

*Full Length Research Paper*

## Aminonaphthoquinones as potential anti-breast cancer agents

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**A small library of compounds, including aminonaphthoquinones, heterocyclic and aromatic compounds, was synthesized to determine their antiproliferative activity against three mammalian cancer cell lines viz:cervical carcinoma (HeLa), breast adenocarcinoma (MCF-7) and T-cell leukaemia (Jurkat). Investigations into the effect of the compounds on coagulation and platelet activity were also undertaken. In particular, the aminonaphthoquinones showed significant antiproliferative activity against the three cell lines, with aminonaphthoquinone4 showing more potent antiproliferative activity when compared to camptothecin (PC). This quinone was also identified as possessing significant antiplatelet activity *in vitro*, by inhibiting both platelet aggregation and platelet adhesion.**

**Key words:** Aminonaphthoquinones, anticancer activity, anticoagulant, antiplatelet.

### INTRODUCTION

Cancer has become a significant global problem as it has one of the highest mortality rates worldwide, second only to cardiovascular disease. More than 50% of new cancer cases and two-thirds of cancer-related deaths occur in developing countries. Not only does this place a burden on health systems, but it also negatively impacts on the economic growth of these countries. It is estimated that between 2009 and 2020, the number of new cancer cases will increase to 16.8 million, and that the majority of cases will occur in Africa ([www.cansa.givengain.org](http://www.cansa.givengain.org)).

Apoptosis is an energy-driven process that affects single cells, and is not accompanied by inflammation (Holdenrieder and Stieber, 2004). Loss of control of apoptosis disrupts the balance between cell proliferation and cell death, leading to diseases such as cancer. Most cancer cells resist this process by using anti-apoptotic

signaling pathways even though genetic and transformational changes are present (Lee et al., 2011). In cancer patients, the aim of medical interventions, such as chemotherapy, is to restore the equilibrium between cell proliferation and cell death. Some chemotherapeutic agents cause cell cycle arrest by inducing cellular damage, whilst insufficient repair causes induction of apoptosis.

The apoptotic signal is transmitted intracellularly by a well-ordered system of initiator and effector caspases. Caspases (cysteine-dependent aspartate-specific acid proteases) activate pro-caspases by cleavage after aspartate sequences. In this way, the signal finally reaches the downstream effector caspases. The key mediator of apoptosis has been identified as caspase 3 (Huerta et al., 2007), and its activation is a typical characteristic of apoptosis (Lee et al., 2011). Other typical biochemical and morphological features of apoptosis include chromatin condensation, DNA fragmentation, budding of cellular membranes, loss of phospholipid asymmetry in the plasma membrane, changes in mitochondrial membrane permeability as well as release of cytochrome c, an inter membrane space

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**Abbreviations:** DC, 0.1% Dimethyl sulfoxide (DMSO) vehicle control; PC, camptothecin.

mitochondrial protein (Krysko et al., 2008).

Two pathways of apoptosis have been identified. The death receptor pathway is activated when the tumour necrosis factor (TNF) ligand binds to its receptor, binding of TNF-related apoptosis inducing ligand (TRAIL) to the TRAIL receptors DR4 and DR5, or binding of the Fas ligand to its receptor. Initiator caspases 8 and 10 are then activated, which in turn activates the effector caspases 3, 6 and 7 (Huerta et al., 2007). The mitochondrial pathway is activated by the release of cytochrome *c* from the mitochondria. Anapoptosome is formed by the interaction of Apoptosis protease activating factor-1 (Apaf-1) with cytochrome *c*, pro-caspase 9 and ATP. Caspase 9 is thereby activated, resulting in cleavage and thus activation of caspases 3, 6 and 7, which effect apoptosis (Huerta et al., 2007). Activation of these effectors ultimately results in apoptosis, immaterial of the stimulus that caused its activation.

It is widely recognized that cancer patients are at a significant risk of thromboembolic complications, which may even present as the first clinical manifestation of the disease. These complications range from pulmonary embolism, deep vein thrombosis to superficial thrombophlebitis and disseminated intravascular coagulation (DIC) (Furie and Furie, 2006; Rickles and Flanga, 2001) and could be linked to irregularities in platelet activation and elevated levels of coagulation proteins such as fibrinogen (Caine et al., 2005). Blood coagulation can be activated in response to inflammatory stimuli, directly by tissue factor released from malignant and normal cells, or indirectly by tissue factor expressed on endothelial cells, monocytes, macrophages and fibroblasts (Furie and Furie, 2006).

In patients with malignancies, serum levels of plasma-soluble fibrin monomer complex, fibrinogen and fibrin degradation products (D-dimer) are elevated. Activated platelets release pro- or anti-antigenic mediators such as vascular endothelial growth factor (VEGF), which is essential for metastasis (Caine et al., 2005). Released growth factors form aggregates with fibrinogen, significantly increasing D-dimer levels and the risk for thrombotic events in these patients (Kvolik et al., 2010).

In patients receiving anticancer chemotherapy, the patient's general condition may be aggravated, coagulative disorders may be worsened, and, despite the overall increased risk for thromboembolic events, increased risk of bleeding complications may occur, due to the toxicity of the chemotherapeutic agents used (Kvolik et al., 2010).

This study was thus aimed at identifying novel compounds within a small library that possesses anti-cancer and anti-coagulation and/or anti-platelet activity. Synthetic compounds from three different classes were investigated. This included the substituted aromatic compounds (Govender et al., 2007; Moleele, 2007; Govender et al., 2007), the three aminonaphthoquinones 4, 5 and 6 and two heterocyclic Compounds 7 and 8 [Ligtenbarg et al., 1998; Shieh et al., 2003] (Scheme 1).

The synthesis of the three most active compounds, the aminonaphthoquinones 4-6, is described in the work. Of interest is that the aminonaphthoquinone class of compounds has found application in this particular field (Zakharova et al., 2011; Das Sarma et al., 2008) although the piperazine-naphthoquinone motif (Das Sarma et al., 2008) has not been utilized frequently as anti-cancer agents.

