 hours				
Treatment	**Sub G1 (%)**	**G0/G1 (%)**	**S (%)**	**G2/M (%)**
Control	2.34	47.45	27.71	22.50
Vehicle	1.22	53.54	22.63	22.61
STX140	13.57	7.44	6.18	72.81
Compound 2 (2µg/ml)	2.32	54.47	21.35	21.86
Compound 3 (2µg/ml)	2.25	52.62	22.51	22.62
Compound 5 (0.5µg/ml)	35.54	36.42	16.74	11.30
Cell Cycle analysis 48 hours				
Treatment	**Sub G1 (%)**	**G0/G1 (%)**	**S (%)**	**G2/M (%)**
Control	2.48	72.32	12.51	12.69
Vehicle	1.43	69.38	13.54	15.65
STX140	30.22	11.40	14.12	44.26
Compound 2 (2µg/ml)	4.30	62.55	19.82	13.33
Compound 3 (2µg/ml)	4.54	51.76	22.10	21.60
Compound 5 (0.5µg/ml)	3.59	63.65	19.48	13.28

6.3.2 Annexin V-FITC/PI

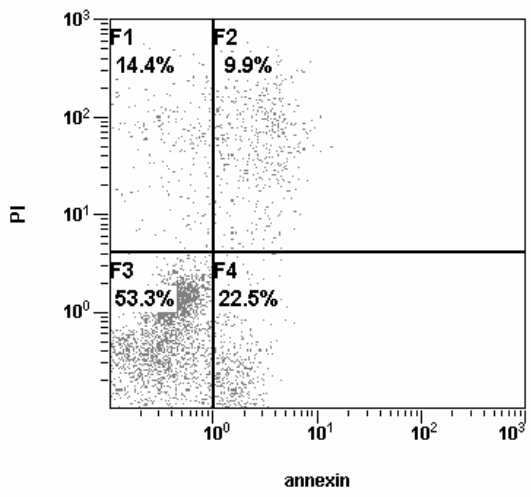
6.3.2.1 Annexin V-FITC/PI staining U937 cells

To confirm apoptosis, Annexin V-FITC/PI staining was done on U937 cells. From the results (Table 6.5) it is evident that there were a number of apoptotic cells observed by all the compounds. However, a large number of cells did undergo necrosis and compound 5 showed the least number of necrotic cells of all the derivatives at 24 and 48 hours (Figure 6.7). At 48 hours the results were more or less the same as at 24 hours. Cisplatin was the only treatment that had a higher percentage apoptotic cells compared to necrotic cells (Figure 6.7).

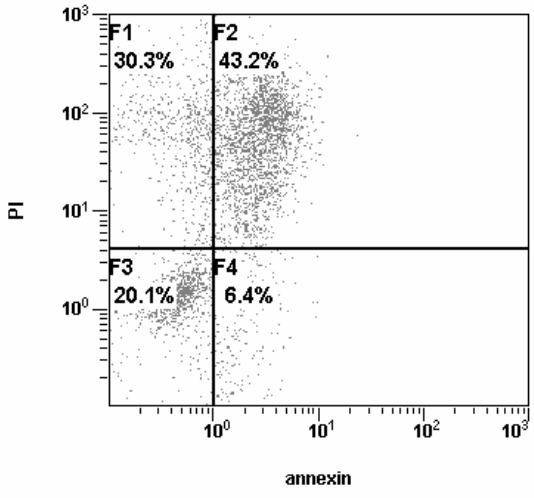


(a)

