



CHAPTER 7

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Cancer is a group of diseases (more than 100 different diseases), characterized by uncontrolled growth and spread of abnormal cells. Each year many more people are newly diagnosed with cancer. Currently cancer is treated by surgery, radiation, hormones and chemotherapy. Both radiation and chemotherapy induce apoptosis that suppress tumour growth. Failure to activate apoptosis is one of the major obstacles to the successful treatment of cancer with drugs. The loss of specificity of current therapies as well as high toxicity towards the non-cancerous cells led to the seeking of a new anticancer drugs from plants which launches the mechanism of apoptosis in the amelioration of this group of diseases.

The ethanol extract of six plant species namely: *Artemisia afra* (leaves), *Centella asiatica* (leaves), *Euphorbia ingens* (stem), *Euclea natalensis* (roots), *Foeniculum vulgare* (seeds), *Hypoxis hemerocallidea* (corms), and *Tulbaghia violacea* (leaves) were tested for its cytotoxicity against four human cancer cell lines at concentrations ranging from 0.78 to 100 µg/ml. From all the ethanol extracts, *Foeniculum vulgare* was selected based on its cytotoxic activity towards HeLa cells, the highest cytotoxicity at 20.73 ± 0.065 µg/ml and DU145 cells at 56.41 ± 0.281 µg/ml, was selected for isolation of the bioactive compound/s.

The ethanol extract of *Foeniculum vulgare* was fractionated using column chromatography with hexane and ethyl acetate at different ratios as eluent. The cytotoxicity of these



fractions was determined. Fraction eight exhibited the highest cytotoxic activity at $20.98 \pm 4.48 \mu\text{g/ml}$. Therefore, this fraction was subjected to further separation by column chromatography. 4-Methoxycinnamyl alcohol and syringin was isolated and its structure was determined by NMR spectroscopic methods. On MCF-7 and DU145 cells, 4-methoxycinnamyl alcohol, were less cytotoxic at 14.21 ± 0.16 and $22.10 \pm 0.14 \mu\text{g/ml}$, respectively. Syringin is less cytotoxic on MCF-7 cells ($21.88 \pm 0.13 \mu\text{g/ml}$) and was not toxic towards the DU145 cells with an IC_{50} of larger than $100 \mu\text{g/ml}$ the highest concentration tested. Both 4-methoxycinnamyl alcohol and syringin was then tested for its cytotoxicity against U937. 4-Methoxycinnamyl alcohol had its highest cytotoxic activity on HeLa cells at $7.82 \pm 0.28 \mu\text{g/ml}$ whereas syringin had its highest cytotoxicity on HeLa cells at $10.26 \pm 0.18 \mu\text{g/ml}$ and therefore these two isolated compounds were selected for cytotoxicity on PBMCs.

Previously 7-methyljuglone was isolated from *E. natalensis* and had toxicity towards cancer cells. Therefore, 7-methyljuglone and its 5-hydroxy, 5-acetoxy-, 5-alkoxy- and 1,2,4,5-tetra-*O*-acetate derivatives were synthesized to establish their structure-activity relationships as well as to see if there is an induction of apoptosis of a few selected derivatives, and to provide insight into possible mode(s) of action of some of the selected derivatives. From the fifty percent inhibitory concentration values it was clear that the 5-hydroxy (8-Halogen) (1-5) group derivatives had the greatest inhibitory effect of all the derivatives. The 1,2,4,5-tetra-*O*-acetate derivatives (16,17,18) had the least anticancer activity of all the derivatives. The only promising result given by these derivatives (1,2,4,5-tetra-*O*-acetate derivatives) is compound 17 (HeLa $8.389 \pm 4.51 \mu\text{g/ml}$ and SNO $9.389 \pm 3.52 \mu\text{g/ml}$). The 5-ethoxy derivatives (8, 10 and 12), had 50% reduction in cell viability at $11.10 \pm 2.00 \mu\text{g/ml}$ and less on all four cancer cell lines, with 10 (without the Cl at the 8 position) having the best cytotoxic activity of the 5-ethoxy derivatives with an IC_{50} value as low as $3.25 \pm 1.24 \mu\text{g/ml}$ on DU145 cells. Compound 7 and the other two 5-methoxy derivatives, 9 and 11, also had activities of between $10.63 \pm 1.56 \mu\text{g/ml}$ and $3.10 \pm 0.76 \mu\text{g/ml}$ on the four cell lines. This time compound 9 had the best activity of the 5-methoxy derivatives on MCF-7 cells with an $\text{IC}_{50} = 3.10 \pm 0.76 \mu\text{g/ml}$. Of all the 5-hydroxy (8-Halogen) group derivatives (1-5), compounds 4 gave the best cytotoxic effect on all the cell lines with IC_{50} values between $2.24 \pm 0.90 \mu\text{g/ml}$ and the lowest value $2.07 \pm 2.106 \mu\text{g/ml}$. The other 5-hydroxy (8-Halogen) derivatives also had good activity on all the cell lines with



