



## Cytotoxicity of synthesized 1,4-naphthoquinone analogues on selected human cancer cell lines

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### ABSTRACT

In an effort to establish new candidates with enhanced anticancer activity of 5-hydroxy-7-methyl-1,4-naphthoquinone scaffold (7-methyljuglone) previously isolated from the root extract of *Euclea natalensis*, a series of 7-methyljuglone derivatives have been synthesized and assessed for cytotoxicity on selected human cancer lines. These compounds were screened in vitro for anticancer activity on MCF-7, HeLa, SNO and DU145 human cancer cell lines by MTT assay. Most of them exhibited significant toxicity on cancer cell lines with lower IC<sub>50</sub> values. The most potent derivative (**19**) exhibited the toxicity on HeLa and DU145 cell lines with IC<sub>50</sub> value of 5.3 and 6.8 μM followed by compound (**5**) with IC<sub>50</sub> value of 10.1 and 9.3 μM, respectively. Structure–activity relationship reveals that the fluoro substituents at position C-8 while hydroxyl substituents at C-2 and C-5 positions played an important role in toxicity.

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### 1. Introduction

Quinone scaffolds are widespread in nature and present in many drugs which are used clinically in the therapy of solid cancer. The cytotoxic effects of these quinones are mainly due to the inhibition of DNA topoisomerase-II.<sup>1,2</sup> Naphthoquinones are widely distributed in plants, fungi and some animals, and most of these are found to exhibit an interesting range of pharmacological properties. 1,4-Naphthoquinone is an important example of quinone family and is used as a raw material in pharmaceutical industries. 1,4-Naphthoquinone and its derivatives exhibited several interesting and different biological responses including antibacterial,<sup>3</sup> antifungal,<sup>4,5</sup> anti-inflammatory, antiplatelet, antiallergic,<sup>6</sup> anti-thrombotic,<sup>7</sup> antiviral,<sup>8</sup> anticancer,<sup>9–11</sup> apoptosis,<sup>12</sup> lipoxigenase,<sup>13,14</sup> radical scavenging<sup>15</sup> and anti-ringworm<sup>16</sup> activities. Recently, 1,4-naphthoquinone derivatives were proved to be human DNA topoisomerase I and II inhibitors.<sup>2</sup> The presence of two carbonyl groups in naphthoquinones that have the ability to accept one or two electrons to form the corresponding radical anion or di-anion species and their acid-base properties is responsible for the various biological activities.<sup>17,18</sup> In another study, it

was found that the presence of hydroxy groups at 5 and 8 positions, which facilitate the tautomerism in the structure of 1,4-naphthoquinone, reduced the electrophilicity of the naphthoquinone ring.

Cancer is the second leading cause of death worldwide despite a major endeavour of research and development in academia and pharmaceutical industry to search for new anticancer agents.<sup>19–21</sup> Although major advances have been made by researchers but the medical need is still largely unmet due to many factors; among which are the lack of selectivity of conventional drugs leading to toxicity, the metastatic spreading, and multi-drug resistance.<sup>22–27</sup> Novel and selective anticancer agents are urgently required due to problems associated with currently available anticancer drugs. The plant *Euclea natalensis* (A. DC.) is used traditionally for the treatment of cancer in South Africa.<sup>28,29</sup> The metabolite 7-methyljuglone (7-MJ) previously isolated from this plant<sup>3</sup>, exhibited anticancer activity. A series of derivatives of 7-MJ were synthesized. Previous studies have shown that mono or dihydroxy substitution (at C-2 or C-5 and C-8 positions in the aromatic ring) of naphthoquinones resulted in higher toxicity as compared to the parent 1,4-naphthoquinone due to increased efficiency of redox cycling.<sup>3</sup> The introduction of a fluorine atom into antibiotic quinolones has been shown to enhance their activity. In view of these previous observations, it was decided to synthesize 7-MJ derivatives in order to establish structure–activity relationship. This paper

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reports the synthesis and cytotoxicity of a series of 5-hydroxy-, 5-alkoxy- and 5-acetoxy-8-substituted-naphthoquinones on selected human cancer cell lines. The mechanism of action was established by cell cycle analysis and structure activity relationship has also been discussed.

## 2. Result and discussion

### 2.1. Synthesis of 1,4-naphthoquinone derivatives

The synthesis of 7-MJ (**1**) and its derivatives **2–19** (Table 1) has been done by the same method that was previously used for the synthesis in our laboratory.<sup>3</sup>

### 2.2. Cytotoxicity

This is the first study that discloses the effects of 7-MJ derivatives on four cancer cell lines, cell cycle, apoptosis and caspase 3/7. To determine the fifty percent inhibitory concentration ( $IC_{50}$ ) values of the compounds, the four adherent cancer cells were treated with several concentrations. All assays were carried out in triplicate. The cytotoxicity results (Table 1) revealed that most of the tested compounds exhibited good activity, mainly against prostate (DU-145), cervical (HeLa) and breast (MCF-7) cell lines while being less active against the oesophageal cancer cell line. Out of 18 derivatives, compounds **2–6** and **19** were found to be potent inhibitors of the growth of HeLa, DU-145 and MCF-7 cancer cell lines at very low concentrations.

