Evaluation of the effect of selected complementary and alternative medicines on the efficacy of cancer chemotherapeutic agents

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of

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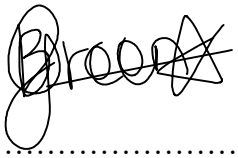
DECLARATION

I, Blake Green, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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Blake Green

On this……22……day of……March……2022

DEDICATION

I dedicate this dissertation to,
my late mother Rhoda Sandra Goldstone
(31-05-1961 to 24-07-2004)
whom I carry forever in my heart,
my dear parents Michael and Caroline Green
for your unconditional love and encouragement through life,
and my sister Leizell Green who has always been my biggest supporter.

ABSTRACT

Breast cancer is the most diagnosed cancer worldwide, and the second leading cause of cancer-related deaths. In Sub-Saharan Africa breast cancer incidence is associated with younger women, late-stage diagnosis, poor prognosis, and limited availability of chemotherapy. Despite the favourable prognosis of certain subtypes of breast cancers, treatment failure occurs as result of drug resistance. Drug resistance is facilitated by the P-glycoprotein (PgP) transporter protein which functions as an efflux pump and is overexpressed in many types of cancer. The upregulation of the inducible protein, haem oxygenase-1 (HMOX1), has been identified as an additional role player in cancer drug resistance. Complementary and alternative medicines (CAMs) are frequently used by cancer patients to improve treatment outcomes and alleviate drug adverse effects. There is a lack of information regarding the effect CAMs may have on the effectiveness of cancer drugs. This study investigates the potential interactions of selected CAMs with chemotherapy drugs using MCF-7 breast cancer cell line as a model.

The MCF-7 breast cancer cells were maintained in culture and the cytotoxic efficacy of the chemotherapy drugs and CAMs were determined by the MTT cell viability assay. Fluorescent stains were used to evaluate morphological changes. The effects of active compounds on the PgP function were determined by the calcein-AM retention assay. Immunofluorescence microscopy was used to evaluate protein expression levels of PgP and of HMOX1 in the MCF-7 cell line

Doxorubicin was most active chemotherapy drug in MCF-7 cells and was selected as the positive control in this study. Of the selected CAMs evaluated, resveratrol, synephrine and caffeine were the most active in MCF-7 cells with IC_{50} values of 80.54 µM, 149.30 µM and 167.40 µM, respectively over 48 hours. In combination treatments of doxorubicin with the active CAMs at IC_{50} concentrations, resveratrol significantly reduced the potency of doxorubicin after 24 hours whilst synephrine enhanced the potency of doxorubicin after 48 hours. Morphological evaluation showed cellular

changes consistent with apoptosis when cells were treated with doxorubicin and resveratrol whilst some cells showed necrosis when exposed to the CAMs.

PgP efflux activity was stimulated at low concentrations of doxorubicin and resveratrol whilst high concentrations of synephrine and caffeine were required after a 24 hour treatment period. During combinations studies, resveratrol enhanced PgP efflux activity in the presence of 0.05 μM and 0.5 μM concentrations of doxorubicin. In contrast, synephrine and caffeine inhibited the PgP efflux activity when used in combination with doxorubicin.

Immunofluorescence showed that doxorubicin strongly induced the expression of PgP protein after both 24 hours and 48 hours. The CAMs had no effects on baseline PgP expression during single-agent treatment. When combined with doxorubicin, resveratrol and caffeine suppressed the doxorubicin increased PgP expression levels after 48 hours. The expression levels of the HMOX1 were induced by doxorubicin and synephrine following single agent treatment. In combination treatments, all the active CAMs suppressed the doxorubicin induced increase in expression of HMOX1.

In summary, synephrine increased the efficacy of doxorubicin. Synephrine and caffeine inhibited doxorubicin associated PgP efflux activity. Resveratrol and caffeine were effective in suppressing the doxorubicin induced increase in expression of PgP. All the CAMs suppressed the doxorubicin induced HMOX1 expression in MCF-7 cells. The data indicate the CAMs investigated in this study may affect the clinical efficacy of doxorubicin. Further investigation is required to elucidate the CAM effects on the efficacy of chemotherapeutic drugs.

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RESEARCH OUTPUTS

Conference Proceedings

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LIST OF ABBREVIATIONS AND ACCRONYMS

ABC	-	Adenosine triphosphate-binding cassette
ABCB1/MDR1	-	Multidrug resistant 1/P-glycoprotein gene
AIDS	-	Acquired immunodeficiency syndrome
AJCC	-	American Joint Committee on Cancer
AP-1	-	Activator protein 1
ATP	-	Adenosine triphosphate
BRCA1	-	Breast cancer gene 1
Calcein-AM	-	Calcein–acetoxymethylester
CAM	-	Complementary and alternative medicine
cAMP	-	Cyclic adenosine monophosphate
DCIs	-	Drug-CAM interactions
DSHEA	-	Dietary Supplement Health and Education Act
ER	-	Oestrogen receptor
GABA	-	Gamma (γ) aminobutyric acid
GLOBOCAN	-	Global Cancer Observatory - Part of IARC
HER1	-	Human epidermal growth factor receptor 1
HER2	-	Human epidermal growth factor receptor 2
HIF-1α	-	Hypoxia-inducible factor–1α
HIV	-	Human immunodeficiency virus
HMOX1	-	Haem oxygenase-1
HSP32	-	Heat shock protein-32

IARC	-	International Agency for Research on Cancer
IC_{50}	-	Inhibitory concentration or potency is the concentration of drug required to achieve a half-maximal effect
MCF-7	-	Michigan Cancer Foundation- 7
MDR	-	Multidrug resistance
MMP	-	Mitochondrial membrane potential
MRP1	-	Multidrug resistance associated protein 1
NF-κβ	-	Nuclear factor kappa beta
NIH/NCCAM	-	National Institutes of Health/National Center for Complementary and Alternative Medicine
Nrf2	-	Nuclear factor erythroid 2 p45-related factor 2
NSCLC	-	Non-small cell lung cancer
PgP	-	P-Glycoprotein
PR	-	Progesterone receptor
RP	-	Relative potency
ROS	-	Reactive oxygen species
SEM	-	Standard error of the mean
TNF	-	Tumour necrosis factor
USA	-	United States of America
US FDA	-	United States Food and Drug Administration

LIST OF SYMBOLS

cm^2	-	Centimeter squared
CO	-	Carbon monoxide
CO_2	-	Carbon dioxide
g	-	Grams
h	-	Hours
KCl	-	Potassium chloride
KH_2PO_4	-	Potassium dihydrogen phosphate
m	-	Meter
mg	-	Milligram
min	-	Min
mL	-	Milliliter
mM	-	Millimolar
ms	-	Millisecond
NaCl	-	Sodium chloride
$NaHPO_4$	-	Disodium hydrogen phosphate
nm	-	Nanometer
rpm	-	Rotations per minute
v/v	-	Volume per volume
w/v	-	Weight per volume
µg	-	Microgram

µL	-	Microliter
µM	-	Micromolar
°C	-	Degree Celsius
%	-	Percent
~	-	Approximately

CHAPTER 1: INTRODUCTION

1.1 Complementary and Alternative Medicines

1.1.1 Concept

Complementary and alternative medicines (CAMs) is generically known as a diverse group of healthcare systems and products, which are not part of standard medical treatment (Pace, 2012; Engler and Li, 2013). These complementary treatment options consist of therapeutic, diagnostic and prevention strategies, which is advocated to complement conventional medicine by contributing to a common whole (Devine and Hayes, 2016).

Complementary medicine is different from alternative medicine, in that complementary medicine is used together with conventional medicine, and alternative medicine is used in place of conventional medicine (Tabish, 2008). Complementary and alternative medicine therapies are poorly understood, and frequently not well researched (Baars *et al.*, 2019; Knecht *et al.*, 2020).

A report published by the Zion Market Research (2017) stated the global dietary supplements (CAM) market was valued at ZAR1.86 trillion in 2016. The report also stated that the vitamins and supplement market in South Africa was valued at ZAR3.8 billion in 2017, showing an increase of R0.9 billion since 2014 (Zion Market Research, 2017). Instead of the development of more effective nutritional supplements, the vast increase in annual retail sales of the nutritional supplement industry is possibly due to exaggerated marketing claims and is not supported by rigorous scientific investigation (Martínez *et al.*, 2012; Temple, 2013).

1.1.2 Types of CAMs

CAMs include herbal therapies, dietary supplements; natural hormones; health and healing practices, such as hypnosis, meditation, yoga, biofeedback, exercise, chiropractic or massage therapy; and non-traditional medical systems, such as acupuncture (Engler and Li, 2013; Devine and Hayes, 2016). The National Institutes of

Health/National Center for Complementary and Alternative Medicine (NIH/NCCAM) have categorised five types of CAM therapies namely: mind-body therapies, biologically-based therapies, manipulative and body-based methods, energy therapies and alternative medical systems (Koithan, 2009). These different types of CAMs are accessed in varying degrees and through various means, either independently by patients or recommended by misinformed medical practitioners (Smith *et al.*, 2014; Paepke *et al.*, 2020). In addition, CAMs use are also driven by cultural, economic and social factors (Devine and Hayes, 2016).

The Dietary Supplement Health and Education Act (DSHEA) gave authority to the United States Food and Drug Administration (US FDA) to regulate dietary supplements, and stated that the products may not claim to diagnose, treat, cure, or prevent any disease (Frankos *et al.*, 2010). Dietary supplements are regulated more like food products, thus the regulations are much less restrictive than for prescription drugs (Baran, 2014). These practices have contributed to doping, mislabelling of supplements as well as contamination of these products by various substances (Schauss, 2012; Mathews, 2018). This raises concern for the wellbeing of consumers of these products and especially cancer patients using CAMs during cancer treatment (Sweet *et al.*, 2016).

1.2 CAM use in cancer patients

Many patients with lifestyle diseases and clinical disorders seek other modalities in an effort to control symptoms and to prevent and treat disease (Krueger *et al.*, 2004). Research have reported that many breast cancer patients use some form of CAM, in addition to normal cancer treatments (Henderson and Donatelle, 2004; Yates *et al.*, 2005; Wanchai *et al.*, 2010). It is reported that patients often use CAMs together with conventional medicine, to assist with the alleviation of adverse effects- whereas others hope for an additional therapeutic effect or even a cure (Drozdoff *et al.*, 2018). It is well known that drugs used to treat cancer may have serious adverse effects (Stenvang *et al.*, 2013; Wellstein *et al.*, 2018).These adverse effects include severe nausea and vomiting, hair loss, bone-marrow suppression, cardiomyopathy, skin toxicity, infertility risk, weight loss and in some cancers weight gain, myalgia, dyspareunia, chronic fatigue, neuropathy and cognitive dysfunction (Miller *et al*., 2016; Palesh *et al*., 2018).

A concern for cancer patients is cancer-related cognitive impairment, an adverse effect that is not fully understood (Ono *et al*., 2015).

Cognitive impairment as an adverse effect of cancer treatment, is commonly known as 'chemofog' or 'chemobrain' (Jansen *et al*., 2011). Prospective research studies suggest this condition may either be an acute effect during pre-treatment or neoadjuvant chemotherapy (Ono *et al*., 2015; Janelsins *et al*., 2017). In an attempt to better understand the cognitive deficits, a review by Selamat *et al.* (2014) reported difficulty in verbal learning, memory, and ability to concentrate in cancer survivors causing a decline in functional performance. Persistent chemobrain has a detrimental impact on the economic, emotional, and interpersonal relationships of cancer patients (Gyöngyvér *et al.*, 2018).

Cancer patients experiencing cognitive dysfunction and cancer-related fatigue are likely to use energy boosting products like coffee and energy-drinks, to enhance their cognitive performance that allows them to sustain normal daily activities (Horneber *et al.*, 2012; Lehrer *et al.*, 2013). In a randomized clinical trial study, guarana (*Paullinia cupana*) which is one of the ingredients in energy drinks, improved cancer-related fatigue in women undergoing chemotherapy for breast cancer (De Oliveira Campos *et al.*, 2011). Energy drinks are in high demand by consumers and recent years have seen the introduction of a wide variety, with various active ingredients (Clauson *et al.*, 2008; Wolk *et al.*, 2012). They often contain high levels of caffeine and other additives such as:- ephedrine and synephrine, that are stimulants (Willson, 2018). A study by Giles *et al.* (2012) evaluated the cognitive effects of glucose, caffeine and taurine found in some energy drinks; and determined that it was caffeine that was responsible for the changes in cognitive performance following consumption.

A retrospective study by Sestak *et al.* (2012) assessed the effects of the hormonal anticancer agents, tamoxifen and anastrozole, on weight gain in postmenopausal women. This study indicated that weight gain occurs primarily within the first 12 months of active treatment in a subset of patients (Sestak *et al.*, 2012). Weight gain during cancer treatment may cause cancer patients to use synephrine-containing slimming aids (Rossato *et al.*, 2011). Campbell and colleagues reported that some energy drinks may contain p-synephrine in combination with the caffeine (Campbell *et*

al., 2013). Since these products are registered as food supplements, patients may consume synephrine and caffeine in high doses (Rossato *et al.*, 2011).

Despite the fact that patients experience the beneficial effects from the use of CAMs, patients do not inform their oncologist that they are using CAMs (Arthur *et al.*, 2013; Knecht *et al.*, 2020). This non-disclosure by patients is deliberate due to the fear of reprisal from their oncologists (Chao *et al.*, 2008; Shelley *et al.*, 2009). When taken during cancer treatment, the use of CAMS may exacerbate the adverse drug reaction of the chemotherapy or may have a direct impact on the efficacy of cancer drugs (Smith *et al.*, 2016; Fasinu and Rapp, 2019; Auxtero *et al.*, 2021). The CAMs reportedly used in cancer is listed on the National Institute of Health's US National Library of Medicine and include curcumin, *Sutherlandia frutescens* (*S. frutescens*), amygdalin and resveratrol (MedlinePlus, 2019). The pharmacological effects of the CAMS: caffeine, synephrine, resveratrol and *S. frutescens* are described in the following sections.

1.2.1 Caffeine

Caffeine (1,3,7-trimethylxanthine) is a central nervous system stimulant of the methylxanthine class, and is used as performance enhancer at low concentrations (Heckman *et al.*, 2010). Caffeine reduces fatigue, acts as an anorexiant, and diuretic (Nall *et al.*, 2016). Primary metabolism of caffeine is by liver enzymes into 1,7-dimethylxanthine (also known as paraxanthine) (Willson, 2018). Caffeine increases the release of various neurotransmitters in different regions of the brain by antagonizing adenosine receptors (A1 and A2A) (Wu *et al.*, 2017).

The adverse effects caused by caffeine consumption depends on the dose and the health status of the consumer (Banerjee *et al.*, 2014). Psychological adverse effects like nervousness and irritation manifest at lower doses of 15 mg/L (Willson, 2018). Caffeine at higher doses of 50 mg/L cause more serious adverse effects in cardiovascular and muscular tissues like palpitations, nausea, tremor, perspiration, and paraesthesia (Willson, 2018). Coffee and energy drinks are known to be the main source of caffeine consumption by the public (Alves and Lima, 2009; Mottram, 2018). A variety of caffeinated energy drinks are commercially available in the market such as Monster, Play, Red Bull, Full, Mother, Wired, Energade, MoFaya, and Reboost (Reissig *et al.*, 2010; Rambe and Jafeta, 2017; Stacey *et al.*, 2017). The caffeine content ranges

between 20.82 to 33.72 mg per 100 mL in commercial energy beverages (Al-Bratty *et al.*, 2020) Other natural stimulants like taurine, ginseng, and guarana are also commonly found in energy drinks (Higgins *et al.*, 2010). In a previous study, Reissig *et al.* (2010) reported a higher caffeine content range between 2.5 to 171 mg per 30 mL in commercially available energy drinks. Findings from this study show that there is variation in caffeine-content of energy drinks available on the market and this raises concern on the regulations of these products (Al-Shaar *et al.*, 2017).

The effects of caffeine are not limited to energy enhancement. Caffeine up-regulated the tumour suppressor proteins (p16, p21, p53 and caveolin-1) and reduced the expression/secretion of various cytokines (IL-6, TGF-β, SDF-1 and MMP-2) which stimulate cancer cell proliferation and invasion in HUVEC and MDA-MB-231 breast cancer cells when treated at 0.2 mM for 1 hour (Al-Ansari and Aboussekhra, 2014). The immune modulatory effects by caffeine is mediated mostly via inhibition of cyclic adenosine monophosphate (cAMP)-dependent phosphodiesterase which leads to increase intracellular accumulation of cAMP (Sabisz and Skladanowski, 2008). Increase d cAMP blocks the Ras/Raf/ERK pathway and decreases cell proliferation at a concentration of 1 mM of cAMP analogue (8-bromoadenosine cyclic monophosphate) as well as inducing the cell cycle inhibitor $p27^{kip1}$ causing cell cycle arrest (Kato *et al.*, 1994; Zhang *et al.*, 2020). These increased cAMP levels decrease the efficiency of removing lethal DNA damage and is beneficial towards anti-tumour activity, whilst this is of concern in normal non-cancerous tissue due to the immunosuppressive effects of cAMP (Raker *et al.*, 2016). Since ephedra-containing performance supplements were banned by the FDA, reformulated energy enhancing supplements contain p-synephrine and caffeine among other alkaloid derivatives (Haller *et al.*, 2008a).

1.2.2 Synephrine

p-Synephrine is a sympathomimetic phenylethylamine derivative that is found citrus fruits, mainly in bitter orange peel, *Citrus aurantium* (Stohs and Ray, 2019). The use of synephrine in dietary supplements for weight loss/management, sports performance, energy, and appetite control has been evaluated (Suntar *et al.*, 2018; Ribeiro *et al.*, 2019). p-Synephrine is structurally related to ephedrine, but the structural differences changes the adrenergic receptor binding characteristics (Rossato *et al.*, 2011). Synephrine therefore has different physiological and pharmacological effect (Rossato *et*

al., 2011; Stohs *et al.*, 2011). There is strong evidence that p-synephrine has lipolytic activity and it is currently marketed as a dietary supplement for weight loss (Hong *et al.*, 2012; Schmitt *et al.*, 2012; Stohs and Badmaev, 2016). p-Synephrine exerts its effects through multiple mechanisms, including its binding to β-3 adrenergic receptors that regulate lipid and carbohydrate metabolism (Stohs, 2017). Niemann and Gay (2003) analysed the synephrine content in products marketed as dietary supplements and demonstrated that the labels do not reflect their true content, synephrine was either absent or present in excess when compared to the quantities indicated on the product labels. In reports of human consumption of synephrine containing supplements there were indications of unwanted cardiovascular effects which included hypertension, tachyarrhythmia, variant angina, cardiac arrest, QT prolongation, ventricular fibrillation, myocardial infarction, and sudden death (Rossato, *et al.*, 2011).

1.2.3 Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenolic phytoalexin stilbene derivative, and is produced in plants by the enzyme stilbene synthase (Ramírez-Garza *et al.*, 2018; Salehi *et al.*, 2018). Resveratrol exists as both cis-(Z) and trans-(E) isomers (Figueiras *et al.*, 2011). Resveratrol transforms from the trans-isomer to the cis-isomer when heated or exposed to ultraviolet irradiation (Sergides *et al.*, 2016). Resveratrol has been used in traditional remedies in belief of the health claims associated with the chemical compound (Gambini *et al.*, 2015). The numerous health benefit claims related to resveratrol is that it functions as an anti-oxidant, anti-aging, cardio-protective, anti-inflammatory and neuro-protective naturally occurring compound (Carter *et al.*, 2014; Salehi *et al.*, 2018). Furthermore, resveratrol has been studied more frequently in laboratory research due to its reported antitumour effects and more clinical studies are needed (Venkatadri *et al.*, 2016; Hernandez-Valencia *et al.*, 2018; Averilla *et al.*, 2019). The reported adverse effects of resveratrol includes nausea, diarrhoea, fatigue, and renal toxicity (Salehi *et al.*, 2018; Shaito *et al.*, 2020).

1.2.4 Sutherlandia frutescens

In South Africa, the Ministry of Health actively promotes the use of traditional medicines as complementary medicine, of which two herbal remedies (*Hypoxis haemrocallidea* and *S. frutescens*) are recommended for the management of many ailments, including cancer (Mills *et al.*, 2005). *S. frutescens* (*Fabaceae*), also known as

the “cancer bush”, is one of the most widely used indigenous plants of southern Africa, and grows in the South West and Northern Cape provinces of South Africa (Faleschini *et al.*, 2013; Aboyade *et al.*, 2014). The herbal plant is widely marketed in South Africa and other countries practicing herbal medicine as *Sutherlandia* tablets or as a leaf decoction or infusion (van Wyk and Albrecht, 2008; Stander *et al.*, 2009).

An *in vitro* study by Faleschini *et al.* (2013) chemically profiled extracts of *S. frutescens* and evaluated the immune modulating activities thereof. Their results showed that the non-polar compounds were more helpful at stimulating the *in vitro* immune system, whilst the polar extracts were found to induce an anti-inflammatory type of response in the HL60 human leukaemia cell line (Faleschini *et al.*, 2013).

The biological activity of *S. frutescens* has been attributed to the main constituents of the extract (Sia, 2004). This being L-canavanine, L-arginine, D-pinitol, and gamma (γ) aminobutyric acid (GABA) compounds (Ntuli *et al.*, 2018). L-arginine is an essential amino acid in protein synthesis and has been reported to exhibit anti-diabetic activity (Akaogi *et al.*, 2006). L-canavanine is a non-proteinogenic amino acid present in most legumes and it has been shown to possess anticancer activity, particularly against pancreatic cancer (Albrecht *et al.*, 2012). GABA is the major inhibitory neurotransmitter in the central nervous system (Free *et al.*, 2018). The sugar D- pinitol (D-chiroinositol analogue) has shown anti-diabetic, hepatoprotective and anti-cachexial activity (López-Sánchez *et al.*, 2018). Rengarajan *et al.* (2014) reported that D-pinitol promoted apoptosis in the MCF-7 breast cancer cell line.

1.3 Breast Cancer

1.3.1 Global Breast Cancer Statistics

The new breast cancer cases reported in 2018 was approximately 2.08 million recorded world-wide (Bray *et al.*, 2018). According to Global Cancer Observatory part of the International Agency for Research on Cancer reported estimates of the worldwide incidence and mortality for all cancers combined for the year 2018 (Ferlay *et al.*, 2019). Breast cancer was the most frequently diagnosed cancer in females in the vast majority of the countries of the world, except in Eastern Africa where cervical cancer surpassed all other cancers (Ferlay *et al.*, 2019). Furthermore, breast cancer was the most frequent

cause of death from cancer in 11 regions of the world, lung cancer in five regions and cervical cancer in the four regions of sub-Saharan Africa.

1.3.2 South African Breast Cancer Statistics

In Southern Africa, South Africa is the greatest contributor to breast cancer mortality and incidence (Cumber *et al.*, 2017). Breast cancer is the second leading cause of cancer-related deaths following cervical cancer in sub-Saharan Africa (Ferlay *et al.*, 2019). The South African National Cancer Registry (2014), shows that breast cancer accounted for the most histologically diagnosed cancer (22 % of all cancers) in South African women (National Cancer Registry, 2019). The incidence and mortality of breast cancer, in South African males and females, was estimated at 182 787 and 78 762, respectively (Ferlay *et al.*, 2019). Harford (2011) reported that the incidence of breast cancer is associated with early age patients and most of the women had advanced-stage disease at the time of diagnosis. Five-year survival for breast cancer was 90% in the United States but 40% in South Africa (Cortes *et al.*, 2020).

1.3.3 Molecular subtypes of breast cancer

Many types of breast cancers in distinct areas of the breast including ducts, the lobules, or surrounding tissue (Rummel *et al.*, 2015). Breast cancer subtypes are determined by the specific cells that are affected (Dai *et al.*, 2015). Based on where the tumour is located in the tissue, breast cancer cells are known as carcinomas found in epithelium, or as sarcomas found in blood vessels and smooth muscle (Sinn and Kreipe, 2013).

Breast cancer has been characterized molecularly into subtypes according to expression of the oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), as well as HER1 and various cytokeratin (eg.CK5/6) (Malhotra *et al.*, 2010; Rivenbark *et al.*, 2013; Dai *et al.*, 2015; Kleibl and Kristensen, 2016). These subtypes are summarised in Table 1.1. The differential expression of these molecular biomarkers provides a clinical classification for breast cancer and dictates therapeutic approaches for treatment (Krop *et al.*, 2017).

The current literature reveals that Luminal A is considered the subtype with best prognosis due to the expression of high levels of ER+/PR+, whereas the Luminal B subtype expresses low levels of ER+/PR+ and either HER2+/- is the more aggressive subtype with poor prognosis (Figueroa-Magalhães *et al.*, 2014).

TABLE 1.1 CHARACTERIZATION OF THE MOLECULAR SUBTYPES ACCORDING TO THE EXPRESSION OF RECEPTORS

Molecular Subtype:	Expression of receptors
Luminal A	ER+/PR+, HER2-, low Ki67
Luminal B	ER+/PR+, HER2+ or HER2-, high Ki67
Triple negative BC or basal-like	ER-/PR- and HER2-, CK5/14
HER2-enriched	ER-/PR- and highly HER2+
Claudin-low	ER-/PR- and HER2-, claudin 3,4,7 and E-cadherin absent
Normal-like	ER+/PR+, HER2-, low Ki67

The luminal A and B subtypes comprise the majority of ER expressing cancers (Skibinski and Kuperwasser, 2015). Luminal A cancers show greater sensitivity to endocrine therapy with little sensitivity to chemotherapy (Liedtke and Kolberg, 2016). Luminal B cancers, on the other hand, demonstrate a greater tendency towards resistance to endocrine therapy, and greater sensitivity to chemotherapy (Liedtke and Kolberg, 2016). The HER-2 positive cancers define a second subtype, having a distinct gene expression profile that is associated with HER-2 gene amplification (Spector *et al.*, 2007). This subtype also has a worse prognosis that is abrogated by treatment with anti-HER-2 therapy (Clavarezza and Venturini, 2008). Together, the basal-like and claudin-low molecular subtypes represent subsets of triple-negative breast cancers, lacking expression of ER and PR (ER- and PR-) and also lacking amplification of *ERBB2* gene (HER-2 negative) (Ismail-Khan and Bui, 2010; Telli, 2016).

Triple negative tumours have poor prognosis (Voduc *et al.*, 2010). This is due to the association of basal-like tumours and Claudin-low tumours with treatment resistance properties attributed by their stem cell like features and mutations in the *TP53* gene which codes for p53 protein and *BRCA1* genes codes for BRCA1 protein (Wahba and El-hadaad, 2015; Bianchini *et al.*, 2016). Claudin-low breast cancers are enriched for markers of epithelial-to-mesenchymal transition and stem cell-like cell features (Prat *et al.*, 2010). The normal-like breast cancers tend to cluster closely with normal breast epithelium in microarray studies (Voduc *et al.*, 2010). It is not yet clear whether this is a distinct molecular subtype of breast cancer or simply a grouping of breast cancers that

are not otherwise classifiable because of contaminating normal epithelium (Turkoz *et al.*, 2013).

1.3.4 Breast Cancer Cell Line Models

Cancer cell cultures grown *in vitro* have been important to study the complexities of tumour biology and provide a platform for drug assessment and improvement (Prat *et al.*, 2013). In the 1970s at the Michigan Cancer Foundation, the MCF-7 cell line was developed from a pleural effusion of a patient with metastatic mammary carcinoma (Levenson and Jordan, 1997; Welsh, 2013). In addition, the cell line possess several characteristics of differentiated mammary epithelium, including the cytoplasmic oestrogen receptor and the capability of forming domes (Comşa *et al.*, 2015). These features are similar to those observed in ER+ tumours, a representative of the Luminal A subtype and a good model for breast cancers that express ER (Lee *et al.*, 2015).

1.4 Breast Cancer Treatment

In treatment of breast cancer, oncologist use a multidiscipline approach which consists of breast surgery, radiation, neoadjuvant therapy (pre-surgery) which aims to reduce the tumour size and/or adjuvant therapy (post-surgery) which reduces the recurrence of tumours post-operatively (Liedtke *et al.*, 2018) .

1.4.1 Surgery and radiation therapy

Early stage breast cancer (Stage 0 - II) is commonly treated by means of surgery and radiation therapy as an adjunct (Liedtke *et al.*, 2018). There are two types of breast cancer surgery available, mastectomy (removal of the entire breast tissue) and breast conservation involves the resection of tumours with minimal loss of normal breast tissue (Warburton *et al.*, 2020). Recurrence in patients treated through surgery and radiation therapy or chemotherapy normally presents as metastatic disease (Tsuji and Plock, 2017).

1.4.2 Chemotherapy

The most active antineoplastic agents against metastatic breast cancer include the anthracyclines, antimetabolites, immunologic therapy, endocrine therapy, DNA alkylating agents, metal ions and antimitotic agents (Pathak *et al.*, 2018; Wellstein *et al.*, 2018). Chemotherapy regimens are typically administered in cycles, usually 3 or 4

weeks, and patient symptoms and performance status are measured with each cycle (Gray *et al.*, 2019).

1.4.2.1 Taxanes and anthracyclines

Taxanes, like docetaxel and paclitaxel, are plant derived which act by binding β-tubulin thus inhibiting the disassembly of microtubules forming aberrant structures (Wellstein *et al.*, 2018). In cell division mitotic spindles are formed by microtubules, and aberrant microtubules cause mitotic-phase cell cycle arrest, and retards the growth of breast cancer tumours (Velasco and Bruna, 2015; Wellstein *et al.*, 2018). Taxanes are used to treat receptor negative breast cancer or breast cancer that has failed to respond to anthracycline therapy (Gradishar, 2012). Common adverse effects associated with taxanes are peripheral neuropathy, leukopenia, neutropenia, fluid retention, hypersensitivity and asthenia (Wellstein *et al.*, 2018).

Anthracyclines include three drugs: doxorubicin, daunorubicin and epirubicin, which are used as first-line therapy for receptor negative breast cancer (Jacquin *et al.*, 2012). These agents act in several ways, which include, intercalation with deoxyribonucleic acids (DNA), complexation with topoisomerase II and DNA and production of free radicals. These mechanisms result in DNA damage and cell death in tumours (Frezza *et al.*, 2018; Pathak *et al.*, 2018). The use of anthracyclines in metastatic breast cancer are associated with adverse effects like, increased and severe cardiotoxicity, nausea, vomiting, alopecia and neutropenia (Mayer and Burstein, 2007). The associated cardiac effects of anthracyclines may limit their use in patients with pre-existing cardiac disease, therefore taxanes are also indicated as first line agents for metastatic breast cancer that is receptor negative (Mcgowan *et al.*, 2017). The extensive use of anthracyclines and taxanes leads to drug resistance and alternative therapy for refractory metastatic breast cancer are used (Edwardson *et al.*, 2013).

1.4.2.2 Antimetabolites and plant alkaloids

Antimetabolites, also known as prodrugs, include purine-pyrimidine antagonist (gemcitabine, 5-fluorouracil and 6-mercaptopurine) and folate antagonist (methotrexate) which are administered in their inactive, and native form (Hassan *et al.*, 2018; Wellstein *et al.*, 2018). Most antimetabolites require an enzymatic conversion into its active form which can directly inhibit enzymes involved in DNA replication or the molecule

incorporates itself into the DNA strand, disrupting DNA repair, and therefore causes S-phase cell cycle arrest (Wellstein *et al.*, 2018). The adverse effects caused by folate antagonists include mucositis, myelosuppression, renal failure, pneumonitis, cerebral dysfunction, skin rash, abnormal liver function, foetal death, and oligospermia (Fernández-Villa *et al.*, 2019). Similar to folate antagonists, the purine-pyrimidine antagonist cause adverse effects like urticaria, teratogenicity, haemolytic or aplastic anaemia, tumour lysis syndrome, neurotoxicity, hand-foot syndrome, and gastrointestinal interference (Ginat, 2015; Wellstein *et al.*, 2018).

Plant alkaloids like camptothecin and irinotecan, are inhibitors of topoisomerases which unwind DNA during replication and have been used to treat a broad spectrum of human cancers (Wellstein *et al.*, 2018). Vinblastine, vincristine and vinorelbine are examples of vinca alkaloids derived from the periwinkle plant *Catharanthus roseus* (Moudi *et al.*, 2013). These compounds have cell cycle-specific activity in the mitotic-phase, where tubulin polymerization is inhibited, and preventing formation of the mitotic spindle causing mitotic-phase cell cycle arrest (Moudi *et al.*, 2013; Levitsky and Dembitsky, 2015). The adverse reactions are broad, including typical side effects of cytotoxic chemotherapeutics, such as myelosuppression, mucositis, fever, anaemia, and alopecia (Thirumaran *et al.*, 2007; Wellstein *et al.*, 2018).

1.4.2.3 Hormone therapy

Hormone therapy is indicated in cases where there is insignificant visceral metastasis, slow disease progression and a long disease free interval (Rugo *et al.*, 2016) . Tamoxifen, which is a partial agonist at the oestrogen receptor is commonly used to treat ER positive breast cancer and given as adjuvant therapy to reduce the risk of recurrence and/or death from breast cancer (Yang *et al.*, 2013; Silva *et al.*, 2018). The primary mechanism of action for tamoxifen is competitive inhibition of oestrogen on the oestrogen-receptor therefore retarding the growth of breast cancer cells (Silva *et al.*, 2018).

In recent years, aromatase inhibitors have become favourable alternatives to tamoxifen due to a better toxicity profile (Kelly *et al.*, 2015; Jameera Begam *et al.*, 2017). Aromatase inhibitors like anastrozole, letrozole and exemestane are frequently prescribed for patients with oestrogen receptor-positive (ER+) breast cancer to control

advanced disease and to prevent relapse after treatment with localized breast cancer (Ma *et al.*, 2015). The aromatase inhibitors act through the inhibition of the aromatase enzyme, a cytochrome P450 enzyme that converts testosterone into oestrogen and progesterone (Thirumaran *et al.*, 2007; Gibson *et al.*, 2009). Therefore, aromatase inhibitors decrease the production of oestrogen and progesterone resulting in the inhibition of growth in hormone receptor positive tumours. Patients treated with aromatase inhibitors have an increased risk of decreased bone density and arthralgia (Servitja *et al.*, 2015).

1.4.2.4 Targeted therapy

Targeted therapy includes monoclonal antibodies like trastuzumab and kinase inhibitors like everolimus (Raval *et al.*, 2016). Trastuzumab is a recombinant monoclonal antibody that binds and blocks the action of HER2. The HER2 is a growth factor receptor belonging to the HER2 family that is overexpressed in breast cancer and linked to progression of tumours (Vu and Claret, 2012).A second agent lapatinib was approved in 2007 for the use in the treatment of breast cancer that overexpresses HER2 and is refractory to trastuzumab (Higa and Abraham, 2007). Lapatinib is a potent reversible and selective tyrosine kinase inhibitor that competitively binds to the tyrosine kinase domain of HER1 and HER2 receptors inhibiting their function (Opdam *et al.*, 2012). Treatment with monoclonal antibodies show acute adverse effects like fever, chills, nausea, dyspnoea, and rashes (Wellstein *et al.*, 2018). More serious adverse effect often caused in treatment using trastuzumab is cardiotoxicity, and combination regimens including doxorubicin will increase the risk of heart failure (Onitilo *et al.*, 2014; Galluzzi *et al.*, 2018).

1.5 Cell death

Four main types of cell death have been identified: apoptosis, necrosis, autophagy, and mitotic catastrophe (Galluzzi *et al.*, 2018). Apoptosis is the preferred cell death in cancer treatment due to reduced incidence and severity of tumour lysis syndrome associated with this type of cell death (Baig *et al.*, 2016; Cohen *et al.*, 2021).

1.5.1 Apoptosis

Apoptosis, also known as programmed cell death, is a controlled process that unicellular and multicellular eukaryotes undergo in response to foreign insults (Kaczanowski,

2016; Galluzzi *et al.*, 2018). Depending on the stimuli, favourable or unfavourable, cell receptors will either stimulate signals to promote growth of the cell or induce cell death (Sever and Brugge, 2015). Apoptosis is executed by the activation of a class of aspartate-specific cysteine-dependent proteases, called caspases, that activate and initiate cleavage of signal transduction proteins, leading to cells death (Shalini *et al.*, 2015). Caspases are synthesized as inactive zymogens that become activated upon cleavage by proteolytic caspases or by oligomerization induced by the formation of large multi-protein complexes (MacKenzie and Clark, 2012). Apoptotic cell death occurs via two main signalling pathways: inducible extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (De Vitto *et al.*, 2018). This type of cell death involves numerous physiological changes in the cell including: cell shrinkage due to the condensation of the cytoplasm, the degradation of the nucleus observed as chromatin condensation, and protrusions of the plasma membrane called blebs caused by interference of the polar charges at the surface of the plasma membrane (Atale *et al.*, 2014). Later stages of apoptosis involve the formation of apoptotic bodies that are usually engulfed by surrounding neighbouring cells or macrophages as part of homeostasis (Nikoletopoulou *et al.*, 2013; Galluzzi *et al.*, 2018).

1.5.2 Autophagy

Autophagy is a process characterised by the digestion of the cells contents in order to provide energy in response to nutrient deprivation and in some cases exposure to chemotherapy (Galluzzi *et al.*, 2018; Yu *et al.*, 2018). There are three main types of autophagy in mammalian cells, microautophagy, chaperone-mediated autophagy, and macroautophagy which is frequently studied (Feng *et al.*, 2014; Yu *et al.*, 2018). The most prominent morphological features observed during autophagy are extensive formation of double membrane autophagic vacuoles and lack of chromatin condensation (Larsson *et al.*, 2016). The formation of autophagosomes, which engulf proteins and other cytoplasmic components, fuses with lysosomes for degradation and either recycles the macromolecules for energy or degrades pro-survival proteins which signal other programmed cell death mechanisms (Yonekawa and Thorburn, 2013; Jung *et al.*, 2020).

1.5.3 Necrosis

Necrotic death, typically an unprogrammed cellular process, characterised by cell swelling and membrane rupture, and typically followed by inflammatory reactions (Los

et al., 2002; Nikoletopoulou *et al.*, 2013). Necrotic cell death has been shown to occur via two regulated pathways: the death receptor pathway which is activated by external stimuli and/or the mitochondrial pathway which is initiated by intrinsic stimuli (Galluzzi *et al.*, 2018). Necroptosis is a common form of regulated necrotic cell death, and activates caspase-dependent signalling pathway whilst this pathway is distinct to apoptosis (Galluzzi *et al.*, 2018; Tang *et al.*, 2019; Yan *et al.*, 2020). The inflammatory cytokine called the tumour necrosis factor (TNF) is known as an external stimulus that signal the cell surface death-receptor, which leads to cross-talk between necroptosis and apoptosis (Nikoletopoulou *et al.*, 2013; Morioka *et al.*, 2014; Tang *et al.*, 2019).

1.5.4 Mitotic catastrophe

Mitotic catastrophe is a cell death occurring during or shortly after a dysregulated or failed mitosis and can be accompanied by morphological alterations such as micronuclei, which are often chromosome fragments in vesicles, and multinucleation resulting from deficient separation during cytokinesis (Vitale *et al.,* 2011). In a recent review, Vitale *et al.* (2017) discussed that mitotic catastrophe cannot execute cell death as definite, but rather that it constitutes an onco-suppressive mechanism involving caspase 2 which interacts cell death pathways. Cell death, accidental or regulated, may occur in multiple forms in response to different stresses (Galluzzi *et al.*, 2018; Tang *et al.*, 2019). The loss of control over single or cross-talks between types of cell death contributes to human diseases such as cancer, neurodegeneration, autoimmune diseases, and infectious diseases (Nikoletopoulou *et al.*, 2013).

1.6 Drug resistance

Despite the many advancements in cancer therapy, the emergence of drug resistance remains a problem (Holohan *et al.*, 2013). Multidrug resistance (MDR) is a phenomenon in which cancer cells exhibit a cross-resistant phenotype against multiple unrelated drugs that are structurally and/or functionally different and have different molecular targets (He and Wei, 2012; Stavrovskaya and Rybalkina, 2018; Tinoush *et al.*, 2020).