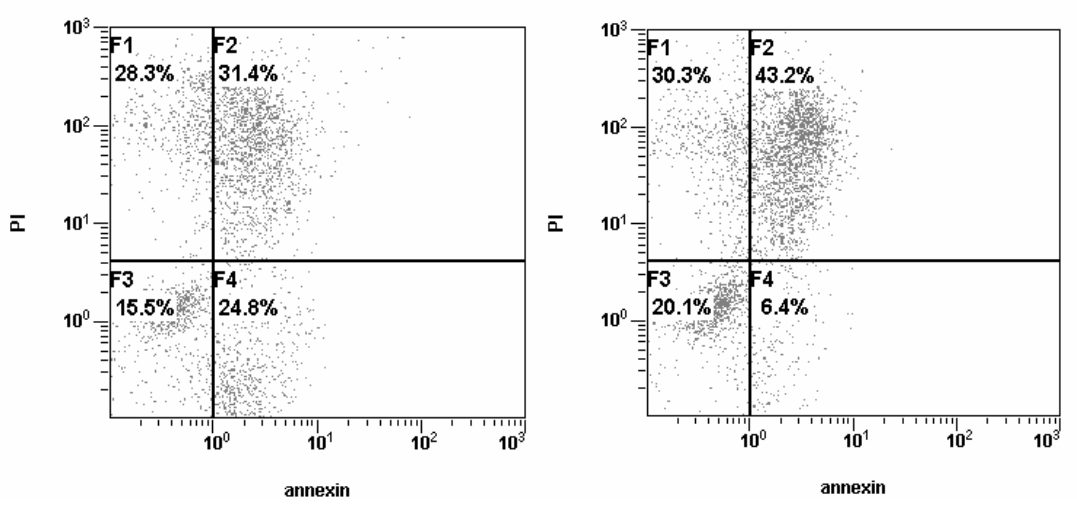
(b)



(c)



(d)



(e) (f)

Figure 6.7 U937 annexin V-FITC/PI stained (a) after 24 hours on exposure to control (b) after 48 hours on exposure to control (c) after 24 hours on exposure to cisplatin (d) after 48 hours on exposure to cisplatin (e) after 24 hours on exposure to compound 5 (f) after 24 hours on exposure to compound 5.

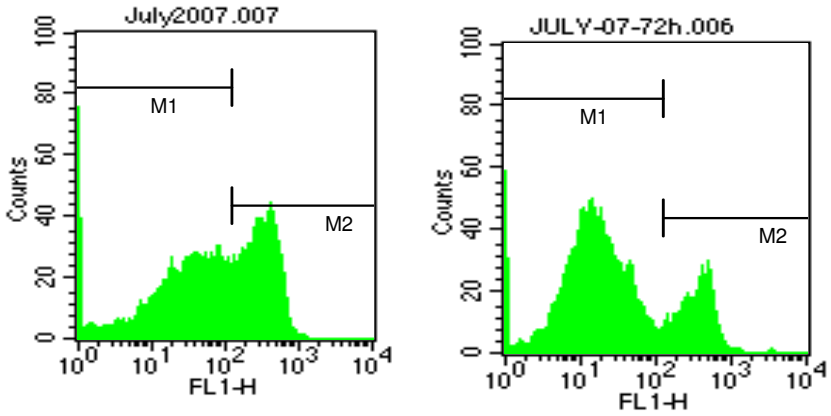
Table 6.5 Summary of U937 annexin V-FITC/PI staining after 24 and 48 hours.

Annexin V-FITC/PI 24 hours				
Treatment	**Viable (%)**	**Apoptotic (%)**	**Late Apoptotic (%)**	**Necrotic (%)**
Control	66.05	6.37	3.08	24.50
Cisplatin (10µM)	40.56	35.00	16.05	8.39
Compound 2 (2µg/ml)	13.52	12.69	12.14	61.65
Compound 3 (2µg/ml)	4.46	9.94	34.80	51.25
Compound 4 (2µg/ml)	6.12	16.49	26.36	51.03
Compound 5 (0.5µg/ml)	14.96	26.75	37.37	20.92