The  $IC_{50}$  values showed (Table 1) that the compounds **2–15** and **19** exhibited very good anticancer activity against all the cell lines. The derivative **19** was the most significant one against cell lines (HeLa:  $IC_{50}$  5.3  $\mu$ M and DU-145:  $IC_{50}$  6.8  $\mu$ M), followed by compounds **5** (DU-145:  $IC_{50}$  9.3  $\mu$ M and SNO:  $IC_{50}$  9.4  $\mu$ M) and **4**

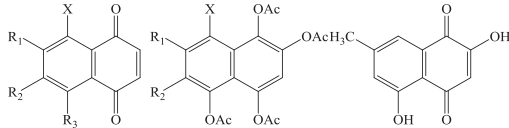
(HeLa:  $IC_{50}$  10.2  $\mu$ M and DU-145:  $IC_{50}$  15.4  $\mu$ M); while the parent compound **1** (7-MJ) was found to be less active than most of the derivatives. The compound **5** also exhibited the toxicity against MCF-7 and HeLa cell lines with  $IC_{50}$  values of 10.0 and 10.1  $\mu$ M, respectively (Comparable to Cisplatin,  $IC_{50}$  10.0  $\mu$ M).

The introduction of a hydroxyl group at position C-2 in **19** resulted in a sixfold increase in the activity as compared to the parent compound **1**. The presence of halogen substituent at C-8 also increased the activity. The cytotoxicity of **6** was found to be greater than **1**; this clearly indicated that the change of position of  $-CH_3$  group from C-7 to C-6, enhanced the activity. The weak activity of derivatives **7–15** (5-Alkoxy or 5-Acetoxy) indicated that the presence of 5-hydroxyl group is necessary for the activity. When the carbonyl group of quinone was converted to acetate, the activity was reduced significantly. Hence, the weakest activity of the tetra-acetate derivatives **16–18** (1,2,4,5-tetraacetate) revealed that the quinone moiety is highly required for the development of new significant leads. Out of all the derivatives, on the basis of their activity, compounds **1–6** and **19** were taken up for further investigation.

### 2.3. Cytotoxicity on human macrophages (U937) and peripheral blood mononuclear cells (PBMCs)

Based on the cytotoxicity data of a few derivatives on most of the cell lines, compounds **1–6** and **19** were further tested on peripheral blood mononuclear cells (PBMCs) and on human macrophages. The comparative toxicity on PBMCs (Table 2, Fig. 1) suggested that compounds **4** and **19** were the least toxic, whilst the other compounds including **1** were found to be more toxic than the other derivatives at the lower concentrations tested. Compounds **4** ( $IC_{50}$ : 188.7  $\mu$ M) and **19** ( $IC_{50}$ : 54.0  $\mu$ M) were not toxic to the PBMCs, suggesting their actions to be specific for tumour

**Table 1**  
List of synthesized 1,4-naphthoquinone derivatives and their cytotoxicity against human cancer cell lines

	Substituents on naphthoquinone scaffold				Cytotoxicity of derivatives on selected cancer cell lines ( $IC_{50}^e$ values are given in $\mu$ M)			
	X	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	MCF-7 <sup>a</sup>	HeLa <sup>b</sup>	SNO <sup>c</sup>	DU 145 <sup>d</sup>
								