Cancer cells may exhibit intrinsic MDR or may acquire MDR during chemotherapy. For intrinsic MDR, cancer cells exhibit resistance to chemotherapy at their initial exposure to the anticancer drug (Wang *et al.*, 2019). However, in acquired MDR, resistance to

chemotherapy occurs during the course of the treatment or upon recurrence of the disease after successful chemotherapy (Alfarouk *et al.*, 2015; Wang *et al.*, 2019). Several host factors are involved in the development of both intrinsic and acquired MDR including: a change in transport of drugs across membranes, genetic changes (increased transcription of oncogenes) or a decrease in target , increased DNA repair mechanisms and changes to the structures of target molecules which confers drug insensitivity (Ray *et al.*, 2017).

In clinical practice, two or more MDR mechanisms often act simultaneously in each cancer type making the design of treatment regimens challenging (Coley, 2008). Research over the past decade focused on enhancing the efficacy of chemotherapy by suppressing or evading these MDR mechanisms including the use of new anticancer drugs that could escape from efflux mechanisms, MDR modulators or chemosensitizers, multifunctional nanocarriers, and RNAi therapy (Breier *et al.*, 2012; Shaffer *et al.*, 2012; Safhi *et al.*, 2017).

Since several factors are involved in the development of both intrinsic and acquired MDR, a clear understanding of these molecular mechanisms is necessary to develop effective treatment regimens. Hypoxia in cancer potentiates multidrug resistance via different cellular pathways such as lost sensitivity to p53-mediated apoptosis, and enhanced P-glycoprotein (PgP) expression. To date, the most widely studied MDR mechanisms are those associated with drug efflux mechanisms involving adenosine triphosphate (ATP)-dependent transmembrane transporter proteins like PgP (Shi *et al.*, 2009; Baguley, 2010; Gillet and Gottesman, 2011; Bugde *et al.*, 2017; Nanayakkara *et al.*, 2018).

In humans, more than 48 MDR genes were identified from the ATP-binding cassette (ABC) transporter superfamily. The most extensively characterized MDR transporters are PgP (*MDR1*/*ABCB1*), multidrug resistance associated protein-1 (*MRP1*/*ABCC1*), and breast cancer resistant protein (*ABCG2*) (Szakács *et al.*, 2006; Shi *et al.*, 2009; Abdullah and Chow, 2013).

1.6.1 P-glycoprotein

The subfamily B member of the ABC transporters, PgP is a 170 kilodalton plasma membrane protein encoded by the *MDR* resistant gene *MDR1*/*ABCB1* and homologue

MDR3/*ABCB4* gene (Rosmorduc and Poupon, 2007; Seelig, 2020). The protein has two identical halves, which consist of two transmembrane domains that form a passage for substrates, two nucleotide-binding domains that bind and hydrolyse ATP, three glycosylation sites and two phosphorylation sites (Ooko *et al.*, 2016; Dewanjee *et al.*, 2017). Many transcription factor-binding sequences, including nuclear factor-κB (NF-κβ) , Y-box binding protein-1, activator protein-1 (AP-1), and hypoxia-inducible factor-1 (HIF-1) are involved in the transcription of the *MDR1* gene (Katayama *et al.*, 2014). The activities of these transcription factors in PgP expression are linked to various signal transduction pathways, and the two main pathways are; the mitogen-activated protein kinase (MAPK), and the phosphatidylinositol 3-kinase (PI3K) pathways vital cell survival signalling pathways (Li *et al.*, 2012). The regulatory mechanisms of PgP mRNA and protein expression have been associated with activation or inactivation of oncogenes (Ras and c-Raf) and transcriptional process, MDR1 mRNA translation into PgP and post translational modification, and protein intracellular trafficking (Szaflarski *et al.*, 2013; Robinson and Tiriveedhi, 2020).

In the human body, PgP protein is found in vital organs like the intestinal tract, brain and spleen to provide protection from toxic substances including drugs (Kim and Chen, 2018). The protein functions as an ATP-dependent efflux pump for drugs and xenobiotics including neutral or positively charged hydrophobic compounds, and is present in tissues involved in the absorption and metabolism of drugs (Dewanjee *et al.*, 2017; Stavrovskaya and Rybalkina, 2018). P-glycoprotein can detect and bind a large variety of anticancer drugs including anthracyclines, epipodophyllotoxins, vinca alkaloids, and taxanes (Mueller *et al.*, 2004; Shaffer *et al.*, 2012).

The hyperactivity and overexpression of PgP protein in cancer has been linked to reduced chemotherapeutic responses and poor clinical outcome leading to resistance to chemotherapy in various cancer types such as leukaemia and breast cancer (Bugde *et al.*, 2017; Tinoush *et al.*, 2020). It is evident from the literature that tumours originating from tissues with naturally high levels of PgP expression may be intrinsically drug resistant, examples are colorectal, kidney, pancreas, and liver carcinoma (Abdallah *et al.*, 2015). The activation of PgP follows after the substrate binds to the binding pocket, one of the PgP nucleotide-binding sites (H or R) is activated, two ATP molecules binds to the nucleotide-binding domain where it is hydrolysed, leading to conformational

change of the protein structure, which causes expulsion of the drug from the cell (Johnson and Chen, 2018; Kim and Chen, 2018).

The FDA (USA) requires documentation regarding potential drug interactions with PgP and several other clinically important transporters prior to approval of any new drug (Giacomini *et al.*, 2010). Several classes of chemicals and clinically approved drugs are known PgP inhibitors for example calcium channel blockers (verapamil), antiarrhythmic drugs (quinidine), antibiotics (cephalosporins), proton pump inhibitors (omeprazole), and hormones (progesterone) positively tested in cellular assays (Abdallah *et al.*, 2015; Brayboy *et al.*, 2018; Lai *et al.*, 2020). Despite the success of PgP inhibitors in vitro, there are no approved drugs available to use clinically to overcome MDR by specifically targeting PgP (Shaffer *et al.*, 2012). The limitation of available PgP inhibitors in clinical use are highlighted and considered as challenges to overcome (Srivalli and Lakshmi, 2012). The challenges of these inhibitor drugs include the undesired toxicity to normal surrounding tissue, the general low potency of these inhibitors, low specificity, and drug interactions (Gillet and Gottesman, 2010).

Research have focused on strategies to overcome PgP-mediated MDR by developing new or modified anticancer drugs, designing delivery systems, and antisense strategies such as ribozymes or small interfering RNAs that down regulate *ABCB1* gene expression (Gillet and Gottesman, 2011; Bugde *et al.*, 2017; Dinić *et al.*, 2019). Since the role of PgP in cancer MDR is well reported, development of selective PgP inhibitors to suppress drug efflux with further assessment of safety and efficacy of these drugs remains relevant (Bugde *et al.*, 2017; Waghray and Zhang, 2018a).

1.6.2 Haem oxygenase-1

Heat shock protein-32 (HSP32), also known as haem oxygenase-1 (HMOX1), is a 32 kilodalton haem-degrading enzyme part of the HSP family (Podkalicka *et al.*, 2017). In mammalian cells, the stress response involves the induction of 4 major members of HSP families named according to their molecular weight: HSP27, HSP60, HSP70 and HSP90 (Lianos *et al.*, 2015) . In comparison to other chaperone molecules, HMOX1 is involved in anti-inflammatory reactions and protects sensitive cells from oxidative stress by degrading toxic haem into free iron, carbon monoxide (CO) and biliverdin (Reed *et al.*, 2010). Furthermore, biliverdin is then reduced into bilirubin, a linear

tetrapyrrole with antioxidant properties (Maines and Panahian, 2001). Expression of HMOX1 is low under normal physiologic conditions, and a variety of stimuli and activated signalling molecules including haem, reactive oxygen species (ROS), nitric oxide species, prostaglandins, cytokines, growth factors such as insulin, and lipopolysaccharide can up-regulate its expression (Liebert *et al.*, 2005).

It is known that HMOX1 is highly expressed in the organs such as the spleen, reticuloendothelial cells of the liver and bone marrow, which are responsible for degrading senescent red blood cells, and HMOX1 in macrophages is involved in the recycling of haemoglobin-haem (Chen *et al.*, 2019). HMOX1 is an integral protein of the smooth endoplasmic reticulum , however it can localize to other compartments including caveolae, mitochondria, and the nucleus where it is thought to mediate signalling functions (Dennery, 2014; Chau, 2015; Mascaró *et al.*, 2021). McAllister *et al.* (2004) reported the HMOX1 mRNA expression is upregulated by oncogenes (e.g., Kaposi sarcoma-herpes virus and BCR/ABL kinase). HMOX1 transcription is controlled by key regulators like activator protein-1 (AP-1), nuclear factor kappa-beta (NF-κβ), hypoxia inducible factor-1 (HIF-1) and nuclear factor erythroid 2 p45-related factor 2 (Nrf2) which show overexpression in many tumours and contribute to chemo-resistance (Barton *et al.*, 2003; McAllister, 2004; Lombaert *et al.*, 2013 and Furfaro *et al.*, 2016).

It was reported that upregulation of HMOX1 expression may play a role in protecting breast cancer cells from chemotherapeutic drug cytotoxicity (Tan *et al.*, 2015). The up-regulation of HMOX1 expression leads to cells evading programmed cell death (Gozzelino *et al.*, 2010). This may result in cancer treatment failure (Wagener *et al.*, 2013). In support of this statement, a study by Shiraishi *et al.* (2018) evaluated the functional significance of HMOX1 expression in cisplatin-induced renal injury. The results obtained from *in vivo* experiments demonstrated that transgenic mice deficient in HMOX1 showed more nephrotoxicity, as assessed by structural and functional changes, compared with wild-type mice treated with cisplatin (Shiraishi *et al.*, 2018). In contrast Shiraishi and co-workers (2018) found that their results from *in vitro* experiments showed that haemin, an inducer of HMOX1, significantly decreased cisplatin-induced cell death in human renal proximal tubule cells.

A study by Petrache *et al.* (2019) demonstrated that conditional overexpression of HMOX1 prevents tumour necrosis factor-α (TNF-α) induced apoptosis in murine fibroblasts. Chen *et al.* (2004) demonstrated that increased expression level of HMOX1 in papillary thyroid carcinoma cells reduced the TNF-α and cycloheximide signal induction of apoptosis. In addition, Gozzelino and colleagues (2010) reported that exogenous administration of a low concentration of CO prevents TNF-α induced apoptosis.

1.7 Research Proposal

1.7.1 Problem statement

The use of CAMs is widespread, and widely used by cancer patients (Wanchai *et al.*, 2010; Tautz *et al.*, 2012; Fox *et al.*, 2013). These products potentially contain biologically active compounds, which may cause interactions with conventional cancer treatments when used concurrently (Jones *et al.*, 2019).

One of the major concerns against the use of CAMs in patients is due to the risk of these products being contaminated (Van Thuyne *et al.*, 2006; Deldicque and Francaux, 2016). This implies that the content of nutritional supplement products and CAM products, to that of the information provided on the label could differ (Petroczi *et al.*, 2011). Gabriels *et al.* (2015) reported that supplement products may be purposefully contaminated and may contain harmful substances as determine using tandem mass spectrometry studies. Substances such as melamine, steroids and pesticide residues may be found in these supplements (Gabriels *et al.*, 2015). CAM products are regulated as food, and the quality of these products are poorly controlled (Schauss, 2012; Baran, 2014). Therefore, cancer patients consuming nutritional supplements may be at risk of ingesting potentially harmful chemicals.

A variety of CAMs have been listed in the National Institute of Health's U.S. National Library of Medicine such as curcumin, *S. frutescens*, quercetin and resveratrol (https://medlineplus.gov/druginfo/herb_All.html). However, the use of CAMs predisposes cancer patients to a high risk of having interactions between the cancer drugs used in chemotherapy and the CAMs they consume (Sheela *et al.*, 2010; Ramos-Esquivel *et al.*, 2017). This may decrease the therapeutic efficacy and/or increase adverse drug reactions compromising the chemotherapeutic outcome (Yap *et al.*, 2009).

1.7.2 Research Aim

This study intends to evaluate the potential anticancer efficacy of individual components of energy drinks and selected complementary medicines and determine their potential interactions with known cancer treatments using MCF-7 breast carcinoma cells.

1.7.3 Research objectives

The study will use the MCF-7 breast cancer cell line and the objectives are as follows:

i. Screening at 2 concentration levels to identify the most active chemotherapeutic agent from three considered and the three most active CAMs from four considered
ii. Determine IC_{50} values of active anticancer drug and CAM compounds.
iii. Determine the IC_{50} values of most active anticancer drug when treated in combinations with the active CAMs.
iv. Evaluate the effects of anticancer drugs and CAM compounds on cancer cell morphology.
v. Evaluate the effects of the active anticancer drug and CAM compounds on the PgP efflux activity.
vi. Evaluate the effects of the active CAMs on the efflux of the most active anticancer during combination treatments
vii. Determine the effect of the active anticancer drug and CAM compounds on the levels of expression of PgP.
viii. Determine the expression levels of PgP in combination treatment of the most active anticancer drug with the active CAMs.
ix. Determine the effects of the active anticancer drug and CAM compounds on the induction of HMOX1 expression levels after single-agent and combination treatment.
x. Determine the expression levels of HMOX1 in combination treatment of the most active anticancer drug with the active CAMs

CHAPTER 2: METHODS

This chapter provides the research methodology, and the design that was applied to attend to the set objectives as presented Chapter 1 Section 1.7.3. All aqueous solutions were prepared using double distilled Milli-Q ™ water unless otherwise stated (Millipore Elix 3 Water Purification System). Sterile laboratory techniques were practiced.

2.1 Solubilisation of compounds

Of the chemotherapeutic drugs, gemcitabine and irinotecan were solubilised in DMSO which is a known organic solvent. Doxorubicin was solubilised in absolute ethanol (BioUltra culture grade, Sigma-Aldrich, Johannesburg South Africa) and all chemotherapeutic drugs were prepared at stock concentrations ≥10 mM. The CAMs were prepared at 40mM stock concentrations. Resveratrol and synephrine were solubilised in absolute DMSO (BioUltra culture grade, Sigma Johannesburg South Africa). Caffeine and amygdalin were soluble in 75% ethanol (v/v), but amygdalin did not remain in solution. Upon gradual heating to a final temperature of 60 °C, amygdalin was completely dissolved. The prepared compounds were stored at 4 °C.

2.2 Extraction of *Sutherlandia frutescens* herb mixture

S. frutescens (Cancer Bush) herb mixture was purchased from a health store in Osfonteinspruit, Northern Cape, South Africa. The herb mixture consisted of dried leaves and stems which were grinded into fine powder. In a beaker, 5g of the fine herb powder stirred in 100mL of methanol (anhydrous grade, Sigma-Aldrich, Johannesburg) to form a mixture. The methanol preparation was stirred overnight at room temperature. Methanol has been used as a solvent for *S. frutescens* extract preparations that have been tested in previous research studies (Tai *et al.*, 2004; Aboyade *et al.*, 2014; Ntuli *et al.*, 2018). The *S. frutescens* methanol extract was filtered, and the filtrate was placed in a fume hood in order to evaporate the methanol. The methanol in the herb mixture did not evaporate after a week incubation period, and this preparation was not used in this study. In another preparation, 5g of the fine powder was constantly stirred in 50 mL of

distilled water heated at 80 °C in a beaker for 30 min. The *S. frutescens* hot water extract is one of the formulations that have been used in studies investigating the herb (Aboyade *et al.*, 2014; Omoruyi *et al.*, 2018). The stirred *S. frutescens* hot water extract was allowed to cool to room temperature and the mixture was filtered into volumetric flask. The hot water extract filtrate was halved into two beakers to test for two considered solvents. The filtrate was evaporated in a fume hood overnight, and the dry residue in both beakers were weighed. The two solvents considered was sterile saline and DMSO. The dry hot water extract residue did not completely dissolve in 5 mL of DMSO. Following solubilisation of the hot water extract in 5 mL of saline, the solution was sterile filtered through a 0.22 µm membrane. The final stock concentrations of the *S. frutescens* hot water extract dissolved in saline was calculated to be 93.96mg/mL and aliquots were stored in a 4 °C refrigerator. The *S. frutescens* hot water extract was thawed slowly to 37 °C and vortexed when used in experiments. A 60% ethanol (w/v) formulation of *S. frutescens* product was purchased from a health store (Dis-Chem retail pharmacy, Johannesburg, South Africa) and was used as one of the herb extract formulations in this study.

2.3 Cell culture

2.3.1 Cell culture initiation

The MCF-7 breast cancer cell line was purchased from the American Type Culture Collection (ATCC) and verified for authenticity by Short tandem repeat analysis by Inqaba Biotechnical Industries, Pretoria, South Africa. The MCF-7 breast carcinoma cells were stored in liquid nitrogen and was rapidly thawed in a water bath at 37 ºC as was needed. Once thawed, the cell suspension was transferred to a 25 cm^2 Greiner bio-one culture flask (Lasec, Johannesburg, South Africa) containing culture medium composed of 1:1 (v:v) Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Johannesburg, South Africa) and Hams F-12 media (Sigma-Aldrich, Johannesburg, South Africa). The culture media was supplemented with 1% (v/v) L-glutamine (Merck, Johannesburg, South Africa), 1% (v/v) sodium pyruvate (Merck), 1% (v/v) non-essential amino acids (Merck), and 20% (v/v) of foetal bovine serum (FBS) (Thermo Fisher Scientific, Johannesburg, South Africa). FBS purchased was heat-inactivated and stored at -20 °C in a refrigerator. The culture media was replaced with fresh media supplemented with 10% (v/v) FBS periodically. The ethics waiver: W-C0CBP-190704-

01, for the use of the MCF-7 breast cancer cells in this study was granted by the Faculty of Health Sciences, University of the Witwatersrand (Appendix A).

2.3.2 Cell culture maintenance

Cells were grown in 75 cm^2 Greiner bio-one culture flasks (Lasec SA, Johannesburg South Africa) containing 20 mL of culture medium incubated at 37 °C and 5% carbon dioxide (CO_2) in the Heracell 150 CO_2 incubator (Thermo Fisher Scientific, Johannesburg South Africa). Culture medium was replaced by washing the cells with phosphate buffered saline (PBS) and adding fresh media every third day for experiment initiation. PBS consisted of 1.37 M NaCl (Merck, Johannesburg South Africa), 27 mM KCl, 100 mM Na_2HPO_4 and 18 mM KH_2PO_4 (Sigma-Aldrich, Johannesburg, South Africa) and was autoclaved at 121 °C before use in the experiments.

2.3.3 Cell sub-culturing

When cells had reached 70% confluency, the culture media was aspirated, and cells were washed once with 3 mL of PBS. The cells were detached from the growth surface by adding 3 mL of 0.25% trypsin-ethylenediaminetetraacetic (Trypsin-EDTA) (Sigma-Aldrich, Johannesburg South Africa) and placing the flask in a 37 °C incubator for 2-3 minutes. When cells were in suspension, culture media was added to inactivate trypsin-EDTA reaction (Iii *et al.*, 1995). Cells were then counted using the trypan blue exclusion assay.

2.3.4 Trypan Blue technique

Trypan blue (Sigma-Aldrich, Johannesburg, South Africa) was dissolved to a final concentration of 0.2% (w/v) in PBS, sterile filtered through a 0.22 µm membrane and stored in a dark box at room temperature. The trypan blue exclusion assay is based on the staining of dead cells by trypan blue due to the loss of integrity of their cell membrane. Therefore, dead cells are stained blue, while live cells are lightly stained on their cell membrane (Strober, 2015). A mixture of 10 µL of 1:1 trypan blue solution to 10 µL to cell suspension was added to a haemocytometer and the cells were counted. Cells were counted on the haemocytometer under a light microscope (100× magnification). Cell numbers counted on each quadrant of the haemocytometer was averaged, and then multiplied by the dilution factor (×2), and by 10^4 to obtain the

number of cells per millilitre, respectively. The counted cells were used for subsequent experiments on that day, and the remaining suspension reseeded for further cell culture.

2.3.5 Cryopreservation

Cells extracted from the base of the flask were placed in a 15 mL falcon tube and centrifuged at 1500 rpm using a Heraeus Megafuge 40 centrifuge with a swing-out Thermo Scientific TX-750 Rotor (Thermo Scientific, Massachusetts, United States of America) for 5 minutes. The supernatant was carefully discarded, and the pellet resuspended in 10 mL of freezing media composed of culture media supplemented with 5% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Johannesburg, South Africa) for preservation. The solution was then aspirated and aliquoted equally into 2 mL cryotubes. The cryotubes were placed for 72h in a -70 °C freezer allowing for the slow decrease in temperature (1 °C per hour) of the freezing solution so as to ensure maximal survival of the cells under cryopreservation before being transferred to liquid nitrogen (Freshney, 2006).

Cryopreservation is useful for the long-term storage of cells in a state that protects the cells from contamination and aging during a period in which they are not used and stored as stock for future use (Freshney, 2006; Pegg, 2009).

2.4 MTT Cell Viability Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Johannesburg, South Africa) was prepared to a 5 mg/mL (w/v in PBS) solution, sterile filtered through a 0.22 µm membrane and stored at 4 °C. The MTT assay distinguishes viable from non-viable cells. Viable cells can transform the yellow tetrazolium salt through mitochondrial succinate dehydrogenase to purple formazan crystals (Mosmann, 1983).

MCF-7 cells were counted and seeded at 1×10^4 cells/well in 96 well-plates, in a volume of 100 µL. These cells numbers were determined to be representative of the linear relationship between cell number and absorbance values in MTT standardisation experiment (Appendix B). Cells were incubated in a humidified incubator at 37 °C and 5% CO_2 overnight to allow attachment to the growth surface. Following attachment in the well plate, cells were treated with 50 and 5 µM per well of test compounds for basic

screening, and a range of concentrations (0.001-8000 µM) for determining the IC_{50} values. The IC_{50} value is a measure of the potency of a drug and is thus the concentration of drug required to achieve the half-maximal effect. The concentration equivalent to the IC_{50} value is recommended for pure compound testing by the National Cancer Institute (Monks *et al.*, 1991). A vehicle control using the same ethanol and DMSO concentration range between 0.0000469 to 7.5% (*v/v)* as in the treatments, were performed. A vehicle control is used in studies in which a substance (e.g., saline or ethanol) is used as a vehicle for a solution of the experimental compound (Johnson and Besselsen, 2002).

Combination studies were performed to assess the effects of the active CAMs on the efficacy of chemotherapy drug/s after 24 hours and 48 hours. The same concentration range used to obtain IC_{50} values for the chemotherapy drugs were used in combination treatments. Cells were treated with the chemotherapy drug in presence of each of the active CAM concentrations equivalent to their IC_{50} values for both 24h and 48h treatment period.

The treated cells were then incubated for 24 hours and 48 hours at 37 ºC and followed by adding MTT solution. Treated and untreated cells were then exposed to 40 µL of MTT solution per well and incubated for 4h at 37 ºC and 5% CO_2. The 96-well plates were centrifuged using the Heraeus Megafuge 40 centrifuge at 1500 rpm for 5 min. The media containing MTT was then completely aspirated from each well, and the formazan crystals present dissolved by addition of 200 µL of DMSO and were shook on a rotatory plate shaker for 5min. The absorbance was read on an iEMS micro-plate reader at a wavelength of 540 nm and a reference wavelength of 690 nm.

The following formula was applied to calculate the cell viability when MCF-7 cells were exposed to test compounds. The blank well only contained DMSO.

Key: Abs - Absorbance

$$\text{Cell Viability (\%)} = \frac{(Average\ Abs\ (treated\ cells) - Average\ Abs\ (Blank))}{(Average\ Abs\ (untreated\ cells) - Average\ Abs\ (Blank))} \times 100$$

Non-linear regression analysis was used to determine the best fit IC_{50} estimates and assumes a minimum (E_0) and a maximum (E_{max}) effect for the select compounds. The inhibitor versus response -variable slope equation was used in dose response curve

analysis of GraphPad Prism (GraphPad Prism version 5 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com). In the combination studies, the dose response curves were analysed using non-linear regression with the Inhibitor vs. normalized response-- Hillslope equal to -1 equation of GraphPad Prism software. Each data point on the graphs were expressed as the mean ± the standard error of the mean (SEM). Data were analysed using Student's t-test followed by Welch's correction test (*post hoc* test for pairwise analysis between groups assuming unequal variances) to determine statistical differences of IC_{50} values. The GraphPad Prism software was used, and the criterion of significance was set at $p < 0.05$.

2.5 Morphology

Cell morphology evaluation involves cell shape, size and structural features visualized using microscopy and provides valuable information regarding cytotoxic effects and cell death mechanisms (Cummings and Schnellmann, 2004; Atale, *et al.*, 2014). Acridine orange (AO), Hoechst 33342 (HO) and propidium iodide (PI) are among the most used fluorescent dyes used to analyse cell culture viability and respectively show specificity for morphological features of living and non-living cell states (Foglieni *et al.*, 2001; Atale *et al.*, 2014). Acridine orange dye stains healthy nuclei and cell cytoplasm an emerald green colour and functions as a lysosomotropic agent which makes it useful to detect acidic vesicular organelles within a cell which are stained red (Atale *et al.*, 2014). The red-fluorescent PI is a DNA-binding dye which can only stain in cells where there is a loss of plasma membrane integrity (Foglieni *et al.*, 2001). Using both AO and PI differentially distinguishes live cells showing normal green nucleus; apoptotic cells show bright green nucleus with condensed or fragmented chromatin; and cells that have died from direct necrosis have a bright red/orange stained nucleus (Renvoizé *et al.*, 1998). Hoechst 33342 is a specific stain for nuclei and can distinguish between apoptotic and non-apoptotic nuclei, showing an increase in fluorescent intensity and specific changes to the structure of the nucleus which include cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (De Vitto *et al.*, 2018).

MCF-7 cells were counted and seeded at 1×10^5 cells/well on sterile cover slips cells within 6-well culture plates with a final volume of 1mL media and incubated overnight at 37°C. For the subsequent day of experiment, the cells grown on coverslips were

treated with doxorubicin and the CAMs at their IC_{80} values (estimated concentration of drug that have an inhibitory effect of 80% of cell population). Following 18h treatment, cells were viewed under an inverted microscope and detached cells from the coverslip were harvested by aspirating the media and centrifugation of suspended cells. Furthermore, attached cells on coverslips were gently washed twice with PBS. The fluorescent dye mixture of Hoechst 33342 (5 µg/mL) (Sigma Aldrich/Merck), propidium iodide (10 µg/mL) (Sigma Aldrich/Merck) and acridine orange (10 µg/mL) (Thermo Fisher, Johannesburg, South Africa) at a ratio of 4:1:2 respectively, was prepared to a final volume of 10mL of PBS. The dye mixture was added at 1.5mL/well and the cells were incubated for 20min in dark conditions at room temperature. Following triple staining, the cells grown on the coverslips were washed with PBS. The coverslips were carefully mounted with cells facing downwards onto a full-size microscope slide with fluoromount solution (Sigma Aldrich/Merck). The cells were viewed on an Olympus CKX41 microscope, using the following Olympus filter cubes sets; U-MNIB3 (Excitation:480/20, Emission: 510/50) for acridine orange, U-MWIG2 (Excitation535/30, Emission: 580 long pass) for propidium iodide and U-MWU2 (Excitation365/10, Emission 420 long pass) for Hoechst 33342. Images were captured with an Olympus DP72 camera and analysed with the Olympus CellSens Software package (Olympus, Tokyo, Japan). The images were optimized for brightness and contrast using identical settings.

2.6 Calcein-AM assay

The activity of the drug efflux pump, ABCB1/PgP, in MCF-7 cells was evaluated using the Vybrant™ Multidrug Resistance Assay Kit (V-13180) (Thermo Fisher Scientific, Johannesburg, South Africa). The assay includes the use of calcein-acetoxymethyl (AM), an ester form of calcein, a non-fluorescent cell-permeable dye. The calcein-AM assay has been shown to identify both substrates and inhibitors of MDR proteins, and is therefore an advantageous assay above others (Pasquier *et al.*, 2013; Tsou *et al.*, 2015; Chang *et al.*, 2019). Upon transport of calcein-AM into viable cells, the cleavage of the lipophilic blocking groups by intracellular esterases in the cytoplasm convert the molecule into a fluorescent form, calcein (Uggeri *et al.*, 2004). Calcein-AM is a substrate of PgP and is rapidly effluxed in cells overexpressing the protein, indicated as a reduced fluorescence in those cells (Homolya *et al.*, 1993). Cyclosporin A and

verapamil were used as positive controls for PgP efflux activity inhibition in the assay (Litman *et al.*, 1997; Uggeri *et al.*, 2004). MCF-7 cells were seeded into sterile black-surfaced 96-well flat bottom plates at a density of 1×10^4 cells/mL per well and incubated overnight at 37°C. This was followed by treating the cells with doxorubicin, and the active CAMs individually at concentration ranges like the concentrations as used in the cell viability assays. Cells were incubated for 24h. On the respective days of the experiment, 100 µL of each positive control (Cyclosporin A and Verapamil) at a range of concentrations (recommended by the Vybrant Assay Kit protocol) being between 0.083 - 61.1 µM was added to the untreated wells and incubated for 30min. After incubation, 50 µL of calcein-AM solution at 0.25 µM (final concentration) was added to all the wells and incubated for a further 30 min at 37 °C. After incubation, the plates were removed and centrifuged at 1500 rpm at room temperature for 10 min. The supernatant was aspirated and 200 µL of ice-cold PBS was added to each well. The calcein fluorescence of the treated and untreated cells in the 96-well plates were measured using a FilterMax™ F5 Multi-Mode Microplate Reader (Molecular Devices, Inc., United States of America) at excitation wavelength of 485 nm and emission wavelength of 535 nm.

The following formula was used to calculate the calcein-AM retention when MCF-7 cells were exposed to test compounds. The blank well only contained PBS.

Key: *Fluor* - calcein-specific fluorescence

$$\text{Calcein-AM retention (\%)} = \frac{(Average\ Fluor\ (treated\ cells) - Average\ Fluor\ (Blank))}{(Average\ Fluor\ (untreated\ cells) - Average\ Fluor\ (Blank))} \times 100$$

Each data point on the graphs were expressed as the mean ± the SEM. Data were analysed using Students' t-test with *post hoc* Welch's correction test to determine statistical differences of calcein-AM retention between the treatments and significance was set at p-value less than 0.05.

2.7 Immunofluorescence

Immunohistochemistry has been proven to be one of the most important techniques for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions, identifying the presence of antibody binding either by direct labelling of the antibody, or by use of secondary labelling method (Ramos-Vara, 2017).

Following trypsination, ~1×10^5 MCF-7 cells were seeded on sterile cover slips within 6-well culture plates, and incubated overnight at 37 °C. The next day, the cells grown on coverslips were treated with test compounds at various effective concentrations, individually and in combination. Following 6h and 18h treatment, for haem oxygenase-1, with 24h and 48h treatment for P-glycoprotein, the MCF-7 cells were fixed by the addition of 1 mL of 3% formaldehyde (Merck, Johannesburg, South Africa) in 1×PBS (v/v) to each well and incubated for 25 min at room temperature. The 3% formaldehyde was aspirated off, and the wells were gently rinsed 3 times with 1×PBS. A diluted detergent solution prepared using 0.25% Triton-X-100 (Thermo Fisher Scientific, Johannesburg, South Africa) (v/v) in 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, Johannesburg, South Africa) in PBS (v/v) was added to each well onto the coverslips, and the cells were permeabilized for 20 min. The coverslips were gently rinsed 3 times with 1 mL 1×PBS. The cells were blocked to avoid non-specific antibody binding (Ramos-Vara, 2017) by adding 500 µL of 1% BSA in PBS, being added as a blocking buffer to each of the cover slips and incubated for 30 min.

2.7.1 P-glycoprotein

The cells grown on coverslips were fixed, permeabilized and blocked as described above at 24h and 48h treatment periods. The cells were treated with doxorubicin, resveratrol, synephrine, caffeine at concentrations equivalent to their IC_{50} values. Cells were treated with combinations of doxorubicin with the CAMs at the concentrations equal to the IC_{50} values for 24h and 48h. The coverslips were transferred with the cells facing upwards onto parafilm placed on a wet thick filter paper. The primary Ab, 0.43 mg/mL mouse anti-human PgP monoclonal Ab (MAS-12854) (Thermo Fisher Scientific, Johannesburg South Africa), was prepared at 1:100 in 0.5% BSA. The primary Ab was added to the cells at 150 µL per coverslip, except for the negative control. The coverslips were placed in the dark at 4 °C overnight. After overnight incubation, the primary Ab was harvested into a microtube, and the coverslips were rinsed three times with PBS in the 6-well plate. The coverslips were mounted on the parafilm. The secondary Ab, 2 mg/mL goat anti-mouse polyclonal Ab (ab150113) (Thermo Fisher Scientific, Johannesburg South Africa), was prepared at 1:800 in 0.5% BSA. The secondary Ab was incubated at 150 µL per coverslip in the dark at room temperature for 1h. Following secondary Ab incubation, the antibody was harvested,

and the coverslips were rinsed in 6-well plate. The attached cells on coverslips were stained with Hoechst 33342 for 20 min. The coverslips were rinsed with PBS and then carefully mounted cells facing downwards onto a full-size microscope slide with fluoromount solution (Sigma-Aldrich, Johannesburg South Africa). The cells were viewed using an Olympus CKX41 microscope fitted with a DP72 digital camera, using the U-MNIB3 (Excitation: 480/20, Emission: 510/50) for Alexa Fluor 488 and U-MWU2 (Excitation: 365/10, Emission: 420 long pass) for Hoechst 33342. Photographs were captured at the same exposure times and analysed with consistent settings using Olympus CellSens software Life Sciences package.

Colocalisation analysis of Alexa Fluor 488 and Hoechst 33342 was performed to determine the correlation of PgP in the nucleus of cells by means of Pearson's Correlation Coefficient using the Olympus CellSens software Life Sciences package. In the overlay photomicrographs, six cells were selected from three images of repeated experiments and the colocalisation feature measured the selected regions of interest for overlap of signals (Dunn *et al.*, 2011; Shihan *et al.*, 2021). The Image J software was used to determine the PgP mean fluorescence intensities of the treated cells captured in the photomicrographs to compare PgP expression levels (Schindelin *et al.*, 2012; Shihan *et al.*, 2021). The mean fluorescence intensities of PgP in ten cells that appear similar in fluorescence signal were selected from two photomicrographs of three repeated experiments. The fluorescence intensities indicating expression levels in arbitrary units represent the mean and the SEM determined using column statistics and statistically compared using Students' t-test on GraphPad Prism software package.

2.7.2 Haem oxygenase-1

The MCF-7 cells were treated for 6h and 18h with doxorubicin, resveratrol, synephrine, caffeine at concentrations equivalent to their IC_{50} values. Cells were treated with combinations of doxorubicin with the CAMs at the concentrations equal to the IC_{50} values for 6h and 18h. The cells were fixed at the specific treatment exposure time, permeabilised and blocked. The coverslips were transferred onto parafilm placed on a wet thick filter paper. The primary Ab, 0.43 mg/mL rabbit HMOX1 polyclonal Ab (PA5-27338) (Thermo Fisher Scientific, Johannesburg South Africa), was prepared at 1:300 in 0.5% BSA. The primary Ab was incubated at 150 µL per coverslip, except for the negative control, in the dark at 4 °C overnight. After overnight incubation, the

primary Ab was harvested into a microtube, and the coverslips were rinsed three times with PBS in the 6-well plate. The coverslips were mounted on the parafilm. The secondary Ab, goat anti-rabbit polyclonal Ab (ab150077) (Thermo Fisher Scientific, Johannesburg South Africa), was prepared at 1:800 in 0.5% BSA. The secondary Ab was incubated at 150 µL per coverslip in the dark at room temperature for 1h. Following secondary Ab incubation, the antibody was harvested, and the coverslips were rinsed in 6-well plate. The attached cells on coverslips were stained with Hoechst 33342 for 20 min. The coverslips were rinsed with PBS and then carefully mounted cells facing downwards onto a full-size microscope slide with fluoromount solution (Sigma-Aldrich, Johannesburg South Africa). The cells were viewed using an Olympus CKX41 microscope fitted with a DP72 digital camera, using the U-MNIB3 (Excitation:480/20, Emission: 510/50) for Alexa Fluor 488 and U-MWU2 (Excitation365/10, Emission 420 long pass) for Hoechst 33342. Photographs were analysed with consistent settings using Olympus CellSens software Life Sciences package.

Colocalisation analysis of Alexa Fluor 488 and Hoechst 33342 was performed to determine the correlation of HMOX1 in the nucleus of cells by means of Pearson's Correlation Coefficient using the Olympus CellSens software Life Sciences package. In the overlay photomicrographs, six cells were selected from three images of repeated experiments and the colocalisation feature measured the selected regions of interest for overlap of signals. The Image J software was used to determine the HMOX1 mean fluorescence intensities of the treated cells as described for PgP expression levels. The mean fluorescence intensities and standard error of the mean were determined using the same statistics performed for PgP on the GraphPad Prism software package

CHAPTER 3: RESULTS

In this study, three drugs used in chemotherapy and four CAMs were selected for experimental investigations. The chemotherapy drugs were doxorubicin, gemcitabine and irinotecan, used in treatment of metastatic breast cancer (Duggan *et al.*, 2011; Cortazar *et al.*, 2012; Lan *et al.*, 2014). The CAMs were resveratrol, synephrine, caffeine, amygdalin and *S. frutescens* which were selected after consulting the Drug, Herbs and Supplements section adapted from the Natural Medicines Comprehensive Database linked to the MedlinePlus online library (https://medlineplus.gov/druginfo/herb_All.htmL). The cytotoxic effects, changes in cell morphology, PgP efflux activity, and the expression levels of PgP and HMOX1 are presented for the most active chemotherapy and the active CAMs, alone and in combination, in the MCF-7 cell line.

3.1.1 Identification of active chemotherapy drugs and CAMs against MCF-7 cells

The effects of the chemotherapy drugs (gemcitabine, irinotecan, and doxorubicin) and the CAMs (amygdalin, resveratrol, synephrine and caffeine) on cell viability was evaluated at concentrations of 50 µM and 5 µM against MCF-7 cells using the MTT assay as described in Chapter 2 section 2.4. This preliminary evaluation was done at 24 hours to estimate the concentration range required to determine the IC_{50} values of the active compounds. Activity of the compounds was expressed as the percentage cell viability compared to an untreated control and is shown in Fig 3.1. Doxorubicin was the most active with percentage cell viability of 27.1 ± 1.4% at 5 µM and 5.4 ± 0.8% at 50 µM. Gemcitabine and irinotecan at 50 µM showed moderate decrease in cell viability with 65.8 ± 4.2% and 52.6 ± 2.8% cell growth, respectively. Both gemcitabine and irinotecan were poorly active at 5 µM with percentage cell viability of 84.2 ± 4.7 % and 74.7 ± 5.9%, respectively. With the exception of amygdalin, the CAMs showed a moderate decrease in cell viability at 5 µM and 50 µM with 80-90% and 65-67%, respectively. The CAMs showed poor activity at 5 µM with a percentage cell viability for treatment with resveratrol at 80.5 ± 6.8%, synephrine at 90.1 ± 6.2%, caffeine at

96.2 ± 4.1%, and amygdalin at 87.6 ± 3.3%. Resveratrol, synephrine and caffeine were more active at 50 µM with a percentage cell viability of 65.8 ± 1.8%, 66.8 ± 3.1% and 65.0 ± 3.2%, respectively. Amygdalin was the least active at 50 µM with percentage cell viability of 80.1 ± 3.8%. Since the chemotherapy drugs and CAMs, except for amygdalin, were active at 50 µM, IC_{50} values were determined for the three cancer drugs, resveratrol, synephrine and caffeine. Amygdalin was excluded due to its poor efficacy.