Annexin V-FITC/PI 48 hours				
Treatment	**Viable (%)**	**Apoptotic (%)**	**Late Apoptotic (%)**	**Necrotic (%)**
Control	81.33	5.70	11.73	1.24
Cisplatin (10μM)	20.72	11.59	32.38	35.31
Compound 2 (2μg/ml)	4.19	3.03	29.91	62.87
Compound 3 (2μg/ml)	2.14	1.53	29.18	67.15
Compound 4 (2μg/ml)	2.19	12.04	33.13	52.64
Compound 5 (0.5μg/ml)	22.00	4.42	33.81	39.77

6.3.2.2 Annexin V-FITC/PI staining MCF-7 cells

Apoptotic cells were defined as the cells positive for Annexin V and negative for propidium iodide (M2 on histogram). With the MCF-7 cells (Table 6.6), compound 5 (Figure 6.8) and 2 gave the highest percentages of apoptotic cells, higher than the positive control 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), and compound 3 a lower percentage of apoptotic cells. At 48 these percentages were however decreasing and compound 3 gave the same result as the positive control.



(a) (b)

Figure 6.8 MCF-7 annexin V-FITC/PI staining of cells treated with compound 5 (a) after 24 hours (b) after 48 hours

Table 6.6 Annexin V-FITC/PI results for MCF-7 cells at 24 and 48 hours.

Annexin V-FITC/PI 24 hours		
Treatment	**Apoptotic (%)**	**Viable, Late Apoptotic and Necrotic (%)**
Control	13.34	86.66
Vehicle	16.45	83.55
STX 140	30.02	69.98
Compound 2	44.95	55.05
Compound 3	20.35	80.65
Compound 5	44.57	55.43
Annexin V-FITC/PI 48 hours		
Treatment	**Apoptotic (%)**	**Viable, Late Apoptotic and Necrotic (%)**
Control	18.94	81.06
Vehicle	17.38	82.62
STX 140	32.84	67.57
Compound 2	22.32	77.68
Compound 3	32.43	67.16
Compound 5	27.42	72.58

6.3.3Caspase 3 and 7

6.3.3.1 Caspase 3 and 7 activity after 24 hours

From table 6.7 it is clear that after incubation cisplatin did activate caspase 3/7 activity, by 6.61 % (Figure 6.9). After incubation, compound 3 and 5 inhibited caspase 3/7 activity (-4.13 % and -3.24 % respectively), while compound 19 did activate caspase 3/7 (7.40 %).