<b>1–15</b>								
<b>16–18</b>								
<b>19</b>								
5-Hydroxy-7-methyl-1,4-naphthoquinone (7-MJ) ( <b>1</b> )	H	Me	H	OH	27.2	66.6	81.4	11.9
8-Chloro-5-hydroxy-7-methyl-1,4-naphthoquinone ( <b>2</b> )	Cl	Me	H	OH	15.7	13.5	17.2	29.5
8-Bromo-5-hydroxy-7-methyl-1,4-naphthoquinone ( <b>3</b> )	Br	Me	H	OH	15.5	11.3	14.9	46.3
8-Fluoro-5-hydroxy-7-methyl-1,4-naphthoquinone ( <b>4</b> )	F	Me	H	OH	20.3	10.2	18.9	15.4
8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone ( <b>5</b> )	Cl	H	Me	OH	10.0	10.1	9.4	9.3
5-Hydroxy-6-methyl-1,4-naphthoquinone ( <b>6</b> )	H	H	Me	OH	15.3	16.4	19.0	15.4
8-Chloro-5-methoxy-7-methyl-1,4-naphthoquinone ( <b>7</b> )	Cl	Me	H	OMe	20.6	19.1	24.6	26.6
8-Chloro-5-ethoxy-7-methyl-1,4-naphthoquinone ( <b>8</b> )	Cl	Me	H	OEt	40.0	19.0	44.2	25.4
5-Acetoxy-8-chloro-7-methyl-1,4-naphthoquinone ( <b>9</b> )	Cl	Me	H	OAc	37.9	26.2	28.8	25.5
5-Acetoxy-7-methyl-1,4-naphthoquinone ( <b>10</b> )	H	Me	H	OAc	14.7	17.2	28.1	24.1
5-Methoxy-7-methyl-1,4-naphthoquinone ( <b>11</b> )	H	Me	H	OMe	15.3	21.1	22.5	30.7
5-Ethoxy-7-methyl-1,4-naphthoquinone ( <b>12</b> )	H	Me	H	OEt	23.7	29.7	25.3	15.0
8-Chloro-5-methoxy-6-methyl-1,4-naphthoquinone ( <b>13</b> )	Cl	H	Me	OMe	16.2	41.5	44.9	36.5
8-Chloro-5-ethoxy-6-methyl-1,4-naphthoquinone ( <b>14</b> )	Cl	H	Me	OEt	32.2	33.2	37.3	26.2
5-Acetoxy-8-chloro-6-methyl-1,4-naphthoquinone ( <b>15</b> )	Cl	H	Me	OAc	18.7	21.8	28.6	12.5
8-Chloro-7-methylnaphthalene-1,2,4,5-tetra-O-acetate ( <b>16</b> )	Cl	Me	H	—	51.1	52.9	56.9	59.6
7-Methylnaphthalene-1,2,4,5-tetra-O-acetate ( <b>17</b> )	H	Me	H	—	82.0	22.4	25.0	64.6
8-Chloro-6-methylnaphthalene-1,2,4,5-tetra-O-acetate ( <b>18</b> )	Cl	H	Me	—	57.5	27.9	53.5	81.6
2,5-Dihydroxy-7-methyl-1,4-naphthoquinone ( <b>19</b> )	—	—	—	—	14.6	5.3	23.2	6.8
Doxorubicin	—	—	—	—	0.66	0.01	0.01	0.01
Cisplatin	—	—	—	—	10.0	10.0	—	—

<sup>a</sup> MCF-7–Breast adenocarcinoma.

<sup>b</sup> HeLa–Cervical epithelial carcinoma.

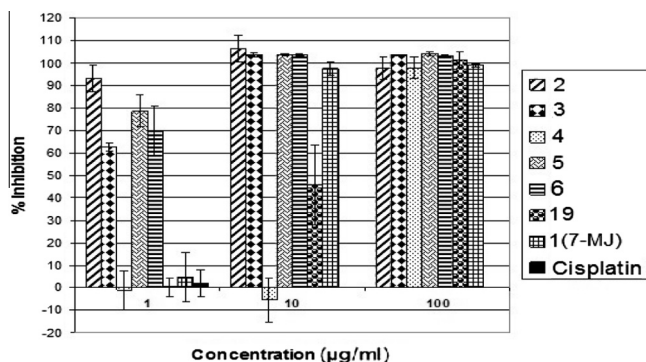
<sup>c</sup> SNO–Oesophageal carcinoma.

<sup>d</sup> DU145–Prostate epithelial carcinoma.

<sup>e</sup>  $IC_{50}$ –fifty percent inhibitory concentration

**Table 2**Cytotoxicity of compounds **1**, **4** and **19** on peripheral blood mononuclear cells

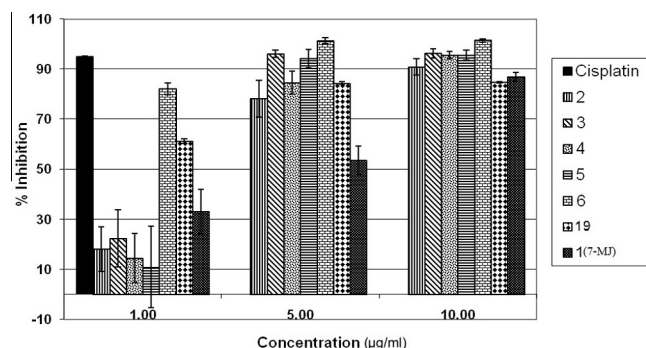
Compound	IC <sub>50</sub> (μM)
<b>1</b>	18.4
<b>4</b>	188.7
<b>19</b>	54

**Figure 1.** Percentage inhibition of Peripheral blood mononuclear cells (PBMCs) by highly active 7-MJ derivatives (Bars and error bars indicate mean  $\pm$  SD of quadruplicates).

cells in contrast to the other compounds tested in this study. The toxicity test on U937 cells (Fig. 2) revealed that the compounds **2–5** were the least toxic on U937 cells.