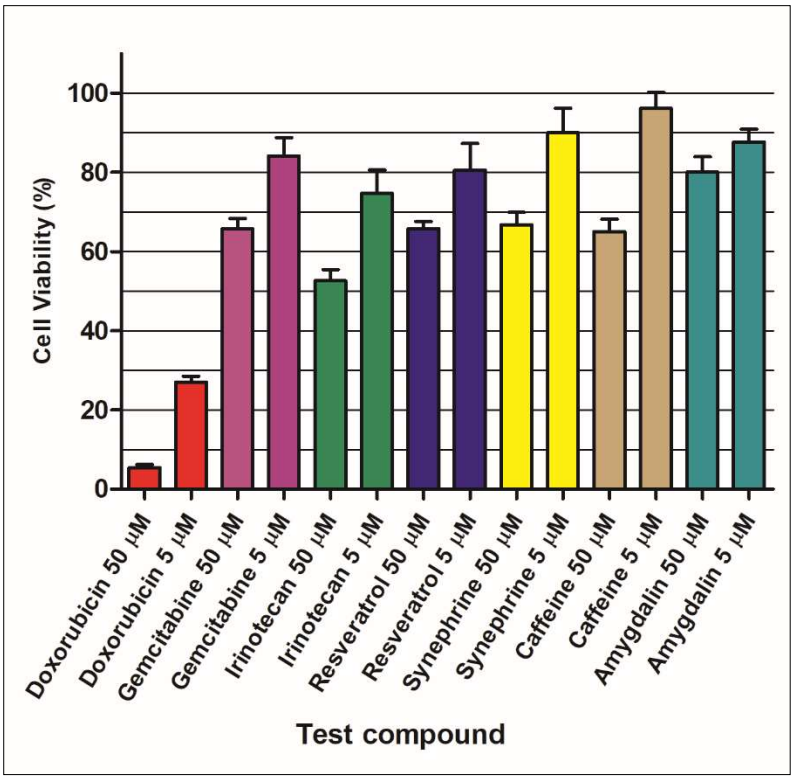


Figure 3.1 The effect of doxorubicin, gemcitabine, irinotecan, and the CAMs resveratrol, synephrine, caffeine and amygdalin on the cell viability of MCF-7 cells. The cells were treated with 50 µM and 5 µM of the test compounds for 24 hours and each column represents the mean of four independent experiments (n=4). The error bars represent the SEM for each concentration.

3.1.2 Determining the IC_{50} values of the chemotherapy drugs and CAMs on MCF-7 cells

Using the MTT assay, MCF-7 cells were treated with a range of concentrations of doxorubicin (0.01-50 µM), gemcitabine (0.1-1500 µM) and irinotecan (0.1-700 µM) for 24 hours and 48 hours. The cells were treated with a range of concentrations of resveratrol (0.05-400 µM), synephrine (1-4000 µM) and caffeine (1-4000 µM) for 24

hours and 48 hours. IC_{50} values were calculated using non-linear regression analysis in the GraphPad Prism software**.**

Experiments were repeated five times and representative dose response curves of the chemotherapy drugs and the CAMS are shown in Figure 3.2. IC_{50} values of the chemotherapy drugs and the CAMs on the MCF-7 cell line are summarised in Table 3.1.

For all compounds tested, the IC_{50} values were lower when the cells were exposed to a 48 hour treatment as opposed to a 24 hour treatment period (Table 3.1). Doxorubicin was the most active of the chemotherapeutic agents with a 48 hours IC_{50} value of 0.74 ± 0.04 μM and was therefore selected to be used as a positive control for the remainder of the study.

Of the three CAMs tested resveratrol was the most active CAM with IC_{50} values of 149.20 ±1.90 μM and 80.54 ± 1.20 μM over 24 hours and 48 hours respectively, whilst synephrine and caffeine were poorly active as is shown in Table 3.1. The IC_{50} value of doxorubicin after 48 hours was much lower compared to the 24 hours treatment period i.e., 0.74 ± 0.04 μM, compared to 1.12 ± 0.19 μM in Table 3.1. The IC_{50} values of the CAMs were however much higher than that of doxorubicin for both treatment periods.

The IC_{50} values of the CAMs- resveratrol, synephrine and caffeine were compared to the IC_{50} value of doxorubicin for both 24 hours and 48 hours treatment periods, to determine if there were any significant differences. The Students' t-test followed by Welch's correction test was used to determine if the differences between the IC_{50} values of the treatments were significant and the p-values are shown in Appendix C. The IC_{50} values of all the CAMs were significantly higher than doxorubicin IC_{50} values for both treatment periods.

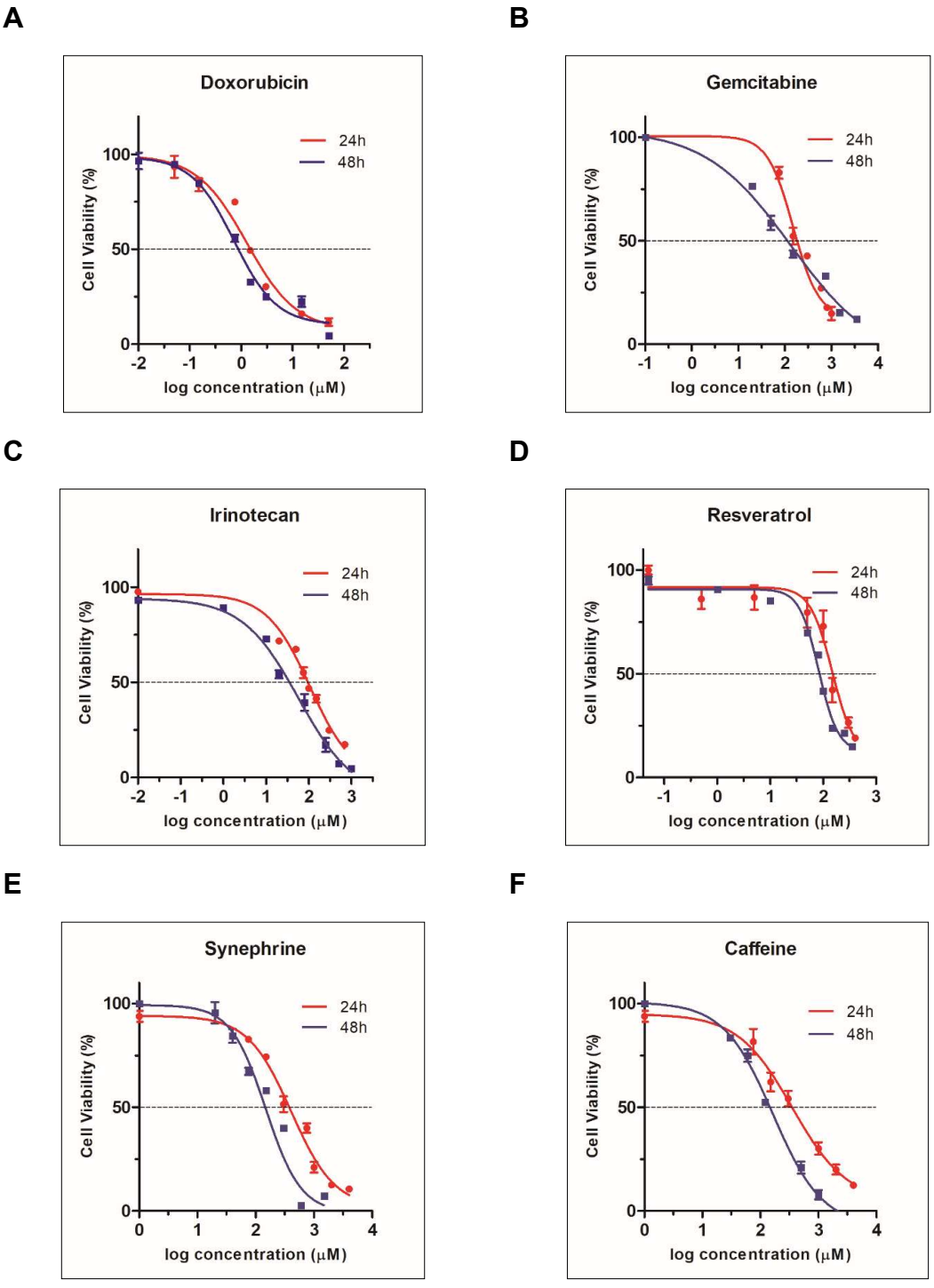
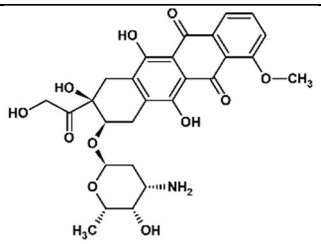
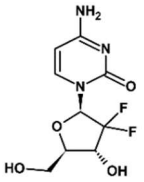
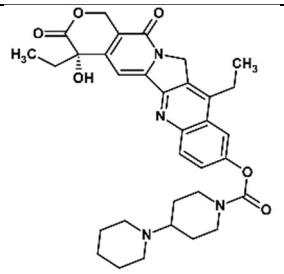
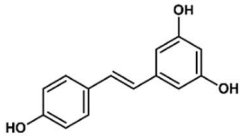
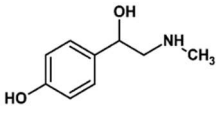
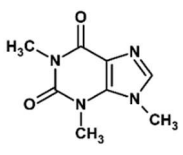


Figure 3.2 Representative dose response curves of doxorubicin, gemcitabine, irinotecan, and the CAMs resveratrol, synephrine and caffeine against MCF-7 cells. The cells were treated with a range of concentrations of (A): doxorubicin between 0.01-50 μM; (B): gemcitabine between 0.1-1500 μM; (C): irinotecan between 0.1-700 μM; (D): resveratrol between 0.05-400 μM, (E): synephrine between 1-4000 μM; and (F): caffeine between 1-4000 μM. The dose response curves are representative of five independent experiments (n=5). Each data point represents the mean cell viability at each concentration and the error bars indicate the SEM.

Table 3.1 The structures, molecular weights and IC_{50} values of the selected chemotherapy drugs and CAMs in the MCF-7 cell line used in this study

Name and Molecular weight of compound	Chemical structure	IC_{50} values (μM)	
		24 hours	48 hours
doxorubicin 579.96 g/mol		1.12 ± 0.19	0.74 ± 0.04
gemcitabine 299.66 g/mol		155.50 ± 4.07	137.40 ± 1.92
irinotecan 623.14 g/mol		102.90 ± 0.83	66.20 ± 1.84
resveratrol 228.24 g/mol		149.20 ± 1.90	80.54 ± 1.20
synephrine 167.21 g/mol		414.10 ± 5.40	149.30 ± 3.20
caffeine 194.19 g/mol		355.60 ± 2.10	167.40 ± 1.90

3.1.3 The effect of *S. frutescens* extracts on the cell viability of MCF-7 cells

S frutescens is indigenous to SA and commonly referred to as the "kankerbossie" or cancer bush. It was one of the CAMs selected from the literature survey on the MedlinePlus library. Commercially available *S. frutescens* products were selected in this study to evaluate their potential efficacy on the MCF-7 cell line. An extract (Phyto-Force) sold in a 60% ethanol formulation was purchased from a health shop (Dis-Chem). Dried leaves and stems were purchased from another health shop (local market).

A *S. frutescens* water extract from the dried leaves and stems were prepared in the laboratory after the leaves were grinded into a fine powder, as described in Section 2.7.1. The dry herb mixture was boiled in distilled water, filtered, evaporated in a fume hood and the solid residue was weighed before dissolving it in sterile saline. This mixture was sterile filtered before use in the MTT experiments. This preparation is referred to as the hot water extract. The two extracts were evaluated against the MCF-7 cell line to determine their effects on cell viability after a 24 hour and a 48 hour treatment period, using the MTT assay.

To obtain IC_{50} values, the MCF-7 cells were treated with the *S. frutescens* extracts at a range of concentrations, 0.015-8.0 % (*v/v*) for the ethanolic extract and 10-8000 µg/mL for the hot water extract. Dose response curves were constructed after 24 hours and 48 hours treatment periods and shown in Fig 3.3. The cells were treated with increasing concentrations of doxorubicin (0.06-28.99 µg/mL) to obtain dose response curves of the positive control in order to directly compare the activity of the *S. frutescens* extracts with doxorubicin. A vehicle control using the same ethanol concentration range between 0.000047-7.50 *v/v* %. as in the treatments, were performed. The experiments were repeated four times and the IC_{50} values are summarised in Table 3.2. Following the 24 hours treatment period, the *S. frutescens* hot water extract had no effect on cell viability and a dose response curve could not be constructed. The *S. frutescens* hot water extract showed activity in the MCF-7 cells with an IC_{50} value of 5700 ± 110.0 µg/mL (5.7 mg/mL) after a 48 hour treatment period as shown in Table 3.2. Doxorubicin showed activity in the MCF-7 cells with an IC_{50} value of 0.43 ± 0.03 µg/mL after 48 hours treatment. In comparison to doxorubicin, the *S. frutescens* hot water extract was poorly active.

The *S. frutescens* ethanol extract concentration was measured in volume-to-volume percentage since the extract was not quantified. Following a 24 hour treatment period, the *S. frutescens* ethanol extract showed activity in the MCF-7 cells at an IC_{50} value of 3.1 ± 0.41 *v*/*v* %. The *S. frutescens* ethanol extract showed increased activity after 48 hours treatment with a lower IC_{50} value of 2.7 ± 0.52 *v*/*v* %. The vehicle control experiments showed ethanol inhibited the growth of MCF-7 cells at concentrations greater than 0.94 *v*/*v* % and 0.28 *v*/*v* % after 24 hours and 48 hours treatment, respectively. This suggests the activity seen in the MCF-7 cells treated with *S. frutescens* extract could be attributed to the ethanol in the preparation. Compared to doxorubicin and irinotecan, the *S. frutescens* extracts showed poor activity against the MCF-7 cell line. Apart from cell morphology experiments, no further work was done with this product.

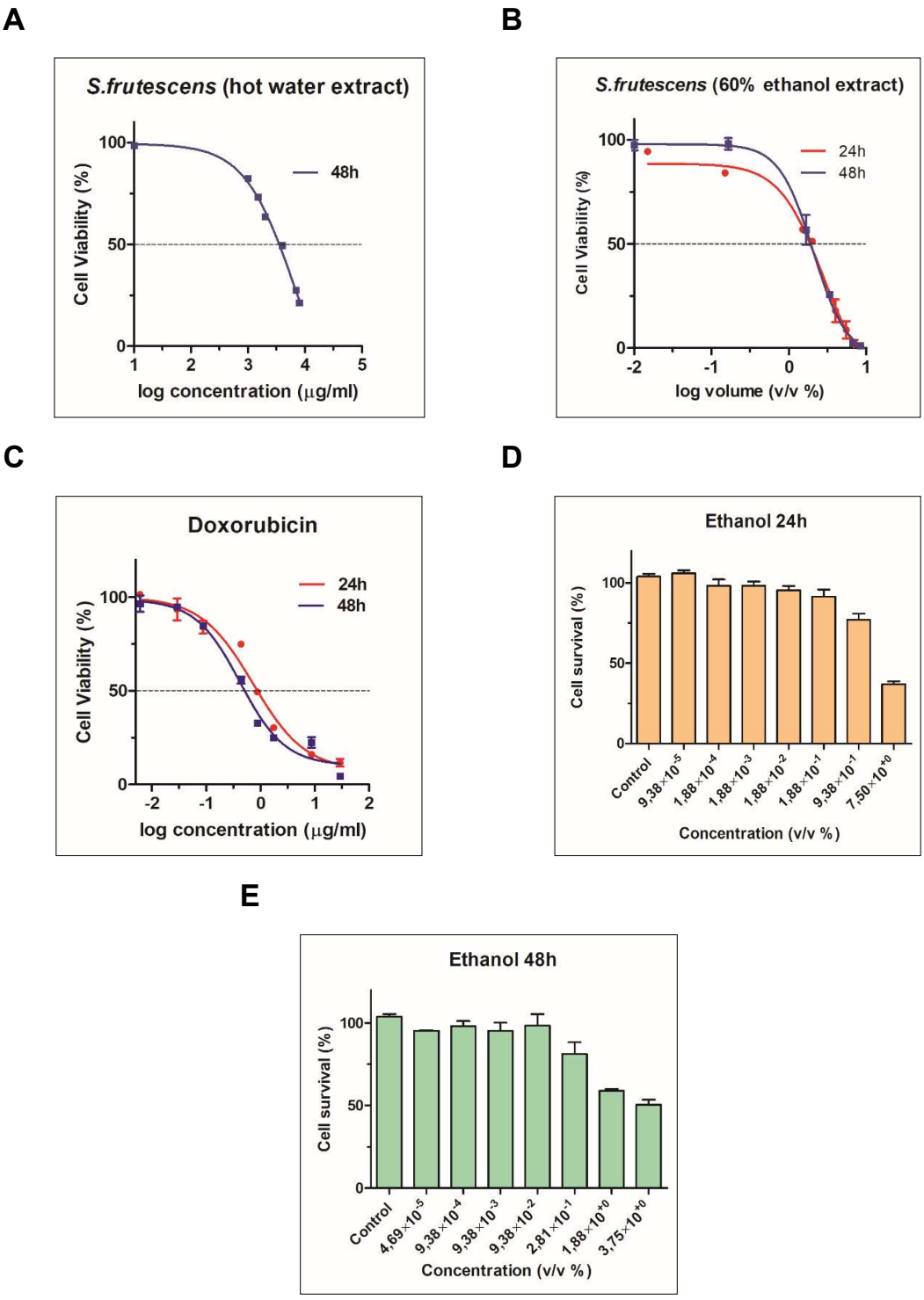
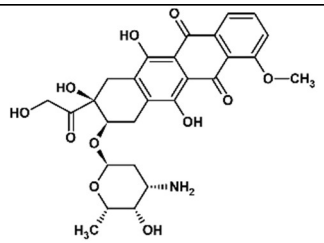


Figure 3.3 Representative dose response curves of *S. frutescens* extracts and doxorubicin tested against the MCF-7 cells showing the low activity of the extracts. Cells were treated with a range of concentrations of (A): *S. frutescens* hot water extract (10-8000 µg/mL); (B): *S. frutescens* 60% ethanol extract (0.015-8.0 *v/v* %); (C): doxorubicin (0.06-28.99 µg/mL); (D) and (E) represent the solvent control-ethanol (0.000047-7.50 *v/v* %.) The dose response curves, and bar graphs are representative of four independent experiments (n=4). Each data point was obtained in quadruplicate and the error bars indicate the SEM.

Table 3.2 The IC_{50} values of *S. frutescens extract* in the MCF-7 cell line at 24 hour and 48 hour treatment periods

Name of compound	Chemical structure	IC_{50} values	
		24 hours	48 hours
doxorubicin		0.65 ± 0.11 (µg/mL)	0.43 ± 0.03 (µg/mL)
S. frutescens (hot water) extract	Impure herb extract, i.e., composed of many constituents	-	5700 ± 110.0 (µg/mL)
S. frutescens (60% ethanol) extract	Impure herb extract, i.e., composed of many constituents	3.1 ± 0.41 (*v*/*v* %)	2.7 ± 0.52 (*v*/*v* %)

3.2 The effect of CAMs on doxorubicin efficacy

Combination studies were performed to ascertain the effects of resveratrol, synephrine and caffeine on the efficacy of doxorubicin in MCF-7 cells over a 24 hour and 48 hour time period. Since the CAMS had high IC_{50} values, their effects on the doxorubicin IC_{50} value were determined using concentrations of CAMs equivalent to their respective IC_{50} values. The MCF-7 cells were treated with the same concentration range of doxorubicin (0.015-60 µM) used to determine its IC_{50} value, in the presence of 157 µM, 423 µM and 354 µM concentrations of resveratrol, synephrine and caffeine for 24 hours treatment period. The MCF-7 cells were treated with doxorubicin concentrations combined with 80.54 µM, 166.20 µM and 167.40 µM concentrations of resveratrol, synephrine and caffeine for 48 hours treatment period. Dose response curves were constructed to determine if the CAMs had an effect on the IC_{50} value of doxorubicin shown in Fig 3.4.

The IC_{50} values were used to calculate the relative potency (RP) for each combination of the CAMs with doxorubicin after 24 hours and 48 h are summarised in Table 3.3. Relative potency of a test compound compared to a reference compound is the ratio of

effective doses (reference divided by test compound) that yield the same mean response (Villeneuve *et al.*, 2000; Bijnsdorp *et al.*, 2011). The accurate use of relative potencies in combination studies requires typical dose response assays of which the response curves for the test compound and the reference compound must have similar asymptotes and the linear slope of the curves should be parallel (Dinse and Umbach, 2011). These requirements were applied to the dose response analysis of this study by using the Inhibitor vs. normalized response-- Hillslope equal to -1 equation of GraphPad Prism software. A Students' t-test was used determine if there were any significant differences in the IC_{50} values of doxorubicin when in combination with the CAMs and the corresponding p-values are shown in Table 3.3.

After a 24 hour treatment period, resveratrol showed a statistically significant increase in the IC_{50} value of doxorubicin (6.93 ± 0.46 μM) and thus it decreased the relative potency of doxorubicin shown in Table 3.3. This change was not observed after 48 hours treatment period. Synephrine showed no significant effects on the doxorubicin after 24 hours treatment period. Following a 48 hour treatment period, synephrine showed a statistically significant decrease in doxorubicin IC_{50} value (0.47 ± 0.02 μM) and therefore increased the relative potency of doxorubicin (Table 3.3). Caffeine showed no significant changes in doxorubicin IC_{50} value after both 24 hours and 48 hours.

When comparing the effects of the three CAMs on the IC_{50} value doxorubicin in MCF-7 cells after 24 hours combination treatment, resveratrol was the most active and it significantly decreased the efficacy of doxorubicin. Following 48 hours treatment, synephrine was the most active CAM in combination treatment compared to resveratrol and caffeine since synephrine significantly increased the doxorubicin efficacy in MCF-7 cells.

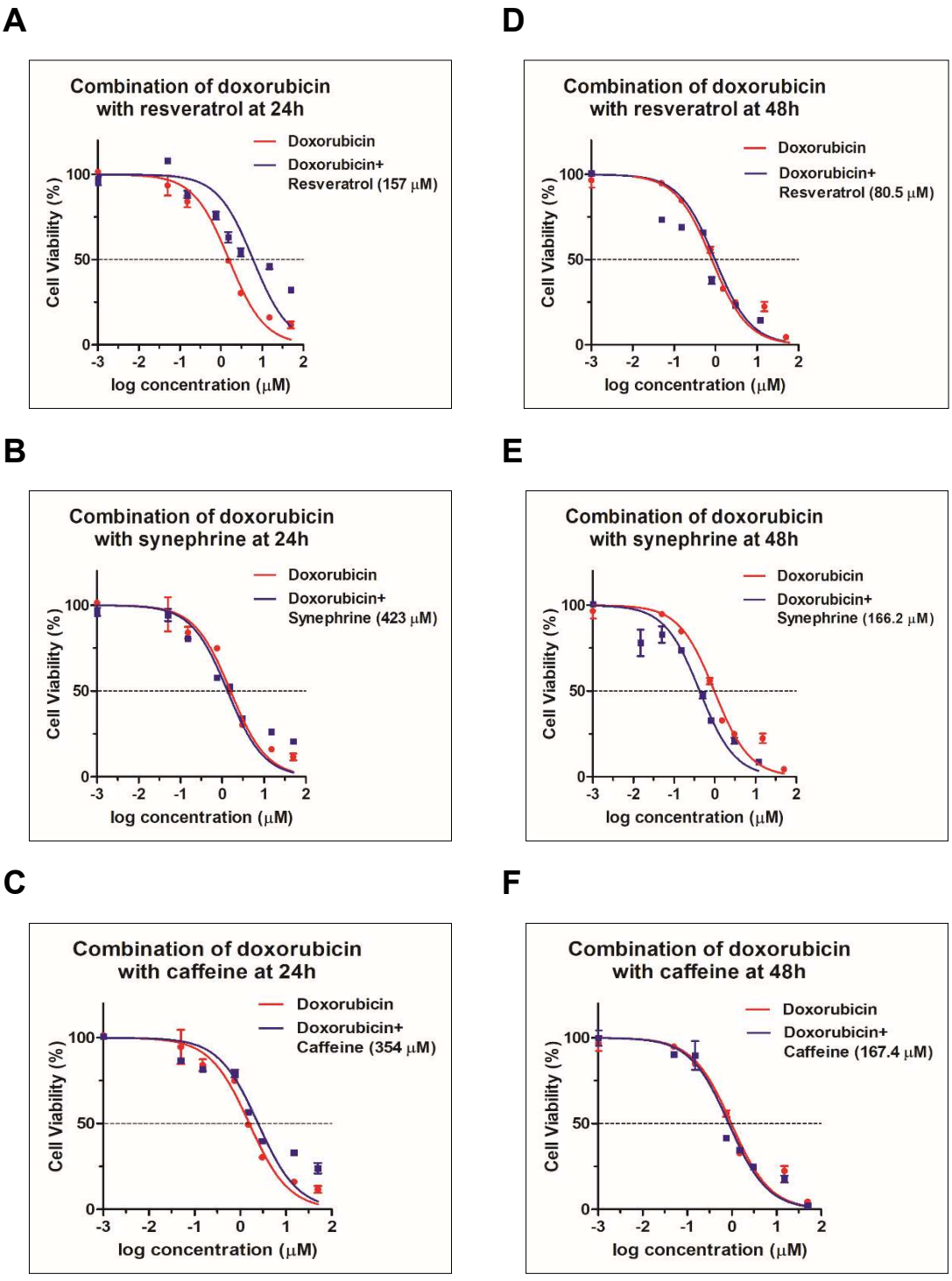


Figure 3.4 Representative dose response curves of combination experiments of doxorubicin and the active CAMs. MCF-7 cells were treated with doxorubicin (0.015-60 μM) and the CAMs A: resveratrol (157 μM), B: synephrine (423 μM) and C: caffeine (354 μM) for 24 hours. Cells were treated with doxorubicin and the CAMs D: resveratrol (80.54 μM), E: synephrine (166.20 μM) and F: caffeine (167.40 μM) for 48 hours. The dose response curves, and bar graphs are representative of five independent experiments (n=5). Each data point was obtained in quadruplicate and the error bars indicate the SEM.

Table 3.3 The IC50 values of doxorubicin when in combination with active CAMs in MCF-7 cell line after 24 hours and 48 hours period. Relative potency of doxorubicin was determined using IC_{50} values. Students' t-test was used to compare IC_{50} values of doxorubicin with combinations.

24 hours				48 hours			
compound	IC_{50} value (μM)	RP	p-value	compound	IC_{50} value (μM)	RP	p-value
doxorubicin (alone)	1.59 ± 0.14	1.0	-	doxorubicin (alone)	0.98 ± 0.09	1.0	-
doxorubicin + resveratrol (157 μM) Fig 3.4 A	6.93 ± 0.46	0.2	0.0074	doxorubicin + resveratrol (80.54 μM) Fig 3.4 D	0.85 ± 0.14	1.2	0.47
doxorubicin + synephrine (166.20 μM) Fig 3.4 B	1.31 ± 0.02	1.2	0.21	doxorubicin + synephrine (166.20 μM) Fig 3.4 E	0.47 ± 0.02	2.1	0.015
doxorubicin + caffeine (354 μM) Fig 3.4 C	1.92 ± 0.29	0.8	0.26	doxorubicin + caffeine (167.40 μM) Fig 3.4 F	0.82 ± 0.05	1.2	0.22

3.3 The effect of the CAMs and doxorubicin on MCF-7 cell morphology

MCF-7 cells were treated with doxorubicin and CAMs at concentrations equivalent to their IC_{80} values obtained after 48 hours. Cells were treated with *S. frutescens* ethanolic extract at the highest concentration where the vehicle control did not show any inhibitory effects i.e., 1.02 *v*/*v* % final concentration of ethanol in extract preparation. Cells were grown on sterile coverslips in six well-plates overnight and the following day the compounds were added to the cells for 18 hours treatment period. Following treatment, the cells were stained using a combination of three fluorescent dyes, acridine orange (AO), propidium iodide (PI) and Hoechst 33342 (HO) to visualise changes in cell morphology as described in Chapter 2 section 2.5. The effects on cell morphology after treatment with doxorubicin and resveratrol are shown in Fig 3.5a; synephrine and caffeine are shown in Fig 3.5b, and *S. frutescens* is shown in Fig 3.5c. Figure 3.5 is split into three sections to better demonstrate the observed changes. The same untreated control images are shown in Fig 3.5 a, b, and c.

Fig 3.5a panels a, b, c, and d, shows the intact untreated control cells, with oval shaped nuclei which was stained a homogenous bright blue fluorescence (due to Hoechst 33342 dye). The cells nuclei and cytoplasm stained emerald-green (due to acridine orange dye). PI did not stain nuclei indicating cells with intact plasma membranes. Fig 3.5a panel e-h shows the changes caused by doxorubicin at concentration of 3.69 µM. The cells appear round shaped, and many cells show decreased staining with HO in Fig 3.5a panel f. The intense blue nuclei in panel f appear to have fragmented nuclei. Both apoptotic and necrotic changes in cells treated with doxorubicin were observed in Fig 3.5a panels g and h. A similar effect was observed in cells treated with resveratrol at concentration of 119.62 µM shown in Fig 3.5a panel j and k. A necrotic nucleus stains a uniform red/orange fluorescence in Fig 3.5a panels h and l, whereas an apoptotic nucleus will show increased bright blue fluorescence and globular or fragmented nuclei, or a bright condensed nucleus as seen in Figure 3.5a panels f and j. Cells treated with resveratrol showed a few nuclei to appear decreased HO staining seen in Fig 3.5a panel j. In addition, the appearance of acidic granules in the perinuclear space of cells treated with resveratrol is shown in Fig 3.5a, panel l. An increase in acidic granules are an indication for cell stress and a preliminary indication for autophagy (Paglin *et al.*, 2001).

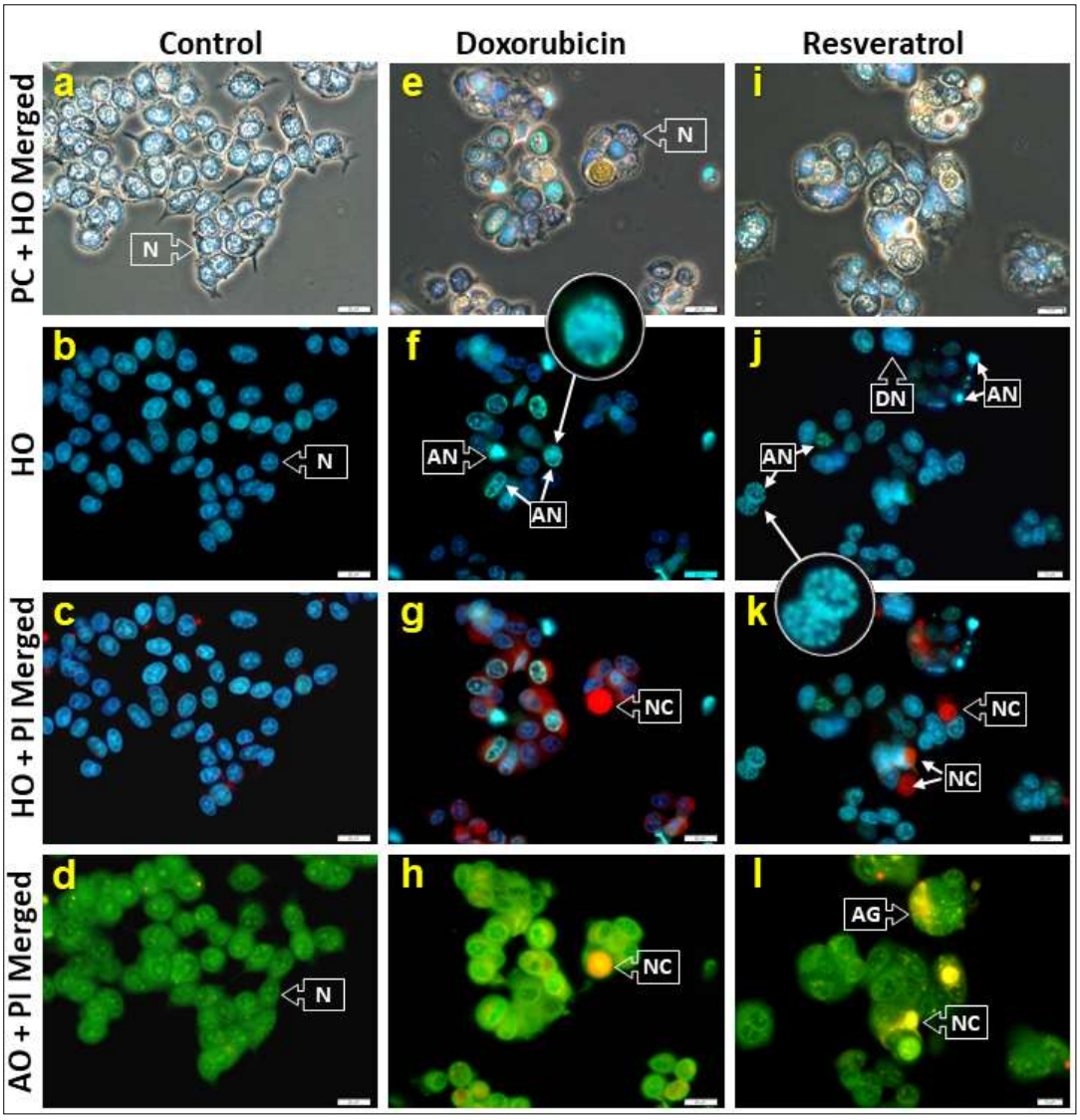


Fig 3.5a Representative photomicrographs showing the effect of doxorubicin and resveratrol on MCF-7cell morphology. Cells were treated for 18 hours. Panels a-d show untreated cells, panels e-h show the effects of 3.69 µM doxorubicin, and panel i-l show the effects of 119.62 µM resveratrol. An Olympus BX41 microscope was used to observe cells and images were captured with a DP72 digital camera (Magnification: 400×; scalebar: 20µm). Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). LEGEND- PC: Phase-contrast; HO: Hoechst 33342; PI: Propidium iodide; AO: Acridine orange; N: nucleus; DN: deformed nucleus; AN: apoptotic nucleus; AG: acidic granules; and NC: necrotic cell

The morphological changes caused by synephrine (398.11 µM) are shown in Fig 3.5b panel e-h. Evidence of apoptotic changes in cells treated with synephrine is shown in a small fraction of cells in the microscopic field, while deformed nuclei and necrotic changes were more prevalent as shown in Fig 3.5b panels f and g. The red and bright

yellow-orange, fluorescent nuclei, indicative of necrosis in the cells treated with synephrine are shown in Fig 3.5b panels g and h. The morphological changes caused by caffeine (457.10 µM) are shown in Fig 3.5b panel i-l. Cell morphology was similar to untreated cells with a small number of cells appearing to be rounded up and stressed a indicated in Fig 3.5b panel i.

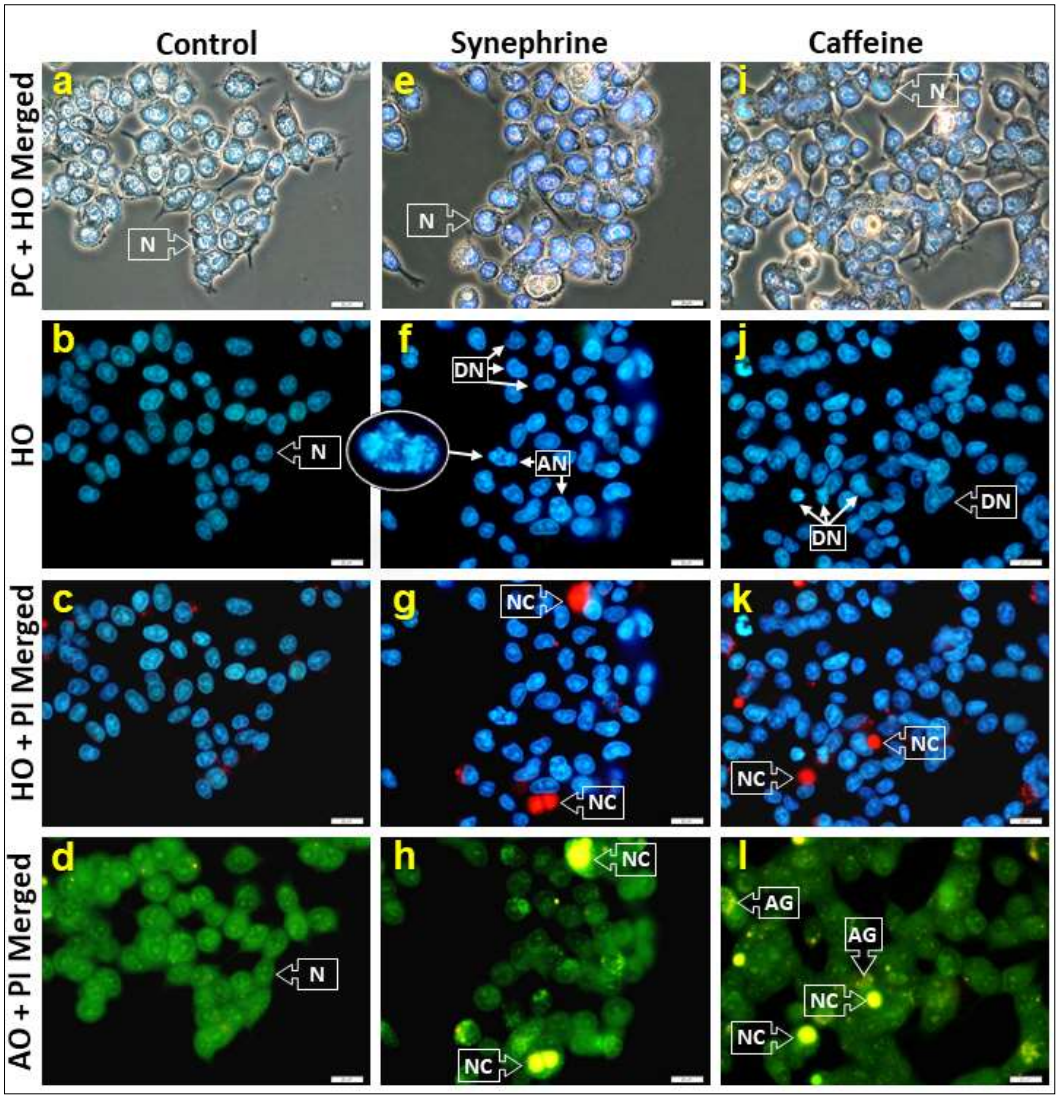


Fig 3.5b Representative photomicrographs showing the effect of synephrine and caffeine on MCF-7 cell morphology. Cells were treated for 18 hours. Panels a-d show untreated cells, panels e-h show the effects of 398.11 µM synephrine, and panel i-l show the effects of 457.10 µM caffeine. An Olympus BX41 microscope was used to observe cells and images were captured with a DP72 digital camera (Magnification: 400×; scalebar: 20µm). Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). LEGEND- PC: Phase-contrast; HO: Hoechst 33342; PI: Propidium iodide; AO: Acridine orange; N: nucleus; DN: deformed nucleus; AN: apoptotic nucleus; AG: acidic granules; and NC: necrotic cell

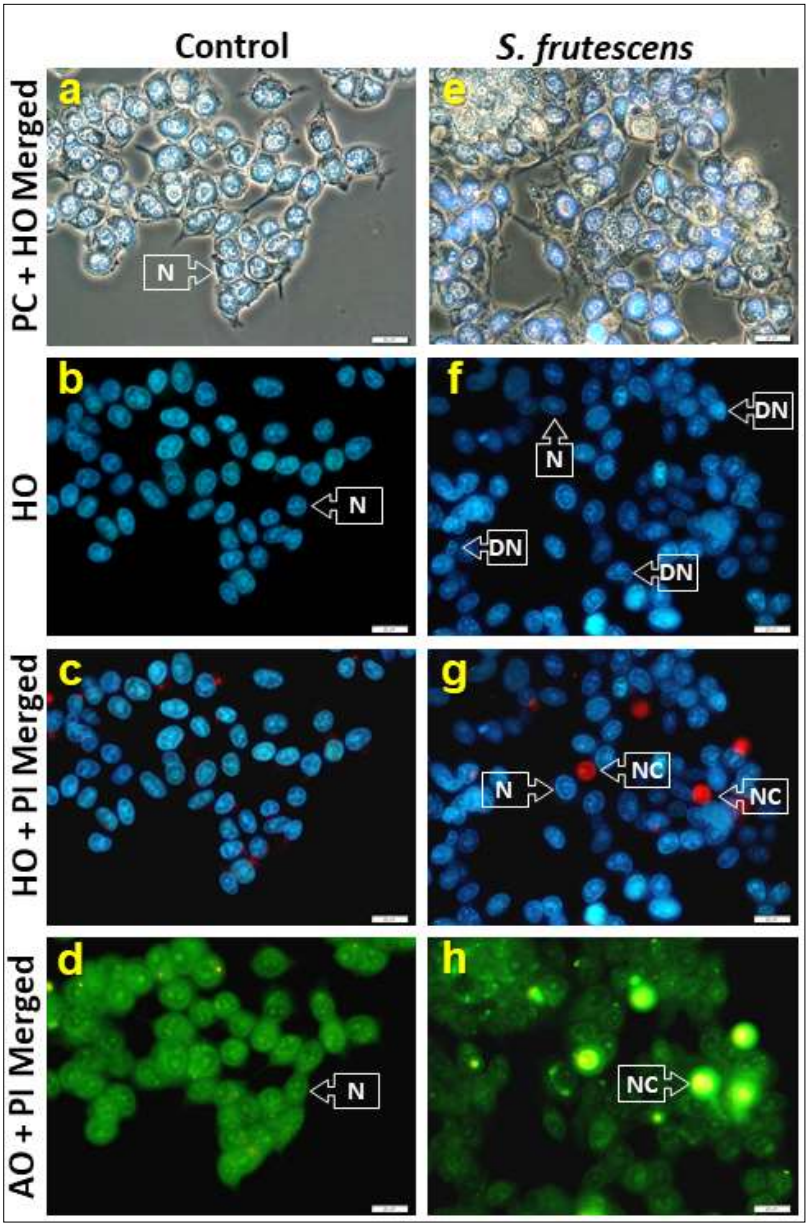


Fig 3.5c Representative photomicrographs showing the effect of *S. frutescens* on MCF-7 cell morphology. Cells were treated for 18 hours. Panels a-d show untreated cells and panels e-h show the effects of 1.7 *v/v* % *S. frutescens* ethanol extract. Olympus BX41 microscope was used to observe cells and images were captured with a DP72 digital camera (Magnification: 400×; scalebar: 20µm). Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). LEGEND- PC: Phase-contrast; HO: Hoechst 33342; PI: Propidium iodide; AO: Acridine orange; N: nucleus; DF: deformed nucleus; and NC: necrotic cell

The HO stain, showed that some nuclei appear deformed in shape shown in Fig 3.5b panel j and necrotic cells were observed in Fig 3.5b panels k and l. Similar to

resveratrol, caffeine increased the appearance of acidic granules in the cytoplasm of cells shown in Fig 3.5b, panel l.