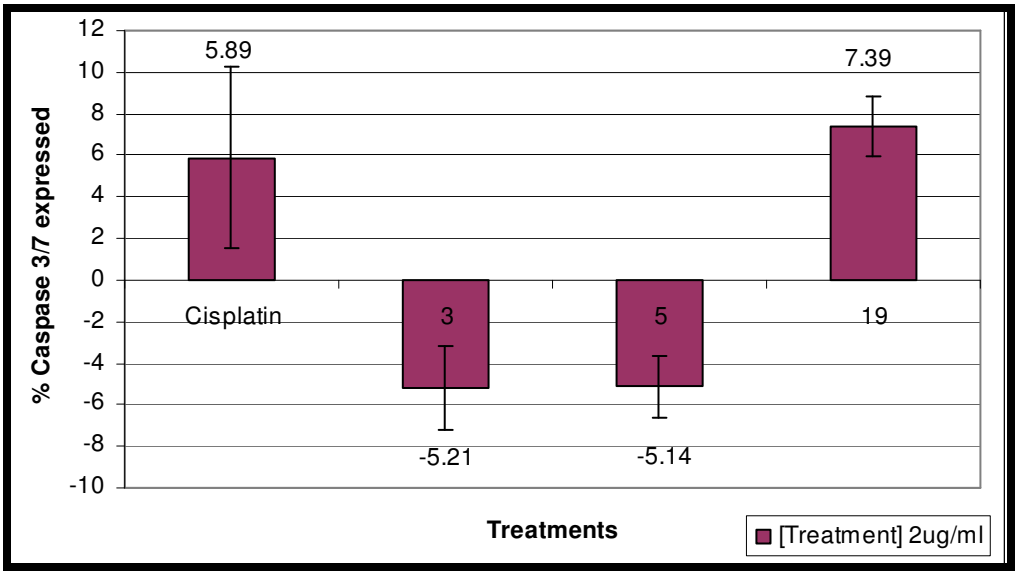


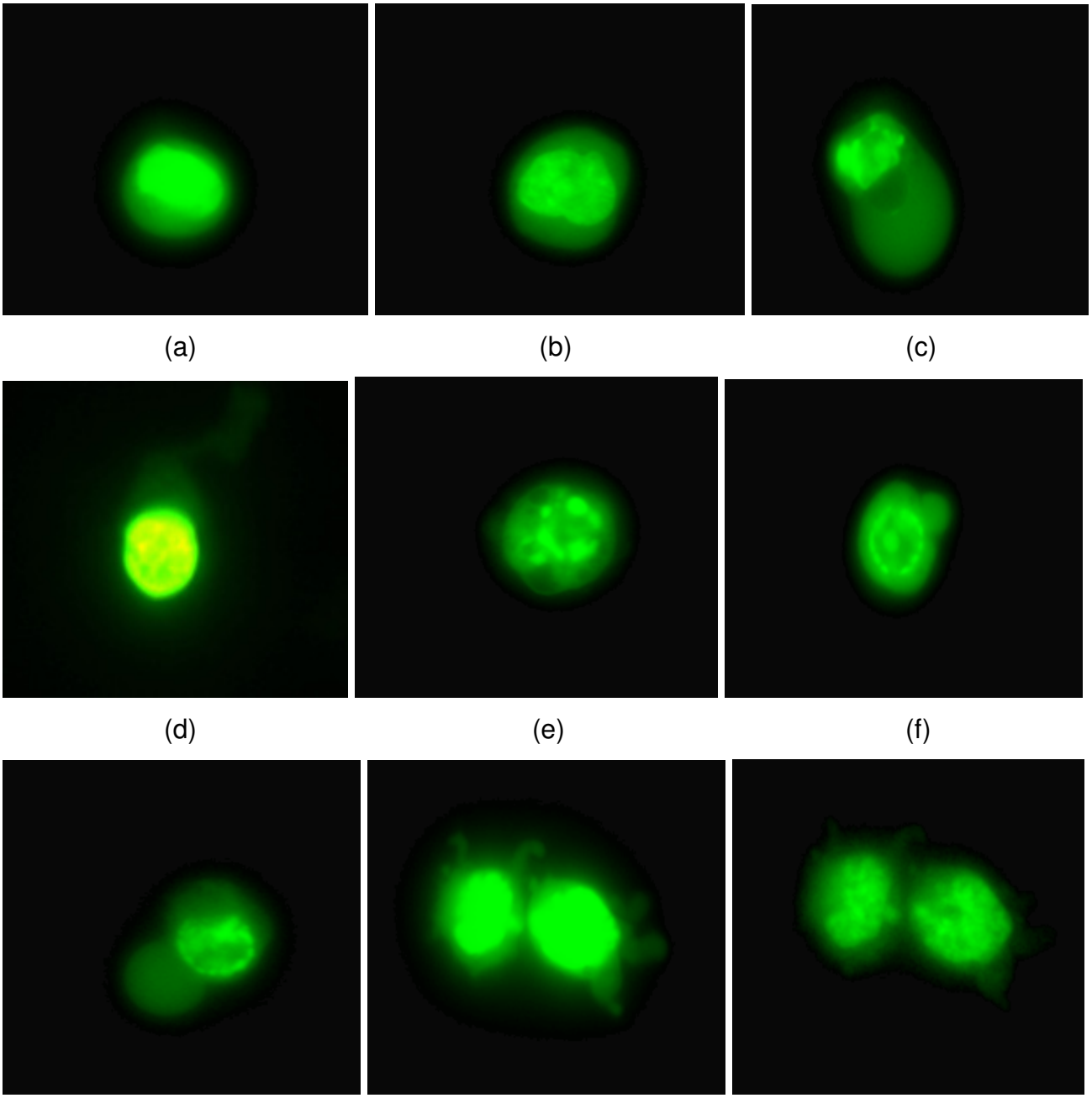
Figure 6.9 Percentage caspase 3/7 expression after a 24 hour treatment in U937 cells (544:620).