## 2.4. Cell cycle analysis

Based on the cytotoxicity data on PBMCs and U937 cells, compounds **2–6** and **19** were further tested for cell cycle analysis. The analysis demonstrated that these compounds, except for compound **19**, caused an accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> phase and thus inhibited the transition of cells into S phase. This was followed by increases in the proportion of cells in sub-G<sub>1</sub> phase of the cell cycle. The major characteristics which have been used to assess apoptosis are the state and content of nuclear DNA. Apoptosis can be readily assessed by flow cytometric quantitation of red fluorescence from the fixed propidium iodide-stained, RNase-treated cells.<sup>30</sup> Apoptotic activity is heralded by sub-G<sub>1</sub> events on DNA histograms.<sup>31</sup> To establish whether the tested compound inhibited cell growth by blocking cells in a certain phase of the cell cycle and/or induced apoptosis, cellular DNA was analysed<sup>32</sup> and stained with PI and the cells were analyzed using flow cytometry.<sup>33</sup> The cell cycle profile (Fig. 3) is represented through 3 independent

**Figure 2.** Percentage inhibition of U937 cells by selected derivatives (Bars and error bars indicate mean  $\pm$  SD of quadruplicates).

experiments on U937 cell lines of compounds **2–6** and **19**. A significant arrest at G<sub>0</sub>/G<sub>1</sub> phase was observed both at 24 and 48 h. Though in the G<sub>0</sub>/G<sub>1</sub> and S phases, cells did not proliferate significantly as compared to the control at 48 h. They increased in sub-G<sub>1</sub> phase (control: 1.5% and compounds **2–6**: 10.9–22.1%). Therefore the compounds **2–6** and **19** arrested the cells in G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle.

There was no increase in the number of apoptotic cells with cisplatin treatment after 12 h, but there was an increase in the G<sub>0</sub>/G<sub>1</sub> phase from 38.0% to 56.0%, and hence a delay in the cell cycle in this phase. Previous reports showed that cisplatin induced a transient G<sub>1</sub> arrest, S phase block and up regulation of p53 and p21<sup>WAF1/CTP1</sup> expression in HepG2, but not HEP3B cells.<sup>34</sup> It might be that either cisplatin delays the cell cycle late in the G<sub>0</sub>/G<sub>1</sub> phase or early in the S phase and as a result there is an overlap between the two phases and therefore a delay in the G<sub>0</sub>/G<sub>1</sub> phase. At 48 h 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 in the G<sub>0</sub>/G<sub>1</sub> phase. This means that, due to the delay in the G<sub>0</sub>/G<sub>1</sub> phase (or early S phase), apoptosis was induced and the cells did not continue further with the cell cycle. Interestingly,  $\beta$ -hydroxyisovaleryl shikonin ( $\beta$ -HIVS) has been reported to arrest cell cycle at G<sub>0</sub>/G<sub>1</sub> phase by suppressing anti-apoptotic proteins.<sup>35</sup> A small increase in the percentage of apoptotic cells by derivatives **2** and **5** at 24 h in sub-G<sub>1</sub> phase, suggested an early induction of apoptosis, which was confirmed by the results at 48 h. At 24 and 48 h there was an increase in the percentage cells in the G<sub>0</sub>/G<sub>1</sub> phase and decrease in all other cell cycle phases, which was expected. With the delay at the G<sub>0</sub>/G<sub>1</sub> phase, the cells could follow through to the other phases of the cell cycle and therefore became apoptotic.