## EXPERIMENTALS

Hydrogen-1 nuclear magnetic resonance ( $^1\text{H}$  NMR) and Carbon-13 NMR ( $^{13}\text{C}$  NMR) spectra were recorded on a Bruker AVANCE 300 spectrometer. All spectra were recorded in  $\text{CDCl}_3$ . All chemical shift values are reported in parts per million (ppm) referenced against TMS which is given an assignment of zero parts per million. Coupling constants (*J*-values) are given in Hertz (Hz). Infrared spectra were recorded on a Bruker Tensor 27 standard system spectrometer. Macherey-Nagel Kieselgel 60 (particle size 0.063 to 0.200mm) was used for conventional silica gel column chromatography with various EtOAc and hexane mixtures as the mobile phase. Thin layer chromatography (TLC) was performed on aluminum-backed Macherey-Nagel AlugramSil G/UV254 plates pre-coated with 0.25mm silica gel 60.

### General experimental procedure for the preparation of aminonaphthoquinones

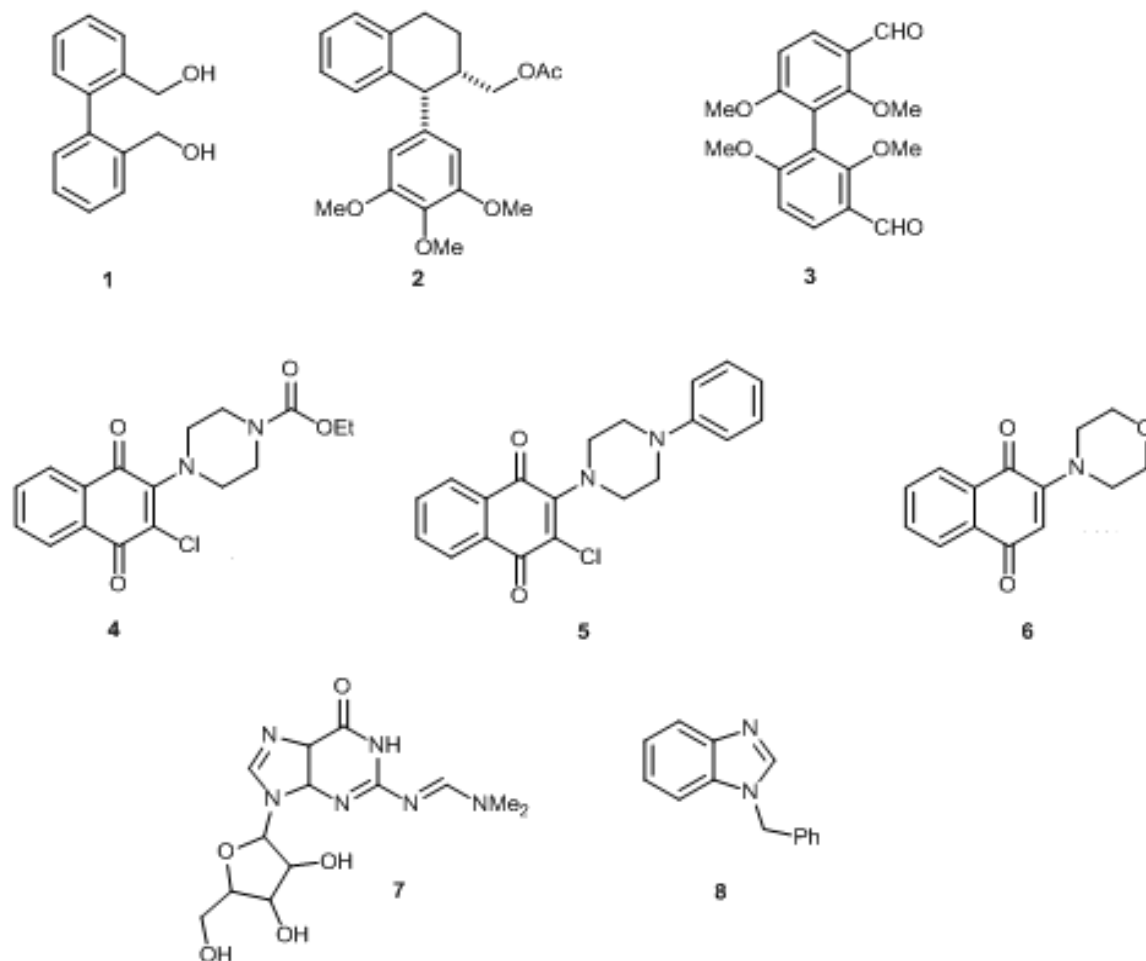
A mixture of the piperazine or morpholine (1eq.), 1,4-naphthoquinone (1eq.), a catalytic amount of  $\text{I}_2$  (10 mol%) and absolute EtOH (2.0  $\text{cm}^3$ ) was sonicated under high-power ultrasound at ambient temperature in a round-bottomed flask open to the atmosphere. The reaction was monitored by TLC until the disappearance of starting materials was noted under ultraviolet (UV)-light. The crude product was evaporated under reduced pressure and the resulting residue was washed with tap water (c.a. 50  $\text{cm}^3$ ) and extracted with dichloromethane (3  $\times$  50  $\text{cm}^3$ ). The combined organic layers were dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered under water pressure and concentrated *in vacuo*. The crude product was then purified by column chromatography on silica gel using ethyl acetate hexane mixtures. The method is described in Liu and Ji (2008).

### Ethyl-4-(3-chloronaphthoquinone)piperazine-1-carboxylate 4 (Scheme 1 No. 4)

Aminonaphthoquinone 4 was formed as a red solid in a yield of 40%; mp 123 to 125°C; IR:  $\nu_{\text{max}}(\text{cm}^{-1})$  1696, 1672, 1647, 1591, 1554, 1427, 1281, 1117, 990, 790 and 683;  $^1\text{H}$  NMR  $\delta_{\text{H}}$  (300 MHz,  $\text{CDCl}_3$ ) 8.08 (d, 1H, *J* = 7.0), 7.99 (d, 1H, *J* = 7.3), 7.65 (quintet, 2H, *J* = 7.0), 4.15 (q, 2H, *J* = 7.0), 3.65-3.68 (m, 4H), 3.54-3.57 (m, 4H), 1.27 (t, 3H, *J* = 7.0);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  180.6, 176.9, 154.3, 148.8, 133.0, 132.1, 130.3, 130.2, 125.8, 125.5, 123.1, 60.5, 50.0, 43.4, 13.5; HRMS cal for  $\text{C}_{17}\text{H}_{17}\text{ClN}_2\text{O}_4$  349.09441 ( $\text{M}^+$  + H) found 349.09496 ( $\text{M}^+$  + H).