Table 6.7 Summary of caspase 3 and 7 results.

Treatment	**Caspase 3/7 activity (% above control)**
Cisplatin	6.61
Compound 3	-4.13
Compound 5	-3.24
Compound 19	7.40

6.3.4 Acridine orange and ethidium bromide nuclear staining U937 and THP-1 cells

Apoptosis was detected by using DNA-binding fluorescent dyes (Gu & Belury, 2005). The U937 and THP-1 cells exhibited marked morphological changes such as: membrane blebbing, dense chromatin and nuclei were fragmented into apoptotic bodies, as compared with the untreated control which did not exhibit these apoptotic characteristics (Figure 6.10 and Table 6.8).



(a) (b) (c)

(d) (e) (f)

(g) (h) (i)

Figure 6.10 (a) Control THP-1 cells viable (b) Control cells with DMSO (c) nuclear fragmentation (d) orange nuclei (e) nuclear fragmentation (f) blebbing (g) dumbbell (h) blebbing (i) nuclear fragmentation.

Table 6.8 Summary of the morphological changes in U937 and THP-1 cells due to exposure to the naphthoquinone derivatives as determined by fluorescence microscopy of nuclear stains, acridine orange and ethidium bromide.

U937 cells			
Treatments	**% Viable cells**	**% Apoptotic cells**	**% Necrotic cells**
Control cells with only medium	98.50	0.23	1.27
Control cells with DMSO	72.77	23.91	3.32
Compound 1	23.79	65.12	11.09
Compound 2	16.50	78.53	4.97
Compound 3	14.59	77.74	7.67
Compound 4	7.30	87.38	5.32
Compound 5	0.67	79.94	19.39
Compound 6	22.41	62.09	15.50
Compound 19	7.53	88.26	4.21
THP-1 cells			
Treatments	**% Viable cells**	**% Apoptotic cells**	**% Necrotic cells**
Control cells with only medium	96.32	0.08	3.60
Control cells with DMSO	84.94	8.44	6.62
Compound 1	4.32	65.03	30.65
Compound 2	10.05	55.26	34.69
Compound 3	5.69	64.33	29.98
Compound 4	6.97	80.59	12.44
Compound 5	3.54	59.25	37.21
Compound 6	2.85	53.18	43.97
Compound 19	8.95	76.07	14.98

6.3.5 DNA fragmentation

A DNA fragmentation assay was used confirm that 4-methoxycinnamyl alcohol did not lead to the gradual accumulation of fragments after exposure U937 cells for 72 hours (Figure 6.11).

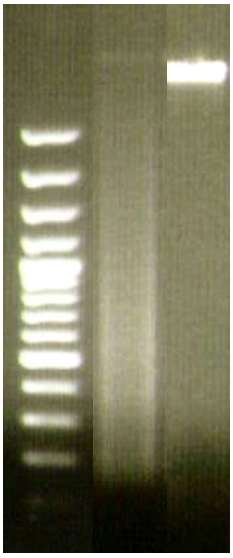


Figure 6.11 First lane loading dye, second lane 4-methoxycinnamyl alcohol with characteristic necrotic smear and third lane control cells without treatment.

6.5Discussion

6.5.1 Cell cycle analysis

Apoptosis assessment by using the state and content of nuclear DNA is one of its main characteristics that are readily assessed with flow cytometric quantitation of red fluorescence from fixed propidium iodide-stained, RNase-treated cells (Warrington *et al.,* 2003). Sub-G1 events on the DNA histograms were where apoptotic activity was heralded (Nicoletti *et al.*, 1991).