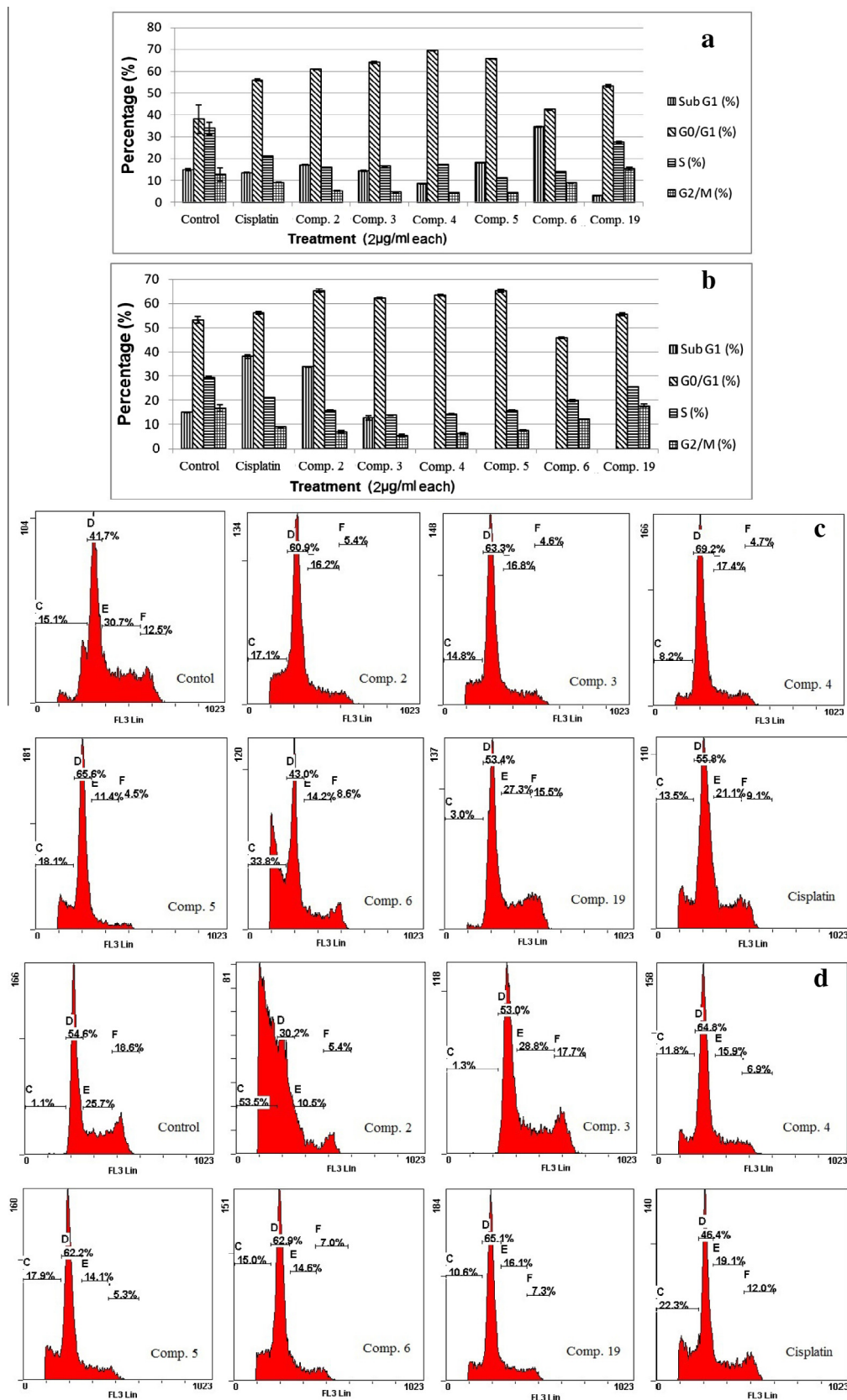
No increase in the percentage of apoptotic cells in sub-G<sub>1</sub> phase but an increase in the G<sub>0</sub>/G<sub>1</sub> phase by derivatives **3** and **4** at 24 h indicated cell cycle arrest which could potentially lead to apoptosis. This was confirmed at 48 h, when both showed increases in the percentage of Sub-G<sub>1</sub>, from 2% to 18% and 2% to 16%, respectively. Again there were delays for both compounds at the G<sub>0</sub>/G<sub>1</sub> 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 tested derivatives, **6** appeared to be the best. It does not delay the cell cycle and might be using another mechanism to induce apoptosis. A much lower number of apoptotic cells by compound **19** at 24 and 48 h suggested that it induced necrosis rather than apoptosis to kill the cells, even at an early stage, which could lead to inflammation. Hence the results would have to be confirmed using another method of apoptosis detection. All the tested compounds, except compound **19**, showed an increase in apoptosis at 48 h, and caused a delay at the G<sub>0</sub>/G<sub>1</sub> phase.