### 2-Chloro-3-(4-phenylpiperazin-1-yl)naphthoquinone 5 (Scheme 1 No. 5)

Aminonaphthoquinone 5 was formed as a red solid in a yield of 58%; mp 112-114°C; IR:  $\nu_{\text{max}}(\text{cm}^{-1})$  1673, 1651, 1593, 1554, 1457, 1305, 1137, 995, 785, 677;  $^1\text{H}$  NMR  $\delta_{\text{H}}$  (300 MHz,  $\text{CDCl}_3$ ) 8.12-8.14 (m, 1H), 8.02-8.05 (m, 1H), 7.66-7.74 (m, 2H), 7.29 (t, 2H, *J* = 7.8 Hz), 6.98 (d, 2H, *J* = 8.0), 6.90 (t, 1H, *J* = 7.3), 3.77 (t, 4H, *J* = 5.0),



**Scheme 1.** The substituted aromatic compounds 1, 2 and 3; the three aminonaphthoquinones 4, 5 and 6 and two heterocyclic compounds 7 and 8.

3.36 (t, 4H,  $J = 4.7$ );  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  181.7, 177.8, 151.1, 149.8, 134.6, 134.1, 131.5, 131.4, 128.4, 126.9, 126.5, 123.2, 120.3, 116.5, 51.2, 49.7; HRMS cal for  $\text{C}_{20}\text{H}_{17}\text{ClN}_2\text{O}_2$  353.10513 ( $\text{M}^+ + \text{H}$ ) found 353.10482 ( $\text{M}^+ + \text{H}$ ).

### 2.1.3 2-Morpholinonaphthoquinone 6 (Scheme 1 No. 6)

Aminonaphthoquinone 6 was formed as a red solid in a yield of 25%; Mp 156-158 °C; IR:  $\nu_{\text{max}}(\text{cm}^{-1})$  1673, 1638, 1588, 1563, 1457, 1390, 1161, 870, 838, 784;  $^1\text{H}$  NMR  $\delta_{\text{H}}$  (300 MHz,  $\text{CDCl}_3$ ) 7.99-8.05 (m, 2H, ArH), 7.63-7.73 (m, 2H, ArH), 6.02 (s, 1H, C=CH), 3.86 (t, 4H,  $J = 4.8$ ,  $\text{OCH}_2$ ), 3.48 (t, 4H,  $J = 4.8$ ,  $\text{NCH}_2$ );  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ) 183.7, 182.9, 153.6, 132.9, 132.7, 132.6, 132.2, 126.7, 125.6, 111.9, 66.4, 49.1, HRMS cal for  $\text{C}_{14}\text{H}_{13}\text{NO}_3$  244.0968 ( $\text{M}^+ + \text{H}$ ) found 244.0965 ( $\text{M}^+ + \text{H}$ ).

### Anticancer activity

Ethical clearance was obtained from the Research Ethics Committee (Human – Clearance no H10-SCI-BCM-014) of the Nelson Mandela Metropolitan University for the collection of blood samples from healthy donors. A stock solution of each compound was made in dimethyl sulfoxide (DMSO) at 10 000  $\mu\text{M}$ , and stored at

-20°C. For each method, a final concentration of 100  $\mu\text{M}$  was used for each synthesized derivative.

### Routine cell maintenance

Breast adenocarcinoma (MCF-7), cervical carcinoma (HeLa) and T-cell leukaemia (Jurkat) cells were routinely maintained in RPMI-1640 media (Lonza Walkersville, Inc.) supplemented with 10% foetal bovine serum (Invitrogen) in a humidified  $\text{CO}_2$  incubator at 37°C.

### Cytotoxicity of the compounds

The MCF-7 and HeLa cells were seeded into 96-well plates at a density of 30 000 cells/well and allowed to attach overnight. The Jurkat cells were seeded at the same density just prior to exposure to the compounds at a final well concentration of 100  $\mu\text{M}$ . Concentration of DMSO per well did not exceed 1%. Camptothecin (PC) was used as a positive control. After a 24-h incubation period, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed, with minor modifications (Mosmann, 1983). Cells were incubated with 5 mg/ml MTT for 3 h at 37°C. Formazan crystals were dissolved in DMSO, and the absorbance

readings taken at 540 nm.

### **Annexin V apoptosis detection assay**

The Annexin V apoptosis detection kit (BioVision) was used as per manufacturer's specifications. Analysis was completed after a 24-h incubation period with the compounds, using the Beckman-Coulter cytomics 500.

### **Caspase 3 assay**

Caspase 3 activity was determined using the CPP32 colorimetric assay kit (BioVision) as per manufacturer's specifications. Cleavage of the substrate DEVD (Asp-Glu-Val-Asp) from the pNA chromophore by caspase 3 allows for the quantitative determination of caspase activity by determining the absorbance of the colorimetric chromophore at 405 nm. Results are reported as fold-increase in activity.

### **Coagulation and platelet studies**

#### **Coagulation tests**

The assays included the prothrombin time (PT) (Tripodi et al., 1992), activated partial thromboplastin time (APTT) (Azevodo et al., 2007) and fibrinogen-C (Krobot et al., 1992) tests and were performed using the CL Analyzer (Instrumentation Laboratory) as per the manufacturer's instructions and calibrated using the calibration plasma (Beckman) provided. All tests included untreated plasma (control), treated (incubated with a final concentration of 100  $\mu$ M compound) and relevant positive controls (aspirin or 0.1 U/ml heparin obtained from Sigma, Germany).

#### **Platelet aggregation and adhesion**

Platelet rich plasma was obtained by the centrifugation of whole blood at 300 $\times$ g for 10min. After the collection of the platelet-rich fraction, the sample was re-centrifuged at 900 $\times$ g for 15min to obtain platelet-poor plasma. A platelet count, aggregation and adhesion experiments were completed as a microtiter plate assay and read at 600nm using a BioTek Power Wave XS reader as described by Bellavite et al. (1994). A microscopic method was used to investigate platelet aggregation and adherence as described by Shahriyary and Yazdanparast (2007).