$IC_{50} = 2.11 \pm 0.67$ and higher (highest $IC_{50} = 12.37 \pm 1.25 \mu\text{g/ml}$) on all four the tested cell lines. Compound 18 was most effective ($IC_{50} = 1.09 \pm 0.22 \mu\text{g/ml}$) while the parent compound 6 was less active than several of these derivatives.

All the selected 7-methyljuglone derivatives: 8-Chloro-5-hydroxy-7-methyl-1, 4-naphthoquinone (1); 8-Bromo-5-hydroxy-7-methyl-1, 4-naphthoquinone (2); 8-Fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone (3); 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone (4); 5-Hydroxy-6-methyl-1,4-naphthoquinone (5); 5-Hydroxy-7-methyl-1,4-naphthoquinone (6); and 2,5-dihydroxy-7-methyl-1,4-naphthoquinone (19) as well as both isolated compounds 4-methoxycinnamyl alcohol and syringin were tested for their cytotoxicity against U937 cells and also peripheral blood mononuclear cells.

On the peripheral blood mononuclear cells, cisplatin, the positive control, had high toxicity on the U937 cells at $5.71 \pm 0.13 \mu\text{g/ml}$. The first isolated compound 4-methoxycinnamyl alcohol had higher cytotoxic activity than cisplatin, with an IC_{50} value of $3.550 \pm 0.18 \mu\text{g/ml}$. At the concentrations of 10 and 100 $\mu\text{g/ml}$ 4-methoxycinnamyl alcohol was comparable to that of cisplatin. Syringin however, had much lower cytotoxicity on the U937 cells than 4-methoxycinnamyl alcohol, at $91.14 \pm 0.63 \mu\text{g/ml}$. 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 $\mu\text{g/ml}$ on the PBMCs when compared to cisplatin. At 100 $\mu\text{g/ml}$, 4-methoxycinnamyl alcohol had the highest toxicity towards the PBMCs. Based on the results on and PBMCs cells, 4-methoxycinnamyl alcohol was selected for further investigation to examine the mechanism which this compound uses to inhibit the cancer cells.

On the U937 cells all the selected derivatives were toxic at 5 and 10 $\mu\text{g/ml}$. Cisplatin, the positive control, had the highest toxicity/percentage of inhibition even at 1 $\mu\text{g/ml}$. 5-Hydroxy-6-methyl-1,4-naphthoquinone (Compound 5) was the most toxic of all the selected compounds, and the other compounds had IC_{50} values between 1 and 5 $\mu\text{g/ml}$ and were therefore less toxic. 8-Chloro-5-hydroxy-6-methyl-1,4-naphthoquinone (Compound 4) were the least toxic (U937 cells) of the selected compounds. On the PBMCs compound 3 and 6 and 19 were the least cytotoxic, whilst the other compounds showed much greater cytotoxicity. Compound 6 had the lowest IC_{50} at 18.40 $\mu\text{g/ml}$ and was followed by



compound 19 IC₅₀ at 53.97 µg/ml, therefore more toxic towards PBMCs), than compound 3 which had an IC₅₀ of 188.70 µg/ml. The IC₅₀ values of the other compounds (compounds 1, 2, 4 and 5) were less than 1,00 µg/ml, the lowest concentration tested, and therefore very cytotoxic towards PBMCs.