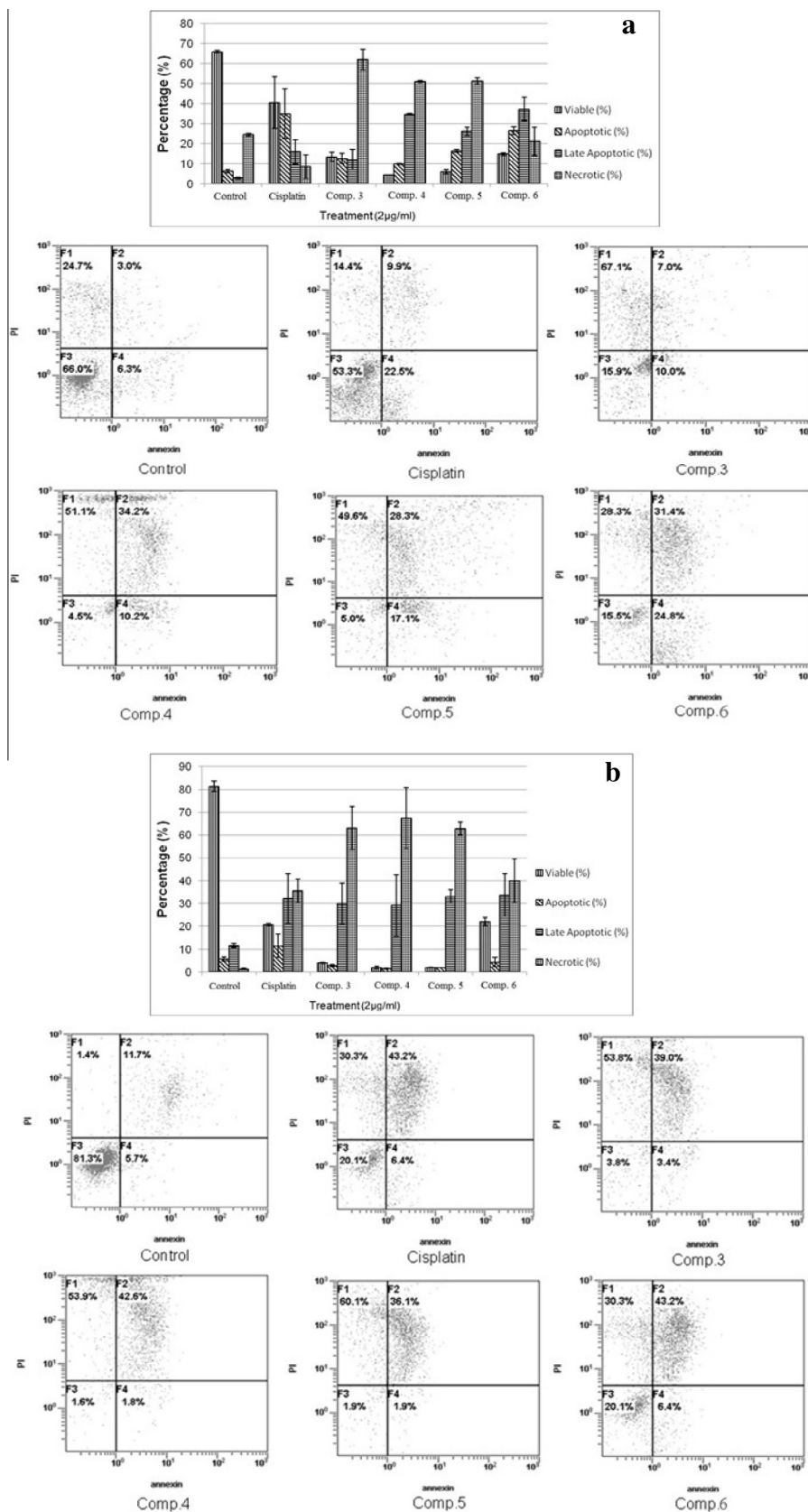
## 2.5. Analysis of apoptosis

On the basis of cell cycle analysis results, compounds **3–6** were further tested for apoptosis. The result (Fig. 4) showed that, the tested derivatives induced more apoptosis at 24 h (early apoptosis) as compared to 48 h (late apoptosis). There is significant difference in the proportions of non-apoptotic cells, and those in early and late apoptosis of cell treated were observed after 24 and 48 h of treatment with the derivatives when compared to the control. The sub-G<sub>1</sub> peak from cell cycle analysis is a convenient tool for getting an indication of apoptosis.

Based on Annexin V/propidium iodide staining, **6** was found to be induce the greatest amount of apoptosis at an early apoptotic stage, which further supported the results of cell cycle with highest percentage cells in the sub-G<sub>1</sub> phase in U937 cells. Compounds **3–5** induced apoptosis significantly in the late apoptotic quadrant after



**Figure 3.** Effect of compounds on the cell cycle progression at different phases of U937 cell line after (3a & 3b) The cell cycle profile is representative of three independent experiments at 24 and 48 h, (3c & 3d) Percentage of cells in different phases; Results represent mean  $\pm$  SEM of three independent experiments.