#### **Analysis of results**

Data is presented as mean  $\pm$  SEM of n=3 experiments. Results were analyzed using student's *t* test or one-way analysis of variance (ANOVA) using MS Excel 2007. A *p* value of <0.05 was considered to be statistically significant.

## **RESULTS AND DISCUSSION**

All compounds were dissolved in DMSO to obtain stock concentrations of 10 000 $\mu$ M. Stock solutions were dissolved in media or assay solution to obtain final working concentrations of 100 $\mu$ M. All cell-based assays used camptothecin (PC) as the positive control, and 0.1% DMSO (DC) as the negative (vehicle) control. Coagulation assays used plasma as a negative control, and aspirin or

heparin as positive controls.

### **MTT cytotoxicity assay**

The MTT assay is a quantitative colorimetric assay for the determination of cell viability and cytotoxicity, based on the activity of mitochondrial succinate dehydrogenase, which is an indicator of metabolically active cells (Brunner and Holst-Hansen, 1998). The 1% DMSO vehicle control did not significantly affect the viability of the cells as it reduced viability to 93.85 $\pm$ 6.6%. PC, which targets and inhibits DNA topoisomerase I (Fujimori et al., 1996), did not show any cytotoxic effects on the MCF-7 cells (104.55 $\pm$ 12.7%). The presence of viable MCF-7 cells could be due to acquired PC-resistance in the form of a highly efficient intrinsic DNA repair mechanism, which counteracts the effect of PC (Fujimori et al., 1996). (Figure 1)

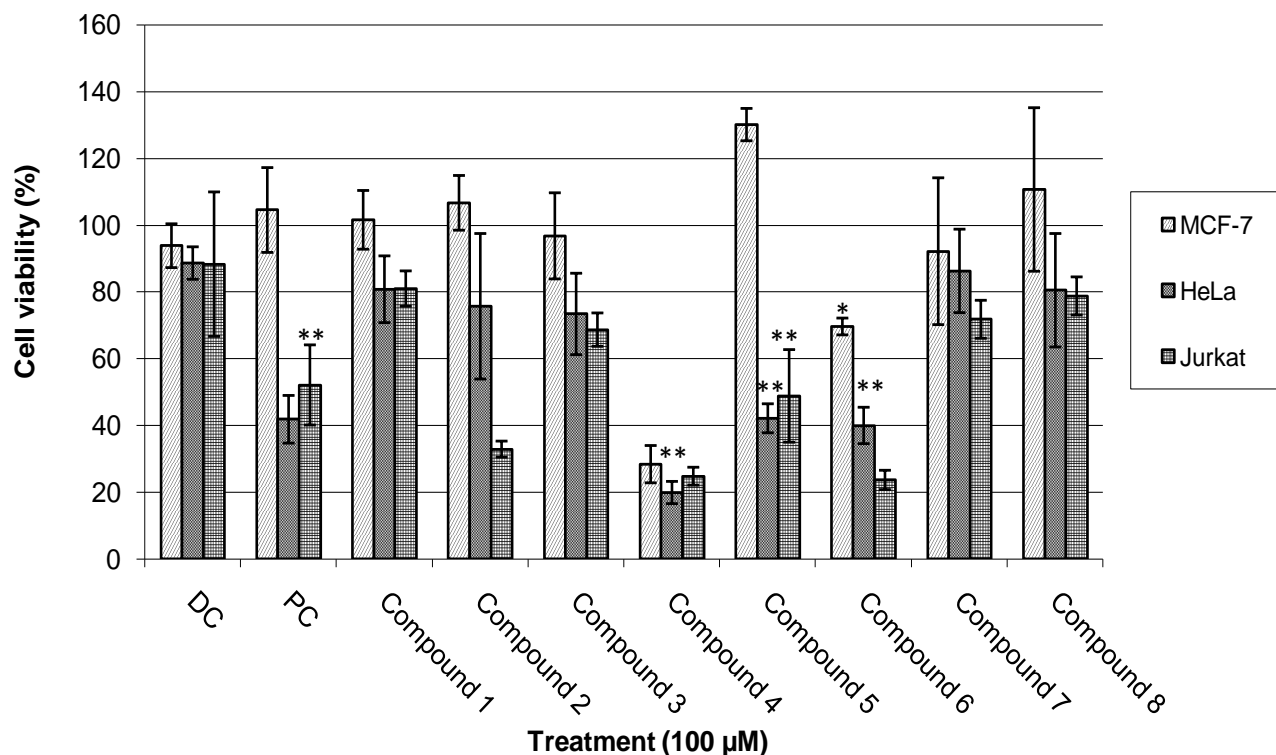
PC significantly decreased the viability of Jurkat and HeLa cells (52.1 $\pm$ 12.0% and 41.8 $\pm$ 7.2%, respectively), as expected, as it has been found that PC inhibits the RNA and DNA synthesis in HeLa cells, causing a reduction in cell viability (Horwitz et al., 1971). Aminonaphthoquinones4 and 6 significantly reduced cell viability of the MCF-7 cells (*p*<0.05) whilst aminonaphthoquinone5 enhanced the proliferative activities of these cells (130.1 $\pm$ 4.9%). The effect of aminonaphthoquinone5 and 6 on HeLa cells was similar to that of PC (*p*>0.05), while aminonaphthoquinone4 significantly reduced cell viability (19.9 $\pm$ 3.3%) when compared to PC (*p*<0.05). PC and aminonaphthoquinone4 had an equivalent effect on cytotoxicity on the Jurkat cells (*p*>0.05), while aminonaphthoquinone5 and 6 reduced cell viability to 48.9 $\pm$ 13.1% and 23.7 $\pm$ 2.9%, respectively.

Based on these results, it was determined that only the aminonaphthoquinones4, 5 and 6 possessed significant cytotoxic activity, although the comparative potencies of these compounds cannot be commented on without the evaluation of the half maximal inhibitory concentration (IC<sub>50</sub>) values. Based on these results, further investigation into the effects on induction of apoptosis was undertaken for these three compounds.

### **Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection**

Fluorescently labeled (FITC) annexin proteins were used as an indicator of the amount of exposed phosphatidylserine (PS) via a flow cytometry-based staining reaction. Propidium iodide (PI), a membrane-impermeable DNA dye, was added simultaneously to the test sample to assist in distinguishing between viable (Annexin V and PI negative), non-viable/necrotic (Annexin V and PI positive) and apoptotic cells (Annexin V positive and PI negative) (Engeland et al., 1997).