Fig 3.5c panel e-h shows the morphological changes caused by *S. frutescens* ethanol extract (1.70 *v*/*v* %). Similar to the effect of caffeine, cells treated with *S. frutescens* ethanol extract appear to have normal cell morphology with a small number of cells appearing to be rounded up and stressed as shown in Fig 3.5c panel e. A few cells appear to have deformed nuclei in Fig 3.5c panel f. *S. frutescens* caused an increase in the formation of necrotic cells shown by red and bright yellow-orange in Fig 3.5c panel g and h.

Morphological changes indicative of apoptosis includes chromatin condensation, nuclear fragmentation, appear fluoresced at higher HO intensity and the formation of apoptotic bodies (De Vitto *et al.*, 2018). In summary, doxorubicin, resveratrol and synephrine were able to induce morphological changes consistent with apoptosis. Changes in cell morphology as a result of necrosis are characterized by round, swollen cells with intense PI stained nuclei (Atale *et al.*, 2014). Cells treated with doxorubicin and the CAMs-resveratrol, synephrine, caffeine and *S. frutescens* show evidence of necrotic cell death. The presence of acidic granules in the cytoplasm in cells treated with resveratrol and caffeine was indicative of possible autophagic cell death. These are preliminary observations and further investigations are required to confirm the data using more specific cell death assessment assays.

3.4 The effect of CAMs and doxorubicin on PgP efflux activity.

The function of the PgP drug efflux pump was measured using the calcein-AM accumulation assay as described in Chapter 2 section 2.6. In cells where PgP is active, calcein-AM is exported out of the cell before it can be hydrolysed by esterases into the fluorescent calcein molecule. A reduced fluorescence signal thus indicates increased PgP activity. Cyclosporin A and verapamil are inhibitors of PgP and were included to determine their effects on PgP activity in the cells and is shown in Figure 3.6. The effect of doxorubicin and the CAMs on PgP activity was determined as well as combinations of the CAMs with doxorubicin. The data is presented in Fig 3.7 to Fig 3.9.

The cells were treated with increasing range of concentrations of verapamil (0.02-61.1 µM) shown in Fig 3.6 A, and cyclosporin (0.0083-24.9 µM) shown in Fig 3.6 B for 30 minutes prior to the end of the treatment period. The range of concentrations of cyclosporin A and verapamil used here was recommended by the Vybrant Multidrug Resistance Assay Kit (V-13180) protocol. Verapamil rapidly inhibited PgP activity in MCF-7 cells after 30 minutes since the calcein-AM levels were increased. In cells treated with verapamil between 6.1 µM and 61.1 µM, PgP activity was inhibited in a dose-dependent manner.

Similarly, after treatment with cyclosporin A, PgP activity was rapidly inhibited in a dose dependent manner but at a range of lower concentrations (0.083 µM-24.9 µM) than that of verapamil. In comparison to verapamil, cyclosporin A was a more potent inhibitor of PgP activity since the calcein-AM levels was higher than the levels seen in verapamil after the same treatment period.

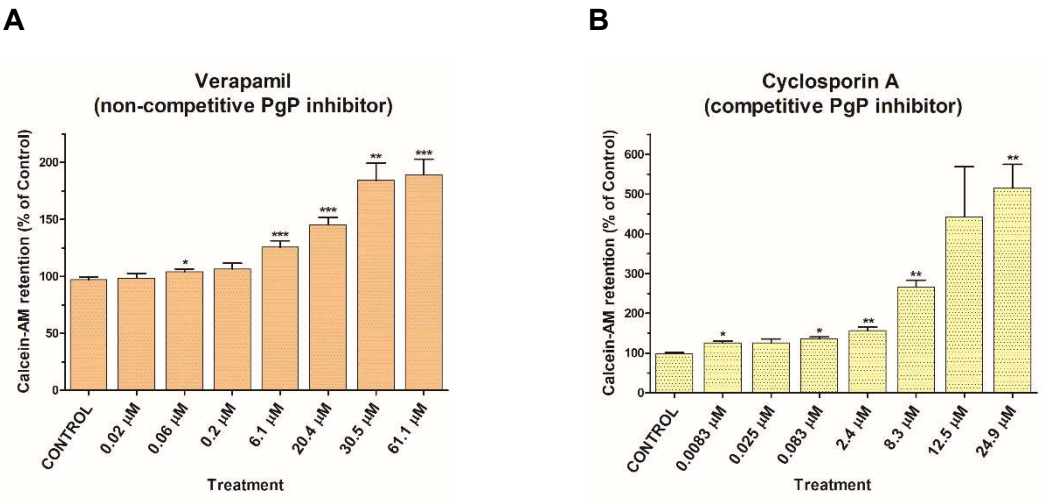


Figure 3.6 The effect of increasing verapamil and cyclosporin A concentrations on PgP activity in MCF-7 cells. The cells were treated with a range of concentrations of A: verapamil (0.02-61.1 µM), and B: cyclosporin A (0.0083-24.9 µM) for 30 minutes. Each bar represents the mean calcein-AM levels obtained in quadruplicate and the error bars indicate the SEM. The bar graphs are representative of four independent experiments (n=4).

3.4.1 The effect of doxorubicin and resveratrol on the PgP efflux activity in MCF-7 cells

Since doxorubicin and resveratrol showed cytotoxic effects in MCF-7 cells at low concentrations, their effects on PgP activity were evaluated at the same range of concentrations. Cells were treated with doxorubicin and resveratrol at 0.1 μM, 0.5 μM and 10 μM for 24 hours. The results are shown in Fig 3.7. Students' t-test was used to determine if any significant differences was observed in calcein-AM levels in treated cells compared to the untreated control and is indicated by asterisks on the bar graphs in Fig 3.7.

After a 24 hour treatment period, doxorubicin at concentrations of 0.1 μM, 0.5 μM and 10 μM significantly increased the PgP efflux activity in MCF-7 cells when compared to the untreated control. Calcein-AM levels was decreased by doxorubicin in a dose dependent manner (Fig 3.7).

Similar to doxorubicin, resveratrol at all three treatment concentrations significantly increased the PgP efflux activity when compared to the untreated control. The calcein-AM levels were decreased but the dose dependent relationship with resveratrol appears less clear (Fig 3.7).

Doxorubicin and resveratrol significantly increased the PgP efflux activity in cells at all three concentrations. Therefore, their effects on calcein-AM levels were statistically compared to determine if there were any significant differences between the two treatments and is summarised in Table 3.4.

Doxorubicin and resveratrol increased the PgP activity of MCF-7 cells in a similar fashion when treated at concentrations of 0.1 μM and 0.5 μM since there were no significant differences between their mean calcein-AM levels. At a concentration of 10 μM, resveratrol showed a statistically meaningful difference when compared to doxorubicin indicating that it activated PgP less than doxorubicin.

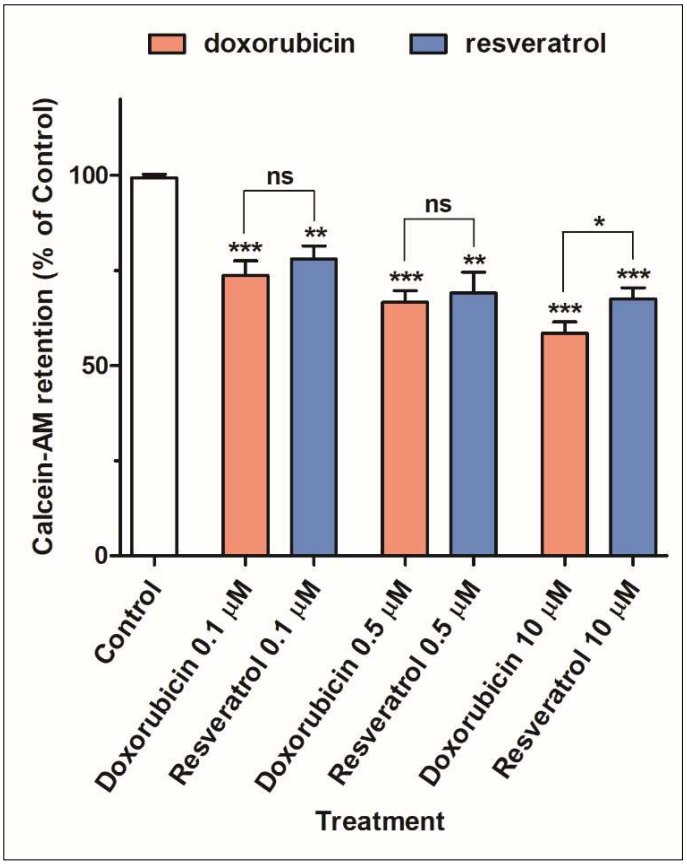


Figure 3.7 Effects of doxorubicin and resveratrol on PgP efflux activity in MCF-7 cells treated for 24 hours. The cells were treated with 0.1 μM, 0.5 μM and 10 μM concentrations of doxorubicin and resveratrol for 24 hours treatment period. Experiments were repeated four times. The bars for each concentration represent the mean calcein-AM accumulation in the treated cells and the error bars indicate the SEM. The bar graphs are representative of four independent experiments (n=4). Students' t-test followed by Welch's correction test were performed to measure statistical difference between treatments and the untreated control cells, as well as between same treatment concentrations. Significance criterion at p-value <0.05 (*), ≤0.01 (**), ≤0.001 (***) or non-significant (ns).

Table 3.4 Statistical evaluation of doxorubicin and resveratrol to determine if differences in PgP activity was significant. Students' t-test followed by Welch's correction test was used for evaluation.

Common variable (Treatment group)	Test variable (Concentration)	p-value	Significance (p<0.05)
resveratrol **compared to** doxorubicin	0.1 µM	0.42	Non-significant
	0.5 µM	0.70	Non-significant
	10 µM	0.047	Significant

3.4.2 The effect of synephrine and caffeine on the PgP efflux activity in MCF-7 cells

Since synephrine and caffeine showed poor cytotoxic activity against the MCF-7 cells, their effects on PgP activity was evaluated at concentrations of 75 µM, 150 µM and 300 µM after a 24 hour treatment period. The results are shown in Fig 3.8. Students' t-test was used to determine if any significant differences in calcein-AM levels in treatments compared to the untreated control indicated by asterisks on the bar graphs.

Synephrine caused a statistically meaningful decrease in the calcein-AM levels in cells treated all three concentrations compared to the untreated cells (Fig 3.8). The PgP activity was significantly increased after treatment with synephrine whilst the dose dependent relationship appears to be unclear.

Similar to synephrine, caffeine caused a statistically significant decrease in calcein-AM levels in cells treated with all three concentrations (Fig 3.8). After 24 hours treatment period, caffeine caused a significant increase in PgP activity in a dose dependent manner. Synephrine and caffeine significantly increased the PgP efflux activity in cells at all three concentrations. Therefore, their effects on calcein-AM levels were statistically evaluated to determine if there were any significant differences between the two treatments and is summarised in Table 3.5. At concentrations of 75 µM and 300 µM, caffeine showed a statistically meaningful difference when compared to synephrine

indicating that it increased the PgP activity more efficiently than synephrine. There was no statistical difference between caffeine and synephrine at a concentration of 150 µM indicating PgP activity was increased in a similar fashion with the two treatments.

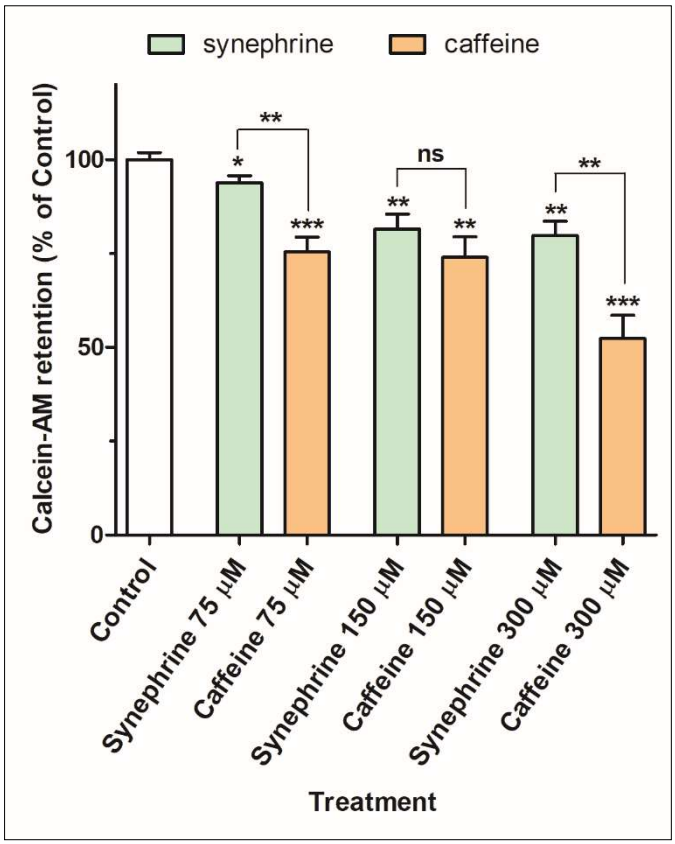


Figure 3.8 Effects of synephrine and caffeine on PgP efflux activity in MCF-7 cells treated for 24 hours. The cells were treated with 75 µM, 150 µM and 300 µM concentrations of synephrine and caffeine for 24 hours treatment period. The bars for each concentration represent the mean calcein-AM accumulation in the treated cells and the error bars indicate the SEM. The bar graphs are representative of four independent experiments (n=4). Students' t-test followed by Welch's correction test were performed to measure statistical difference between treatments and the untreated control cells, as well as between same treatment concentrations. Significance criterion at p-value <0.05 (*), ≤0.01 (**), ≤0.001 (***) or non-significant (ns).

Table 3.5 Statistical evaluation of synephrine and caffeine to determine if differences in PgP activity was significant. Students' t-test followed by Welch's correction test was used for evaluation.

Common variable (Treatment group)	Test variable (Concentration)	p-value	Significance (p<0.05)
caffeine **compared to** synephrine	75 µM	0.0022	Significant
	150 µM	0.2821	Non-significant
	300 µM	0.0027	Significant

3.4.3 The effect of doxorubicin in combinations with CAMs on PgP efflux activity in MCF-7 cells

The effects of CAMs on the doxorubicin induced PgP efflux activity in MCF-7 cells was assessed after 24 hours treatment. Doxorubicin is the positive control, and the compound is active at low concentrations below 1 µM. Cells were treated with 0.05 µM, 0.5 µM and 1 µM of doxorubicin alone and in combinations with CAMs at a constant concentration of resveratrol (157 µM), synephrine (423 µM) and caffeine (354 µM) for 24 hours. The results are shown in Fig 3.9 and the statistical comparisons are summarised in Table 3.7. The statistical comparison of all treatments with the untreated control is summarised in a table in Appendix F.

At a concentration of 0.05 µM, doxorubicin caused a statistically meaningful decrease in calcein-AM levels when compared to the untreated control thus increasing PgP activity. In combination treatments with 0.05 µM of doxorubicin, resveratrol significantly increased the PgP activity in comparison to the untreated control. In contrast to resveratrol, synephrine and caffeine inhibited the PgP activity in the presence of 0.05 µM of doxorubicin to close to that of the untreated control i.e., there were no significant differences in calcein-AM levels compared to the untreated control.

At a concentration of 0.5 µM, doxorubicin caused a statistically meaningful decrease in calcein-AM levels similar to the levels seen at doxorubicin concentration of 0.05 µM. In combinations with resveratrol, the PgP activity was significantly increased compared to

the untreated control and there was a statistically meaningful difference when compared to doxorubicin alone indicating resveratrol enhanced the doxorubicin induced PgP activity.

In combinations with synephrine, the PgP activity was significantly increased when compared to untreated control. There was no significant difference in calcein-AM levels in combinations with synephrine when compared to doxorubicin alone indicating inhibition of doxorubicin induced PgP activity but to a lesser extent than in presence of 0.05 µM of doxorubicin.

Caffeine caused a statistically meaningful inhibition of PgP activity in the presence of 0.5 µM of doxorubicin to close to that of the untreated control i.e., there were no significant differences in calcein-AM levels compared to the untreated control.

At a concentration of 1 µM, doxorubicin caused a statistically meaningful decrease in calcein-AM levels thus indicating an increase in PgP activity in dose dependent manner. In combinations with 1 µM of doxorubicin, resveratrol and synephrine had no statically meaningful effect on doxorubicin induced PgP activity however the PgP activity was significantly increased compared to the untreated control. Caffeine still inhibited the doxorubicin induced activity to close to that of untreated control albeit less inhibition than that of the lower doxorubicin concentrations.

In single-agent treatment, doxorubicin showed a significant decrease in calcein-AM levels in treatment with all three concentrations. This indicates doxorubicin activated PgP efflux and in a dose dependent manner.

In combination treatment with doxorubicin, resveratrol enhanced PgP activity in a dose dependent manner and a statistically meaningful increase in activity at a concentration of 0.5 µM of doxorubicin. This effect was lost at a concentration of doxorubicin at 1 µM.

Synephrine showed a statistically meaningful increase in doxorubicin induced calcein-AM levels at a doxorubicin concentration of 0.05 µM i.e., concentration lower than its IC_{50} value after 24 hour treatment. This indicates synephrine inhibited doxorubicin associated PgP activity. This effect was lost at a doxorubicin concentration of 1 µM and synephrine showed inhibition of PgP activity in a doxorubicin dose dependent manner.

Caffeine caused a statistically meaningful increase in doxorubicin induced calcein-AM levels at all three doxorubicin concentrations tested in combinations. It appears there was no dose dependent effect in combination treatment with caffeine however the PgP activity in the presence of all three doxorubicin concentrations was not significantly different to the untreated control. This indicates caffeine inhibited doxorubicin associated PgP activity close to that of untreated MCF-7 cells after 24 hours.

In summary, doxorubicin, and resveratrol both increased PgP activity in a similar fashion at concentrations of 0.1 μM and 0.5 μM while doxorubicin showed more increased activity at 10 μM. Synephrine and caffeine showed a similar increase in PgP activity at a concentration of 150 μM while caffeine caused a greater increase in PgP activity at concentrations of 75 μM and 300 μM. Doxorubicin was the most potent activator of PgP efflux in comparison to the CAMs. Out of the three CAMs evaluated, resveratrol was the most potent activator of PgP. Following 24 hours combination treatments, resveratrol enhanced the doxorubicin induced PgP efflux activity while synephrine and caffeine inhibited the PgP activity caused by doxorubicin. Out of all three CAMs assessed in combination treatments, caffeine was the most effective inhibitor of doxorubicin induced PgP activity.

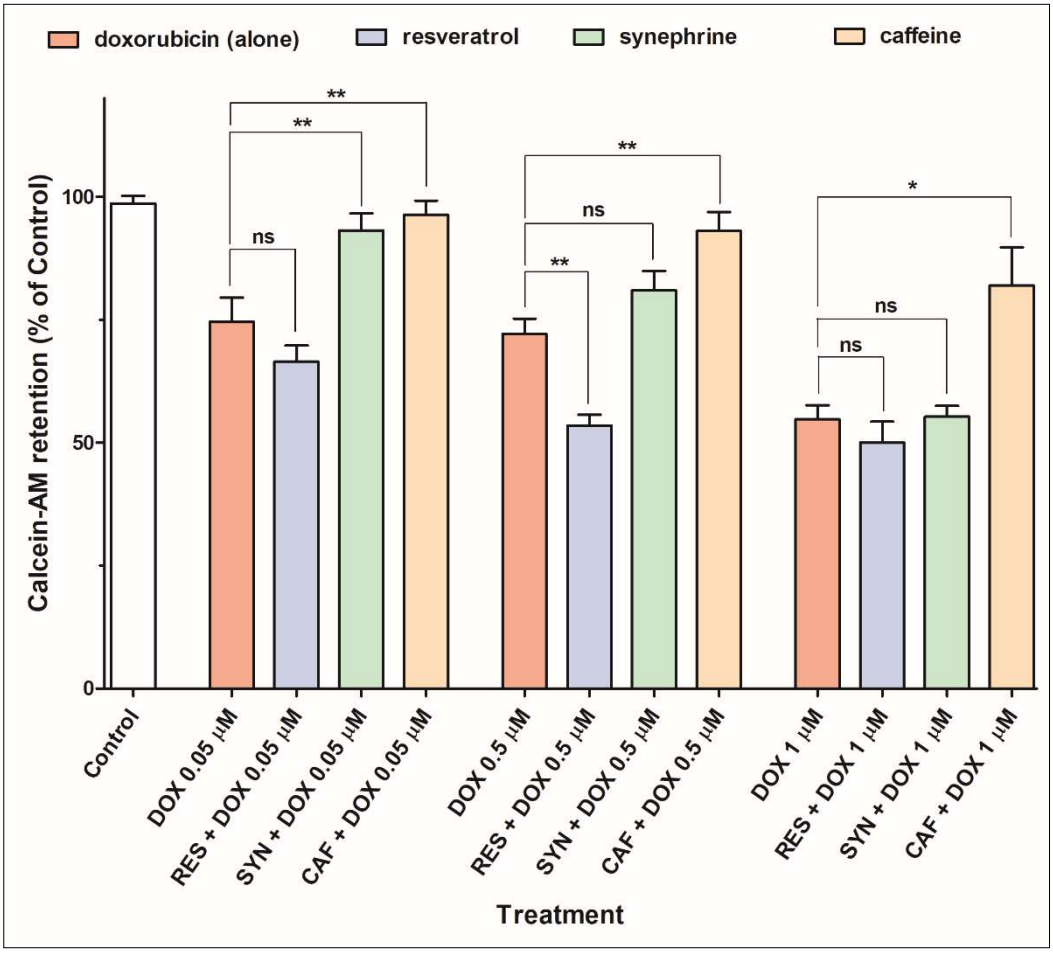


Figure 3.9 The effects of CAMs on doxorubicin induced PgP activity in MCF-7 cells after a 24 hour treatment period. Cells were treated with concentrations of doxorubicin at 0.05 μM, 0.5 μM and 1 μM in the presence of resveratrol (157 μM), synephrine (423 μM) and caffeine (354 μM) for 24 hours. The bars for each concentration represent the mean calcein-AM level in the cells and the error bars indicate the SEM. The bar graphs are representative of four independent experiments (n=4). Students' t-test followed by Welch's correction test were performed to measure statistical difference between doxorubicin alone and in combination with CAMs at the same treatment concentrations. Significance criterion at p-value <0.05 (*), ≤0.01 (**), ≤0.001 (***) or non-significant (ns). Legend: DOX = doxorubicin, RES = resveratrol, SYN = synephrine, CAF = caffeine

Table 3.6 Statistical comparison of CAMs effects on the doxorubicin mean calcein-AM accumulation in cells after 24 hours treatment as determined with the Students' t-test followed by Welch's correction test.

Common variable (doxorubicin concentration)	Test variable (Combination treatment)	p-value	Significance (p<0.05)
0.05 μM	resveratrol + doxorubicin	0.19	Non-significant
	synephrine + doxorubicin	0.009	Significant
	caffeine + doxorubicin	0.003	Significant
0.5 μM	resveratrol + doxorubicin	<0.0001	Significant
	synephrine + doxorubicin	0.097	Non-significant
	caffeine + doxorubicin	0.001	Significant
1 μM	resveratrol + doxorubicin	0.37	Non-significant
	synephrine + doxorubicin	0.88	Non-significant
	caffeine + doxorubicin	0.011	Significant

3.5 The effect of CAMs and doxorubicin on the expression levels of PgP protein in the MCF-7 cell line

The effects of CAMs and doxorubicin on PgP protein expression levels were evaluated using the immunofluorescence technique as described in Chapter 2 section 2.7.1. The PgP primary antibody was a mouse anti-human PgP monoclonal antibody (MAS-12854) prepared at 1:100 and the secondary antibody was a goat anti-mouse polyclonal antibody (ab150113) conjugated with Alexa Fluor 488 prepared at 1:800 for PgP protein detection. With the exception of resveratrol, cells were treated with the CAMs and doxorubicin at concentrations equivalent to their IC_{50} values for 24 hours and 48 hours (Fig 3.10 and Fig 3.11). Resveratrol was evaluated at both an IC_{50} and an IC_{80} concentration since it was the most potent CAM with an IC_{50} value below 100 μM. Thereafter, cells were treated with doxorubicin in combinations with CAMs at the concentrations indicated for 24 hours and 48 hours (Fig 3.12 and Fig 3.13). Combination treatments were performed to determine if the three CAMs affect the doxorubicin induced PgP protein expression. The photomicrographs were captured using identical camera settings for each experiment and processed with consistent settings using the Olympus CellSens software package. Fluorescence intensity was measured using Image J software (Schindelin *et al.*, 2012; Shihan *et al.*, 2021) as described in Chapter 2 section 2.7.1. The Students' t-test was used to statistically compare mean fluorescence intensities of cells for significant differences in PgP protein expression levels. Localization of PgP protein in the nucleus was analysed using the signal from the HO stain and the Alexa Fluor 488 using the co-localisation feature of Olympus CellSens software. The selected cells for analysis are shown in Appendix G and Appendix H. Results are expressed as a Pearson's Correlation Coefficient (PCC) with a value of -1 being a perfect negative correlation and +1 being a perfect positive correlation and 0 denoting absence of relationship (Adler and Parmryd, 2010; Dunn *et al.*, 2011). In Table 3.7, the grading scale used to measure strength of correlation is assigned to PCC values and was adapted from Mukaka (2012) and Chao (2018). The PgP fluorescence intensities and colocalisation results are summarised in Tables 3.8 to 3.11.

Table 3.7 Grading scale for interpreting a size of a correlation coefficient

PCC (negative or positive)	Interpretation of correlation
Less than 0.29	Negligible
0.30–0.49	Weak/low
0.50–0.69	Moderate
0.70–0.89	Strong
0.90–1.00	Very strong/high

3.5.1 The PgP protein expression levels after treatment with doxorubicin and the CAMs for 24 hours

The MCF-7 cells were treated with doxorubicin at 1.12 μM, synephrine at 423 μM, caffeine at 354 μM, resveratrol at 157 μM and 181.27 μM for 24 hours. The results are shown in Fig 3.10. The analysis of the mean fluorescence intensities and localisation of PgP protein expression is summarised in Table 3.8.

The untreated cells had a weak green fluorescence signal indicating low baseline PgP protein expression (Fig 3.10 panel d). Cells had a typical polygonal cell morphology and the HO stain showed blue oval shaped nuclei (Fig 3.10 panels a and b).

Doxorubicin strongly induced the expression of PgP protein and appeared to be found in the nucleus (Fig 3.10 panels g and h). Image J analysis of fluorescence intensity indicate a 20 fold increase in PgP protein when compared to the untreated control cells (Table 3.8). Colocalisation analysis indicate a strong Pearson's Correlation (0.76) between PgP and HO signals confirming PgP expression occurred in the nucleus of the cells treated with doxorubicin.

Cells treated with resveratrol at 157 μM showed a weak green fluorescence signal indicating low PgP protein expression similar to the baseline expression levels (Fig 3.10 panel l). At the higher concentration of 181.27 μM, resveratrol showed no change in PgP protein expression (Fig 3.10 panel p). Measurement of fluorescence intensity using Image J indicated no statistically meaningful effects of resveratrol at both treatment

concentrations on the expression levels of PgP protein (Table 3.8). This indicates that resveratrol did not affect the expression levels of PgP protein after 24 hours.
Cells treated with synephrine at 423 µM, cells showed no changes in PgP protein expression when compared to the untreated cells (Fig 3.10 panel t). Image J analysis of cells treated with synephrine indicated no significant changes in the PgP fluorescence intensity when compared to the untreated cells (Table 3.8). This indicated synephrine did not affect the expression levels of PgP protein after 24 hours.

Similar to synephrine, cells treated with caffeine at 354 µM showed no changes in the PgP protein expression levels (Fig 3.10 panels x). Image J analysis of fluorescence intensity indicated no significance change in expression levels of PgP protein when compared to the untreated cells (Table 3.8).

Colocalisation analysis results showed an overall weak Pearson's Correlation between PgP and HO signals in treatments with all the CAMs confirming a poor expression of PgP protein in the nucleus.

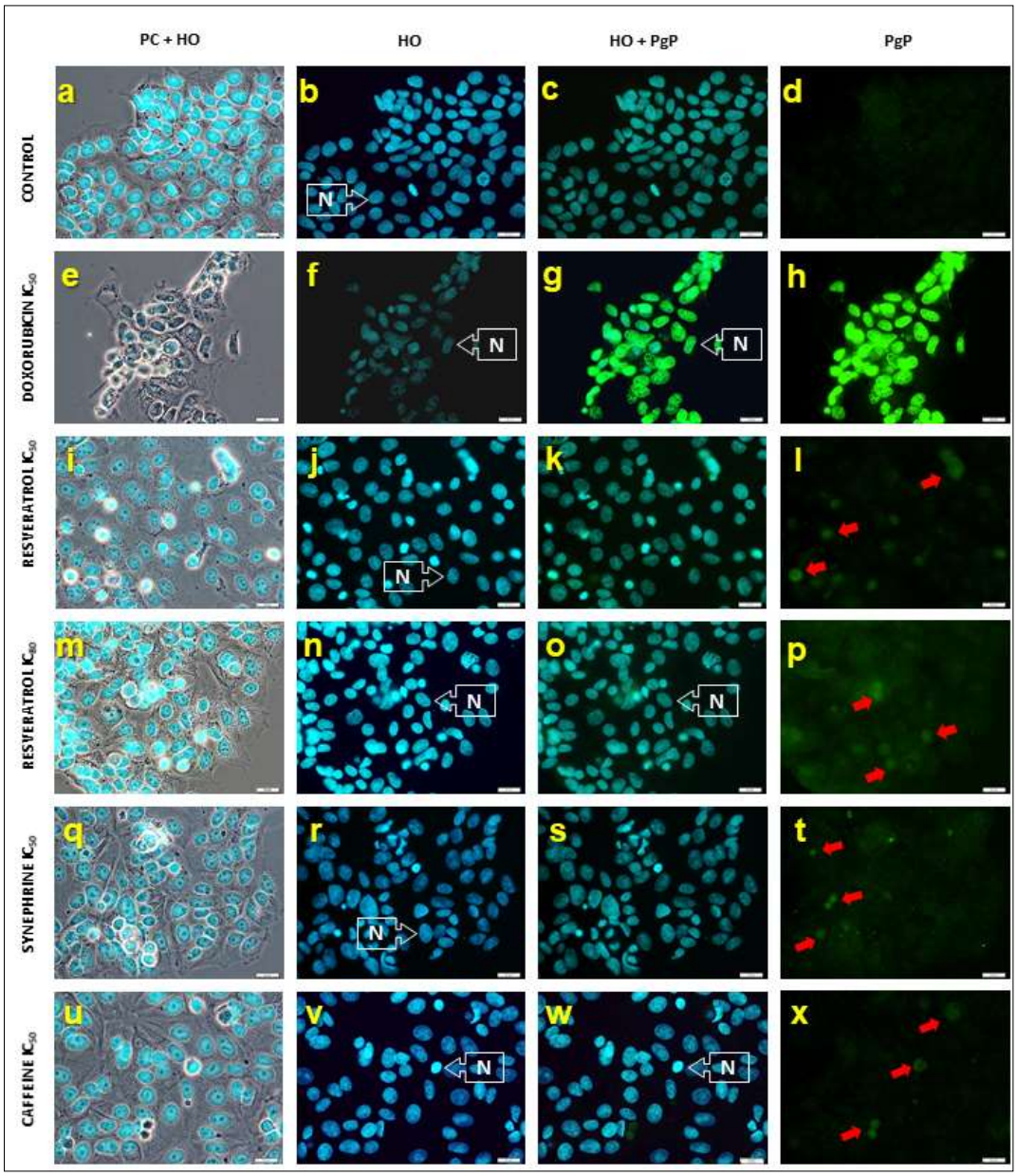


Figure 3.10 PgP protein expression levels in MCF-7 cells following treatment with doxorubicin, resveratrol, synephrine and caffeine for 24 hours. Cells were treated doxorubicin at 1.12 µM (e-h), synephrine at 423 µM (q-t) and caffeine at 354 µM (u-x) for 24 hours. Cells were treated with resveratrol at concentrations of 157 µM (i-l), and 181.27 µM (m-p). The cells were viewed using an U-MNIB3 filter (Excitation: 480/20, Emission: 510/50) for PgP expression detection by Alexa Fluor 488 and an U-MWU2 filter (Excitation: 365/10, Emission: 420 long pass) for the nucleus counterstained using HO. Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). An Olympus BX41 microscope was used to observe cells and images were captured with a DP72 digital camera (Magnification: 400×; scalebar: 20µm). Legend: N = nucleus, and "Arrows" = Cells with high fluorescence signal

Table 3.8 PgP protein mean fluorescence intensities and localisation in MCF-7 cells treated with doxorubicin, resveratrol, synephrine and caffeine for 24 hours. Student t-test followed by Welch's correction test was used to determine if differences between treatments and untreated control were significant.

Test variable	Mean fluorescence intensity (AU)	Significance p<0.05	Cells expressing PgP in the nucleus (PCC)
Untreated group (control)	3.37 ± 0.24	-	0.18
doxorubicin (1.12 µM)	77.49 ± 1.13	Significant p=0.0003	0.76
resveratrol (157 µM)	3.92 ± 0.43	Non-significant p= 0.89	0.25
resveratrol (181.27 µM)	4.61 ± 0.15	Non-significant p= 0.092	0.16
synephrine (423 µM)	4.12 ± 0.36	Non-significant p=0.59	0.23
caffeine (354 µM)	3.12 ± 0.15	Non-significant p=0.11	0.14

3.5.2 The PgP protein expression levels after treatment with doxorubicin and the CAMs for 48 hours

The MCF-7 cells were treated with doxorubicin at 0.74 µM, synephrine at 166.20 µM, caffeine at 167.40 µM, resveratrol at 80.40 µM and 119.60 µM for 48 hours. The results are shown in Fig 3.11. The analysis of the fluorescence intensities and localisation of PgP protein expression is summarised in Table 3.9. After 48 hours, the untreated cells maintained a low PgP protein expression indicated by the weak green fluorescence (Fig 3.11 panel d). Similar to the 24 hour period, after 48 hours doxorubicin at 0.74 µM strongly induced the expression levels of PgP protein which appears to be localised in the nucleus, in Fig 3.11 panels g and h. The cells appear stressed, growing on top of each other and the nuclei appear condensed in size (Fig 3.11 panels e and f).

Doxorubicin strongly induced the PgP protein expression levels when compared to the untreated control cells (Table 3.9). Colocalisation analysis indicated a high (0.90) Pearson's Correlation between PgP and HO signals confirming PgP expression still occurred in the nucleus at 48 hours.

In treatment with all the CAMs, the expression of PgP appear unchanged when compared to the untreated control (Fig 3.11 panels l, p, t, and x). Similar to 24 hours, at 48 hours measurement of PgP mean fluorescence intensity by Image J indicated no significant differences in the PgP fluorescence intensities between all the CAMs and the untreated control cells (Table 3.9). This indicates resveratrol at both concentrations, synephrine and caffeine did not affect the PgP expression levels after 48 hours. Colocalisation analysis indicated a weak (0.0-0.49) Pearson's Correlation between PgP and HO signals confirming PgP expression occur less prominent in the nucleus of cells treated with the CAMs for 48 hours.

In summary, doxorubicin was a strong inducer of PgP expression levels after both treatment periods while the all the CAMs had no meaningful effects on the expression of PgP protein. Colocalisation analysis indicated PgP occurred in the nuclei of cells treated with doxorubicin confirmed by a strong Pearson's Correlation and the expression of PgP in the nucleus was found less prominent in CAM treatments.

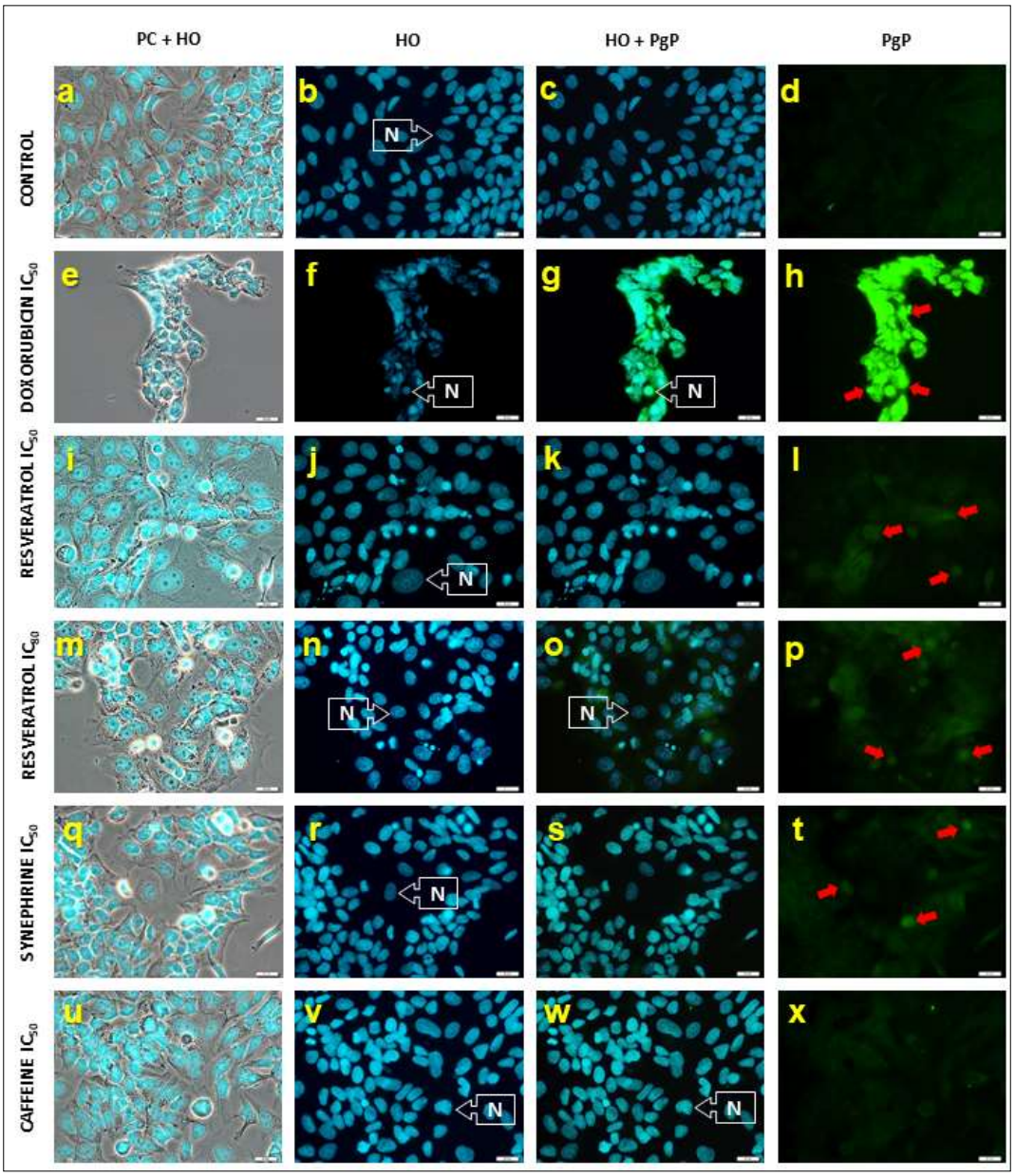


Figure 3.11 PgP protein expression levels in MCF-7 cells following treatment with doxorubicin, resveratrol, synephrine and caffeine for 48 hours. Cells were treated with doxorubicin at 0.74 µM (e-h), synephrine at 166.20 µM (q-t) and caffeine at 167.40 µM (u-x) for 48 hours. Cells were treated with resveratrol at concentrations of 80.40 µM (i-l), and 119.60 µM (m-p). The cells were viewed using an U-MNIB3 filter (Excitation: 480/20, Emission: 510/50) for PgP expression detection by Alexa Fluor 488 and an U-MWU2 filter (Excitation: 365/10, Emission: 420 long pass) for the nucleus counterstained using HO. Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). An Olympus BX41 microscope was used to observe cells and images were captured with a DP72 digital camera (Magnification: 400×; scalebar: 20µm). Legend: N = nucleus, and "Arrows" = Cells with high fluorescence signal

Table 3.9 PgP protein mean fluorescence intensities and localisation in MCF-7 cells treated with doxorubicin, resveratrol, synephrine and caffeine for 48 hours. Student t-test followed by Welch's correction test was used to determine if differences between treatments and untreated control were significant.