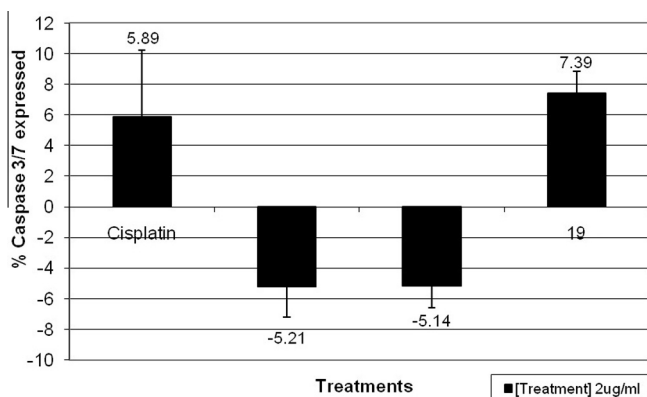


**Figure 4.** Apoptosis analysis of U937 cells exposed for 24 h (**4a**) and 48 h (**4b**). Double staining was used to distinguish between viable, early apoptotic, late apoptotic and necrotic cells. The lower left quadrant shows the viable cells, the upper left shows necrotic cells, the lower right shows the early apoptotic cells while the upper right shows late apoptotic cells.



**Table 3**  
Activation (%) of caspase 3/7

Compounds	Caspase 3/7 activity (% above control)
Cisplatin	5.89
Compound <b>4</b>	–5.21
Compound <b>6</b>	–5.14
Compound <b>19</b>	7.39



**Figure 5.** Activation (%) of caspase 3/7.

24 h, suggesting that apoptosis occurred very soon after exposure and shorter incubation time may have been better. This is supported by the very low percentage of viable cells seen after only 24 h. The compounds **2–5** and **19** significantly inhibited the growth of HeLa and DU-145 cells in vitro, possibly by either down-regulation of antiapoptotic Bcl-X<sub>L</sub> and up-regulation of proapoptotic Bax or by increasing the p21<sup>waf1/cip1</sup> protein, which is involved in cell cycle arrest in G<sub>1</sub> or G<sub>2</sub> phase.<sup>36</sup> Copeland et al. has reported that 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone exhibits cytotoxicity on androgen-dependent and -independent prostate cancer cell lines inducing apoptosis by arresting cell cycle at G<sub>1</sub> phase.<sup>37</sup> The cell cycle alterations indicate that cell cycle arrest is one of the primary mechanisms responsible for the anticancer activity of synthesized 7-MJ derivatives in HeLa and DU-145 cells.

### 2.6. Caspase 3/7 activity

Based on the cell cycle analysis, compounds **4**, **6** and **19** were further tested for induction of apoptosis. The induction of apoptosis was confirmed by induction of caspases. It is clear from Table 3 that after incubation cisplatin and compound **19** activated caspase 3/7 activity by 6.6% and 7.4%, respectively, while compounds **4** and **6** (Fig. 5) inhibited caspase 3/7 activity by –4.1% and –3.2%, respectively. No activation of caspase 3/7 by compounds **4** and **6** suggests that they induce apoptosis through a different pathway. Surprisingly, compound **19** activated caspase 3/7, although no apoptosis was seen during cell cycle analysis. It is possible that the induction and execution of apoptosis by **19** is slower than the other compounds (may be more time is required for inducing apoptosis). The increase in G<sub>0</sub>/G<sub>1</sub> observed at 24 h from 38.0% to 53.1% might be the first indication of cell cycle arrest that could lead to apoptosis induction.

### 3. Conclusions

The compounds, 2,5-dihydroxy-7-methyl-1,4-naphthoquinone (**19**), 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone (**5**), 8-fluoro-5-hydroxy-7-methyl-1,4-naphthoquinone (**4**) and 5-hydroxy-6-methyl-1,4-naphtho-quinone (**6**) were found to be

more cytotoxic and specific against human cervical epithelial carcinoma (HeLa), prostate epithelial carcinoma (DU-145) and breast adenocarcinoma (MCF-7) cancer cell lines as confirmed in cell cycle, apoptosis and caspase 3/7 activation as compared to the other derivatives. The derivative 2,5-dihydroxy-7-methyl-1,4-naphthoquinone (**19**) was found to be the most potent among all; though not inducing apoptosis up to 48 h suggested that may be slow induction occurred, which had to be tested for longer time. The derivative **5** (8-chloro-5-hydroxy-6-methyl-1,4-naphthoquinone) and **6** (5-hydroxy-6-methyl-1,4-naphtho-quinone) could be further modified to reduce the toxicity.