An example of typical scatter plots obtained (Figure 2A)



**Figure 1.** The evaluation of cytotoxicity of the compounds against the cell lines. Percentage cell viability, as assessed by the MTT assay, of various cell lines following 100 μM treatment with the compounds (n=3) \*p < 0.05 relative to DMSO control (DC). \*\*p < 0.05 relative to the camptothecin control (PC).

indicates that, as expected, 98.5% of untreated MCF-7 cells remained viable, by allowing for the exclusion of PI and the absence of binding of Annexin-V FITC. An increase in PI and Annexin-V FITC fluorescence due to the presence of apoptotic and necrotic cells induced by physical damage as well as apoptosis via PC treatment is shown in Figure 2B. On treatment with aminonaphthoquinone4, 86.8% of MCF-7 cells underwent late apoptotic events, while 5.1% were in early apoptosis (Figure 2C).

Percentage values representing the physiological state of differentially treated cells were extrapolated from triplicate Annexin-V scatter plots and used to construct Table 1, which show the percentage of cells with specific physiological characteristics.

Viable cells are regarded as Annexin-V FITC and PI negative, apoptotic cells are FITC positive and PI negative, while necrotic cells are FITC negative and PI positive.

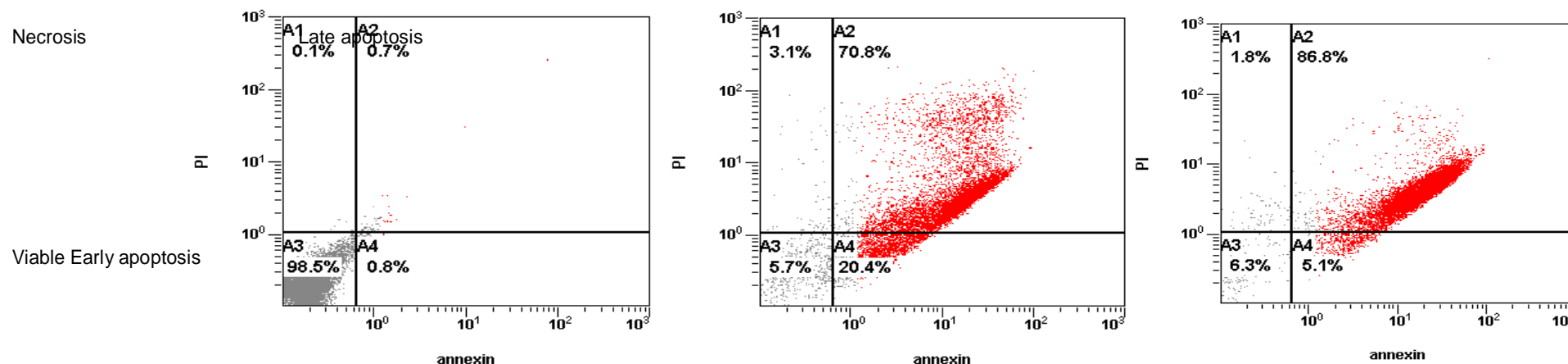
Viable cells maintain intact cell membranes, thus excluding the PI from entering the cell. Results obtained for Annexin-V FITC staining of untreated Jurkat and HeLa cells were similar (p>0.05) to those obtained for MCF-7 cells.

Aminonaphthoquinone4-treated MCF-7 cells showed significantly decreased cell viability indicative of late apoptosis (89.8±1.3%), which was greater than that

noted for cells exposed to the positive control (p < 0.05). PC caused 71.1±3.1% of the MCF-7 cell population to undergo late apoptosis and 14.9±2.8% to undergo early apoptosis.

The disparity between the effects of PC on cell viability and induction of apoptosis in MCF-7 cells can be due to the fact that the MTT assay quantifies the activity of succinate dehydrogenase within the mitochondria, while the Annexin-V assay determines the loss of plasma membrane asymmetry, which occurs in two different regions of the cell.

Aminonaphthoquinone4 induced 57.9 ± 3.1% of HeLa cells to undergo cell death via late apoptosis when compared to the control (p < 0.05), with a relatively small percentage of cells in the early apoptotic stage (2.4±0.8%). This correlates to the MTT results where aminonaphthoquinone4 induced the greatest cytotoxicity, surpassing values obtained for the positive control as well as the other compounds. The viability of aminonaphthoquinone4-treated cells (26.1±3.6%) was comparable to the viability of the PC-treated HeLa cells (26.7±1.3%) (p> 0.05). As expected, aminonaphthoquinones5 and 6 had less of an effect on cell death than aminonaphthoquinone4, which is comparable to MTT results indicating that aminonaphthoquinone4 is a more potent cytotoxic compound than aminonaphthoquinones5 and 6. At 24 h,



**Figure 2.** Annexin V-FITC apoptosis detection.(A), Typical flow cytometry scatter plots of untreated MCF-7 cells; (B), cells treated with 100 μM camptothecin; (C), cells treated with 100 μM aminonaphthoquinone4.

the percentages of cells in the early apoptotic stage under the influence of aminonaphthoquinone5 (4.6±1.0%) and 6(16.0±3.9%) are significantly higher than foraminonaphthoquinone4, further illustrating the greater potency of this compound in inducing apoptosis in a shorter period of time.

Both aminonaphthoquinones5 and 6 decreased Jurkat cell viability by ±70%. Within a 24-hperiod, aminonaphthoquinone4caused 1.7 ± 0.1% of Jurkat cells to enter early apoptosis, with 94.2±2.0% of Jurkat cells undergoing late apoptosis, which is significantly higher (p<0.05) than the 45.4±2.8% induced by PC.

Aminonaphthoquinone 4therefore shows a greater apoptosis-inducing effect than the positive control in Jurkat cells. Aminonaphthoquinones 5 and 6 alsoinduced a significantly greater amount of apoptosis in Jurkat cells when compared to the positive control (p<0.05), but not to the extent of aminonaphthoquinone4. In addition, necrosis was an insignificant contributor to cell death.