Test variable	Mean fluorescence intensity (AU)	Significance p<0.05	Cells expressing PgP in the nucleus (PCC)
Untreated group (control)	4.38 ± 0.22	-	0.04
doxorubicin (0.74 μM)	77.60 ± 0.88	Significant p=0.0004	0.90
resveratrol (80.40 μM)	3.68 ± 0.35	Non-significant p= 0.14	0.16
resveratrol (119.60 μM)	4.56 ± 0.38	Non-significant p=0.69	0.39
synephrine (166.20 μM)	4.21 ± 0.23	Non-significant p=0.61	0.28
caffeine (167.40 μM)	4.16 ± 0.16	Non-significant p=0.43	0.20

3.5.3 The doxorubicin induced expression levels of PgP protein after treatment with CAMs in combination for 24 hours

The effects of doxorubicin combined with CAMs on PgP expression was assessed after 24 hours. Cells were treated with doxorubicin (1.12 μM) alone and in combinations with resveratrol (157 μM), synephrine (423 μM) and caffeine (354 μM). The results are shown in Fig 3.12. Analysis of the fluorescence intensities and localisation of PgP protein expression is summarised in in Table 3.10.

Doxorubicin strongly induced the PgP expression and appear to be found in the nucleus (Fig 3.12 panels g and h). Image J analysis indicate a high percentage of cells (91.80 %) with high fluorescence intensity at 77.49 ± 1.13 AU while the remainder percentage of cells had a low fluorescence intensity at 32.90 ± 1.43 AU (Table 3.10).

When doxorubicin was combined with resveratrol for 24 hours, many cells had a round appearance and seemed almost detached from the growth surface (Fig 3.12 panel i). The expression of PgP protein remained localised in the nucleus, however a large number of the nuclei appear to be deformed (Fig 3.12 panels j and k). The percentage of cells with high intensity fluorescent staining was decreased to 25.05 % and resveratrol showed a small decrease in PgP intensity of these cells at 62.15 ± 2.93 AU when compared to doxorubicin alone (Table 3.10). The remainder of the cells i.e., 74.95% showed a statistically meaningful decreased PgP fluorescence intensity than cells treated with doxorubicin alone (Table 3.10). Image J analysis of fluorescence intensity of the cells showed that resveratrol treated cells showed a significant decrease in PgP fluorescence intensity (Table 3.10). Colocalisation analysis of PgP and HO signals showed a strong positive Pearson's Correlation (0.74) confirming PgP remained localised in the nucleus when compared to cells treated with doxorubicin alone. Resveratrol was able to decrease doxorubicin induced expression levels of PgP protein in the high percentage of cells after 24 hours.

Cells treated with synephrine and doxorubicin in combination showed a similar PgP intensity fluorescent staining to doxorubicin alone and appear to be found in the nucleus (Fig 3.12 panels o and p). In measurement of fluorescence intensity with Image J analysis, the high percentage of cells (89.41 %) showed no statistically meaningful difference in the fluorescence intensity of cells when compared to doxorubicin alone and only a small percentage of cells (10.6 %) showed a decrease in intensity (Table 3.10). This indicates that synephrine did not affect the doxorubicin induced expression of PgP protein after a 24 hour treatment period.

Evaluation of the cells treated with doxorubicin and caffeine in combination showed a similar fluorescence intensity staining to that of doxorubicin alone in the majority of the cells and a small number of cells appeared to have a lower intensity of PgP (Figure 3.12 panel t). Image J analysis of fluorescence intensity showed no significant change in the intensity of the majority of the cells (73.42 %) whilst the remainder 26.58 % of the cells had a decreased fluorescence intensity which was less intense than the cells of low intensity in doxorubicin alone (Table 3.10). This indicates that caffeine was able to decrease the doxorubicin induced expression of PgP in combination treatment i.e., more

than 20% of the cells appear to have low fluorescence intensity however the overall expression levels remained unchanged.

Similar to the 24 hour treatment period, after 48 hour treatment with all the CAMs in combination with doxorubicin caused PgP to localise in the nucleus as confirmed by a strong Pearson's Correlation between PgP and HO signals.

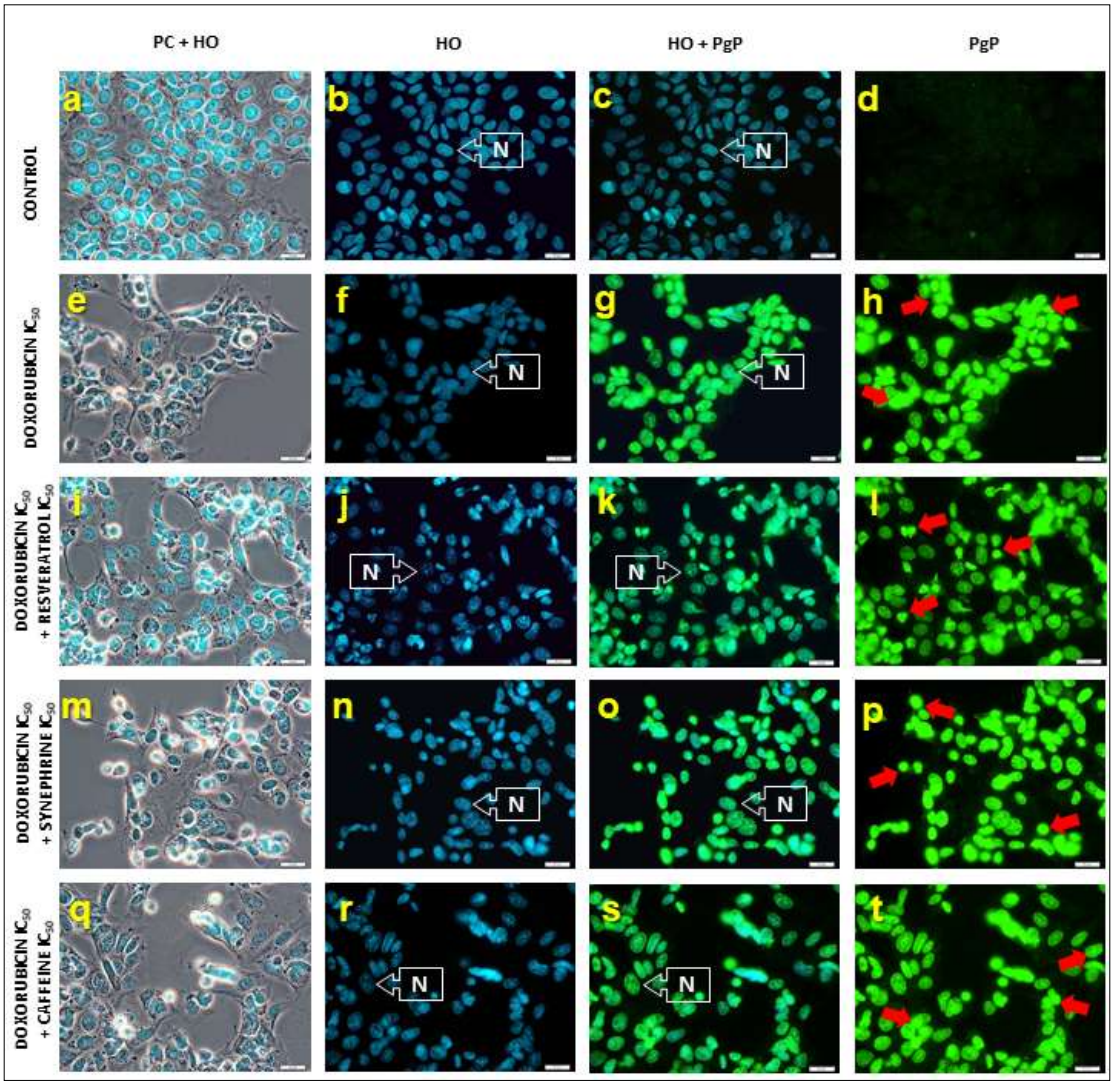


Figure 3.12 PgP protein expression levels in MCF-7 cells following treatment with doxorubicin and combinations with active CAMs after 24 hours. The MCF-7 cells were treated with doxorubicin at 1.12 µM (e-h) alone and in combinations with resveratrol at 157 µM (i-l), synephrine at 423 µM (m-p) and caffeine at 354 µM (q-t) for 24 hours. The cells were viewed using a U-MNIB3 filter (Excitation: 480/20, Emission: 510/50) for PgP expression detection by Alexa Fluor 488 and an U-MWU2 filter (Excitation: 365/10, Emission: 420 long pass) for the nucleus counterstained using HO. Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). An Olympus BX41 microscope was used to observe cells and images were captured with a DP72 digital camera (Magnification: 400×; scalebar: 20µm). Legend: N = nucleus, and “Arrows” = Cells with high fluorescence signal

Table 3.10 PgP mean fluorescence intensities of MCF-7 cells treated with doxorubicin and in combinations with the CAMs for 24 hours. Student t-test followed by Welch's correction test was used to determine if differences between doxorubicin and combination treatments were significant.

Test variable	Cells with high fluorescence signal		Cells with low fluorescence signal		Overall Significance (p<0.05)	Cells with PgP in the nucleus (PCC)
	% of cells	Mean fluorescence intensity (AU)	% of cells	Mean fluorescence intensity (AU)		
Untreated group (control)	0	-	100	3.83 ± 0.32	-	0.18
doxorubicin (alone)	91.80	77.49 ± 1.13 *	8.20	32.90 ± 1.43	-	0.76
resveratrol + doxorubicin	25.05	62.15 ± 2.93	74.95	31.03 ± 1.73 *	Significant p<0.0002	0.74
synephrine + doxorubicin	89.41	78.70 ± 2.27 *	10.59	43.34 ± 2.14	Non-significant p=0.66	0.80
caffeine + doxorubicin	73.42	76.62 ± 1.84 *	26.58	27.45 ± 0.36	Non-significant p=0.71	0.88

Asterisk (*): indicates the statistical comparisons of the mean fluorescence intensities in the high percentage of cells

3.5.4 The doxorubicin induced expression levels of PgP protein after treatment with CAMs in combination for 48 hours

The effects of doxorubicin combined with CAMs on PgP expression was assessed after 48 hours. Cells were treated with doxorubicin (1.12 μM) alone and in combinations

with resveratrol (80.54 μM), synephrine (166.20 μM) and caffeine (167.40 μM). The results are shown in Fig 3.13. The analysis of the fluorescence intensities and localisation of PgP protein expression is summarised in in Table 3.11.

The expression of PgP protein appears to have remained localised to the nuclei in all the treatments (Fig 3.13 panels g, k, o, and s). This was confirmed by colocalisation analysis of PgP and HO staining which showed a strong Pearson's Correlation (0.85-0.94) in all treatments (Table 3.11). Doxorubicin treated cells appeared smaller in size than the untreated control cells but showed a high fluorescence intense PgP signal (Fig 3.13 panels e and h). Doxorubicin caused a high percentage of cells (94.28 %) to have an intense PgP signal of 77.60 ± 0.88 AU and a low percentage of cells (5.72 %) with a weak fluorescence signal of 32.72 ± 0.24 AU (Table 3.11).

When combined with doxorubicin, resveratrol caused a significant decrease in the percentage of cells with high PgP intensity with only around 17.72 % of the cells –

showing an intense PgP signal but lower intensity than that of doxorubicin (Table 3.11). In the majority of the cells (82.28 %), resveratrol dramatically decreased the fluorescence intensity of PgP i.e., the mean fluorescence intensity in cells treated with resveratrol 19.78 ± 1.29 AU and doxorubicin alone 70.28 ± 3.86 AU showed strong significant differences.

Synephrine caused a significant decrease in the percentage of cells with high PgP intensity to 75.12 % and the PgP fluorescence intensity was significantly lower in these cells at 62.31 ± 0.83 AU than that of doxorubicin alone (Table 3.11). The remainder 24.88 % of cells with low PgP intensity was significantly increased, and the PgP signal was more intense in these cells at 50.67 ± 2.10 AU than the low PgP intensity cells in treatment with doxorubicin alone. Synephrine was able to significantly decrease the overall PgP expression caused in treatment with doxorubicin, however the low intensity PgP cells had a higher PgP intensity than all of the cells treated with resveratrol combination.

Caffeine caused a small decrease in the percentage of cells with high PgP intensity to 84.41 % and the PgP fluorescence intensity was significantly lower in these cells at 53.09 ± 0.50 AU than that of doxorubicin alone (Table 3.11). The remainder 15.59 %

of cells with low PgP intensity was significantly increased, however the PgP signal was less intense in these cells with 22.81 ± 0.62 AU than the low PgP intensity cells in treatment with doxorubicin alone (Table 3.11). Caffeine significantly decreased the doxorubicin induced expression of PgP albeit less effective than in combinations with resveratrol.

Similar to the 24 hour treatment period, after 48 hour treatment PgP appeared to be localised in the nucleus in cells treated with all the CAMs in combination with doxorubicin as confirmed by a strong Pearson's Correlation between PgP and HO signals.

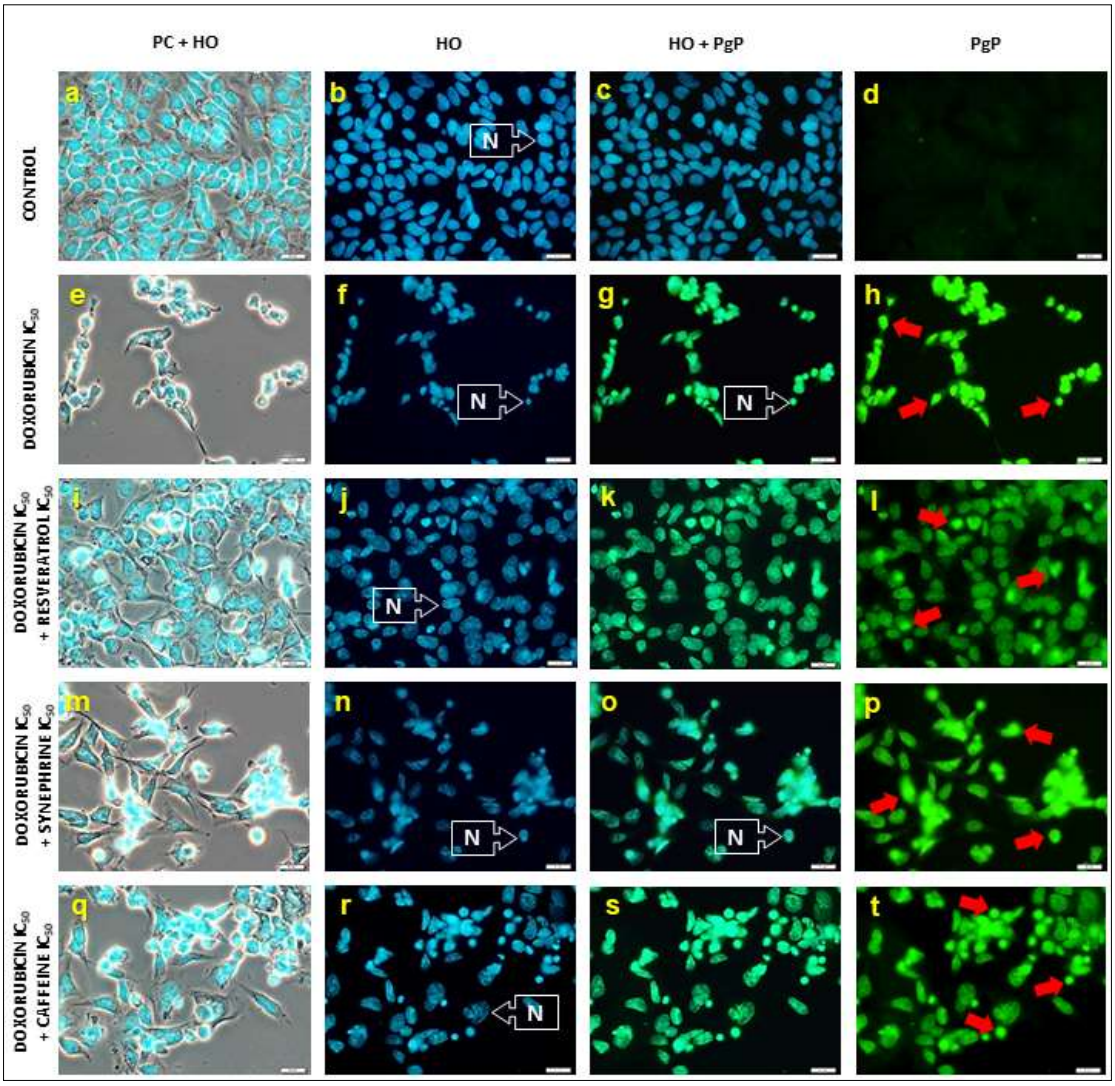


Figure 3.13 PgP protein expression levels in MCF-7 cells following treatment with doxorubicin and combinations with active CAMs for 48 hours. The MCF-7 cells were treated with doxorubicin at 0.74 µM (e-h) alone and in combinations with resveratrol at 80.54 µM (i-l), synephrine at 166.20 µM (m-p) and caffeine at 167.40 (q-t) for 48 hours. The cells were viewed using an U-MNIB3 filter (Excitation: 480/20, Emission: 510/50) for PgP expression detection by Alexa Fluor 488 and an U-MWU2 filter (Excitation: 365/10, Emission: 420 long pass) for the cells nuclei counterstained using HO. Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). An Olympus BX41 microscope was used to observe cells and images were captured with a DP72 digital camera (Magnification: 400×; scalebar: 20µm). Legend: N = nucleus, and “Arrows” = Cells with high fluorescence signal

Table 3.11 PgP mean fluorescence intensities of MCF-7 cells treated with doxorubicin and in combinations with the cams for 48 hours. Student t-test followed by Welch's correction test was used to determine if differences between doxorubicin and combination treatments were significant

Test variable	Cells with high fluorescence signal		Cells with low fluorescence signal		Overall Significance ($p<0.05$)	Cells expressing PgP in the nucleus (PCC)
	% of cells	Mean fluorescence intensity (AU)	% of cells	Mean fluorescence intensity (AU)		
Untreated group (control)	0	-	100	4.38 ± 0.22	-	0.04
doxorubicin (alone)	94.28	77.60 ± 0.88 *	5.72	32.72 ± 0.24	-	0.90
resveratrol + doxorubicin	17.72	45.57 ± 1.26	82.28	19.78 ± 1.29 *	Significant $p<0.0001$	0.85
synephrine + doxorubicin	75.12	62.31 ± 0.83 *	24.88	50.67 ± 2.10	Significant $p<0.0001$	0.93
caffeine + doxorubicin	84.41	53.09 ± 0.50 *	15.59	22.81 ± 0.62	Significant $p<0.0001$	0.94

Asterisk (*): indicates the statistical comparisons of the mean fluorescence intensities in the high percentage of cells

In summary, of the three CAMs, resveratrol suppressed the doxorubicin induced expression of PgP protein after 24 hours whilst after a 48 hour period all the CAMs significantly suppressed the induced expression of PgP. The expression of PgP protein was confirmed to be localised in the nucleus in all combination treatments after both treatment periods.

3.6. The expression levels of HMOX1 in the MCF-7 cell line

HMOX1 is induced in cells during oxidative stress and protects cells from free radical damage (Gozzelino *et al.*, 2010). It was reported that HMOX1 expression levels are increased at early time periods after signal induction between 0 and 12 hours *in vivo* (Agarwal *et al.*, 1995; Yet *et al.*, 1997; Pellacani *et al.*, 1998). In this study, the effects of CAMs and doxorubicin on HMOX1 expression levels after 6 hours and 18 hours treatment were evaluated using the immunofluorescence technique as described in Chapter 2 section 2.7.2. The HMOX1 primary antibody was a rabbit HMOX1 polyclonal antibody (PA5-27338) prepared at 1:300 and a secondary antibody was a goat anti-rabbit polyclonal antibody (ab150077) conjugated with Alexa Fluor 488 prepared at 1:800 was used for protein detection. Cells grown on coverslips were treated with the CAMs and doxorubicin at concentrations equivalent to their IC_{50} values for 6 hours and 18 hours treatment periods, with the exception of resveratrol which was also evaluated at an IC_{80} value as it was the most potent CAM (Fig 3.14 and Fig 3.15). Thereafter, cells were treated with doxorubicin in combinations with CAMs at the concentrations indicated for 6 hours and 18 hours (Fig 3.16 and Fig 3.17). Combination treatments were performed to determine if the three CAMs affect the doxorubicin induced HMOX1 expression levels. The photomicrographs were captured at the same exposure times and processed with consistent settings using the Olympus CellSens software package. Fluorescence intensity was measured using Image J software and the results are summarised in Tables 3.13 to 3.16. The Students' t-test was used to evaluate if the differences in HMOX1mean fluorescence intensities of the treated cells were significant. Localization of HMOX1 in the nucleus was analysed using the signal from the HO stain and the HMOX1 by the co-localisation feature of Olympus CellSens software. The selected cells for analysis are shown in Appendix I and Appendix J. Results are expressed as a Pearson's Correlation Coefficient in Tables 3.13 to 3.16.

3.6.1 The expression levels of HMOX1 after treatment with doxorubicin and the CAMs for 6 hours

The effects of doxorubicin, resveratrol, synephrine and caffeine on the expression levels of HMOX1 in the MCF-7 cells after 6 hours treatment period is shown in Fig 3.14 and Table 3.12. The untreated cells showed normal cell morphology, with oval shaped

nuclei stained blue (Fig 3.14 panel b). HMOX1 expression level appears located in the nucleus in Fig 3.14 panel c.

The expression of HMOX1 appear to be increased in cells treated with doxorubicin and occurs mainly in the nucleus (Fig 3.14 panels g and h). A few cells appear to have HMOX1 predominantly localised to the cytoplasm in Fig 3.14 panels g and h. Statistical analysis indicate that doxorubicin significantly increased the HMOX1 expression levels in cells after 6 hours treatment when compared to untreated control (Table 3.12). The small percentage of cells (~10.95%) that appeared to have HMOX1 expression predominantly in the cytoplasm as confirmed by a weak Pearson's Correlation (0.35) between HMOX1 and HO signals. The majority of the cells (~89.05%) had a high Pearson's Correlation (0.92) between HMOX1 and HO signals confirming that HMOX1 occurred mainly in the nucleus.

In treatments with resveratrol at 157 µM and 181.27 µM, expression of HMOX1 appear to be increased shown by a stronger fluorescence signal and appear to be localised in nucleus (Fig 3.14 panels k, l, o, and p). Resveratrol significantly increased the HMOX1 expression levels after 6 hour treatment with 157 µM and 181.27 µM concentrations when compared to the untreated cells (Table 3.12). A few cells appear to have round condensed, bright blue nuclei with surrounding cell nuclei appearing c-shaped and deformed as indicated by the HO stain (Fig 3.14 panels j and n). Similar to doxorubicin, a small number of cells appear to have HMOX1 found mainly in the cytoplasm after treatments with resveratrol in Fig 3.14 panels k, l, o and p. A small percentage of cells in resveratrol treatment with 157 µM (~7.51%) and with 181.27 µM (~7.08%) appeared to have HMOX1 expression predominantly in the cytoplasm since the Pearson's Correlation was negligible (0.16 and 0.08, respectively) between HMOX1 and HO signals indicating HMOX1 was not found in the nucleus. Resveratrol caused the majority of the cells (~92%) treated at 157 µM and 181.27 µM to have HMOX1 expression the nucleus confirmed by a strong Pearson's Correlation (0.89 and 0.83, respectively) between the HMOX1 and HO signals.

In the cells treated with synephrine, HMOX1 expression levels appear to be increased and remained localised mainly in the nucleus (Fig 3.14 panels s and t). A few cells appear to have HMOX1 localised mainly in the cytoplasm after treatment with

synephrine in Fig 3.14 panels s and t. Synephrine significantly increased the HMOX1 expression levels in comparison to the untreated cells (Table 3.12). The small percentage of cells (~14.54%) showed HMOX1 expression occurred in the cytoplasm as confirmed by a negligible Pearson's Correlation (0.14) between HMOX1 and HO signals. The remainder of the cells (~85.46%) have HMOX1 expression localised mainly in the nucleus confirmed by a high Pearson's Correlation (0.94) between HMOX1 and HO signals.

The expression of HMOX1 appear to be decreased in cells treated with caffeine since poor green fluorescence signal is observed as shown in Fig 3.14 panel x. Caffeine significantly suppressed the HMOX1 expression levels in cells after 6 hours treatment in comparison to the untreated cells i.e., mean fluorescence intensities are 17.76 ± 0.50 AU and 21.98 ± 0.38 AU, respectively (Table 3.12). A few cells appear to have bright blue, condensed nuclei while the nuclei of neighbouring cells appeared deformed, hook-shaped in the HO stain shown in Fig 3.14 panel v. Similar to all the treatments, a small percentage of cells (~20.92%) showed that HMOX1 expression did not localise in the nucleus as confirmed by a negligible Pearson's Correlation (0.22) between HMOX1 and HO fluorescence signals. The majority of the cells (~79.08%) showed a strong Pearson's Correlation (0.94) between HMOX1 and HO confirming HMOX1 was predominantly found in the nucleus.

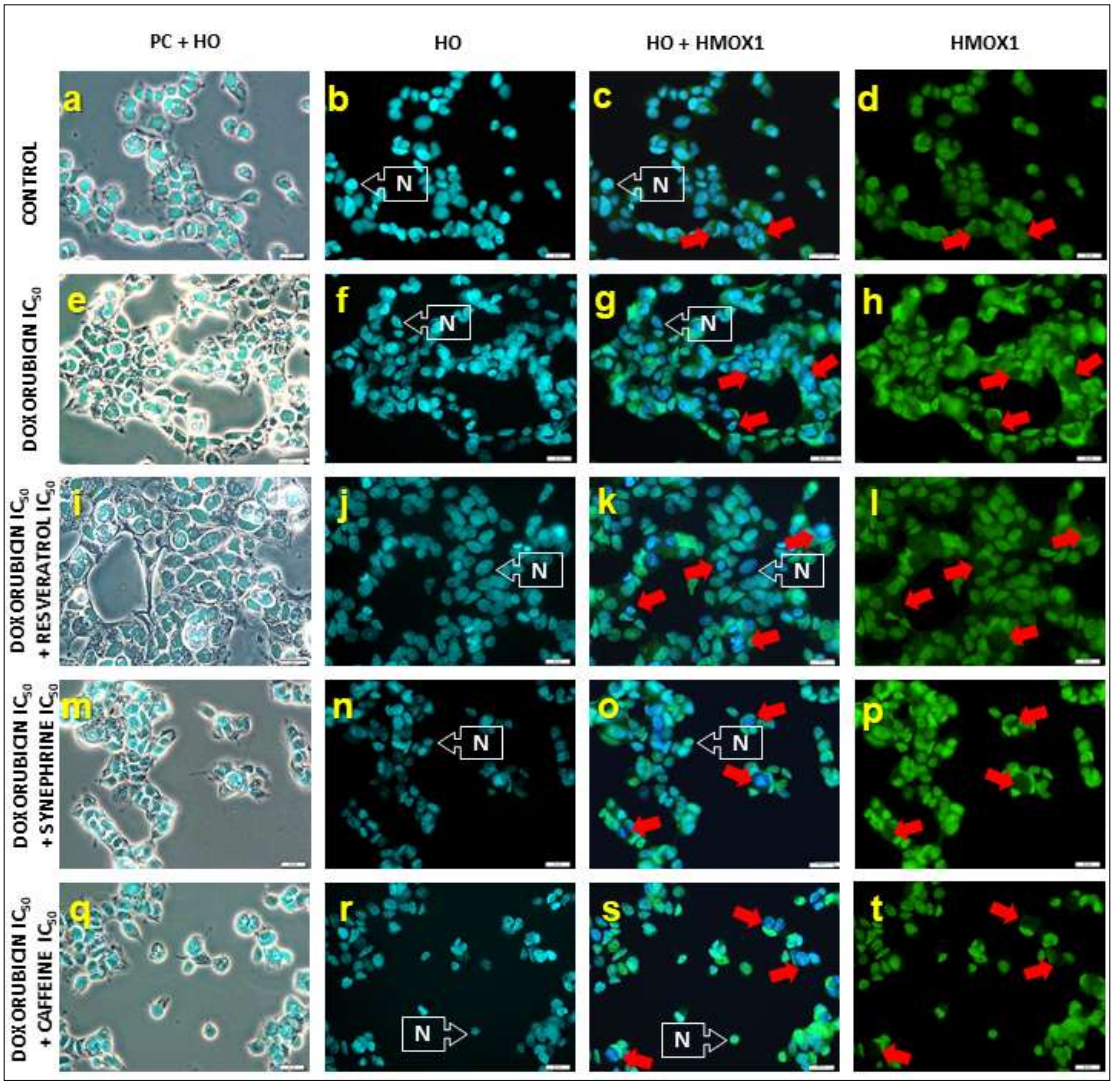


Figure 3.14 HMOX1 protein expression levels in MCF-7 cells following treatment with doxorubicin, resveratrol, synephrine and caffeine after 6 hours. Cells were treated with doxorubicin at 1.12 µM (e-h), synephrine at 423 µM (q-t) and caffeine at 354 µM (u-x) for 6 hours. Cells were treated with resveratrol at concentrations of 157 µM (i-l), and 181.27 µM (m-p). The cells were viewed using an U-MNIB3 filter (Excitation: 480/20, Emission: 510/50) for HMOX1 expression detection by Alexa Fluor 488 and an U-MWU2 filter (Excitation: 365/10, Emission: 420 long pass) for the cells nuclei counterstained using HO. Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). Photomicrographs were captured using an Olympus BX41 microscope fitted with a DP72 digital camera and analysed using Olympus CellSens software (Magnification: 400× and Scale bar: 20 µm). Legend: N = nucleus, and "Arrows" = cells with HMOX1 predominantly in the cytoplasm

Table 3.12 HMOX1 mean fluorescence intensities and localisation in MCF-7 cells treated with doxorubicin, resveratrol, synephrine and caffeine for 6 hours. Student t-test followed by Welch's correction test was used to determine if differences between treatments and untreated control cells were significant.

Test variable	Mean fluorescence intensity (AU)	Significance p<0.05	Cells expressing HMOX1 in the nucleus		Cells expressing HMOX1 in the cytoplasm	
			Fraction of cells (%)	PCC	Fraction of cells (%)	PCC
Untreated group (control)	21.98 ± 0.38	-	91.31	0.92	8.69	0.39
doxorubicin (1.12 μM)	43.44 ± 1.42	Significant p<0.0001	89.05	0.92	10.95	0.35
resveratrol (157 μM)	26.40 ± 0.37	Significant p<0.0001	92.49	0.89	7.51	0.16
resveratrol (181.27 μM)	28.28 ± 0.76	Significant p=0.0001	92.92	0.83	7.08	0.08
synephrine (423 μM)	26.87 ± 0.51	Significant p<0.0001	85.46	0.94	14.54	0.14
caffeine (354 μM)	17.76 ± 0.50	Significant p<0.0001	79.08	0.84	20.92	0.22

3.6.2 The expression levels of HMOX1 after treatment with doxorubicin and the CAMs for 18 hours

The effects of doxorubicin, resveratrol, synephrine and caffeine on the expression levels of HMOX1 in the MCF-7 cells after 18 hours treatment period is shown in Fig 3.15 and Table 3.13. After 18 hours, the untreated cells showed normal cell morphology with oval nuclei stained blue in Fig 3.15 panel a and b. Similar to 6 hours period, the expression levels of HMOX1 appear localised in the nucleus of the untreated cells Fig 3.15 panel c and d.

Similar to the 6 hour treatment period, doxorubicin strongly induced the expression of HMOX1 and appears to be found mainly in the nucleus (Fig 3.15 panels g and h) and the HMOX1 fluorescence intensity was significantly increased in comparison to the untreated cells (Table 3.13). A few cells appear to have HMOX1 localised predominantly in the cytoplasm shown in Fig 3.15 panels g and h. The small percentage of cells (~10.07%) showed that HMOX1 expression may have occurred in the nucleus as confirmed by a moderate Pearson's Correlation (0.56) between the HMOX1 and HO signals indicating HMOX1 was found in the nucleus while the remainder of the cells (~89.93%) had a high Pearson's Correlation (0.92) indicating HMOX1 mainly occurred in the nucleus.

Resveratrol treatments at 157 μM and 181.27 μM showed no change in the baseline expression of HMOX1 which appeared to be localised in the nucleus (Fig 3.15 panels k, l, o, and p). This was confirmed by Image J analysis showing resveratrol had no significant effects on the HMOX1 fluorescence intensities after 18h treatment when cells were exposed to both 157 μM and 181.27 μM. (Table 3.13).

This indicates resveratrol had no effect on HMOX1 expression levels at 18 hours. Further evaluation of resveratrol treatment showed a small percentage of cells at 157 μM (~16.08 %) and at 181.27 μM (~15.49 %) had a negligible to low Pearson's correlation (-0.15 and 0.34, respectively) between HMOX1 and HO signals indicating HMOX1 did not localise in the nucleus of these cells. The remainder of the cells (80%) treated with resveratrol at 157 μM and at 181.27 μM had a strong Pearson's Correlation (0.90 and 0.87, respectively) between the HMOX1 and HO signals confirming HMOX1 occurred mainly in the nucleus.

Similar to the 6 hours treatment period, cells treated with synephrine appear to have increased expression of HMOX1 and remained localised in the nucleus after 18 hours treatment period (Fig 3.15 panel t). Synephrine significantly increased the HMOX1 fluorescence intensity when compared to the untreated cells (Table 3.13). The small percentage of cells (~14.54%) show HMOX1 expression did not localise in the nucleus as confirmed by a negative and low Pearson's Correlation (-0.41) between HMOX1 and HO signals in cells treated with synephrine. The majority of the cells (~85.46%) have a high Pearson's Correlation (0.91) between the HMOX1 and HO signals indicating that HMOX1 occurred mainly in the nucleus.

The expression of HMOX1 appear to be increased in cells treated with caffeine was found in the nucleus in Fig 3.15 panels w and x. Caffeine significantly increased the HMOX1 expression levels in cells after 18 hours treatment in comparison to the untreated cells (Table 3.13). Similar to treatments with doxorubicin, a small percentage of cells (~10.07%) show HMOX1 expression did not localise in the nucleus as confirmed by a negligible Pearson's Correlation Coefficient (0.29) in colocalisation analysis. The majority of the cells (~89.93%) have HMOX1 expression localised in the nucleus confirmed by a strong Pearson's Correlation (0.94) between the HMOX1 and HO signals.

In summary, doxorubicin was the strongest inducer of HMOX1 expression after both treatment periods. Resveratrol treated at 157 μM and 181.27 μM significantly increased HMOX1 expression levels after 6 hours while both concentrations of resveratrol had no effects after 18 hours. Synephrine treated at 423 μM caused a small yet significant induction of HMOX1 expression levels after both treatment periods. Caffeine treated at 354 μM caused a small, yet significant decrease in the expression levels of HMOX1 after 6 hours while at after 18 hours treatment a small increase in HMOX1 expression levels was observed. Further evaluation showed a small percentage of cells appeared to have HMOX1 expression predominantly in the cytoplasm as confirmed by weak Pearson's Correlation between HMOX1 and HO signals indicating HMOX1 did not occur in the nucleus. These cells showed a meaningful increase in number after 6 hour treatment with synephrine and caffeine while after 18 hour treatment synephrine and resveratrol caused an increase.

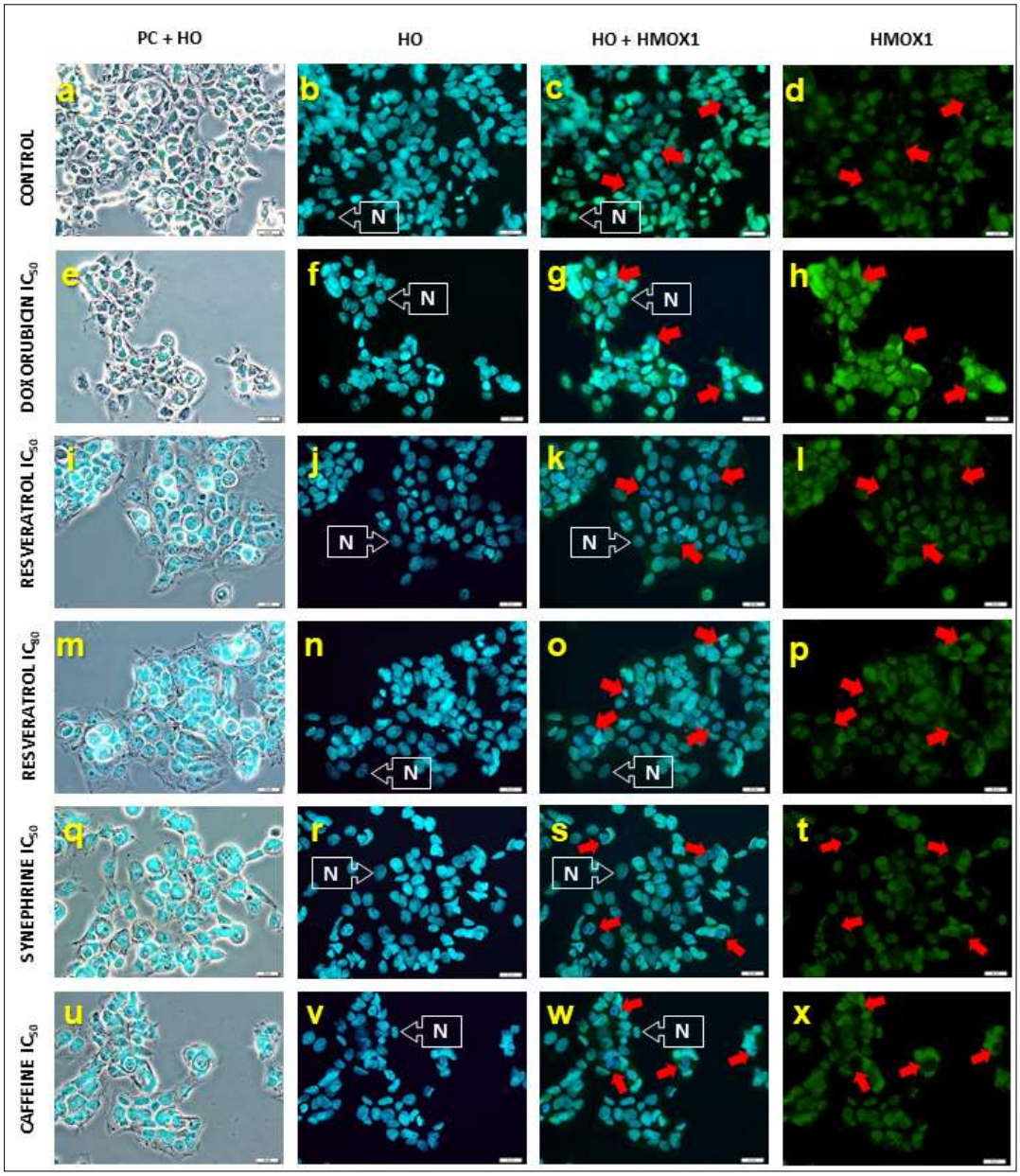


Figure 3.15 HMOX1 protein expression levels in MCF-7 cells following treatment with doxorubicin, resveratrol, synephrine and caffeine after 18 hours. Cells were treated with doxorubicin at 1.12 µM (e-h), synephrine at 423 µM (q-t) and caffeine at 354 µM (u-x) for 18 hours. Cells were treated with resveratrol at concentrations of 157 µM (i-l), and 181.27 µM (m-p). The cells were viewed using an U-MNIB3 filter (Excitation: 480/20, Emission: 510/50) for HMOX1 expression detection by Alexa Fluor 488 and an U-MWU2 filter (Excitation: 365/10, Emission: 420 long pass) for the nucleus counterstained using HO. Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). Photomicrographs were captured using an Olympus BX41 microscope fitted with a DP72 digital camera and analysed using Olympus CellSens software (Magnification: 400× and Scale bar: 20 µm). Legend: N = nucleus, and “Arrows” = cells with HMOX1 predominantly in the cytoplasm

Table 3.13 HMOX1 mean fluorescence intensities and localisation in MCF-7 cells treated with doxorubicin, resveratrol, synephrine and caffeine for 18 hours. Student t-test followed by Welch's correction test was used to determine if differences between treatments and untreated control cells were significant.