To establish whether or not the naphthoquinones inhibited cell growth by blocking cells in a certain phase of the cell cycle and/or induce apoptosis, cellular DNA was analysed (Peng *et al*., 2006) and stained with PI and the cells analyzed using flow cytometry (Koduru *et al.,* 2007).

6.5.1.1 Cell cycle analysis U937 cells

There was no increase in the number of apoptotic cells with cisplatin treatment after 24 hours, but there was an increase in the G0/G1 phase from 38.20 % to 56.02 %, and therefore a delay in the cell cycle at this phase. Previous reports showed a delay in the S phase (Qin and Ng, 2002) but it can be that cisplatin delays the cell cycle late in the G0/G1 phase or early in the S phase and with the results there is an overlap between the two phases and therefore a delay was found in the G0/G1 phase. At 48 hours cisplatin showed an increase in the number of apoptotic cells. The lower percentages found at all the other phases of the cell cycle were expected because of the increase at G0/G1. This means that, due to the delay in the G0/G1 phase (or early S phase), apoptosis was induced and the cells did not continue further with the cell cycle.

Compounds 1 and 4 did show a small increase in the percentage of apoptotic cells at 24 hours, suggesting an early induction of apoptosis. This was confirmed at 48 hours. At both 24 and 48 hours there was an increase in the percentage cells in the G0/G1 phase and a decrease in all the other cell cycle phases, which was expected. With the delay at the G0/G1 phase, the cells cannot follow through with the other phases of the cell cycle and therefore became apoptotic.

Compounds 2 and 3 did not show any increase in the percentage of apoptotic cells at 24 hours when they were compared with the control. Despite this there were percentage increases for both at the G0/G1 phase, indicating cell cycle arrest which could potentially lead to apoptosis. This was confirmed at 48 hours, when compounds 2 and 3 both showed increases in the percentage of SubG1, from 1.53 % to 17.78 % and 1.53 % to 15.76 %, respectively. Again there were delays for both compounds at the G0/G1 phase and as a consequence, a decrease in all the other phases because these cells could not continue through the cell cycle and therefore became apoptotic.

Of all the compounds tested, compound 5 appeared to be the best. Even from 24 hours a good increase was observed in the percentage of apoptotic cells, the same was found at 48 hours. There was no increase at any of the other cell cycle phases which might show that this compound does not delay the cell cycle at all and may use another mechanism to induce apoptosis. Therefore, this compound could be considered for further investigation.

The least active compound was 19. At 24 hours a much lower number of apoptotic cells was observed, and at 48 hours the same number as for the control were found. This could mean that this compound induces necrosis rather than apoptosis to kill the cells, even at an early stage, and therefore, this could lead to inflammation. If this is the case, this compound is not a good choice for further investigation; however, the results would have to be confirmed using another method of apoptosis detection.

All the compounds except compound 19 showed an increase in apoptosis at 48 hours and all for them caused a delay at the G0/G1 phase, except compound 5. This was expected because all these compounds have the same chemical structure except for the functional groups, confirming the results.

The cell cycle analysis of 4-methoxycinnamyl alcohol indicates that it did not arrest the cells at any of the cell cycle stages and no increase in apoptotic cell death was observed. This suggests that 4-methoxycinnamyl alcohol possibly induced necrotic cell death to kill the cells but further investigation is needed to verify this.

6.5.1.2 Cell cycle analysis MCF-7 cells

The positive control, 2-methoxyoestradiol-3,17-O,O-bis-sulphamate STX140, showed an increase in the G2/M phase, and therefore, a delay in the cell cycle at this phase. Lower percentages found at the other phases of the cell cycle were expected because of the increase at G2/M. This means that, due to the delay in the G2/M phase, apoptosis was induced and the cells did not continue further with the cell cycle.