MTT and Annexin-V results indicate that aminonaphthoquinone4 has the greatest cytotoxic and apoptotic effects on all three of the cell lines tested, as compared to PC, thereby displaying potential as a lead anticancer compound.

### Caspase 3 activity

Caspase 3 exists as a pro-enzyme within the cell, and is activated during apoptosis. These proteases possess the intrinsic enzymatic ability to cleave intracellular protein substrates at the C-terminal end of the aspartate residue, a vital process during apoptosis. Determination of protein concentration in cytosolic extracts allowed for standardization of the amount of protein in each caspase 3 sample analyzed. Caspase 3 fold-increase was determined for cell lines exposed to compounds for 6 and 24 h (Figure 3). Although caspase 3 activity is presented for the MCF-7 cells, it should be noted that caspase 3

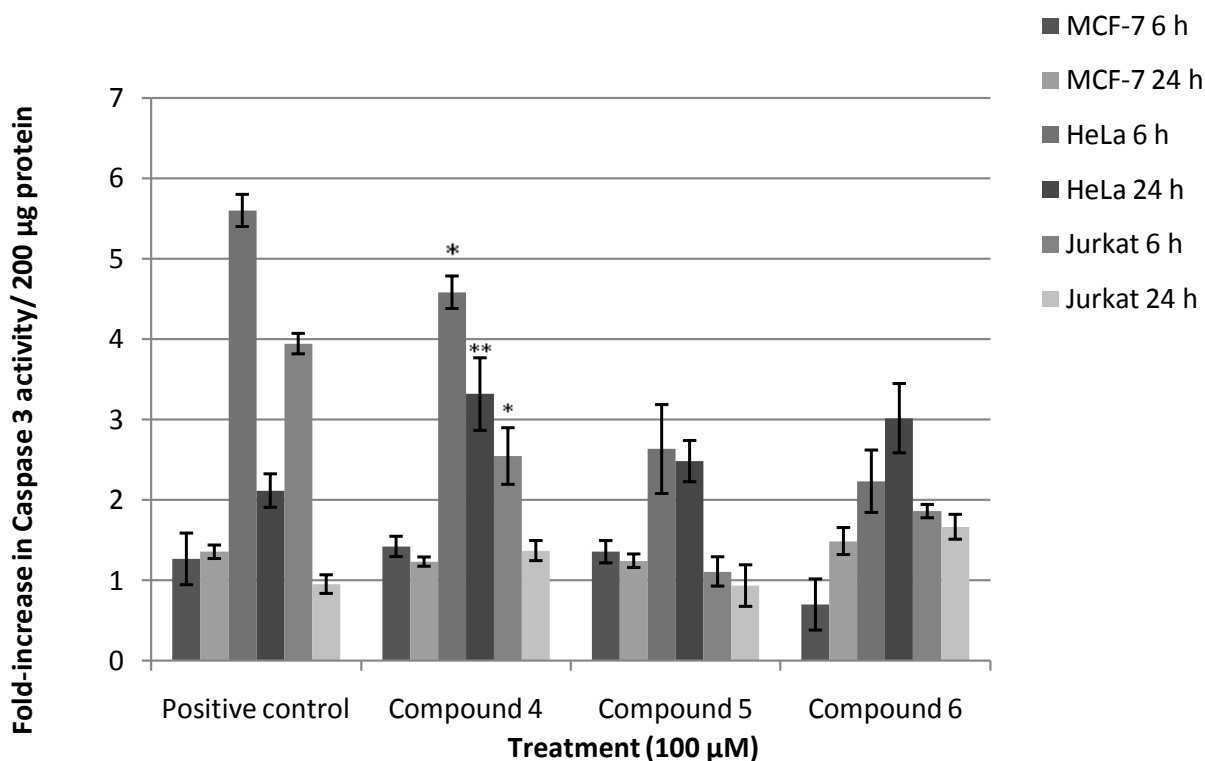
isnot expressed in MCF-7 cells due to a 47-base pair deletion within exon 3 of the CASP-3 gene (Janicke, 2008). The fold-increase represented in the graph is due to the cleavage activity of other effector enzymes, since the DEVD substrate used in the quantification of caspase 3 is a substrate which may be cleared by other effector caspases such as the closely-related caspase 7 (Janicke, 2008), or bystander cleavage of the substrate may have occurred. It should however be noted that DEVD is the preferred substrate of caspase 3.The fold-increase in caspase 3 activities was significantly greater in HeLa and Jurkat cells after being exposed to aminonaphthoquinones4 for 6 hours when compared to 24 h exposure (p < 0.05). This was as expected due to effector caspase activation being an early apoptotic process (Rudin and Thompson, 1997).

Activation of caspase 3 indicates that apoptosis was induced after 6 h via all the test compounds in HeLa and Jurkat cells, while aminonaphthoquinone4 and 6 caused significantly

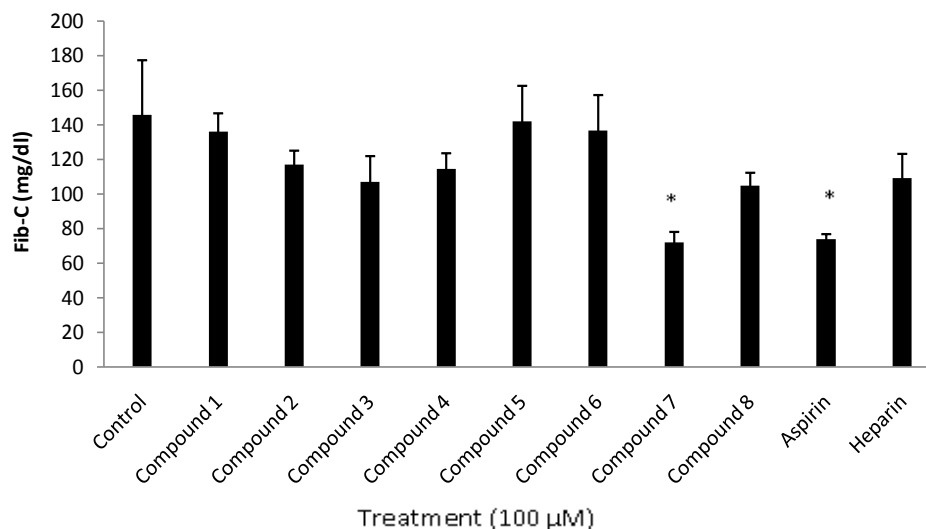
**Table 1.** Annexin-V results<sup>a</sup> showing percentage of MCF-7 cells with specific physiological state under the influence of various experimental conditions (n = 3), using the Annexin-V apoptosis detection kit.