Test variable	Mean fluorescence intensity (AU)	Significance p<0.05	Cells expressing HMOX1 in the nucleus		Cells expressing HMOX1 in the cytoplasm	
			Fraction of cells (%)	PCC	Fraction of cells (%)	PCC
Untreated group (control)	18.32 ± 0.16	-	90.80	0.89	9.2	0.71
doxorubicin (1.12 μM)	42.09 ± 0.77	Significant p<0.0001	89.93	0.92	10.07	0.56
resveratrol (157 μM)	18.33 ± 0.38	Non-significant p=0.98	83.92	0.90	16.08	-0.15
resveratrol (181.27 μM)	19.97 ± 0.87	Non-significant p=0.12	84.51	0.87	15.49	0.34
synephrine (423 μM)	20.95 ± 0.62	Significant p=0.0092	85.46	0.91	14.54	-0.41
Caffeine (354 μM)	23.66 ± 0.73	Significant p=0.0008	89.93	0.94	10.07	0.29

3.6.3 The doxorubicin induced HMOX1 expression levels after treatment with CAMs in combination for 6 hours

The effects of resveratrol, synephrine and caffeine on the doxorubicin induced expression levels of HMOX1 in the MCF-7 cells after 6 hours are shown in Fig 3.16. The mean fluorescence intensities and colocalisation analysis data is summarised in

Table 3.14. Doxorubicin strongly induced the expression of HMOX1 and appear to be found predominantly in the nucleus (Fig 3.16 panels g and h). Statistical analysis indicate that doxorubicin caused a meaningful increase in the HMOX1 expression levels in cells after 6 hours (Table 3.14).

The expression of HMOX1 appear to be decreased in cells treated with resveratrol combined with doxorubicin since poor green fluorescence signal is observed and remained localised in the nucleus as shown in (Fig 3.16 panels k and l). In combination treatments, resveratrol significantly decreased the HMOX1 fluorescence intensity in cells when compared to doxorubicin alone i.e., statistical meaningful differences in the mean fluorescence intensities of 31.63 ± 0.56 AU and 43.44 ± 1.42 AU (Table 3.14). This indicates that resveratrol is able to decrease doxorubicin induced expression levels of HMOX1 after 6 hours treatment. In treatment with resveratrol, the percentage of cells (~11.30%) that appear to have HMOX1 expression predominantly in the cytoplasm was marginally increased compared to doxorubicin alone and a negligible Pearson's Correlation (0.17) between HMOX1 and HO signals confirmed that HMOX1 expression did not occur in the nucleus (Table 3.14). Therefore, the high percentage of the cells (~88.70%) appeared to decrease and had a strong Pearson's Correlation (0.89) between HMOX1 and HO signal confirming HMOX1 occurred mainly in the nucleus.

Cells treated with synephrine combined with doxorubicin showed a similar HMOX1 intensity fluorescent staining to doxorubicin alone and appear to be found in the nucleus (Fig 3.16 panels o and p). A few cells appear to have HMOX1 predominantly localised to the cytoplasm in Fig 3.16 panels o and p. In measurement of fluorescence intensity with Image J analysis, there were no statistically meaningful differences in the fluorescence intensity of cells when compared to doxorubicin alone (Table 3.14).

Therefore, an increased percentage of cells (~94.14%) appeared to have HMOX1 in the nucleus and this was confirmed by a strong Pearson's Correlation (0.89) between HMOX1and HO signals. This indicates that synephrine did not affect the doxorubicin induced expression of HMOX1 protein after a 6 hour treatment period. In treatment with synephrine, the small percentage of cells (~5.86%) that appeared to have HMOX1 expression predominantly in the cytoplasm have decreased and had a negligible

Pearson's Correlation (0.09) between the HMOX1 and HO signals indicating HMOX1 was not localised in the nucleus.

Evaluation of the cells treated with doxorubicin and caffeine in combination showed a decreased HMOX1 fluorescence intensity compared to that of doxorubicin alone while HMOX1 appear to remain in the nucleus of the majority of the cells (Figure 3.16 panels s and t). Image J analysis of fluorescence intensity showed a small and significant decrease in the HMOX1 intensity compared to the cells treated with doxorubicin alone i.e., meaningful difference between fluorescence intensities of 37.71 ± 0.61 AU and 43.44 ± 1.42 AU (Table 3.14). This indicates that caffeine was able to decrease the doxorubicin induced expression of HMOX1 during combination treatment. Similar to doxorubicin, a small percentage of cells (~10.65%) showed HMOX1 expression predominantly in the cytoplasm as confirmed by a poor Pearson's Correlation (0.09) between the HMOX1 and HO signals indicating HMOX1 was not located in the nucleus. The majority of the cells (~89.35%) had a strong Pearson's Correlation (0.76) between the HMOX1 and HO signals confirming that HMOX1 is mainly located in the nucleus.

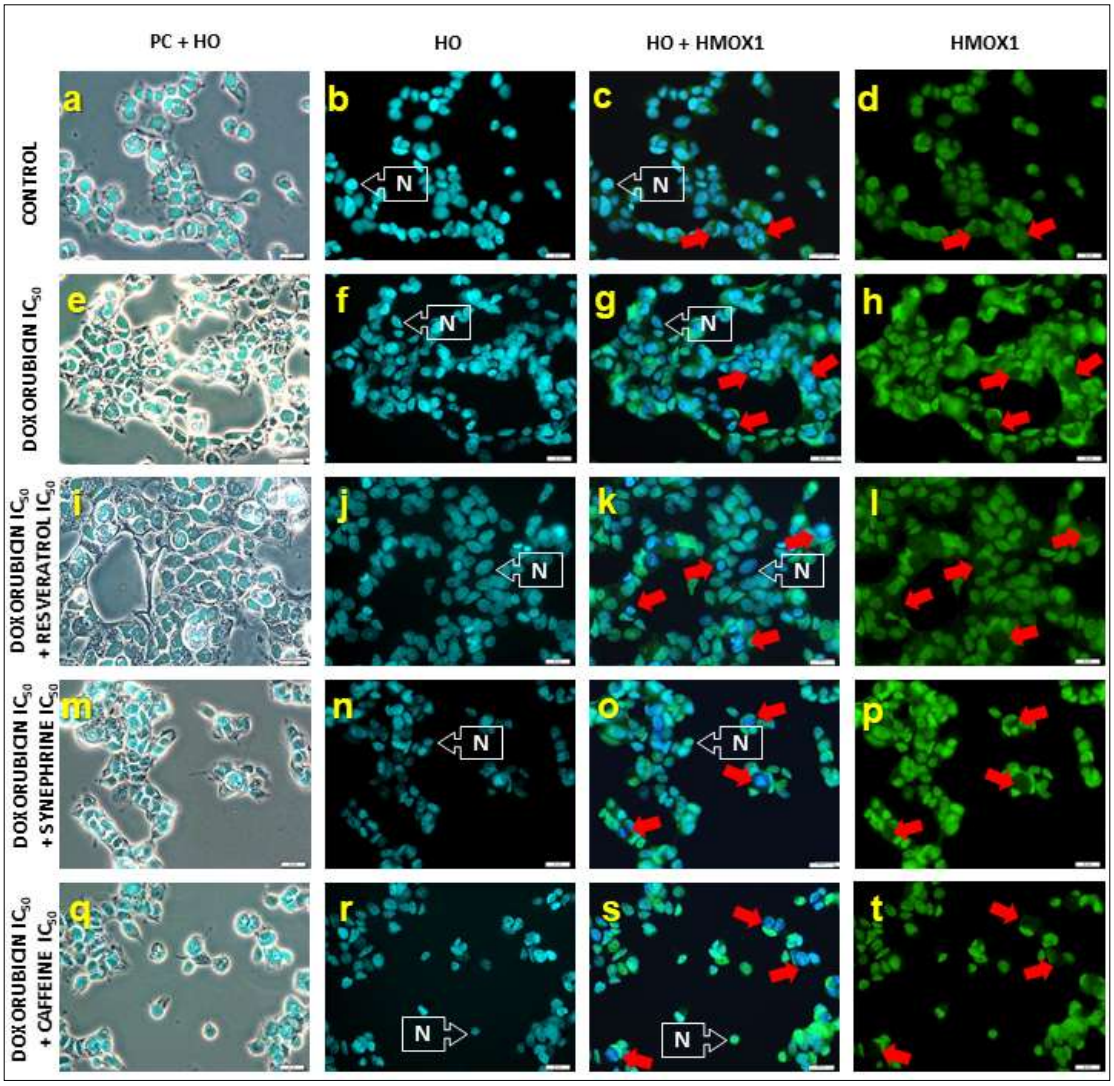


Figure 3.16 The effects of doxorubicin and in combinations with CAMs on HMOX1 expression levels in MCF-7 cells after 6 hours. The MCF-7 cells were treated with doxorubicin at 1.12 µM (d-f) alone and in combinations with resveratrol at 157 µM (g-i), synephrine at 423 µM (j-l) and caffeine at 354 µM (m-o) for 6 hours. The cells were viewed using a U-MNIB3 filter (Excitation: 480/20, Emission: 510/50) for HMOX1 expression detection by Alexa Fluor 488 and an U-MWU2 filter (Excitation: 365/10, Emission: 420 long pass) for the nucleus counterstained using HO. Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). Photomicrographs were captured using an Olympus BX41 microscope fitted with a DP72 digital camera and analysed using Olympus CellSens software (Magnification: 400× and Scale bar: 20 µm). Legend: N = nucleus, and “Arrows” = cells with HMOX1 predominantly in the cytoplasm

Table 3.14 HMOX1 mean fluorescence intensities and localisation in MCF-7 cells treated with doxorubicin and in combinations with the CAMs for 6 hours. Student t-test followed by Welch's correction test was used to determine if differences between combination treatments and doxorubicin alone were significant.

Test variable	Mean fluorescence intensity (AU)	Significance p<0.05	Cells expressing HMOX1 in the cytoplasm and nucleus		Cells expressing HMOX1 in the cytoplasm	
			Fraction of cells (%)	PCC	Fraction of cells (%)	PCC
Untreated group (control)	21.98 ± 0.38	-	91.31	0.92	8.69	0.39
doxorubicin (alone)	43.44 ± 1.42	-	89.05	0.92	10.95	0.35
resveratrol + doxorubicin	31.63 ± 0.56	Significant p=0.0002	88.70	0.89	11.30	0.17
synephrine + doxorubicin	44.27 ± 1.12	Non-significant p=0.65	94.14	0.89	5.86	0.09
caffeine + doxorubicin	37.71 ± 0.61	Significant p=0.0099	89.35	0.76	10.65	0.20

3.6.4 The doxorubicin induced HMOX1 expression levels after treatment with CAMs in combination for 18 hours

The effects of resveratrol, synephrine and caffeine on the doxorubicin induced expression levels of HMOX1 in the MCF-7 cells after 18 hours are shown in Fig 3.17. The HMOX1 mean fluorescence intensities and colocalisation analysis data is

summarised in Table 3.15. The expression of HMOX1 protein appears to have remained localised to the nuclei in all the treatments (Fig 3.17 panels h, l, p, and t). This was confirmed by colocalisation analysis of HMOX1 and HO signals which showed a strong Pearson's correlation (0.82-0.96) in all treatments indicating HMOX1 occurred mainly in the nucleus (Table 3.15). Doxorubicin treated cells appeared smaller in size than the untreated control cells but showed a high fluorescence intense HMOX1 signal Fig 3.17 panels e and h).

Cells treated with doxorubicin combined with resveratrol appeared to have dramatically decreased in HMOX1 expression levels similar to the untreated control cells when compared to doxorubicin alone and appeared to be found in the nucleus (Fig 3.17 panels k and l). Image J analysis showed that resveratrol significantly decreased the HMOX1 fluorescence intensity when compared to doxorubicin alone and close to that of the untreated control i.e., differences in the mean fluorescence intensities of 18.16 ± 0.34 AU and 42.09 ± 0.77 AU were statistically meaningful as shown in Table 3.15.

This indicates that resveratrol was able to inhibit the doxorubicin induced expression levels of HMOX1 after 18 hours treatment. A small percentage of cells (~11.30%) appear to have HMOX1 expression predominantly in the cytoplasm and was confirmed by a negligible Pearson's Correlation (0.36) between HMOX1 and HO signals. The remainder of the cells (~88.70%) had a strong Pearson's Correlation (0.82) between HMOX1 and HO signals indicating HMOX1 occurred mainly in the nucleus.

In treatment of synephrine combined with doxorubicin, the expression of HMOX1 appeared to be decreased in cells and remained localised in the nucleus (Fig 3.17 panels o and p). Synephrine caused a significant decrease in the HMOX1 fluorescence intensity when compared to doxorubicin alone albeit a smaller decrease than that of resveratrol combinations (Table 3.15). Synephrine was able to significantly decrease the HMOX1 expression in cells treated with doxorubicin for 18 hours. Further evaluation of slides showed a small percentage of cells (~11.02%) appeared to have HMOX1 expression predominantly in the cytoplasm and was confirmed by a weak Pearson's Correlation (0.31) between the HMOX1 and HO signals. The majority of the cells (~88.98%) appeared to have HMOX1 expression in the nucleus and as confirmed

by a high Pearson's Correlation (0.96) between the HMOX1 and HO signals indicating HMOX 1 localised mainly in the nucleus.

Similar to synephrine, cells treated with doxorubicin combined with caffeine appeared to have a decreased HMOX1 fluorescence signal which appeared localised in the nucleus after an 18 hour treatment as shown in Fig 3.17 panels s and t. Image J analysis showed a significant decrease in HMOX1 fluorescence intensity when cells were treated with combinations of caffeine with doxorubicin (Table 3.15). This indicates caffeine was able to decrease the doxorubicin induced expression levels of HMOX1 after 18 hours treatment. There was an increase in the percentage of cells that predominantly expressed HMOX1 in the cytoplasm (~15.79%) which was confirmed by a weak Pearson's Correlation (0.36) between HMOX1 and HO signals. This indicated that HMOX1 was not only located in the nucleus. The percentage of the cells that appeared to have HMOX1 expression in the nucleus (~84.21%) was decreased when compared to doxorubicin alone where a Pearson's Correlation (0.91) between HMOX1 and HO signals confirmed that HMOX1 was mainly located mainly in the nucleus.

In summary, resveratrol and caffeine decreased the doxorubicin induced expression levels of HMOX1 after treatment for 6 hours while after 18 hours the induced expression of HMOX1 was decreased by all of the CAMs when combined with doxorubicin. Out of the three CAMs combined with doxorubicin, resveratrol showed the greatest effect since the HMOX1 fluorescence intensity of these treated cells was the lowest of all the CAMs treatments. A small percentage of cells in combination treatments of doxorubicin and the CAMs showed HMOX1 expression predominantly in the cytoplasm. However, in the remainder of cells a high percentage HMOX1 was predominantly found in the nucleus. The cells with HMOX1 expression predominantly in the cytoplasm was decreased in combination treatment with synephrine (~6%) after 6 hours compared to doxorubicin alone while after 18 hours combination with caffeine (~16%) caused an increase.

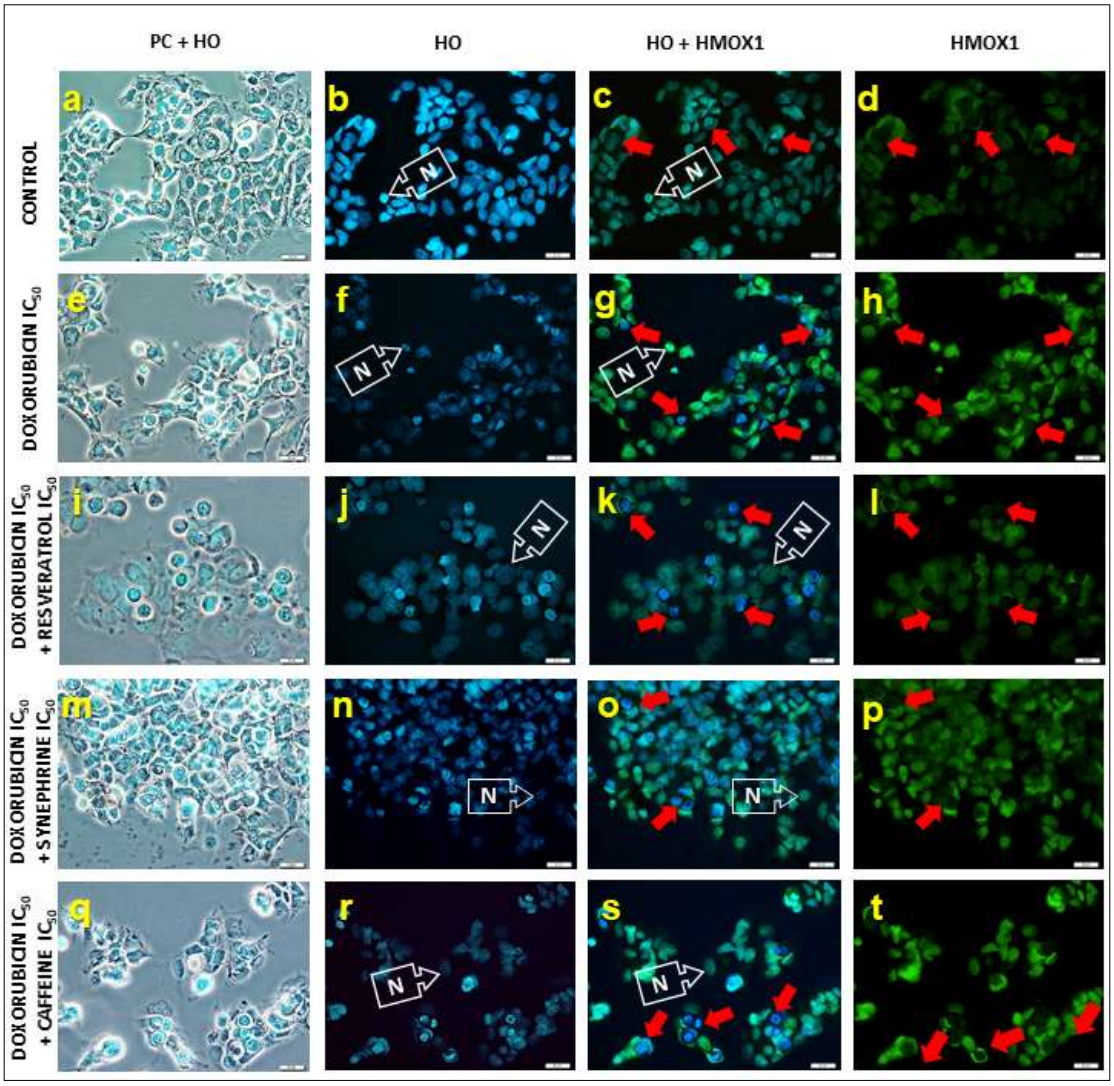


Figure 3.17 The effects of doxorubicin and in combinations with CAMs on HMOX1 expression levels in MCF-7 cells after 18 hours. The MCF-7 cells were treated with doxorubicin at 1.12 µM (d-f) alone and in combinations with resveratrol at 157 µM (g-i), synephrine at 423 µM (j-l) and caffeine at 354 µM (m-o) for 18 hours. The cells were viewed using a U-MNIB3 filter (Excitation: 480/20, Emission: 510/50) for HMOX1 expression detection by Alexa Fluor 488 and a U-MWU2 filter (Excitation: 365/10, Emission: 420 long pass) for the nucleus counterstained using HO. Each photomicrograph is representative of at least four microscopic fields and three independent experiments (n=3). Photomicrographs were captured using an Olympus BX41 microscope fitted with a DP72 digital camera and analysed using Olympus CellSens software (Magnification: 400× and Scale bar: 20 µm). Legend: N = nucleus, and “Arrows” = cells with HMOX1 predominantly in the cytoplasm

Table 3.15 HMOX1 mean fluorescence intensities and localisation in MCF-7 cells treated with doxorubicin and in combinations with the CAMs for 18 hours. Student t-test followed by Welch's correction test was used to determine if differences between combination treatments and doxorubicin alone were significant.

Test variable	Mean fluorescence intensity (AU)	Significance p<0.05	Cells expressing HMOX1 in the nucleus		Cells expressing HMOX1 in the cytoplasm	
			Fraction of cells (%)	PCC	Fraction of cells (%)	PCC
Untreated group (control)	18.32 ± 0.16	-	90.8	0.89	9.20	0.71
doxorubicin (alone)	42.09 ± 0.77	-	89.03	0.92	10.07	0.56
resveratrol + doxorubicin	18.16 ± 0.34	Significant p<0.0001	88.70	0.82	11.30	0.36
synephrine + doxorubicin	32.60 ± 0.77	Significant p<0.0001	88.98	0.96	11.02	0.31
caffeine + doxorubicin	32.75 ± 0.75	Significant p<0.0001	84.21	0.91	15.79	0.36

CHAPTER 4: DISCUSSION

Complementary and alternative medicine include practices that are not typically part of conventional medical care and includes dietary supplements which contain chemical compounds and/or isolated constituents from herbal materials (World Health Organisation, 2004; Devine and Hayes, 2016). A report by Zion Market Research (2017) stated that the global dietary supplements market was value at ZAR1.86 trillion and was expected to reach ZAR3.08 trillion rand in year of 2022. In addition, the dietary supplement market in South Africa was valued at ZAR3.8 billion in 2017, recording an increase of R0.9 billion since 2014 (Zion Market Research, 2017). The use of CAMs has steadily increased, particularly among patients diagnosed with cancer (Johnson *et al.*, 2018).

Cancer patients are known to use CAMs while undergoing chemotherapy and radiation therapy (Wanchai *et al.*, 2010; Tautz *et al.*, 2012; Sweet *et al.*, 2016; Drozdoff *et al.*, 2019). Cancer patients seek out CAMs in an attempt to alleviate adverse effects associated with cancer treatment or in hope of achieving a possible increased therapeutic effect or a miracle cure (Spadacio and Barros, 2008; Lopes *et al.*, 2017; Drozdoff *et al.*, 2018). Patients use alternative treatments without the knowledge of their treating physician or oncologist (Wang *et al.*, 2015; Sweet *et al.*, 2016)

Research interest of CAMs increased in the past two decades and numerous reports are found in the scientific literature indicating their potential usefulness as either preventative and/or therapeutic agents (Harvie, 2014; Dariya *et al.*, 2019; Knecht *et al.*, 2020; Tinoush *et al.*, 2020). Robust scientific data supporting anecdotal evidence regarding the efficacy of most CAMs remain elusive. Importantly, there is limited data available regarding the potential harmful effects CAMs may have and their potential to interfere with conventional cancer therapy is overlooked (Yap *et al.*, 2010; Devine and Hayes, 2016; Buckner *et al.*, 2018). and the potential interactions of select CAMs on the efficacy of doxorubicin in the MCF-7 cell line.

4.1 Cytotoxicity of chemotherapeutic drugs and the CAMs towards MCF-7 cell line

Breast cancer is treated with various classes of cancer drugs which include anthracyclines, alkylating agents, antimetabolites, small molecule inhibitors and monoclonal antibodies (Wellstein *et al.*, 2018). Three anticancer drugs (doxorubicin, gemcitabine, and irinotecan) were selected as potential positive controls for this study. The CAMs were selected after consulting the Drug, Herbs and Supplements section adapted from the Natural Medicines Comprehensive Database linked to the MedlinePlus online library (https://medlineplus.gov/druginfo/herb_All.htmL). They were resveratrol, synephrine, caffeine, amygdalin, and commercially available preparations of *S. frutescens.* As reported in Section 3.1.2, doxorubicin was the most active chemotherapeutic drug against MCF-7 cells with an IC_{50} value in the low micromolar range and therefore selected for further studies. It is also a cornerstone of breast cancer treatment regimens. When the four CAMs were initially evaluated for their ability to inhibit cell growth, amygdalin showed no efficacy at both concentrations of 5 µM and 50 µM. The three CAMs: resveratrol, synephrine and caffeine showed a decreased change in cell viability between concentrations of 5 µM and 50 µM. Therefore, the three CAMs were selected for further experiments and no further work was performed on amygdalin. The two formulations of *S. frutescens*: a prepared hot water extract and a purchased ethanolic extract, were included in this study to evaluate the effects on cell viability. The hot water extract showed no activity while the ethanolic extract was poorly active. Apart from cell morphology evaluation, *S. frutescens* extracts were not included in further experiments.

4.1.1 Doxorubicin

Doxorubicin was the most active and therefore was selected as the positive control. Doxorubicin of the anthracycline drug class, is widely used against a wide range of cancers, such as haematological malignancies, lung cancer, and breast cancer (Yang *et al.*, 2014; Wellstein *et al.*, 2018). Doxorubicin directly intercalates with the double-strand DNA and inhibits the DNA topoisomerase II enzyme (Tacar *et al.*, 2013; Wellstein *et al.*, 2018). Doxorubicin was active in the MCF-7 cell line with low IC_{50} values of 1.12 µM and 0.75 µM after 24 hours and 48 hours, respectively. A study by Tomankova *et al.* (2015) reported a doxorubicin IC_{50} value of 1.2 µM in MCF-7 cells

and Chen *et al.* (2016) reported an IC_{50} value of 1.1 μM after 24 hour treatment using the MTT assay. These findings are consistent with the effects of doxorubicin seen in this study after the same treatment period. Studies assessing the effect of doxorubicin in the MCF-7 cell line after 48 hours have reported cytotoxicity IC_{50} values of approximately 0.2 μM (Chen *et al.*, 2016; Shafiei-Irannejad *et al.*, 2018). In contrast to these findings, Long *et al.* (2015) reported a higher IC_{50} value of doxorubicin at 0.71 μM in the MCF-7 cell line after 48 hour treatment . The findings stand in agreement with the results of this study, as a similar IC_{50} value of doxorubicin after 48 hours was found.

In clinical treatment of cancer patients, doxorubicin is administered via an intravenous infusion and a peak plasma concentration of 6.7 μM has been documented (Liston and Davis, 2017). The IC_{50} values of doxorubicin after 24 hours and 48 hours reported in this study are far below the clinical C_{max}, thus the *in vitro* cytotoxicity of doxorubicin is within the range of concentrations for specific-toxicity where off-target toxicity is limited (Liston and Davis, 2017). The common doxorubicin adverse effects include stomatitis, mucositis, diarrhoea, alopecia, myelosuppression and cardiomyopathy (Prathumsap *et al.*, 2020). Cardiotoxicity associated with doxorubicin by the increased ROS production and signalling pathways via death receptors like TNF receptor 1 mediate apoptosis in cardiomyocytes (Zhao and Zhang, 2017).

Doxorubicin cardiotoxicity may present in acute form which is characterized by abnormal electrocardiographic changes, including ST-wave segment alterations, arrhythmias, and acute myocardial damage due inflammation of external membrane of the heart (Wellstein *et al.*, 2018). Another form of doxorubicin associated cardiotoxicity is chronic, cumulative and dose-related which has long term persistence of myocardial damage and possible late-onset years after treatment with congestive heart failure (Wellstein *et al.*, 2018; Rawat *et al.*, 2021). This is of great concern in patients with predisposing cardiac risks such pre-existing cardiac disease, age >65 years, hypertension, hyperlipidaemia, radiation and concomitant therapy with trastuzumab and cyclophosphamide (Cardinale *et al.*, 2020).

4.1.2 Gemcitabine

Gemcitabine is a pyrimidine nucleoside analogue anticancer drug used in pancreatic and breast cancer treatment regimens (Wellstein *et al.*, 2018). In clinical application, gemcitabine has shown a peak plasma concentration of 89.30 μM in cancer patients (Liston and Davis, 2017). Gemcitabine showed poor cytotoxic activity in the current study with IC_{50} values at 155.5 μM and 137.4 μM after 24 hours and 48 hours treatment, respectively. Other studies reported IC_{50} values of gemcitabine below 20 μM (Robinson *et al.*, 2004; S. Wu *et al.*, 2014; Alkhatib and Al-Saedi, 2017). The poor cytotoxicity in the current study suggests gemcitabine-resistance in our MCF-7 cell line or a faulty gemcitabine preparation. Resistance to gemcitabine include decreased phosphorylation of the nucleotide by kinases, high expression of drug efflux pumps, the expression of inhibitory microRNAs, and faulty membrane bound nucleoside transporters (Damaraju *et al.*, 2003; Jordheim *et al.*, 2005; Jia and Xie, 2015). Any of these mechanisms can explain the high IC_{50} values of gemcitabine observed in the MCF-7 cell line used in this study. Since the data did not corelate with published values, gemcitabine was therefore not further used in the study.

4.1.3 Irinotecan

Irinotecan (CPT-11) is a chemotherapeutic drug used in the treatment of advanced colorectal tumours, and metastatic breast cancer. (Chabner *et al.*, 2011; Lan *et al.*, 2014). Irinotecan is administered as an intravenous infusion with a peak plasma concentration of 5.78 μM (Liston and Davis, 2017). First-pass metabolism of irinotecan after administration occurs in the liver via hepatic carboxyesterase-2 cleavage of the carbamate of CPT-11 to form the active metabolite SN-38 (Brunton *et al.*, 2011; Wellstein *et al.*, 2018). This active metabolite inhibits DNA ligation resulting in double-stranded DNA breaks and cellular death (Marsh and Hoskins, 2010; Jandu *et al.*, 2016). Irinotecan was poorly active against the MCF-7 cell line presumably because the cells are deficient in the activating enzymes (Keyvani-Ghamsari *et al.*, 2017; Cheng *et al.*, 2020).

4.1.4 Resveratrol

Resveratrol is a polyphenolic phytoalexin with numerous reported health benefits in the scientific and non-scientific literature (Cottart *et al.*, 2010; Rius *et al.*, 2010; Carter *et al.*, 2014; Peñalva *et al.*, 2018). The clinical importance of resveratrol remains elusive

with many conflicting reports found in the peer reviewed scientific literature (Salehi *et al.*, 2018). In this current study resveratrol showed poor cytotoxicity towards the MCF-7 breast cancer cell line with IC_{50} values of 149.2 µM and 80.5 µM after 24 hours and 48 hours, respectively. An *in vitro* study by Costa *et al.* (2021) reported an IC_{50} value of resveratrol at 238 µM in MCF-7 cells after 24 hours treatment period. Leon-Galicia *et al.* (2018) reported an IC_{50} value of resveratrol at 101.1 µM after 48 hours treatment in the MCF-7 cell line. Resveratrol is very sensitive to degradation by exposure to oxygen, light, temperature and oxidative enzymes which can account for the variation in IC_{50} values (Davidov-Pardo and McClements, 2015).

A study by Sergides *et al.* (2016) evaluated the pharmacokinetic properties and safety of Evelor tablets which contain trans-resveratrol. A 500 mg single oral dose was administered to healthy persons and a very low peak plasma concentration at 0.312 µM after 1.3 hours was recorded. However, resveratrol glucuronide and sulphate metabolite levels were much higher- 17.89 µM at 3.05 hours and 6.64 µM at 2.78 hours, respectively. This reported peak plasma concentration of resveratrol is much lower than the IC_{50} value of the present study after a 48 hour period, which indicates that resveratrol is unlikely to reach clinically relevant plasma concentrations. Given that cancer drugs are frequently used at IC_{99} concentrations clinically to achieve maximal cell kill, it is not likely that resveratrol will be a clinically useful cancer treatment. The reported adverse effects of resveratrol includes nausea, diarrhoea, fatigue, and renal toxicity (Salehi *et al.*, 2018; Shaito *et al.*, 2020). In addition, the use of resveratrol is hampered by its low bioavailability (Walle, 2011). Poor oral bioavailability of resveratrol is due to rapid and extensive metabolism in the intestine and liver, as well as metabolism by colonic bacteria, leading to low resveratrol plasma concentrations (Muñoz *et al.*, 2015).

4.1.5 Synephrine

p-Synephrine is the most active substance in *Citrus aurantium* and other *Citrus* species, extracted from the peel of unripe fruits of the plant (Stohs, 2017; Suntar *et al.*, 2018). Synephrine is mainly used as a weight loss supplement and is likely to be consumed by breast cancer patients who have gained weight during their treatment (Schmitt *et al.*, 2012; Sestak *et al.*, 2012; Müller *et al.*, 2019) The present study showed that synephrine was poorly active against MCF-7 cells after 24 hours and 48 hours with IC_{50} values of

414.10 ± 5.40 μM and 149.30 ± 3.20 μM, respectively. The few studies reported using synephrine in *in vitro* experiments have not reported IC_{50} values in cancer cell lines (Roh *et al.*, 2014; Stohs, 2017; Suntar *et al.*, 2018; Xu *et al.*, 2018). A pharmacokinetic study conducted in humans showed a peak plasma concentration lower than 2 ng/mL at 2 hours after a single oral dose of 21 mg of synephrine (Haller *et al.*, 2008b). This suggests that excessively high doses would be required to achieve a cytotoxic plasma concentration indicated synephrine in the present study. There is no data available in medical literature reporting the plasma concentration of synephrine after high dose use. Consumption of synephrine has been associated with a risk of myocardial infarction. A case report of a previously healthy 24-year-old male with no family history or cardiac risk factors presented with an ST-segment-elevation myocardial infarction after consuming the Nutrex Lipo-6x® which contains synephrine (Thomas *et al.*, 2009). Unnikrishnan *et al.* (2018) reported a twenty-two-year-old male with no cardiovascular risk factors presented with an elevation of ST-segment indicative of myocardial infarction. The patient confirmed the use of two weight- loss products (Performix™ stim-free; Performix SST) which contained synephrine, caffeine and octopamine (Unnikrishnan *et al.*, 2018). Synephrine was shown to act on adrenergic receptors due to its structural similarities to ephedrine, causing effects like peripheral and coronary vasoconstriction, increased heart rate, and hypertension (Schmitt *et al.* 2012). These effects warrant extreme caution of using synephrine-containing products by the public and is a major concern when patients have undiagnosed cardiovascular risk factors (Stohs, 2017). Since some breast cancer treatment regimens including tamoxifen as an anticancer agent demonstrate the occurrence of weight gain, it is likely that patients may use synephrine-containing slimming aids (Rossato *et al.*, 2011; Sestak *et al.*, 2012). Patients previously treated with doxorubicin or undergoing current doxorubicin treatment may be at increased risk for cardiovascular events since doxorubicin is known for its cardiovascular adverse effects (Unnikrishnan *et al.*, 2018; Rawat *et al.*, 2021).

4.1.6 Caffeine

Caffeine is naturally occurring psychostimulant commonly found in coffee and energy drinks and is primarily metabolized into paraxanthine, in the liver via the CYP1A2 enzyme (Alsabri *et al.*, 2018; Mottram, 2018). Caffeine has recently received attention as a potential anticancer treatment (Popović *et al.*, 2018; Tej and Nayak, 2018; Shojaei-

Zarghani *et al.*, 2020; Liu *et al.*, 2021). It is likely that cancer patients experiencing cognitive dysfunction and cancer-related fatigue may use energy boosting products to enhance their cognitive performance to sustain normal daily activities (Horneber *et al.*, 2012; Lehrer *et al.*, 2013). The proposed mechanism of action of caffeine as stimulant is inhibition of cAMP-phosphodiesterase, causing an increase in intracellular cAMP (Barnes, 2018). Caffeine at a concentration of 50 µM was shown to disrupt multiple DNA damage-responsive cell cycle checkpoints in budding yeast and HeLa cells after 2 hours (Tsabar *et al.*, 2015). In the current study caffeine showed poor cytotoxic activity against MCF-7 cells after 24 hours and 48 hours treatment with IC_{50} values of 355.6 µM and 167.4 µM, respectively.

A study by Martínez-sLópez *et al.* (2014) administered a coffee product containing 70.69 mg of caffeine to adult men and women, resulting in a peak plasma concentration of 10.50 µM after 1.2 hours. Haller *et al.* (2008) reported that a two-capsule dose of energy supplement, containing 303.8 mg of caffeine resulted in a peak plasma concentration of 30.89 µM, much lower than the IC_{50} values in this study. This suggests that high amounts of caffeine-containing products (~2 g caffeine content) would need to be consumed to achieve a C_{max} similar to the concentration equal to the IC_{50} value in this study. Caffeine has dangerous toxic effects above doses of 400 mg (Turnbull *et al.*, 2016). Toxic side effects that are associated with caffeine plasma concentrations above 15 mg/mL (~77 µM) include paradoxical cardiovascular effects (bradycardia or tachycardia), gastrointestinal and psychological effects whilst plasma concentrations of 80 mg/mL (~411.96 µM) are lethal mainly due to cardiotoxic effects of caffeine (Banerjee *et al.*, 2014; Willson, 2018).

Chemotherapy drugs like doxorubicin, cyclophosphamide and imatinib which may be used in combination for breast cancer treatment cause adverse effects like gastrointestinal symptoms (vomiting and diarrhoea) and more seriously cardiotoxicity (haemorrhagic myocardial necrosis and cardiomyopathy) which may persist as long-term events (Wellstein *et al.*, 2018). Similar to synephrine, caffeine overuse, especially in energy drink consumption, is a concern for patients safety with diagnosed and undiagnosed cardiac risk factors (Banerjee *et al.*, 2014; Rawat *et al.*, 2021).

4.1.7 *S. frutescens*

S. frutescens commonly known as the cancer bush, is an indigenous medicinal plant extensively used in South Africa by an array of healers, and lay persons (Mills *et al.*, 2005; Van Wyk, 2015). The recommended therapeutic dose of *S. frutescens* reported in humans is 9 mg/kg/day, which is equivalent to two capsules each containing 300 mg dried leaf powder (Aboyade *et al.*, 2014). The active constituents may contribute to an array of anecdotal pharmacological effects which includes anti-inflammatory and antioxidant activities. These have been reported in several *in vitro* and *in vivo* studies (Chinkwo, 2005; Ntuli *et al.*, 2018; Zonyane *et al.*, 2020).

The current study evaluated two preparation of commercially available *S. frutescens* for activity against MCF-7 cells. The prepared *S. frutescens* hot water extract showed poor cytotoxic effects when tested against MCF-7 cells with a high IC_{50} value of 5.7 mg/mL in comparison to doxorubicin IC_{50} value of 0.43 µg/mL after 48 hours. Traditional healers use dried plant materials grinded into powder, which is usually infused in hot or boiling water and allowed to cool in temperature prior to administration (Aboyade *et al.*, 2014). Teas, decoctions, capsules and tablets of the plant leaves are commercially available at health shops and local markets (Müller *et al.*, 2013; Wilson *et al.*, 2015). Stander *et al.* (2007) reported that prepared *S. frutescens* 70% ethanol extract had an IC_{50} value of 1.5 mg/mL in MCF-7 cells after 24 hours. This low concentration of *S. frutescens* may be attributed to the ethanol solvent effects of the preparation whilst our study prepared a hot water extract.