## 4. Material and methods

### 4.1. Cell culture

Four human cancer cell lines, breast adenocarcinoma (MCF-7), cervical epithelial carcinoma (HeLa), oesophageal carcinoma (SNO) and prostate epithelial carcinoma (DU-145) (Highveld Biological, SA) were maintained in culture flasks in complete Minimum Essential Medium, Eagle supplemented with 10% fetal bovine serum (Highveld Biological, SA), in a humidified 5% CO<sub>2</sub> incubator at 37 °C. Upon reaching confluence, the cells were trypsinized (0.25% trypsin containing 0.01% EDTA) for 10 min at 37 °C and then stopped by the addition of complete medium. About 1 × 10<sup>5</sup> of the viable cells were then re-suspended in complete medium. U937 cells were maintained in culture flasks in complete RPMI 1640 medium (Sigma, Germany) supplemented with 10% fetal bovine serum (Delta Bioproducts, SA), containing 25 mM HEPES and 2 mM glutamine, in a humidified 5% CO<sub>2</sub> incubator at 37 °C.

### 4.2. Cytotoxicity assay

Cytotoxicity of the adherent cells was measured by the XTT (Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). The cancer cells (100 µl) were seeded at 1 × 10<sup>5</sup> per ml onto a microtiter plate and incubated for 24 h to allow the cells to attach to the bottom of the plate. A dilution series was made for the compounds (0.1–100 µg/ml), which was added to the microtiter plate and incubated for 48 h. The XTT reagent was added to a final concentration of 0.3 mg/ml and incubated for 1–2 h. After incubation the absorbance of the colour complex was quantified at 490 nm using an ELISA plate reader with a reference wavelength set at 690 nm. The 50% cell survival (IC<sub>50</sub>) was analysed by using GraphPad Prism (version 4) from the concentration-effect relationship.

### 4.3. Peripheral blood mononuclear cells (PBMCs)

Blood was obtained from healthy adult volunteers. PBMCs were separated with BD Vacutainer™ CPT™ cell preparation tubes containing sodium heparin. The method was followed as published<sup>38</sup> but with different final concentrations tested (1, 10 and 100 µg/ml) using a fluorescence read at 560<sub>Ex</sub>/590<sub>Em</sub> using a Fluoroskan Ascent FL fluorometer (ThermoLabSystems, Finland).

### 4.4. Cell cycle analysis

Compounds with the lowest IC<sub>50</sub> values were further investigated for the mechanism of action. U937 cells were seeded in complete RPMI 1640 medium at a density of 2 × 10<sup>6</sup> cells per millilitre in flasks. After a recovery period of 24 h, the selected compounds (**1–6**) were dissolved in DMSO (2 µg/ml) and added to the cells.

Cisplatin (10  $\mu$ M) was added as positive control and DMSO as negative control. The flasks were further incubated for 24 h. The Coulter<sup>®</sup> DNA Prep<sup>™</sup> Reagents Kit (Beckman Coulter) was prepared as per kit instructions and the method was followed as published,<sup>38</sup> with the difference of 500  $\mu$ l lysis buffer added and 1 ml of propidium iodide (PI) added. The cells were analysed on a Beckman-Coulter FC500 Flow Cytometer (Miami, FL, USA). The respective software was used for the acquisition and analysis of data and the percentage of cells in each phase was determined in the gated population of singlet cells.

#### 4.5. Analysis of apoptosis

The 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. The reported method<sup>38</sup> was followed, the samples were analysed on a Beckman Coulter FC500 flow cytometer (Miami, FL, USA).

#### 4.6. Caspase 3/7 activity assay

The Caspase 3/7 detection kit (Biomol) was used and the reagents were prepared as described in the kit. To evaluate caspase 3 activities, the U937 cells and compounds were prepared for the cell cycle analysis experiments. The compounds were added to the cells and control prepared and incubated for 24 h. The flasks were mixed gently by hand so that the cells mixed well since they tend to settle while standing. 2.0 ml of RPMI 1640 medium containing cells and compounds, positive control, and negative control was removed from each flask and transferred into separate 2 ml Eppendorf tubes. The Eppendorf tubes were centrifuged and 290  $\mu$ l of RPMI 1640 medium was added and the cell suspension was transferred to a black microtiter plate. At least  $2 \times 10^5$  cells per 100  $\mu$ l aliquot was added per well. Ten microlitres of the 30 $\times$  CR (DEVD)<sub>2</sub> working solution was added to the 290  $\mu$ l treated cells and mixed gently by taking in and expelling the cells with a pipette. The black microtiter plate was wrapped in foil and incubated for 2 h at 37 °C under 5% CO<sub>2</sub>. The cells tend to settle and were gently re-suspended every 20 min to ensure that the CR (DEVD)<sub>2</sub> was evenly dispersed among all the cells. The plates were read with ThermoLabsystem Fluoroskan AscentFL fluorescence reader at the wavelengths of 544:620 nm and 544:590 nm.

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#### Supplementary data

Supplementary data (synthetic methodologies of the compounds) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2014.06.013>.

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