Cancer cell line		Physiological state of cells (%)			
		Viable	Early apoptotic	Late apoptotic	Necrotic
Control (DC)	MCF-7 cells	78.3 ± 0.9	9.8 ± 0.9	5.2 ± 0.7	6.6 ± 0.8
	HeLa cells	78.3 ± 0.9	11.1 ± 2.3	6.9 ± 0.5	3.5 ± 0.6
	Jurkat cells	78.6 ± 1.4	11.3 ± 3.6	5.1 ± 1.1	4.9 ± 0.4
Camptothecin (PC)	MCF-7 cells	8.6 ± 3.6	14.9 ± 2.8	71.1 ± 3.1	5.4 ± 0.2
	HeLa cells	26.7 ± 1.3	14.8 ± 3.1	40.0 ± 0.9	18.4 ± 2.6
	Jurkat cells	26.7 ± 1.3	14.8 ± 3.1	40.0 ± 0.9	18.4 ± 2.6
Aminonaphthoquinone 4	MCF-7 cells	5.2 ± 0.3	4.1 ± 0.9**	89.8 ± 1.3 <sup>#</sup> **	0.7 ± 0.2
	HeLa cells	26.1 ± 3.6	2.4 ± 0.8**	57.9 ± 3.1 <sup>#</sup> **	13.6 ± 1.3
	Jurkat cells	3.7 ± 0.9	1.7 ± 0.1**	94.2 ± 2 <sup>#</sup> **	0.3 ± 0.05
Aminonaphthoquinone 5	MCF-7 cells	29.7 ± 2.3	6.3 ± 2.4**	63.5 ± 1.6**	0.4 ± 0.08
	HeLa cells	35.2 ± 2.1	4.6 ± 1.0**	47.4 ± 3.4**	12.7 ± 1.1
	Jurkat cells	31.7 ± 1.9	43.6 ± 2.8**	24.2 ± 3.3**	0.4 ± 0.02
Aminonaphthoquinone 6	MCF-7 cells	9.4 ± 0.8	2.8 ± 0.9**	82.6 ± 0.7**	5.0 ± 0.8
	HeLa cells	38.1 ± 4.7	16.0 ± 3.9	33.6 ± 5.8	12.2 ± 0.7
	Jurkat cells	30.8 ± 1.9	32.6 ± 2.7**	28.2 ± 1.6**	8.3 ± 0.7

<sup>#</sup>p < 0.05 when compared to the 0.1% control (DC); \*\*p < 0.05 when compared to the positive control (PC) for early and late apoptotic cells.<sup>a</sup>, values indicated represent mean ± standard deviation.



**Figure 3.** Caspase 3 activity. Fold-increase in caspase 3 activity in various cell lines exposed to compound treatment for 6 and 24 h (n = 3) \*p < 0.05 when compared to 24 h exposure, \*\*p < 0.05 when compared to the positive control.



**Figure 4.** Conversion of fibrinogen to fibrin using the Fib-C test. Fib-C tests determining the effect on the concentration of fibrinogen released ( $n = 3$ ). \* $p < 0.01$  relative to the control.

higher caspase 3 levels after 24 h treatment in HeLa cells ( $p < 0.05$ ).

### Coagulation activity

The PT and APTT tests were completed to evaluate the inhibitory effects of the compounds on the extrinsic and intrinsic pathways of coagulation, respectively. Prolongation of PT and APTT would indicate an inhibition of one or more of the coagulation factors in the respective coagulation pathway. None of the library of compounds tested displayed any significant prolongation of these clotting times (results not shown) when compared to a positive control.

Thrombin converts fibrinogen to fibrin in plasma, which is evaluated using the Fib-C test. An increase in Fib-C levels is indicative of cardiovascular disease, and can also be used as an indicator of disseminated intravascular coagulation, which can lead to anaemia or death. The most significant effect on Fib-C levels was noted for the heterocyclic compound 7 (a guanosine derivative), which was equivalent to the effect of the positive control, aspirin (Figure 4). Hyperplatelet activity leads to excess aggregation and adhesion, leading to thrombus formation. Compounds 2, 3, 5 and 8 displayed a higher percentage of platelet inhibition relative to aspirin for the concentrations tested (Figure 5). These were also observed and confirmed using microscopy (Figure 6). Only aminonaphthoquinone 4 displayed significant inhibition of platelet adhesion (Figure 7).

From the small library of compounds tested for the anticoagulant and antiplatelet properties, the results indicate that none of the compounds have significant

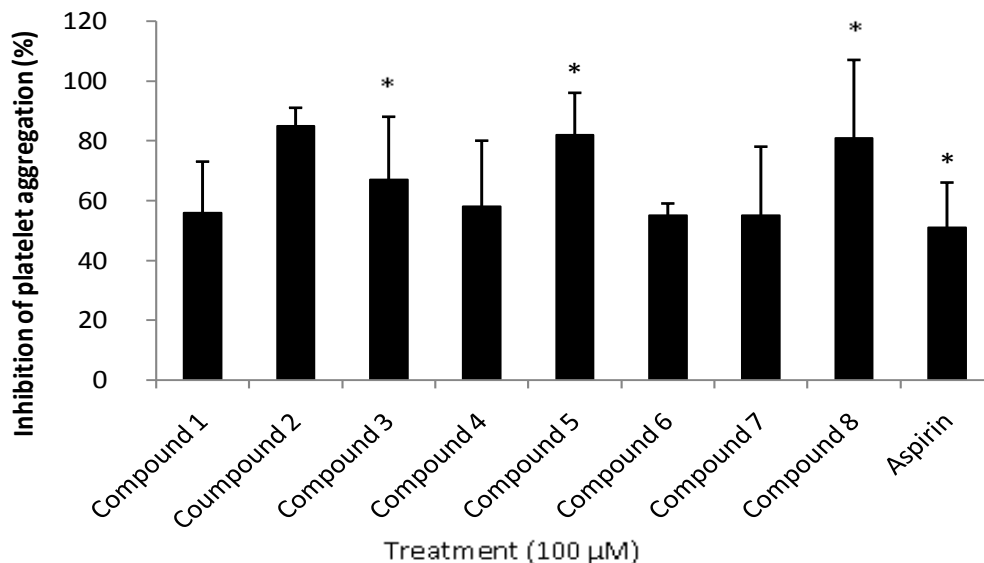
anticoagulant properties. The aminonaphthoquinones have been found to selectively inhibit platelet aggregation with aminonaphthoquinone 4 being the only compound to significantly inhibit both platelet aggregation and adhesion.