The purchased *S. frutescens* 60% ethanol extract in this study showed poor cytotoxicity in the MCF-7 cells where the vehicle control contributed to the observed cytotoxic effects. The IC_{50} values of this formulation were at concentrations of 3.1% (*v*/*v*) and 2.7% (*v*/*v*) over 24 hours and 48 hours exposure, respectively. The variable activity in the reported studies and in this current study, suggest that product obtained commercially has dubious activity. *S. frutescens* is regulated more like food products, thus the regulations are much less restrictive than for products such as prescription drugs (Baran, 2014). The lack of quality control of CAMs being sold on the commercial markets may explain the poor activity the dry leaves *S. frutescens* hot water extract had in the MCF-7 cells and potential impurities of the herb mixture (leaves and stems) containing *S. frutescens*. The two *S. frutescens* extract formulations showed poor

cytotoxic effects in MCF-7 cells and apart from the evaluation of cell morphology, no further work was undertaken with the product.

Summary

In summary, the select CAMs: resveratrol, synephrine and caffeine showed poor cytotoxicity towards the MCF-7 cells with high IC_{50} values. The two *S. frutescens* extract formulations showed poor cytotoxic effects in MCF-7 cells. This suggests that the CAMs have no potential clinical application, considering high doses of the CAMs which would be required to achieve a plasma concentration similar to an IC_{99} value of this study to exert complete inhibitory effects. The IC_{90} or IC_{99} concentration is a value determined using dose response curve analysis which indicates when near complete inhibitory effects are observed, and this concentration is approximately 10 fold greater than its IC_{50} concentration (Swinney, 2011). The consequence of CAM intake at high dosages would lead to off-target toxic effects which could be harmful to the patients. However, in scientific literature, survey data show that it is likely that cancer patients will use CAMs. Therefore, this current study evaluated the effects of the selected CAMs on doxorubicin activity towards the MCF-7 cell line.

4.2 The effects of active CAMs on the efficacy of doxorubicin in the MCF-7 cell line

The potential for interactions of CAMS with chemotherapeutic drugs are poorly described in the medical literature (Buckner *et al.*, 2018; Fasinu and Rapp, 2019). The current study aimed to determine if the CAMs investigated in this study showed any effects on the activity of doxorubicin (Section 3.2).

4.2.1 Resveratrol

Following combination treatments with doxorubicin, resveratrol showed possible antagonistic effects in MCF-7 cells after a 24 hour treatment indicated by the statistically meaningful increase in the IC_{50} value of doxorubicin from 1.59 µM to 6.93 µM. After 48 hours of treatment with resveratrol combined with doxorubicin, the IC_{50} value of doxorubicin was decreased from 0.98 ± 0.09 µM to 0.85 ± 0.14 µM but this change was not statistically significant. In contrast to the current study, Al-Abd *et al.* (2011) showed resveratrol in combination with doxorubicin increased the potency of the

doxorubicin showing IC_{50} concentrations ranging from 0.12 to 0.34 µM in MCF-7 cells. In the study Al-Abd *et al.* (2011), cells were treated for 72 hours, therefore a direct comparison is not possible. The use of resveratrol in combination treatment with doxorubicin showed variability in its effects on the efficacy of doxorubicin and these effects are poorly understood (Leon-Galicia *et al.*, 2018; Salehi *et al.*, 2018).

Doxorubicin is known to cause cardiotoxicity is an adverse effect (Tacar *et al.*, 2012; Prathumsap *et al.*, 2020). Patients are likely to use resveratrol due to the claims of health benefits related to its anti-oxidant, anti-aging, cardio-protective, anti-inflammatory and neuro-protective effects (Salehi *et al.*, 2018). In a few clinical trials evaluating the effects of resveratrol over the past decade, reported contrasting results on the oxidative stress and inflammation possibly due to the effects of the dose consumed, the gut microbiota status, the health status, and the bioavailability of the compound (Ramírez-Garza *et al.*, 2018). This current study showed that 157 µM concentration of resveratrol decreased the cytostatic effects of doxorubicin during combination treatment for 24 hours in this study. However, it would not be possible to achieve such a high plasma concentration of resveratrol during 'normal' consumption. Resveratrol cause an array of adverse effects; gastrointestinal symptoms including nausea, flatulence, bowel motions, abdominal discomfort, loose stools, and liver dysfunction at doses of 2.5 g or more per day (Brown *et al.*, 2010). Since patients suffer from many gastrointestinal adverse effects during chemotherapy treatment, the use of resveratrol taken concurrently with doxorubicin should be cautioned.

4.2.2 Synephrine

In this study synephrine potentiated the cytotoxic effects of doxorubicin against the MCF-7 cells after 24 hours combination treatments since the IC_{50} value of doxorubicin was decreased from concentrations of 1.59 µM to 1.31 µM. Following 48 hours, synephrine enhanced the efficacy of doxorubicin since the doxorubicin IC_{50} concentrations decreased from 0.98 µM to 0.47 µM. However only the 48 hour treatment period was statistically significant. Synephrine is widely used in weight management products in combination with caffeine. (Inchiosa, 2011; Stohs *et al.*, 2012). Breast cancer patients frequently experience weight gain following their cancer treatment and may therefore seek out such slimming aids. Currently there are no report on potential interactions between synephrine and doxorubicin. Synephrine has been

shown to cause adverse effects like peripheral and coronary vasoconstriction, increased heart rate, and hypertension (Schmitt *et al.*, 2012). These adverse effects may exacerbate the cardiotoxicity associated with doxorubicin treatment, causing serious clinical manifestations in patients, and fatality (Thomas *et al.*, 2009; Unnikrishnan *et al.*, 2018; Prathumsap *et al.*, 2020).

4.2.3 Caffeine

In combination treatment with caffeine for 24 hours, the cytotoxic effect of doxorubicin against MCF-7 cells was reduced since the IC_{50} value increased from 1.59 ± 0.14 μM to 1.92 ± 0.29 μM. In contrast to the 24 hour period, after 48 hours treatment caffeine potentiated the cytotoxicity of doxorubicin with a decreased IC_{50} concentration from 0.98 ± 0.09 μM to 0.82 ± 0.05 μM. However, the effects of caffeine on doxorubicin IC_{50} values after both treatment periods were not statistically meaningful. A study by Hill *et al.* (2011) showed caffeine at a concentration of 1.03 mM decreased doxorubicin cytotoxicity against MCF-7 cells after 48 hour treatment using the MTT cell viability assay. These effects were seen at 24 hours in our study and opposite effects were observed at 48 hours. Hall *et al.* (2017) reported preliminary findings that caffeine increased the cytotoxicity of doxorubicin in SH-SY5Y neuroblastoma cells. Caffeine enhanced doxorubicin cytotoxic effects in Ehrlich cells *in vitro* (Kakuyama and Sadzuka, 2001). Sabisz and Skladanowski (2008) reported on the ability of caffeine to enhance the cytostatic effects of anticancer drugs through an inhibitory effect on DNA repair. High doses of caffeine are known to cause sympathomimetic effects which increases risk of cardiac adverse events (Willson, 2018). The high concentrations of caffeine required to increase doxorubicin efficacy would further compromise the cardiac function of patients since they may already suffer from doxorubicin associated cardiotoxicity. Therefore, patients using high caffeine-content energy drinks or supplements concurrent with chemotherapy regimens including doxorubicin may face serious cardiac complications. Consuming high caffeine content energy drinks and supplements may exacerbate the risk of serious cardiac events in patients treated with doxorubicin.

In combinations with the IC_{50} concentrations of resveratrol, synephrine and caffeine, the MCF-7 breast cancer cells showed more sensitive to doxorubicin after 48 hours treatment and resveratrol decreased doxorubicin efficacy after 24 hours treatment.

4.4 The effects of CAMs and doxorubicin on cell morphology

Morphology of MCF-7 cells treated with doxorubicin and CAMs at concentrations equivalent to their IC_{80} values was studied after 18 hour treatment. The *S. frutescens* ethanolic extract was also investigated at the highest concentration tested where the vehicle control did not show any inhibitory effects. The morphology of the cells was evaluated using the vital stains, AO, HO and PI (Section 3.3). Changes to cell morphology using fluorescence microscopy provide valuable information regarding cytotoxic effects and provides preliminary information regarding cell death mechanisms (Atale *et al.*, 2014; Galluzzi *et al.*, 2018).

Apoptosis is a programmed cell death mechanism, in which signalling pathways are activated under normal physiological or in stress response conditions without damage to neighbouring cells (Atale *et al.*, 2014; Kaczanowski, 2016). Apoptotic cells display distinct morphological features such as cell shrinkage, chromatin condensation, membrane blebbing and nuclear fragmentation (Galluzzi *et al.*, 2018). Necrosis, characterized by cell and organelle swelling, loss of ion gradients, increased cell membrane permeability with loss of cell membrane integrity, and the release of intracellular contents into the extracellular environment (Nikoletopoulou *et al.*, 2013; Galluzzi *et al.*, 2018). Necrotic cell death causes an uncontrolled inflammatory response leading to the clinical manifestation of tumour lysis syndrome (Labbé and Saleh, 2008). Autophagy is a pro-survival stress response and type of cell death characterized by increased autophagic vacuolization of the cytoplasm (Feng *et al.*, 2014; Galluzzi *et al.*, 2018). Acridine orange permeates both viable and non-viable cells and emits a green fluorescence when bound to DNA. In addition, it causes acidic granules to fluoresce red and yellow, and acts as a preliminary indicator for autophagy (Paglin *et al.*, 2001).

4.4.1 Doxorubicin

In this study, cells treated with doxorubicin at concentration of 3.7 µM showed evident fragmented nuclei and condensation of nuclear material as seen with the HO stain. These characteristics are consistent with apoptotic cell death. Doxorubicin caused the majority of cells to appear rounded and condensed. Doxorubicin mechanism of action targets the nucleus where the drug intercalates with DNA, and inhibits the topoisomerase II enzyme, leadings to apoptosis (Wellstein *et al.*, 2018). Pilco-Ferreto

and Calaf (2016) demonstrated that doxorubicin at range of concentration between 1-8 μM induced apoptosis in MCF-7 cells after 24 and 48 hour treatment periods. Elgart and colleagues (2018) reported that Hoechst 33342 compete with doxorubicin for binding the DNA minor groove. This would cause a decrease in the HO fluorescent signal, which was observed in majority of the MCF-7 cells treated with doxorubicin in this study. Doxorubicin has an innate red fluorescence and similarly PI dye, making the dye binding difficult to interpret (Yang *et al.*, 2014; Shah *et al.*, 2018). In the cells treated with doxorubicin cell membranes appeared to be intact when viewed in phase contrast mode.

4.4.2 Resveratrol

The current study showed morphological characteristics that are consistent with apoptotic and necrotic cell death after 18h treatment with 119.62 μM of resveratrol. The formation of acidic granules stained red and yellow in the cytoplasmic space of some cells treated with resveratrol, which is a preliminary indicator of the induction of autophagy. There are several studies in the literature that supports resveratrol associated apoptotic cell death (Khaleel *et al.*, 2016; Takashina *et al.*, 2017; El-Readi, S. Eid, *et al.*, 2019). Park *et al.* (2016) demonstrated that resveratrol at a concentration of 50 μM induces autophagy by directly inhibiting the mTOR-ULK1 pathway and decreased the viability of MCF-7 cells.

4.4.3 Synephrine

Cells were treated with 398.1 μM of synephrine for 18 hours. Evidence of apoptotic changes and deformed nuclei were present in a small fraction of cells after 18 hours. A few cells showed no HO signal with only a PI signal indicating necrotic cell death. This was likely a non-specific toxic effect caused by the high concentration of synephrine. PI outcompeted the HO stain for DNA binding and therefore HO was not visible in these cells. There are no studies in the literature reporting on apoptosis or necrosis in cells treated with synephrine. However, a few studies have reported apoptosis associated with *Citrus aurantium* peel extracts in cancer cell lines (Lee, 2012; S. H. Lee *et al.*, 2015). The data from the current study does not confirm apoptotic cell death although some apoptotic nuclei were observed. The very high concentrations most probably caused mostly necrosis.

4.4.4 Caffeine

After a 18 hour treatment with caffeine at 457.1 µM, small number of cells nuclei appeared deformed when stained with HO and necrotic death was observed by the yellow-orange nuclei in AO and PI stains. Niknafs (2011) reported caffeine caused apoptotic and necrotic cell death in MCF-7 cells when treated alone at a concentration of 1 µM, or in combination with cisplatin at 3 µM. In support of the mentioned study, Machado et al. (2020) reported caffeine induced necrosis and more predominantly apoptosis in MCF-7 cells treated with concentrations of 5 mM and 10 mM after 24 hour treatment. These concentrations are 11-fold and 22-fold higher than the concentration in the current study and is not clinically achievable. Similar to resveratrol, caffeine showed a small number of cells with yellow-red acidic granules in the cytoplasm indicative of autophagy. Sinha et al. (2014) demonstrated that caffeine at a concentration of 1.5 mM caused a downregulation of mTOR signalling and induced autophagy in HepG2 cells. In the current study, cells treated with caffeine showed no meaningful morphological changes in the majority of the MCF-7 cells after 18 hours, however necrosis and possible autophagy was observed in a very small percentage of cells.

4.4.5 *S. frutescens*

Cells were treated with 1.7 % (v/v) of *S. frutescens* ethanolic extract for 18 hours. Similar to all the CAMs, *S. frutescens* ethanolic extract caused the cells to show increased formation of rounded cells with red and bright yellow-orange nuclei. These observations indicate the loss of membrane integrity, allowing PI permeation into the dying cell, and these characteristics are consistent with necrosis. Stander *et al.* (2009) showed that a prepared *S. frutescens* hot water extract (10 mg/mL) caused morphological changes in MCF-7 cells consistent with apoptosis at 72 hours. In the scientific literature, studies have shown *S. frutescens* extracts induce apoptosis in various cancer cell lines; CaCo2 colorectal cells, SKNBE(2) neuroblastoma cells and A375 melanoma cells (Leisching *et al.*, 2015; van der Walt *et al.*, 2016; Omoruyi *et al.*, 2018). The concentration used to elicit these effects in the Stander *et al.* (2009) study are excessively high when the current study considers that doxorubicin at 0.002 mg/mL causes 80% of cells to die after a 48 hour treatment. In the present study the increased necrotic cell death caused by *S. frutescens* ethanolic extract may be attributed to the presence of ethanol in its preparation. Since this was a commercial preparation and

registered as a food supplement, there is no requirement for quality control in terms of "efficacy" and there may be huge variations in batches of raw materials, depending on the growing conditions (Albrecht *et al.*, 2012; Baran, 2014). This study showed *S. frutescens* hot water extract was not active and indicates the unreliability of 'health products' available to the public.

4.5 The effects of doxorubicin and the active CAMs on PgP efflux activity

Resistance to chemotherapy drugs in cancer may occur through several mechanisms, including reduced uptake and increased efflux of anticancer compounds in tumours (Leonard *et al.*, 2003; Baguley, 2010; Ray *et al.*, 2017). P-glycoprotein, is a plasma membrane protein part of the ABC transporter family and encoded by the *MDR1* gene (Stavrovskaya and Rybalkina, 2018). The function of PgP as an efflux pump and its role in drug resistance is well documented (Waghray and Zhang, 2018; Robinson and Tiriveedhi, 2020). The induced expression of PgP may be within a few hours in response to toxic insults (Collett *et al.*, 2004; Prenkert *et al.*, 2009). Inhibition of transmembrane PgP efflux of drugs enhances their binding ability to their intracellular targets, and improves the efficacy of the drug (Chang *et al*., 2019). Several clinically used drugs are known as inhibitors of PgP protein that have shown success *in vitro*; including cyclosporin A and verapamil used in this study (Nanayakkara *et al.*, 2018; Robinson and Tiriveedhi, 2020). Cyclosporin A is a calcineurin inhibitor used in immunosuppression, and verapamil is a calcium channel blocker used in cardiovascular disease (Eschenhagen, 2018; Krensky *et al.*, 2018). The known PgP inhibitors are limited in clinical use to reverse MDR, mainly due to their innate mechanism of action, the associated adverse effects, and their inability to discriminate between PgP protein expressed at normal sites in the human body and that found in cancerous tissue (Gillet and Gottesman, 2010; Callaghan *et al.*, 2014). To date there are no approved selective PgP inhibitors available in clinical use to overcome MDR (Nanayakkara *et al.*, 2018). In this study the PgP efflux activity was evaluated in MCF-7 cells treated with doxorubicin and the active CAMs, alone, and the CAMs in combination with doxorubicin (Section 3.4). Cyclosporin A and verapamil are competitive and non-competitive inhibitors of PgP, respectively and were used to demonstrate dose-dependent inhibition of PgP protein (Giacomini and Sugiyama, 2018).

Since doxorubicin and resveratrol showed cytotoxic effects in MCF-7 cells at low concentrations, their effects on PgP activity were evaluated at the same range of concentrations at 0.1 μM, 0.5 μM and 10 μM for 24 hours (Section 3.4.1). Synephrine and caffeine showed poor cytotoxic activity against the MCF-7 cells, therefore their effects on PgP activity was evaluated at a concentration of 75 μM, 150 μM and 300 μM after a 24 hour treatment period (Section 3.4.2).

4.5.1 Doxorubicin

Doxorubicin was the most potent activator of PgP function since calcein-AM accumulation was decreased in MCF-7 cells in a dose dependent manner at concentrations of 0.1 μM, 0.5 μM and 10 μM after 24 h treatment Doxorubicin is a known substrate of PgP which explains the decrease in calcein-AM accumulation (Finch and Pillans, 2014). Despite the doxorubicin-mediated increase in PgP activity after 24 hours, the low micromolar IC_{50} concentration of doxorubicin at this time period supports its effectiveness in treatment of MCF-7 cells.

4.5.2 Resveratrol

Similar to doxorubicin, resveratrol significantly increased PgP activity at all three concentrations after 24 hours. The calcein-AM levels were decreased but the dose dependent relationship with resveratrol was less clear. A study by Junco *et al.* (2013) demonstrated that resveratrol did not inhibit rhodamine 123 efflux in skin carcinoma cells (Ca3/7) and skin papilloma cells (MT1/2) after a 24 hour treatment period. The effects of resveratrol on PgP activity when treated alone, particularly in the MCF-7 cell line has not been well reported. However, a few studies have reported that combination treatment with resveratrol inhibited PgP protein activity and expression in various cancer cell lines, thus enhancing the drug efficacy (Choi *et al.*, 2009; Huang *et al.*, 2014; Khaleel *et al.*, 2016; Wang *et al.*, 2016). These findings suggest that resveratrol may have dual effects on PgP activity which may be dependent on treatment exposure period, cancer cell line, and single-agent or combination treatment.

4.5.3 Synephrine

In the current study, MCF-7 cells treated with synephrine at concentrations of 75 μM, 150 μM and 300 μM showed a statistically meaningful decrease in the calcein-AM levels with an unclear dose dependent relationship. Nabekura *et al.* (2008) evaluated the effects of Auraptene and synephrine on PgP activity in KB-C2 human cervical

cancer cells and reported that synephrine had no effect at a concentration of 50 µM after 2 hours. Nabekura *et al.* (2008) reported that synephrine had no effect similar to the results in the current study. However, the study assessed PgP activity at a shorter treatment period and at lower concentrations of synephrine than the current study making a direct comparison redundant.

4.5.4 Caffeine

Similar to of synephrine, caffeine caused a statistically significant decrease in calcein-AM levels in cells treated with all three concentrations and no dose response effect was observed. Since synephrine and caffeine significantly increased the PgP efflux activity in cells at the same three concentrations, their effects on calcein-AM levels were statistically compared. In comparison to synephrine, caffeine caused significantly lower calcein-AM retention levels at a concentration of 75 µM and 300 µM indicating that it stimulated PgP activity more than synephrine. Abuznait *et al.* (2011) reported that caffeine treated at concentrations of 25 µM, 50 µM and 100 µM increased the PgP activity in LS-180 colorectal cancer cells after a 48 hour treatment period. The findings by Abuznait *et al.* (2011) are similar to the results of the present study albeit lower treatment concentrations and longer treatment period.

4.5.5 The effects of the CAMs on the doxorubicin induced PgP activity

Several studies have reported that combined doxorubicin and PgP modulators with multiple targets is a promising strategy to improve chemotherapeutic efficacy without the need of high dose or additional cytotoxic drugs in the therapeutic regimen (Sharom, 2014; El-Readi, S. Y. Eid, *et al.*, 2019). In the current study the effects of CAMs at a constant concentration of resveratrol (157 µM), synephrine (423 µM) and caffeine (354 µM) on the doxorubicin induced PgP activity were evaluated after combination treatment for 24 hours. Cells were treated with 0.05 µM, 0.5 µM and 1 µM of doxorubicin alone and in combinations with CAMs (Section 3.4.3).

4.5.5.1 Resveratrol

In combination treatment with doxorubicin, resveratrol enhanced PgP activity in a dose dependent manner and a statistically meaningful increase in PgP activity at a concentration of 0.5 µM of doxorubicin. This effect was lost at a concentration of doxorubicin at 1 µM. Studies reported that a PgP substrate may have the same or

distinct binding sites on PgP from another substrate (Shapiro and Ling, 1997). This will cause competition between substrates that bind identical sites or have an additive effect on PgP activity when substrates bind different sites (Chufan *et al.*, 2015). In our study, the stimulation of resveratrol on PgP efflux activity of doxorubicin and may be due to the lipophilic characteristics of resveratrol (Gambini *et al.*, 2015). The results of the current study indicate it is likely that resveratrol may decrease the clinical therapeutic efficacy of doxorubicin in patients when resveratrol is used concurrently during treatment with doxorubicin.

4.5.5.2 Synephrine

Synephrine prevented the doxorubicin induced loss of calcein-AM at all concentrations tested. At a concentration of 0.05 μM the doxorubicin induced PgP activity was inhibited completely. At a concentration of 0.5 μM, synephrine still showed inhibitory effects on doxorubicin induced PgP activity. However, at 10 μM of doxorubicin, synephrine effect was lost and doxorubicin induced PgP efflux of calcein-AM was not significantly inhibited by synephrine. Doxorubicin at a concentration of 10 μM is much higher than its reported peak plasma concentration and thus has no clinical relevance. The inhibition of doxorubicin PgP efflux activity in combination treatment with synephrine showed opposite effects when treated as a single agent. There are no studies reported in the medical literature that showed synephrine inhibited PgP efflux activity. Acute intake of synephrine showed the capacity to increase lipid metabolism in a rat *in vivo* model and in human subjects, consequently increased fatty acid oxidation, ATP levels and exert thermogenic effects (Gutiérrez-Hellín and Del Coso, 2016; da Silva-Pereira *et al.*, 2017; Maldonado *et al.*, 2018). The high concentration of synephrine likely increased heat stress when combined with doxorubicin and may have denatured the PgP protein compromising its efflux function and thus an increase in calcein-AM accumulation. This indicates synephrine would increase the intracellular doxorubicin accumulation and enhance doxorubicin efficacy. The high dose of synephrine required to have beneficial effects during doxorubicin treatment is not achievable at acute intake; however, long term use of high dose may have serious synephrine adverse effects in patients who are vulnerable to doxorubicin cardiotoxicity.

4.5.5.3 Caffeine

Caffeine in combination with doxorubicin caused a statistically meaningful increase in calcein-AM levels at all three doxorubicin concentrations tested. The PgP activity was similar to untreated cells at the three doxorubicin concentrations indicating the complete inhibition of doxorubicin induced PgP efflux. Kakuyama and Sadzuka (2001) reported that doxorubicin reduced cAMP levels in Ehrlich ascites carcinoma cells. Caffeine restored cAMP levels and decreased the efflux of doxorubicin *in vitro* (Kakuyama and Sadzuka, 2001). This suggests that 354 μM of caffeine inhibited the PgP activity caused by doxorubicin treatment to baseline levels and indicate an increased accumulation of doxorubicin in MCF-7 cells. High amounts of caffeine intake will be needed to achieve the beneficial effect and will not be clinically feasible due to caffeine associated cardiotoxicity. Despite the inhibitory effects of caffeine on the doxorubicin increased PgP efflux activity at 10 μM, this concentration is clinically irrelevant during treatment with doxorubicin. The inhibitory effects of caffeine on doxorubicin PgP efflux are in contrast to the caffeine stimulatory effects on PgP efflux activity when used as a single agent. Earlier studies showed that the formation of drug-caffeine stacking complexes, especially DNA intercalator drugs (doxorubicin) which often possess aromatic molecules, via Van der Waal force interactions and increases the accumulation of intracellular drug concentration (Larsen *et al.*, 1996; Evstigneev *et al.*, 2006). This may likely occur at high concentrations of caffeine during combinations. Similar to the Abuznait *et al.* (2011) study, caffeine activated PgP efflux in single-agent treatment with concentrations below its IC_{50} value in the current study. Caffeine may have dual effects of PgP efflux activity which is likely dose dependent, showing inhibitory effects at high concentrations and stimulatory effects at low concentrations.

Summary

In summary, doxorubicin, and resveratrol both increased PgP activity in a similar fashion at low treatment concentrations. Synephrine and caffeine showed a similar increase in PgP activity at high treatment concentrations while caffeine caused a greater increase in PgP activity. Doxorubicin was the most potent activator of PgP efflux in comparison to the CAMs. Doxorubicin is a known PgP substrate. In combination treatment with doxorubicin, synephrine and caffeine showed inhibition of PgP activity which may lead to an increase in doxorubicin intracellular accumulation. This may

result in a clinically beneficial effect. Combination studies showed that resveratrol increased doxorubicin associated PgP efflux activity causing a decrease in intracellular doxorubicin accumulation. These effects of resveratrol in combination with doxorubicin on PgP activity correlate with the cell viability studies in Section 4.2, where resveratrol decreased doxorubicin efficacy after 24 hours treatment.

4.6 The PgP protein expression in MCF-7 cells treated with doxorubicin and the CAMs

The hyperactivity and overexpression of PgP protein is often found in cancer and is associated with cancer chemotherapy resistance (Nanayakkara *et al.*, 2018; Waghray and Zhang, 2018). *MDR1* gene transcription is regulated by NF-κβ, Y-box binding protein-1, AP-1, and HIF-1 transcription factors; that are linked to signal transduction pathways the MAPK, and the PI3K pathways (Li *et al.*, 2012; Katayama *et al.*, 2014) The overexpression of PgP may occur by both selection of resistant cells, and induction of PgP expression during exposure to substrates *in vitro* (Breier *et al.*, 2012). Therefore, the effects of doxorubicin and CAMs, alone and in combination, on PgP protein expression and subcellular location was evaluated (Section 3.5).

4.6.1 Doxorubicin

In this study, treatment with doxorubicin at a concentration of 1.12 μM strongly induced PgP protein expression in MCF-7 cells and was found to be located primarily in the nucleus after 24 and 48 hours. Doxorubicin-induced drug resistance in breast cancer is well reported (Smith *et al.*, 2006; Huang *et al.*, 2014; Zhao *et al.*, 2016). Doxorubicin damages cell DNA and activates NF-κβ via the degradation of its inhibitor molecule, IκBα (Tergaonkar *et al.*, 2003). Doxorubicin at a concentration of 0.02 μM has shown to up-regulate MDR1 and NF-κβ mRNA and protein expression levels in MCF-7 cells (Fang *et al.*, 2014). Active PgP is primarily associated with the plasma membrane, however small portion of PgP is expressed in the nucleus often in cancer cells (Calcabrini *et al.*, 2000; Meschini *et al.*, 2000). The meaning of this is not clear since PgP protein is required to be localized to the plasma membrane to function as an efflux pump. This was further explored and later studies showed that PgP is glycosylated in some tumour cells and active in the nuclear membrane, where it functions to keep target drugs out of the nucleus, thereby contributing to tumour cell survival (Szaflarski *et al.*, 2013). Kuzma-Richeret *et al.* (2011) reported PgP protein localized in the nuclear

membrane of MCF-7 cells. An earlier study reported the expression of inactive PgP in the nucleus was due to inhibited maturation of the protein by processing mutations which prevented PgP from translocating to the plasma membrane (Loo and Clarke, 1999). Doxorubicin-induced DNA damage has shown to inhibit ribosome proteins thus compromising protein post translation modification in the cell ER (Halim *et al.*, 2018). Since doxorubicin was active against MCF-7 cells in this study, it is likely that an immature form of PgP was found in the nucleus.

4.6.2 Resveratrol

In the present study, single-agent treatment with resveratrol at an IC_{50} and IC_{80} concentration did not affect the PgP expression levels in MCF-7 cells after a 24 hour and 48 hour treatment. Combination studies showed that resveratrol (157 μM and 80.5 μM) suppressed doxorubicin induced PgP expression after a 24 hour and a 48 hour treatment. Kim *et al.* (2014) reported that resveratrol (50 μM) decreased the doxorubicin induced PgP protein expression level and enhanced the cytotoxicity of doxorubicin in MCF-7-doxorubicin resistant and MDA-MB231 cells as determined by immunohistochemistry and MTT cell viability experiments for 24 hours treatment period. Huang *et al.* (2014) also reported a decrease in PgP mRNA expression levels and PgP protein expression in MCF-7-doxorubicin resistant cells. In contrast to these findings, resveratrol increased the doxorubicin induced efflux activity of PgP after 24 hours. An increase in PgP protein expression does not necessary translate to increased PgP activity.

4.6.3 Synephrine

Synephrine treatment at an IC_{50} concentration showed no effects on PgP expression in MCF-7 cells after 24 hours and 48 hours. In combination treatment with doxorubicin, synephrine showed no effect on doxorubicin induced PgP expression levels after 24 hours but it did suppress doxorubicin induced PgP expression after 48 hours. A study using BALB/c mice showed synephrine (5 mg/kg) reduced lipopolysaccharide-induced acute lung injury by suppressing the degradation of IκBα, and the phosphorylation of NF-κβ (Wu *et al.*, 2014). It is likely that synephrine decreased the doxorubicin induced PgP expression by suppressing the NF-κβ transcription factor activation mediated by doxorubicin DNA damage.

4.6.4 Caffeine

Caffeine had no effects on the expression levels of PgP protein in MCF-7 cells after 24 hours and 48 hours. The doxorubicin induced PgP expression levels were significantly suppressed in cells after combination treatment with caffeine for 48 hours. It was reported that the MAPK/NF-κβ pathway mediates doxorubicin-induced PgP expression in resistant MCF-7 cells and subsequently alters the cellular pharmacokinetics of doxorubicin (Zhang *et al.*, 2012). A study by Zhao *et al.* (2019) demonstrated that caffeine markedly decreased the phosphorylation levels of MAPK and NF-κB pathway members in liposaccharide induced macrophages. This may explain the inhibitory effects of caffeine on induced expression of PgP protein observed after 48 hours.

Summary

In summary, the untreated control MCF-7 cells showed poor PgP protein expression. Treatment with doxorubicin caused a major increase in PgP protein expression, located primarily in the nucleus after 24 hours and 48 hours treatment periods. The CAMs treatments by themselves did not induce PgP expression after the two treatment periods. When used in combination, resveratrol reduced the doxorubicin associated PgP expression after both treatment periods. Both caffeine and synephrine caused a small but statistically significant decrease in doxorubicin induced PgP expression. In all combination treatments, PgP remained localised in the nucleus. This indicates that the post translational processing and transport to the cell membrane was compromised by doxorubicin.

4.7 Effects of doxorubicin and CAMs on HMOX1 expression in MCF-7 cells

HMOX1 is frequently overexpressed in cancer and its expression is further increased in response to radiation and chemotherapy (Podkalicka *et al.*, 2017; Shiraishi *et al.*, 2018; Z. Zhang *et al.*, 2020). HMOX1 protein is found in various intracellular compartments, including the mitochondria, and the nucleus, where it can mediate signalling functions (Dennery, 2014; Luu Hoang *et al.*, 2021). Under oxidative stress conditions, *HMOX1* transcription is induced (Chen *et al.*, 2019). HMOX1 protects against the accumulation of free radicals, which leads to cell death (Podkalicka *et al.*, 2017; Chen *et al.*, 2019). The cytoprotective potential of HMOX1 in cancer attenuates the efficacy of drug treatment, making it an important target protein in chemo-resistance (Zhe *et al.*,

2015; Pei *et al.*, 2018). Therefore, the effects of doxorubicin and CAMS on HMOX1 expression and subcellular location was evaluated in this study (Section 3.6.1 and Section 3.6.2).

HMOX1 expression in untreated MCF-7 cells was moderate and primarily located in the nucleus of the cells. Gandini *et al.* (2019) reported that nuclear localisation of HMOX1 correlated with higher tumour grade in breast cancer. HMOX1 mRNA expression levels was high in the MCF-7 cell line as determined using real time polymerase chain reaction assay (Bahmani *et al.*, 2011). However, studies have shown that there is poor correlation between expression levels of mRNA and protein expression levels (Buccitelli and Selbach, 2020). This discrepancy is often attributed to post-transcriptional, translational and protein degradation regulation (Vogel and Marcotte, 2012; Buccitelli and Selbach, 2020).

4.7.1 Doxorubicin

Doxorubicin was the most effective inducer of HMOX1 compared to the CAMs since the baseline HMOX1 expression were significantly increased from moderate to high levels after 6 hours and 18 hours. HMOX1 expression was found to be primarily located in the nucleus. Zhu *et al.* (2015) reported that HMOX1 mRNA expression levels in MDA-MB231 breast cancer cells was high and the protein expression levels of HMOX1 were significantly increased in these cells after treatment with doxorubicin for 48 hours. In addition, the study used transfected short interfering RNA which downregulated the mRNA expression levels of HMOX1 and enhanced the cytotoxicity of doxorubicin in MDA-MB-231 breast cancer cell line (Zhu *et al.*, 2015). Doxorubicin is known to increase ROS production (Kim *et al.*, 2006), explaining the increase in HMOX1 protein expression observed in this study. Further evaluation of HMOX1 subcellular localisation used the colocalisation analysis on computer software to determine the correlation between HMOX1 and HO signals. In treatment with doxorubicin and all the CAMs, a small number of cells appear to have HMOX1 expression predominantly in the cytoplasm as confirmed by the weak Pearson's Correlation between HMOX1 and HO signals indicating HMOX1 was not present in the nucleus of these cells. The majority of the cells showed HMOX1 in the nucleus as confirmed by a strong Pearson's Correlation. These results confirm the transport of HMOX1 from the nucleus into the cytoplasm of the small percentage of cells after treatment with doxorubicin.

A study by Lin *et al.* (2007) showed that the transport of HMOX1 into the nucleus was mediated by C-terminal cleavage of the protein, and nuclear localisation of HMOX1 was associated with the reduction of its enzymatic activity. The HMOX1 expression in the nucleus was found to be enzymatically inactive and in its truncated form which is able to alter its own transcription via activating AP-1 (Lin *et al.*, 2008). These findings may support the mechanism by which baseline levels of HMOX1 are maintained in the nucleus, and only a small fraction of cells have HMOX1 transported to the cytoplasm when the cell is under oxidative stress conditions.

4.7.2 Resveratrol

Resveratrol treatment at concentrations of 157 µM and 181.27 µM increased the HMOX1 expression levels in MCF-7 cells after 6 hours but this effect was not observed after 18 hour treatment. Costa *et al.* (2021) reported that after 24 hour treatment with 50 µM of resveratrol for 24 hours the HMOX1 mRNA levels were significantly increased ROS levels, and it inhibited casein kinase 2, leading to a decrease in cell viability and decreased mitochondrial function in MCF-7 cell line. Iwasaki *et al.* (2014) reported that HMOX1 mRNA levels were significantly increased in Jurkat cells as well as induced Nrf2 nuclear accumulation in K562 erythroleukaemia cells after treatment with 50 µM of resveratrol for 24 hours as determined by quantitative real time-polymerase chain reaction and chromatin immunoprecipitation assay. These findings of resveratrol associated with an increase in HMOX1 indicate a cellular stress response mechanism was activated to protect the cells from oxidative stress. This suggest that the cytoprotective effect of resveratrol may contribute to resistance of cancer to radiotherapy and chemotherapy. Despite reports that have shown resveratrol function in the protection mechanisms from oxidative stress via HMOX1 induction *in vitro* and *in vivo* (Sinha *et al.*, 2016; Funes *et al.*, 2020), its clinical implication remains unknown.

4.7.3 Synephrine

Synephrine treatment at 423 µM significantly increased the baseline HMOX1 expression levels in the MCF-7 cells after 6h and 18h exposure. In the work of Ribeiro and colleagues (2019), synephrine treatment at 2 µM, 20 µM and 200 µM induced high accumulation levels of intracellular ROS in HepG2 cells after 6 hours exposure. The findings of this study suggest that synephrine induced oxidative stress in HepG2 cells which likely increased the expression levels of HMOX1 and decreased the cytotoxic

effects of synephrine. This may provide an explanation for the poor efficacy of synephrine since high IC_{50} values were observed in the present study with the MCF-7 cell line.

4.7.4 Caffeine

Caffeine significantly suppressed the HMOX1 expression levels in MCF-7 cells after 6 hours exposure and increased the expression levels after 18 hours of treatment. Azam *et al.* (2003) reported both antioxidant and prooxidant properties of caffeine in molecular experiments using calf thymus DNA. Their results indicate that caffeine and its metabolites may contribute to the overall anti-oxidant and cytoprotective properties of caffeine-containing products (Azam *et al.*, 2003; Endesfelder *et al.*, 2019). Azam *et al.* (2003) reported caffeine showed pro-oxidant effects particularly in the presence of metal ions like copper, leading to the production of ROS and DNA damage. However, in the current study the potential of caffeine as an antioxidant could explain the decreased expression levels of HMOX1 at 6 hours. After 18 hours treatment, caffeine induced oxidative stress which increased the expression levels of HMOX1 and decreased the cytotoxic effects of caffeine. This may contribute to the poor anti-cancer efficacy of caffeine as evident by the high IC_{50} values obtained in cell viability studies.

4.7.5 The effects of the CAMs on the doxorubicin induced expression levels of HMOX1

Since doxorubicin showed meaningful effects on the expression levels of HMOX1 in MCF-7 cells after 6 hour and 18 hour treatment, combination studies as described earlier were pursued. This study investigated the effects of resveratrol, synephrine and caffeine on the doxorubicin induced expression levels of HMOX1 after combination treatment (Section 3.6.3 and Section 3.6.4). Excessive ROS and insufficient antioxidants lead to inflammatory tissue injury (Yang *et al.*, 2018). Cancer cells are highly metabolically active and hypoxic cells which tend to increase ROS formation which damages DNA and is involved in cell survival, therapeutic resistance, and progression (Vera-Ramirez *et al.*, 2012). Doxorubicin causes ROS which leads to excessive oxidative stress and cell death.

In this study the combinations with resveratrol (157 μM) and caffeine (354 μM) significantly suppressed the doxorubicin induced expression levels of HMOX1 after 6h

and 18h. Resveratrol in particular caused a complete inhibition after 18 hours. This suggests that resveratrol and caffeine suppressed the cytoprotective response of HMOX1 to doxorubicin treatment. In this study, resveratrol and caffeine may have increased the oxidative stress caused by doxorubicin, enhancing its cytotoxic effects in MCF-7 cells and similar effects have been reported (Azam *et al.*, 2003; Costa *et al.*, 2021a). However, this effect of resveratrol and caffeine did not increase the doxorubicin efficacy since the IC_{50} values of doxorubicin were increased after combination treatment for 24 hours. This stands in agreement with the complexity of cellular responses to xenobiotics (Mackowiak *et al.*, 2018).

During combination studies, synephrine at a concentration of 423 µM suppressed the doxorubicin induced HMOX1 expression levels after 18h combination treatment while no effect was observed at 6 hours. Wu *et al.* (2014) found that *p*-synephrine significantly reduced anti-inflammatory cells and ROS in lipopolysaccharide-induced acute lung injury of mice showing anti-oxidant effects. However, this cannot be expected in responses of cancer cells. This study suggests that synephrine decreased the cytoprotective response of HMOX1 induced by doxorubicin thereby increasing doxorubicin associated oxidative damage to cancer cells. These results complement the cell viability studies results showing an increased doxorubicin potency during combinations with synephrine.

Further evaluation of HMOX1 subcellular localisation in cells treated with doxorubicin in combination with the CAMs, a small number of cells showed HMOX1 expression predominantly in the cytoplasm similar to doxorubicin treated as single-agent. The majority of the cells showed HMOX1 expression in the nucleus. These results confirm the transport of HMOX1 from the nucleus into the cytoplasm of the small percentage of cells followed by treatment with doxorubicin and the CAMs, alone or in combination. Despite the decreased doxorubicin induced HMOX1 expression, it remained predominantly located in the nucleus. This effect remains unclear due to the enzyme protein needed in the cytoplasm to function to reduce free radical accumulation.