Despite no increase in the percentage of apoptotic cells for compounds 2 and 3 at 24 hours, there were percentage increases for both at the G0/G1 phase, indicating cell cycle arrest which could potentially lead to apoptosis. This was confirmed at 48 hours, where both compounds showed increase in the percentage of SubG1, from 2.48 % to 4.30 % and 2.48 % to 4.54 %, respectively. But this time there were delay for both compounds at the G2/M phase and as a consequence, a decrease in the other phases because these cells could not continue through the cell cycle and therefore, became apoptotic.

Of all the compounds tested, compound 5 appeared to be the best. Even from 24 hours it showed a good increase in the percentage of apoptotic cells, from 2 34 % to 35.54 %, showing that this compound induces apoptosis even faster than the positive control, STX140. There was a small increase from 2.48 % to 3.59 % at 48 hours. There were no increases for any of the other cell cycle phases which might show that compound 5 does not delay the cell cycle at all and may use another mechanism to induce apoptosis. Therefore, this compound is the best of all the compounds tested and should be considered for further investigation.

None of the compounds significantly affected the cell cycle, but 2 and 5 caused a rapid induction of apoptosis (24 hours) with compound 3 less potent and slightly delayed (48 hours). All three compounds appear to only induce apoptosis transiently.. Based on the cell cycle data and the lack of any obvious changes in morphology, at least at this concentration, these compounds do not interact with microtubules. The relative lack of toxicity of these compounds at 2 µg/ml in this study was slightly surprising, since, based on the IC_{50} values, greater cell kill might be expected. It could however, be because the cells were trypsinized. It would be interesting to carry out similar tests over a longer time period, since 72 to 96 h is the 'standard' assay time for most cell proliferation experiments and, the shorter proliferation time assay used in this case, may be overestimating the efficacy of the compounds.

6.5.2 Annexin V-FITC

6.5.2.1 Annexin V-FITC/PI staining U937 cells

The sub-G1 peak from cell cycle analysis is a convenient tool to get some indication of apoptosis. However, results need to be confirmed with other methods. The annexin V-FITC dual labelling experiment on U937 cells was done to confirm and also to distinguish between apoptosis and necrosis. It can be seen that the percentage viable cells was already quite low after 24 hours, with relatively high percentage apoptosis. The increase in percentage viability of the control cells from 66.05 % after 24 hours to 81.33 % after 48 hours can be ascribed to handling during subculturing. At 24 hours, 24.50 % of the control cells were necrotic, indicating physical damage. This percentage decreased to 1.24 % at 48 hours, showing that the necrotic cells present at 24 hours have died and are not detected by the flow cytometer due to discriminator settings to ignore particles that are too small.

Cisplatin gave the highest percentage apoptosis after 24 and 48 hours, with compound 5 being the second best, supporting the results obtained from cell cycle analysis where compound 5 had the highest percentage cells in the sub-G1 phase. It should be noted that compound 5 was tested at a concentration four times lower than the other compounds, due to its high toxicity on U937 cells and this makes the results even more significant. Compounds 2, 3 and 4 did not induce apoptosis to any significant degree, although the relatively high percentages seen in the late apoptotic quadrant after 24 hours may suggest that apoptosis induction occurred very soon after exposure and shorter incubation times may have been better in this case. This is supported by the very low percentage of viable cells seen after only 24 hours. The high percentages of necrotic cells observed for compounds 2, 3 and 4 raises some concern as this indicates that not all the cells were killed through induction of apoptosis. This could lead to serious complications such as inflammation in patients.

6.5.2.2 Annexin V-FITC/PI staining MCF-7 cells

The positive control (STX140) gave the highest percentage apoptosis only after 48 hours. Compounds 2 and 5 caused rapid induction of apoptosis at 24 h with an increase from 13.34 % to 44.95 % and 13.34 % to 44.57 % for both compounds, whereas, compound 3 was less potent and slightly delayed at 48 hours. All the compounds except STX140 and compound 3 showed a reduction in apoptosis showing that these compounds have a much quicker induction time than STX140.