### Conclusion

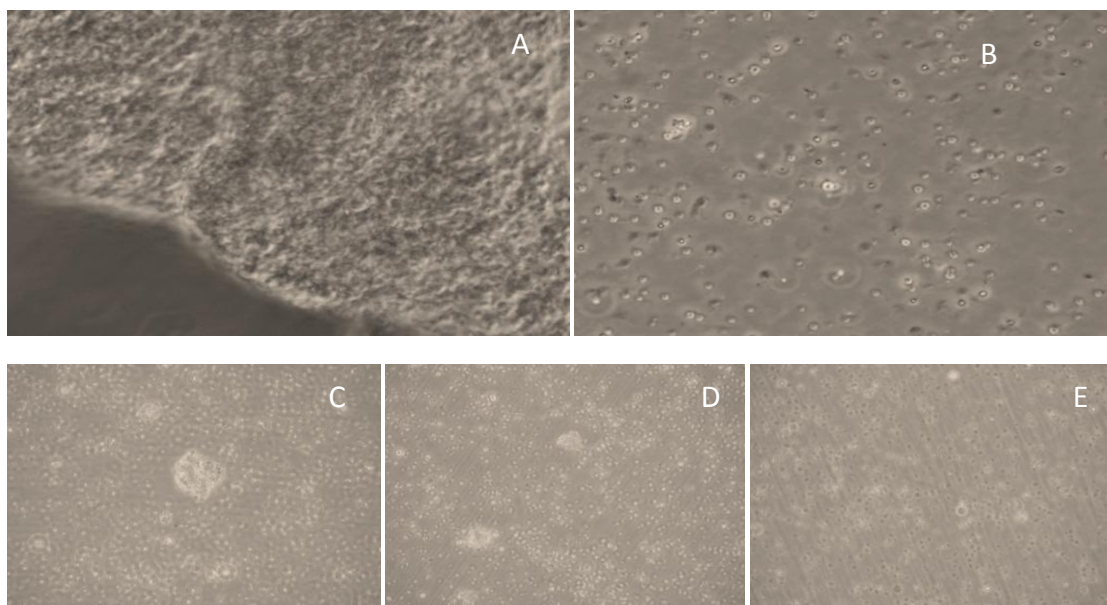
Cancer-related deaths are becoming an increasing economic burden. There has been a plethora of evidence since 1865, that coagulation and platelet abnormalities stimulate tumour angiogenesis and metastasis (Erpenbeck and Schön, 2010). Platelet hyperactivity has been associated with an increased risk of metastasis. Tumours are known to induce platelet activation to acquire metastatic competence. Platelet-derived angiogenesis stimulators further enhance the process (Sabrkhany et al., 2011). Pre-clinical experimentation has noted a correlation between the increased risk of metastasis with increased platelet activity (Brooks et al., 2010). The study thus undertaken was aimed at finding novel synthetic compounds which not only had potential anticancer activity but an associated decreased platelet activity.

The investigation of three classes of novel synthetic compounds allowed for the successful analysis of their effect on apoptotic markers in various cell lines, identifying aminonaphthoquinones as a possible class of compound which may be further developed as anticancer agents. In particular it was found that aminonaphthoquinone 4, was the only compound to significantly inhibit both platelet aggregation and adhesion, and thus holds the most promise as a novel





**Figure 5.** The effect of the compounds on platelet aggregation. The percentage inhibition of platelet aggregation relative to the control as measured in a 96-well microtiter plate ( $n = 3$ ). (\* $p < 0.01$ ) relative to the positive control, aspirin.



**Figure 6.** The effect of the compounds on platelet aggregation using microscopy.

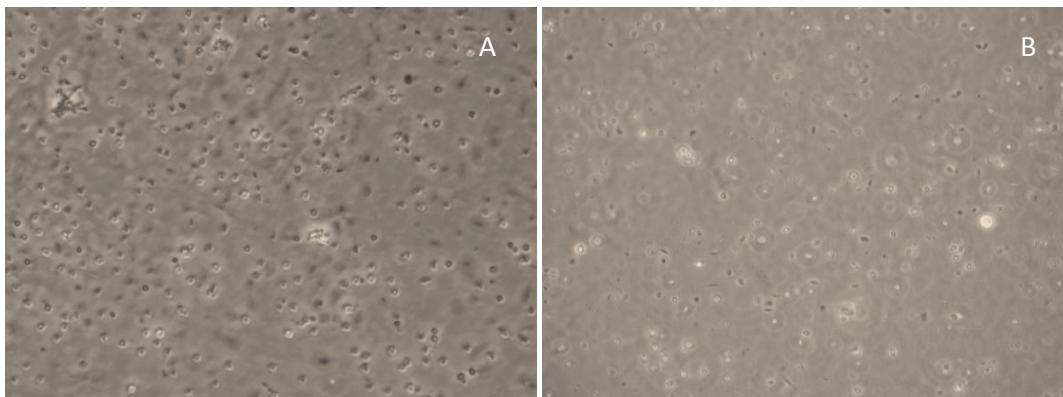
Platelet aggregation in a 10 cm<sup>2</sup> culture dish. (A) Positive control indicating thrombin induced platelet aggregation; (B) control platelets which are not aggregated; and platelets treated with the various selected compounds prior to thrombin-induced platelet aggregation; (C) substituted aromatic compound 1; (D) heterocyclic compound 8; (E) aminonaphthoquinone 6.

anticancer agent.

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**Figure 7.** The effect of the compounds on the ability of platelets to attach to a surface using microscopy. Platelet adhesion using extracellular matrix coated plates. (A) An example of a control with positive adhesion; (B) an example of aminonaphthoquinone $4$  inhibiting platelet adhesion

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