Summary

In summary, the untreated control MCF-7 cells showed moderate HMOX1 protein expression. Treatment with doxorubicin caused an obvious increase in expression levels

of HMOX1, located predominantly in the nucleus. Resveratrol at two concentrations was able to increase HMOX1 expression levels after 6 hours. Resveratrol is able to induce oxidative stress and this effect is of concern to patients with pre-existing inflammation symptoms. Synephrine and caffeine when treated alone had small meaningful effects on HMOX1 expression but may not affect the oxidative stress associated with cancer. When used in combination, resveratrol and caffeine decreased the doxorubicin induced expression levels of HMOX1 after treatment for 6 hours while after 18 hours the induced expression levels of HMOX1 were decreased by all of the CAMs in combination treatment. Resveratrol and caffeine showed a suppressed oxidative response caused by doxorubicin which may be beneficial during treatment with doxorubicin. In all combination treatments, HMOX1 remained localised in the nucleus. The meaning of this is not clear since HMOX1 is reported inactive in the nucleus and is required to be localized to the cytoplasm to function as an oxidative stress-reducing enzyme. Further investigations are needed to confirm the function of HMOX1 in cellular responses to oxidative stress caused during treatment with doxorubicin and the CAMs since the HMOX1 expression was increased after single-agent treatments.

CHAPTER 5: CONCLUSION

The purpose of this study was to evaluate the potential interactions of CAMs with chemotherapy drugs using a breast cancer cell line, MCF-7, as a model. In comparison to doxorubicin, the CAMs were poorly active against the MCF-7 cells. The most active CAMs: resveratrol, synephrine and caffeine were selected to investigate their effect on the activity of doxorubicin. In addition, two commercially available *S. frutescens* extracts were investigated in this study and showed no activity against the MCF-7 cell line. The effects of the active CAMs on doxorubicin efficacy, cell morphology, PgP efflux activity, and expression levels of PgP and HMOX1 protein in the MCF-7 cell line were presented and discussed in Chapter 3 and Chapter 4.

5.1 Doxorubicin

Doxorubicin was used as the positive control in this study instead of gemcitabine and irinotecan due to its low IC_{50} concentration of 0.74 μM over 48 hours treatment. Morphological changes in cells treated with doxorubicin were consistent with apoptotic cell death. Doxorubicin increased the PgP efflux activity, and this effect is expected due to doxorubicin being a known PgP substrate. Further evaluation showed a major increase in PgP expression higher than baseline caused by doxorubicin after two treatment periods. Doxorubicin induced PgP was located in the cell nucleus and would unlikely contribute to the efflux activity since PgP needs to be located at the cell plasma membrane to execute its efflux function. The low doxorubicin IC_{50} concentrations in this study are encouraging. Following two treatment periods, doxorubicin induced increased expression of HMOX1 protein much higher than baseline and was primarily located in the nucleus. In some of the cells, HMOX1 was not found in the nucleus instead it was located predominantly in the cell cytoplasm. HMOX1 is needed in the cytoplasm to execute its function as a haem-degrading enzyme during oxidative stress. It is likely the HMOX1 expressed in the nucleus may be its inactive form and may have had no effects on protecting the cells against oxidative stress.

5.2 Resveratrol

Resveratrol was the most active CAM with an IC_{50} concentration of 80.54 μM over 48 hours which indicates poor clinical efficacy. When combined with doxorubicin, resveratrol increased the IC_{50} value indicating an antagonistic effect. This may be of clinical importance in patients using resveratrol while being treated with doxorubicin. Apoptotic and necrotic cell death was observed in cells treated with resveratrol. Further investigations showed that resveratrol enhanced PgP efflux activity when used alone and in combination with doxorubicin. The increase of PgP activity would decrease the intracellular accumulation of doxorubicin and offers an explanation for the loss of doxorubicin potency. Doxorubicin is a known PgP substrate, and the added increase in PgP efflux activity caused by resveratrol may have increased efflux of doxorubicin. The high resveratrol IC_{50} value is not impossible to achieve in patient plasma concentration if taken regularly and may then impact doxorubicin and other drugs that are substrates for PgP. Resveratrol had no effects on PgP protein expression when used alone but did suppress the doxorubicin induced increased expression during combination treatment and remain located in the nucleus. Despite the decrease of PgP, the protein expression levels were still high, and it is unlikely to affect the PgP efflux activity. Resveratrol was able to increase HMOX1 protein expression above baseline when used alone but did suppress the doxorubicin induced increased expression during combination treatment. In addition, HMOX1 remained located in the nucleus and would likely be an inactive form. This indicates that resveratrol may have had no effect on the oxidative stress caused by doxorubicin. The data obtained in this study indicate that resveratrol may have an impact on doxorubicin treatment and that patients should be actively cautioned against its use.

5.3 Synephrine

Synephrine showed poor efficacy against the MCF-7 cells with a high IC_{50} concentration of 149.3 μM after 48 hours indicating unlikely clinical relevance. When combined with doxorubicin, synephrine decreased doxorubicin IC_{50} concentration implying increased efficacy. This may be beneficial in patients using synephrine during treatment with doxorubicin. However, the large amount of synephrine consumption required to achieve a beneficial effect may cause serious adverse effects. Furthermore,

synephrine increased PgP efflux activity as a single-agent but did supress the doxorubicin induced PgP activity during combination treatment. The decrease in PgP activity would increase the intracellular accumulation of doxorubicin and offers an explanation for albeit small, increased doxorubicin potency. Synephrine had no effects on PgP protein expression but did suppress the doxorubicin induced increased PgP expression during combination treatment. However, despite the decrease of PgP protein levels, the expression levels were still much higher than baseline and remained located in the nucleus. It is unlikely that the high expression levels of PgP affected the efflux of doxorubicin since synephrine increased the doxorubicin efficacy during combination treatment. Synephrine induced HMOX1 expression when used alone but did suppress the doxorubicin induced expression during combination treatment, HMOX-1 was located primarily in the nucleus with a small number of cells that expressed HMOX1 in the cytoplasm when combined with doxorubicin. It is likely that synephrine caused oxidative stress in cells when used alone and therefore induced HMOX1 expression. Moreover, the doxorubicin induced suppression of HMOX1 may have enhanced doxorubicin oxidative stress this may explain the small increase in doxorubicin potency during combination treatment. Synephrine is the primary ingredient in bitter orange extract and is a found in many weight-management supplements. Caution should be advised with the use of synephrine in cancer patients with risks for cardiovascular disease due to its associated adverse effects including cardiotoxicity and death. This is especially true of doxorubicin treated patients who are at high risk for cardiovascular adverse events.

5.4 Caffeine

Caffeine was poorly active against the MCF-7 cells with a high IC_{50} concentration of 167.40 μM over 48 hours which is indicates no clinical relevance of the CAM compound. Caffeine would have to be consumed at toxic dose levels to achieve any clinical effect on cancer cells and likely cause cardiac adverse events or death. During combination treatment, caffeine showed no meaningful effect on the doxorubicin efficacy. Caffeine was able to induce PgP efflux activity when used alone. Moreover, caffeine suppressed the doxorubicin induced PgP activity close to that of normal PgP activity. This suggests caffeine may cause increased doxorubicin accumulation in cells. However, an increase in doxorubicin potency could not be demonstrated in combination

studies. After single agent treatment, caffeine showed no effect on PgP expression but was able to suppress the doxorubicin induced expression levels of PgP. Despite caffeine suppressing the doxorubicin induced expression, the PgP expression levels remained higher than baseline levels and PgP remained in the nucleus. Therefore, it is unlikely that the high expression levels of PgP affected the efflux of doxorubicin since caffeine had no effect on doxorubicin efficacy during combination treatment. The expression levels of HMOX1 were initially suppressed, and thereafter increased by caffeine as a single agent. This indicates that caffeine may have dual effects on HMOX1 expression. During combination treatment similarly to synephrine, caffeine suppressed the doxorubicin induced expression and HMOX1 was expressed in nucleus and only a small fraction of the cells similarly to synephrine only a few cells showed the cytoplasmic location of HMOX1. These effects indicate that caffeine suppressed the doxorubicin induced HMOX1 expression and likely enhanced oxidative stress damage. The use of caffeine as an inhibitor of PgP protein in drug resistance is limited to its high dose intake required to achieve these effects in a clinical setting, thereby increasing the risk of cardiac adverse effects.

Summary

This study showed no potential clinical efficacy of the CAMs; resveratrol, synephrine, caffeine and *S. frutescens* as indicated by the poor cytotoxicity against the MCF-7 cell line. Resveratrol decreased the doxorubicin efficacy, which may be due its enhanced activation of PgP efflux. Synephrine increased the doxorubicin efficacy but at a high concentration used in combination treatment. Caffeine was able to inhibit doxorubicin efflux but at a high concentration. All three active CAMs suppressed expression of the drug resistance associated proteins, PgP and HMOX, during combination treatment.

5.5 Limitations of this study

The current study used a breast cancer cell line, MCF-7, in laboratory experiments to complete the research objectives. Thus, the results of this study cannot be directly extrapolated to clinical setting. This study did not measure a variety of combinations using fixed and variable ratio concentrations in MCF-7 cells. Such assays are critical to elucidate the doxorubicin IC_{50} concentrations when combined with the CAMs.

5.6 Future investigations

Collectively, the data collected in this study showed that doxorubicin efficacy is enhanced by the active CAMs. More combination studies are required to fully investigate whether the CAM and doxorubicin interactions are additive, antagonistic, or synergistic at various exposure periods. Preliminary morphological results could be further investigated using specific cell death assays to understand the mechanism of cell death in MCF-7 cells treated with the CAM compounds.

Research from the PgP activity studies indicate that it would be beneficial to explore the effects of more treatment concentrations of CAMs, especially low concentrations of caffeine since it showed effective inhibition of doxorubicin efflux and include PgP inhibitors and substrates using many combinations. Furthermore, information on the effects of the specific CAMs used in the current study on PgP expression and activity in MCF-7 cells should be further explored.

Studies frequently evaluate the mRNA levels of PgP and equate their findings to drug resistance. However, poor correlation between mRNA transcripts and protein expression is reported. This warrants further investigation of PgP at epigenetic, transcriptional, post-transcriptional, translational, and post-translational levels to better understand the drug transporter protein.

Exploring the expression of endoplasmic reticular stress transmembrane proteins namely PERK, ATF6, and IRE1 in MCF-7 cells treated with CAMs to further investigate the involvement of HMOX1 and other stress response proteins. Further investigations arising from this study should include evaluation of various cell lines and *in vivo* animal models using MCF-7 cells as a xenograft.

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APPENDIX

Appendix A: Research ethics approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Human Research Ethics Committee (Medical)

Research Office Secretariat:
Faculty of Health Sciences, Phillip Tobias Health Sciences Building, 3rd Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown, 2193
Private Bag 3, Wits 2050
Office email: HREC-Medical.ResearchOffice@wits.ac.za
Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CBP-190704-01 04/07/2019

TO WHOM IT MAY CONCERN:

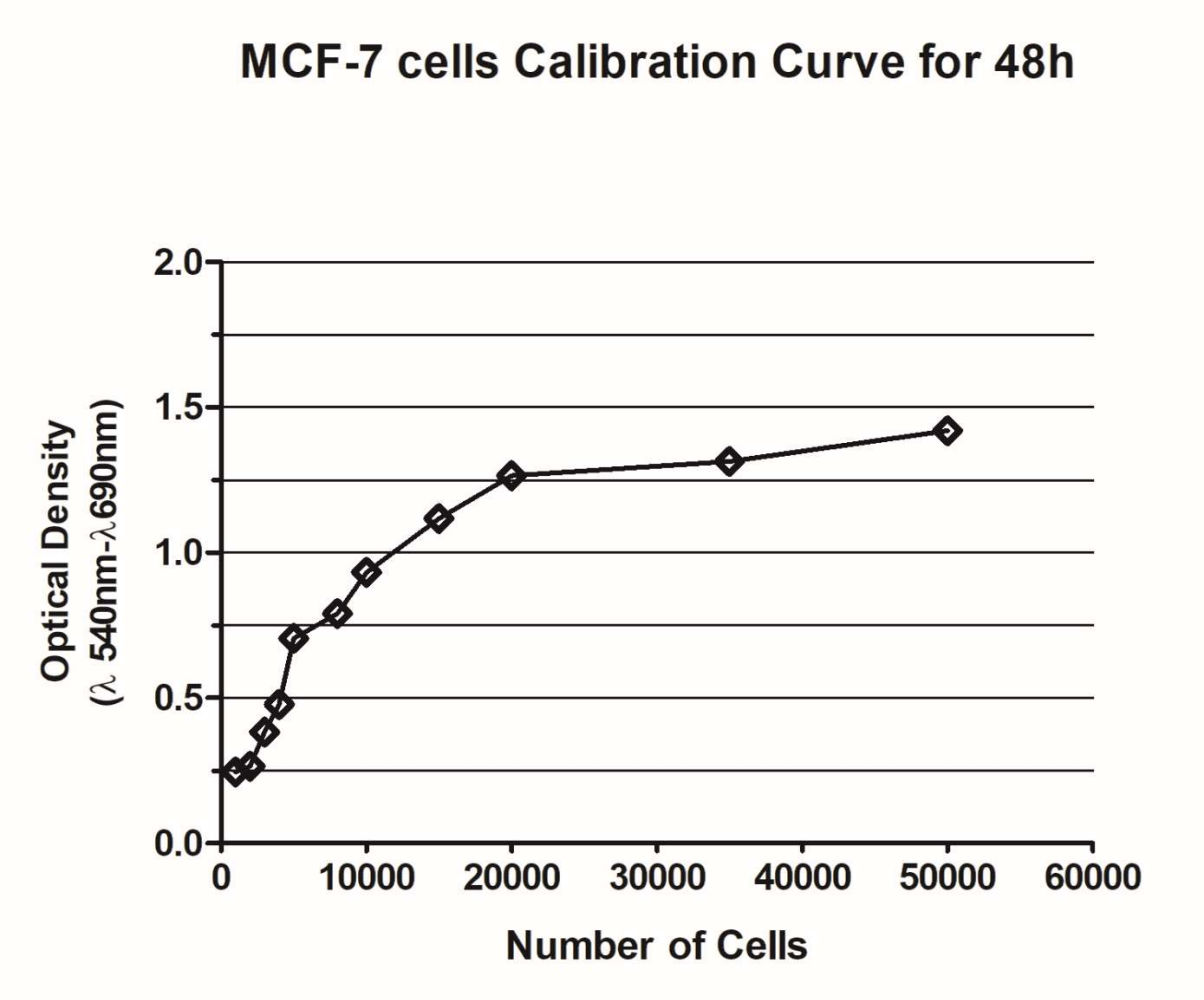
Waiver:	This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)
Investigator:	Mr Blake Green et al (Student no. 1872512)
Supervisors:	Dr G. Gabriels and Dr L. Harmse
Department:	Pharmacy and Pharmacology
Project title:	Evaluation of the effect of selected complementary and alternative medicines on the efficacy of cancer chemotherapeutic agents
Reason:	In vitro laboratory study. Using the MCF-7 human breast cancer cell line. No human participants or tissues will be involved in the study.

Dr CB Penny

Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu, Mapula Ramaila, Josh Ndlangamandla and Rhulani Mkansi

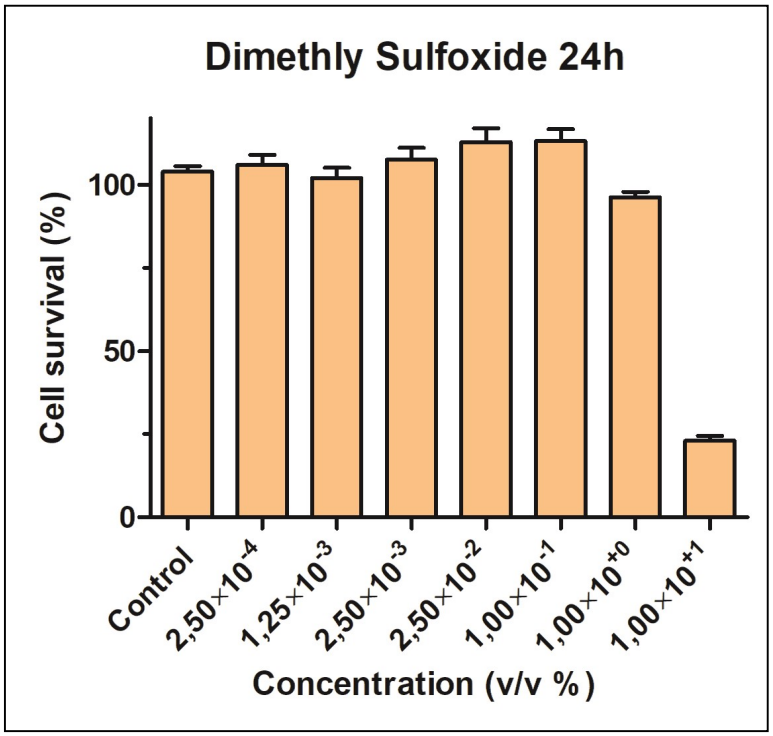
Appendix B: Spectrophotometry standard curve of MCF-7 breast cancer cell numbers after 48 hours

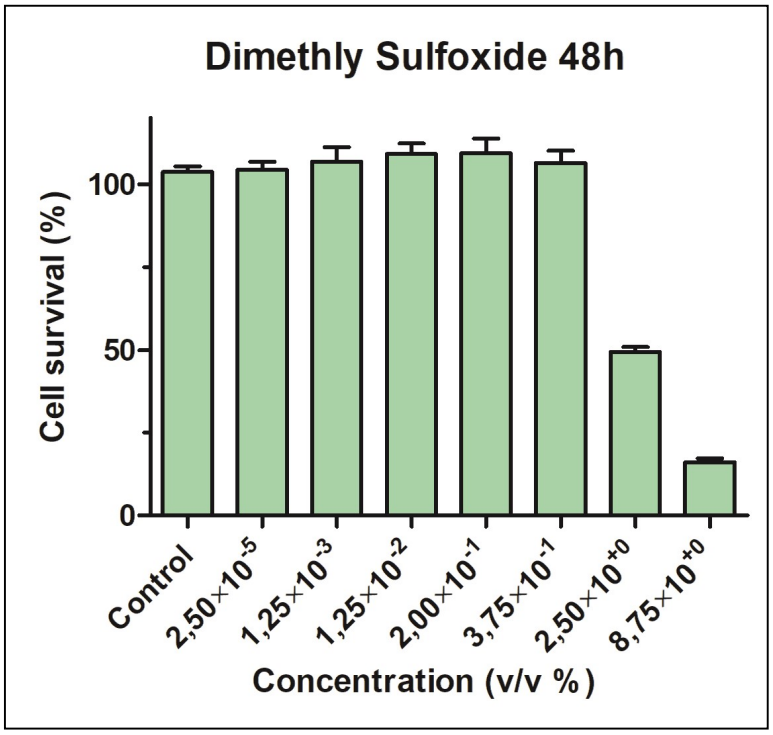


Appendix C: Statistical comparison of the IC_{50} values of doxorubicin and the active CAMs after 24h and 48h treatment periods

Common variable (time)	Test variable	p-value	Significance (p<0.05)
24h	doxorubicin **and** resveratrol	0.0002	Significant
	doxorubicin **and** synephrine	0.0002	Significant
	doxorubicin **and** caffeine	< 0.0001	Significant
48h	doxorubicin **and** resveratrol	< 0.0001	Significant
	doxorubicin **and** synephrine	< 0.0001	Significant
	doxorubicin **and** caffeine	0.0001	Significant

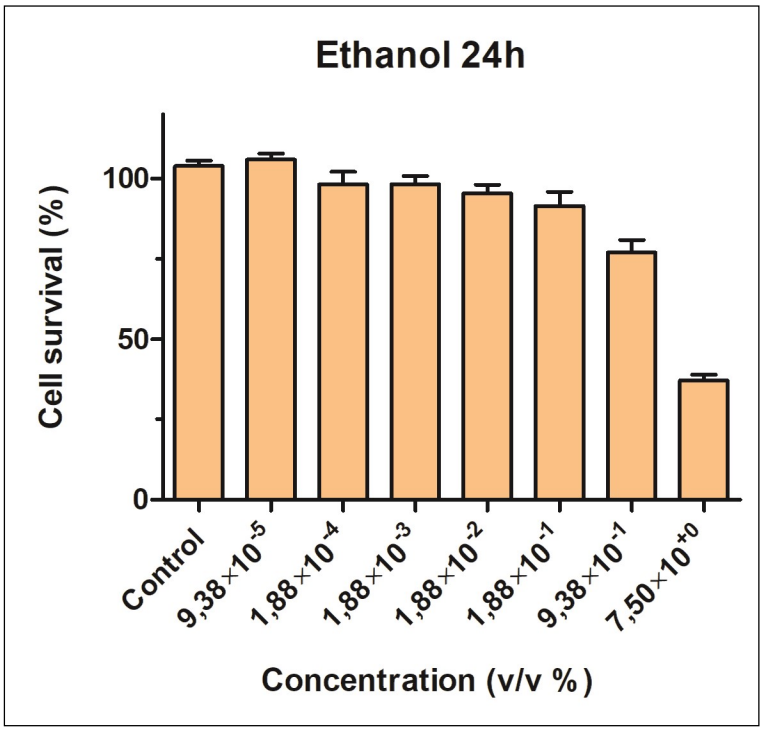
Appendix D: The effects of DMSO on MCF-7 breast cancer cells survival after 24h and 48h exposure

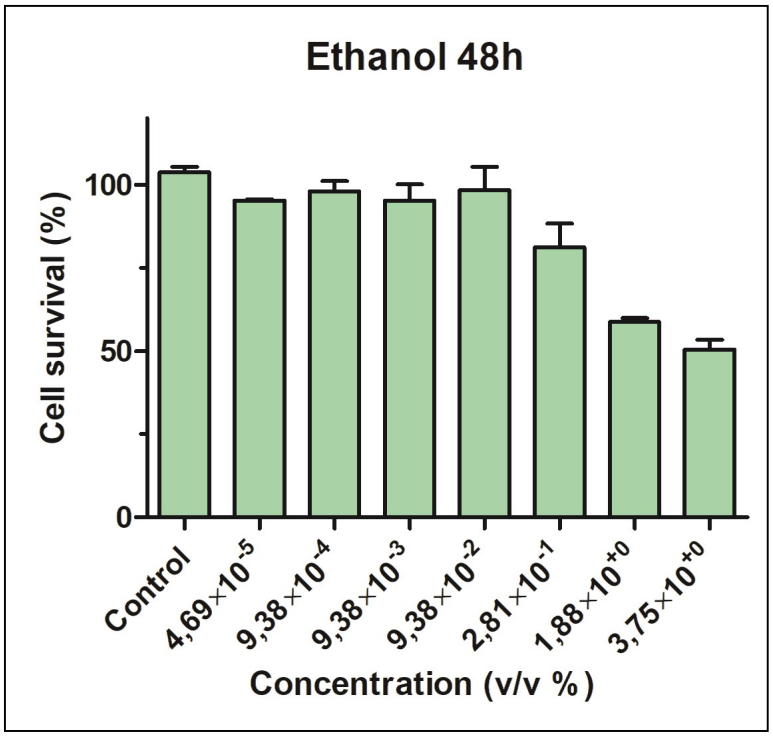




MTT cytotoxicity assay was used to determine cell survival of MCF-7 breast cancer cells and three experiments were repeated three times (n=3). The data is represented in the bar graphs as the mean and SEM.

Appendix E: The effects of ethanol on MCF-7 breast cancer cells survival after 24h and 48h exposure





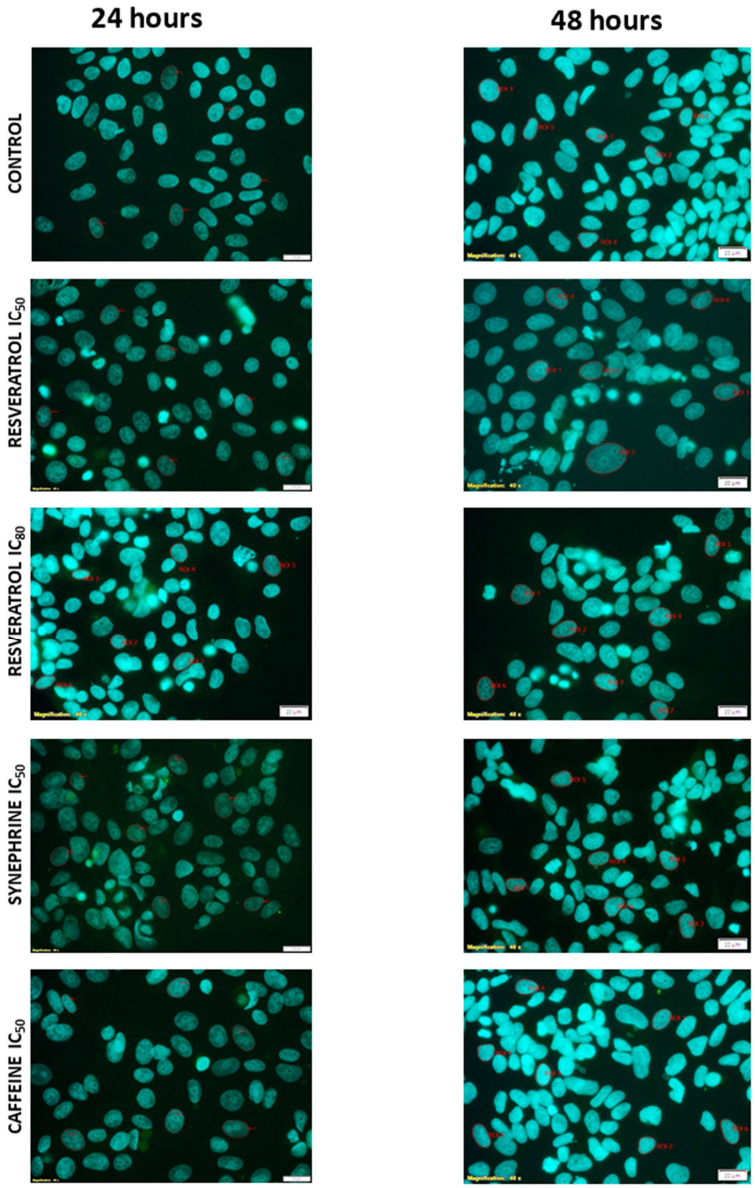
MTT cytotoxicity assay was used to determine cell survival of MCF-7 breast cancer cells and experiments were repeated three times (n=3). The data is represented in the bar graphs as the mean and SEM.

Appendix F: Statistical comparison of the mean calcein-AM accumulation in treatment groups against the untreated control after 24 hours treatment

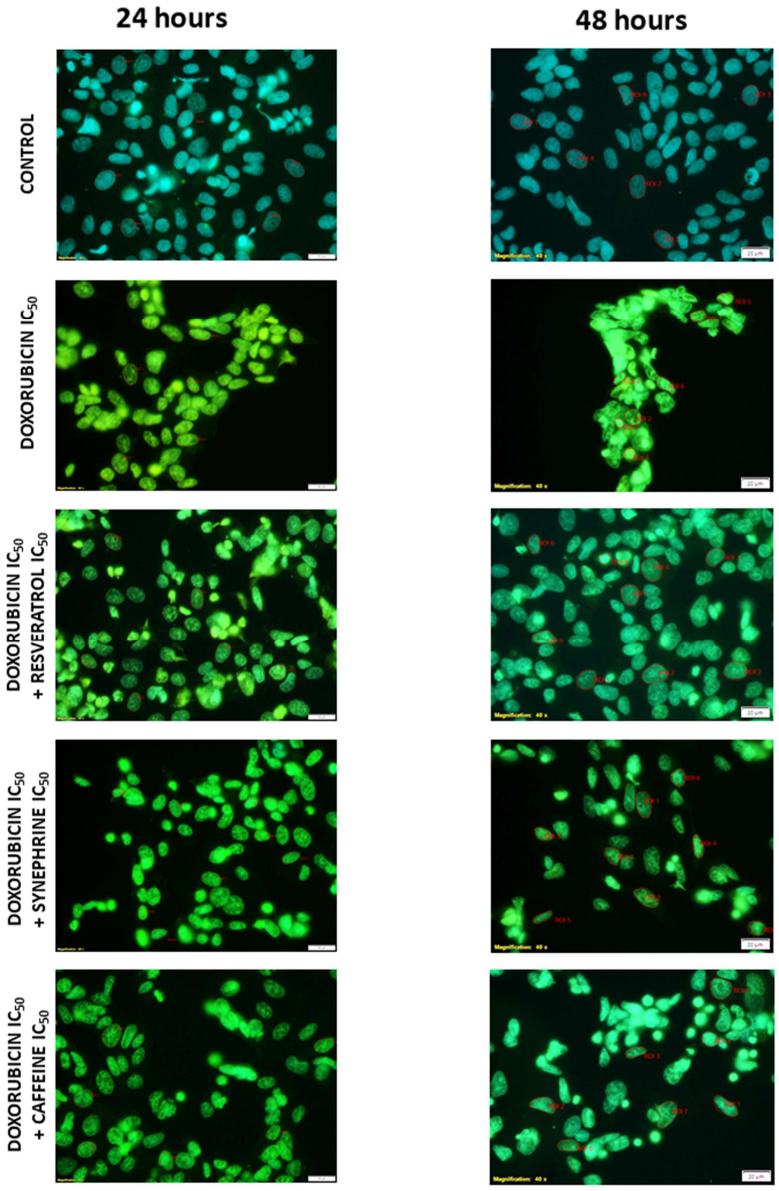
Determined using the Students' t-test followed by Welch's correction test.

Treatment concentration	Test variable (Treatment group)	p-value	Significance ($p<0.05$)
0.05 μM	doxorubicin	0.0016	Significant
	resveratrol + doxorubicin	<0.0001	Significant
	synephrine + doxorubicin	0.84	Non-significant
	caffeine + doxorubicin	0.49	Non-significant
0.5 μM	doxorubicin	<0.0001	Significant
	resveratrol + doxorubicin	<0.0001	Significant
	synephrine + doxorubicin	0.0019	Significant
	caffeine + doxorubicin	0.22	Non-significant
1 μM	doxorubicin	<0.0001	Significant
	resveratrol + doxorubicin	<0.0001	Significant
	synephrine + doxorubicin	<0.0001	Significant
	caffeine + doxorubicin	0.073	Non-significant

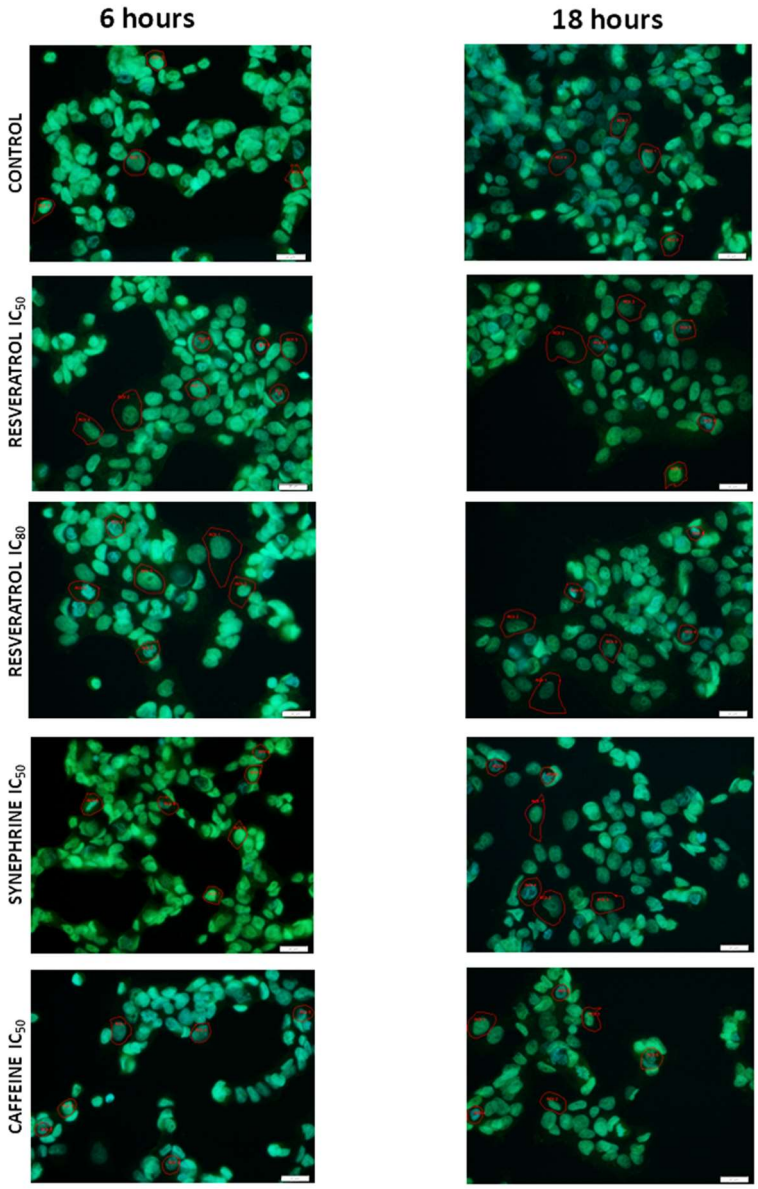
Appendix G: The selected whole frame and ROIs used in colocalisation analysis of PgP in MCF-7 breast cancer cells treated with active CAMs after 24 hours and 48 hours



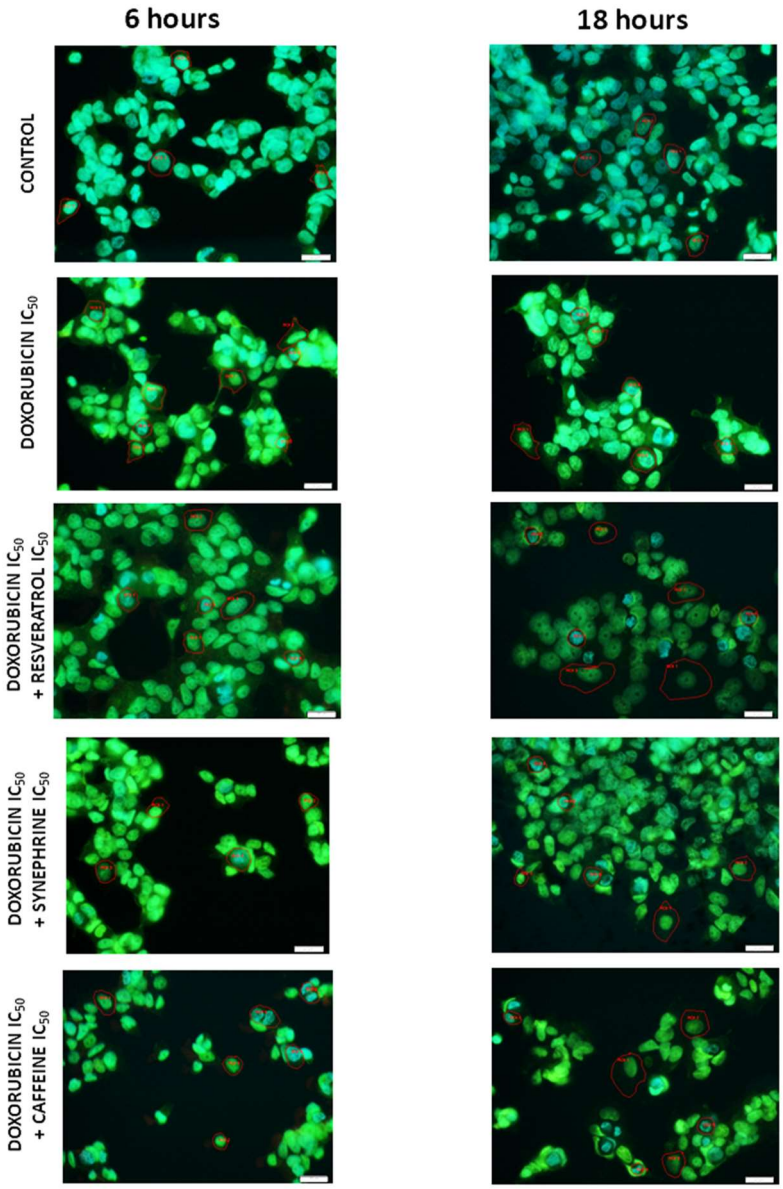
Appendix H: The selected whole frame and ROIs used in colocalisation analysis of PgP in MCF-7 breast cancer cells treated with doxorubicin and combination with CAMs after 24 hours and 48 hours



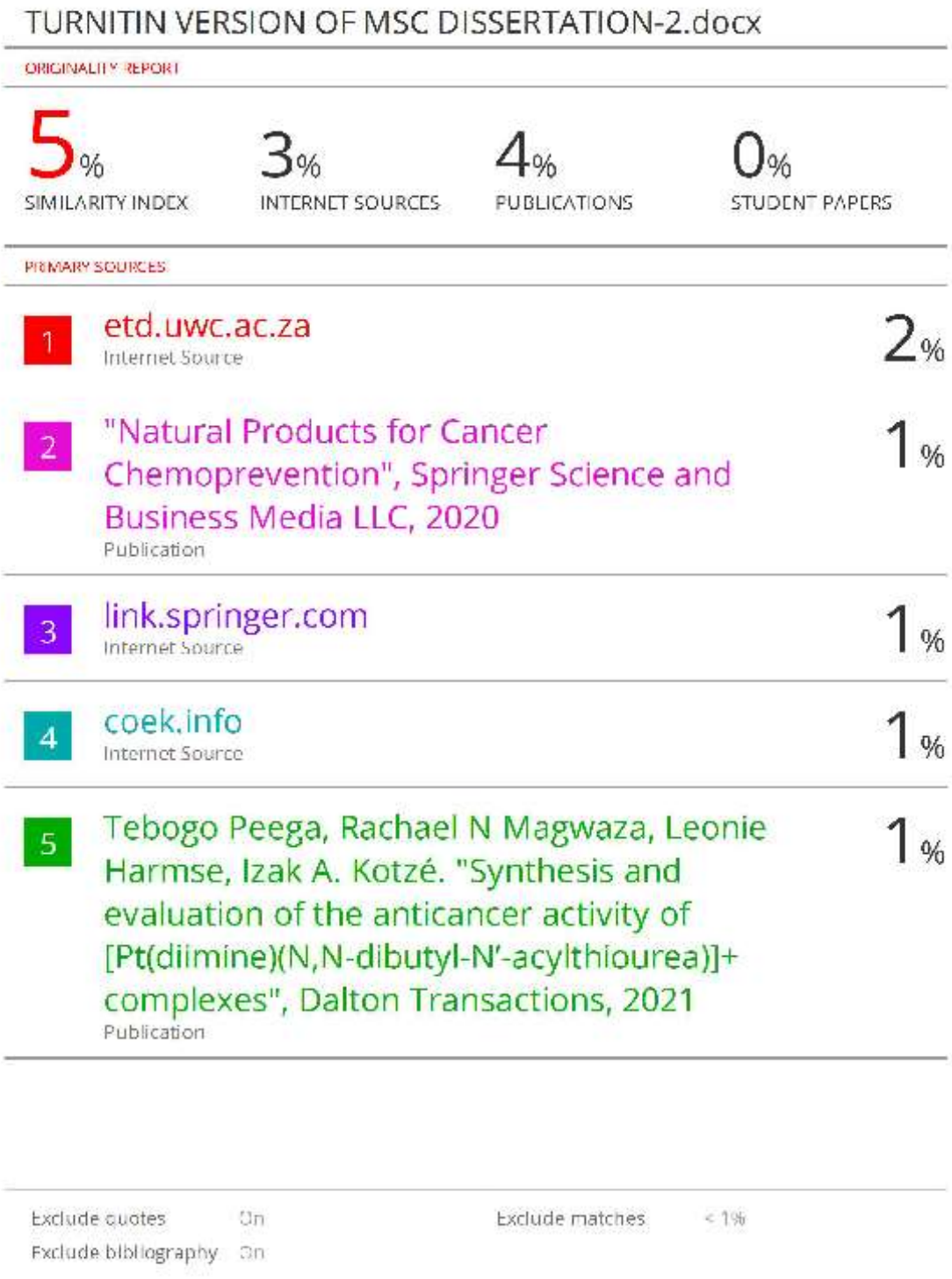
Appendix I: The selected whole frame and ROIs used in colocalisation analysis of HMOX1 in MCF-7 breast cancer cells treated with active CAMs after 6 hours and 18 hours



Appendix J: The selected whole frame and ROIs used in colocalisation analysis of HMOX1 in MCF-7 breast cancer cells treated with doxorubicin and combination with CAMs after 6 hours and 18 hours



Appendix K: Turnitin Report



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