At 24 hours, cisplatin cell cycle analysis, 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 1.5% to 13.5%, 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 as the control at 48 hours. There were increases in the percentage apoptotic cells for both 1 and 4 from 1.5% to 11.6% and 1.5% to 10.9%, 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 increases in the percentage of SubG1, from 1.5% to 17.7% and 1.5% to 15.7%, respectively. Compound 5 showed, at 24 hours, a good increase in the percentage of apoptotic cells, from 15.0% to 34.4%, and at 48 hours there was an increase from 1.5% to 22.1%. With compound 18, a much lower number of apoptotic cells were observed, from 15.0% to 3.1%, at 24 hours, and at 48 hours almost the same number as for the control were found. 4-Methoxycinnamyl alcohol cell cycle analysis, indicate that it did not arrest the cells at any of the cell cycle stages and no increase in apoptotic cell death was observed, possibly suggesting necrotic cell death.

For the MCF-7 cell cycle analysis, at 24 hours and 48 hours, the positive control, 2-methoxyoestradiol-3,17-O,O-bis-sulphamate (STX140), showed an increase in the number of apoptotic cells from 2 % to 13 % and 2% to 30 %, respectively, and in the G2/M phase from 24% to 74% at 24 h and from 14% to 45 % at 48 h. 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% to 4% and 1% to 4%, 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% to 35%.

To confirm apoptosis, Annexin V-FITC/PI staining was done on U937 cells. From the results it was 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. 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 cell compared to necrotic cells.

With the MCF-7 cells, compound 5 and 2 gave high 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 and 72 hours these percentages were however decreasing.

The activation of caspase 3 is one of the most common hallmarks of apoptosis although not all apoptotic pathways necessarily activate this enzyme. It was clear that after incubation cisplatin did activate caspase 3/7 activity, 6.6%. After incubation, compound 3 and 5 inhibited caspase 3/7 activity (-4.1 and -3.2 respectively), while compound 18 did activate caspase 3/7, 7.4%.

Nuclear staining showed that the derivatives exhibit various characteristic features of apoptotic cell death such as blebbing, dense chromatin and nuclear fragmentation, whereas 4-methoxycinnamyl alcohol revealed a typical necrotic smear on the agarose gel.

Considering the findings from cell cycle analysis, caspase 3/7 activation, annexin V-FITC dual labelling and toxicity towards cancer cells and PBMCs together, 5-hydroxy-6-methyl-1,4-naphthoquinone (5) is the most toxic and exerts its effects through the induction of apoptosis. The apoptotic pathway that is activated appears not to involve caspase 3 activation. Unfortunately, 5-hydroxy-6-methyl-1,4-naphthoquinone (5) was also most toxic to the non-cancerous peripheral blood mononuclear cells and therefore has the potential to lead to serious toxicity in patients. Other compounds, such as 8-fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone (3) and 2,5-dihydroxy-7-methyl-1,4-naphthoquinone (18), were much less toxic to PBMCs but were also less potent in killing the cancer cell lines. There should be a balance between efficiency and general toxicity and therefore 8-fluoro-5-hydroxy-7-methyl-1, 4-naphthoquinone (3) and 2,5-dihydroxy-7-methyl-1,4-naphthoquinone (18) may

be better candidates than the more potent, but toxic, 5-hydroxy-6-methyl-1,4-naphthoquinone (5). 4-Methoxycinnamyl alcohol, alternatively had necrotic cell death activity.

7.2 Recommendations for future work

Further studies are required to understand the effect of different functional group substitutions of the isolated compounds and what effect it might have on the activity as well as the mode of inhibition. For the naphthoquinone derivatives and the isolated compounds the following could also be performed: the activation of the other caspases, inhibition of topoisomerase, microtubule inhibition effect, tyrosine kinase inhibition, the synergistic effect of the naphthoquinone derivatives with doxorubicin or other combined therapies, stability of the formulation as well as absorption of naphthoquinones in the human body.

Even though a large number of molecules exhibit anticancer activity *in vitro*, only few are able to induce anticancer activity without killing normal cells in clinical trials. There is however, a large gap between *in vitro* and *in vivo* studies. Therefore, new strategies are needed for discovering new anticancer agents that validate their efficacy and safety.



CHAPTER 8

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