6.5.3 Caspase 3/7 activity after 24 hours

The activation of caspase 3 is one of the most common hallmarks of apoptosis although not all apoptotic pathways necessarily activate this enzyme. The caspase 3/7 assay results show that cisplatin caused activation of these enzymes above the levels detected in untreated control cells, as expected (Table 6.7). Compounds 3 and 5 did not lead to the activation of caspase 3/7, despite the fact that apoptosis was detected during cell cycle analysis. This suggests that compounds 3 and 5 induce apoptosis through a different apoptotic pathway. Surprisingly, compound 18 did activate caspase 3/7, although no apoptosis was seen during cell cycle analysis. It is possible that the induction and execution of apoptosis by compound 18 is slower than with the other compounds, and although caspase 3/7 activity was increased at 24 hours, no increase was observed in the number of apoptotic cells at 24 or 48 hours. The increase in G0/G1 observed at 24 hours from 38.20 % to 53.17 % might be the first indication of cell cycle arrest (U937 cells) that could lead to apoptosis induction.

6.5.4 Acridine orange and ethidium bromide nuclear staining U937 and THP1 cells

Under the fluorescence microscope live cells were fluorescing green because of the acridine orange and the dead cells fluoresced orange because of the ethidium bromide. The U937 and THP-1 cells exhibited condensed chromatin, fragmented nuclei and the appearance of apoptotic bodies, resulting from exposure to the selected naphthoquinone

derivatives. From the results on the U937 cells (Table 6.8), the control cells had high percentage viable cells (98.50 %). The few necrotic cells, in the control, might have been caused by damage to the cells when they were transferred to the plates. Compounds 1 and 6 had the lowest percentages of apoptotic cells, 65.12 % and 62.09 %, respectively. These two compounds (1 and 6) had high percentages of viable cells (23.79 % and 22.41 %) compared to the 7.53 % of compound 19. Compounds 1 and 6 also had high percentages of necrotic cells (11.09 % and 15.50 %) when compared to several of the other derivatives. A much lower percentage of necrotic cells were found for compound 19, 4.21 %. Compounds 4 and 19 had the highest percentages of apoptotic cells, 87.38 % and 88.26 % respectively. The lowest percentage of viable cells 0.67 % and the highest percentage of necrotic cells (19.39 %), of all the derivatives, were from compound 5 with 79.94 % apoptotic cells. Of all the derivatives, compound 19 had the least percentage of necrotic cells at 2.21 %. Both compounds 2 and 4 had few necrotic cells and moderate apoptotic cells when compared to the other derivatives.

From the THP-1 results (Table 6.8) a high percentage viable cells were found with the control cells that were treated only with medium, 96.32 %, and also few necrotic cells as with the U937 control cells. The DMSO containing cells also had a high percentage of viable cells (84.94 %) with few apoptotic and necrotic cells, 8.44 % and 6.62 % respectively. Compound 4 had showed to have the highest percentage of apoptotic cells 80.59 % together with 12.44 % necrotic cells. Where the other remaining compounds 1, 2, 3, 5, 6 and 19 had fewer apoptotic cells than the aforementioned compounds and had more necrotic cells, 65.03 %, 55.26 %, 64.33 %, 59.25 %, and 53.18 % apoptotic cells and 30.65 %, 34.69 %, 29.98 %, 37.21 % and 43.97 %, in that order.

6.5.5 DNA fragmentation

The purpose of DNA fragmentation during apoptosis is still uncertain, but it is said to be probably one of the most useful tests for apoptosis. To determine whether the selected isolated compound, 4-methoxycinnamyl alcohol, induced U937 cells death via apoptosis. When the U937 cells were cultured with the 4-methoxycinnamyl alcohol (5 µg/ml) for 12, 24, 48, 72 and 96 hours and it revealed a typical necrotic smear on the agarose gel, confirming our cell cycle results.

6.6 References

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