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Synergistic cytotoxicity of *Solanum aculeastrum* with 4-hydroxytamoxifen in the MCF-7/TAMR-1 breast adenocarcinoma cell line

by

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Declaration

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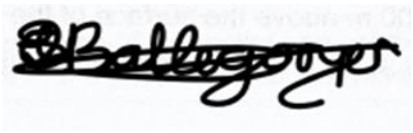

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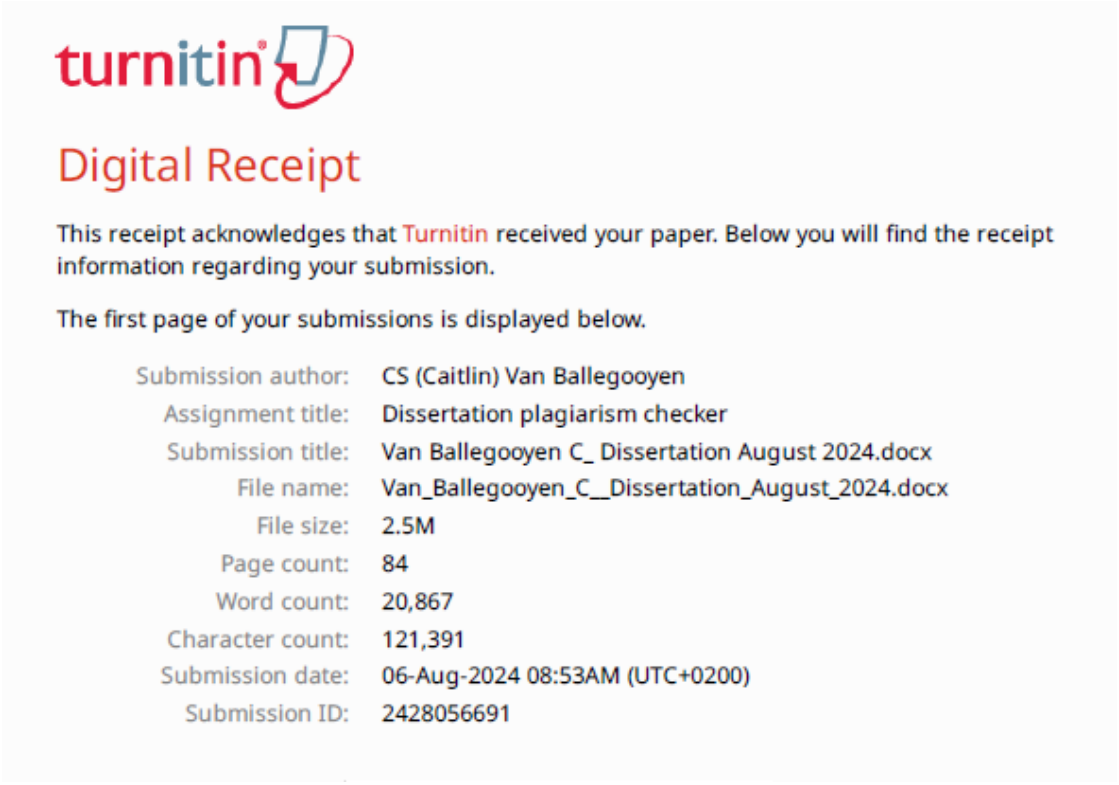

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Dedication

To my entire family and friends for the continuous support, motivation, and confidence that you provided me throughout the completion of my studies and for helping me overcome the multitasking challenges I faced for the duration of this degree.

To my supervisor and co-supervisor for their continuous support, empathy and most importantly their patience, especially during the write-up process of this research study.

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Executive Summary

Introduction: Breast cancer, the leading cause of death in woman worldwide, is characterised by its receptor status. Most patients (70%) are diagnosed with oestrogen receptor (ER)-positive breast cancer. Tamoxifen, a selective oestrogen receptor modulator (SERM), is the preferred chemotherapeutic treatment, however, breast cancer cells may become resistant to its active metabolite (4-hydroxytamoxifen [4-OH-TAM]). Chemoresistance may be due to alterations to ER, reducing chemoresponsiveness. Herbal medicines that are used for the treatment of cancer, such as *Solanum aculeastrum* (soda apple), may provide a combinational effect to reduce breast cancer cell growth and viability. The study assessed the synergistic cytotoxicity of *S. aculeastrum* with 4-OH-TAM in tamoxifen-resistant MCF-7 breast adenocarcinoma cells.

Methods: Tentative phytochemical identification was conducted using ultra-performance liquid chromatography. Inherent cytotoxicity of methanol (ME) and hot water (HWE) extracts alone and in combination with 4-OH-TAM was assessed at 24 h, 48 h and 72 h via sulforhodamine B staining and resazurin conversion assays. Combinational effects were determined using a checkerboard assay and CompuSyn. The effect of the most synergistic combination on cell viability (fluorescence microscopy [Hoechst, acridine orange and propidium iodide]), redox status (reactive oxygen species [ROS] and reduced glutathione [GSH] levels via dichlorofluorescein and monochlorobimaine fluorescence. respectively) and ER-expression (Western blotting) was assessed after 24 h exposure.

Results: A chromatographic fingerprint was produced. The methanol (ME) and hot water extract (HWE) displayed phytochemicals in each extract with solamargine, solasodine, solasonine, and solaculine. Dose-dependent cytotoxicity was observed after 24 h for 4-OH-TAM, ME, and HWE. The most synergistic combination was 10.05 µg/mL HWE and 5.83 µg/mL 4-OH-TAM, with a combinational index of 0.78289. The combination displayed significantly ($p<0.001$) reduced cell density (76.65%) The combinational treatment induced cellular blebbing and rounding, suggesting apoptosis in comparison to individual treatments. The combination abolished ROS levels after 24 h treatment, with a 30% decrease in GSH levels. A non-significant decrease in relative ER-α expression was observed after combinational treatment.

Discussion: The combination showed signs of apoptosis with similar findings been reported for steroidal alkaloids identified in *S. aculeastrum*. The generation of ROS contributes to apoptosis of breast cancer cells, alongside a reduction of GSH availability. Although ROS was not increased after 24 h, the GSH levels reduced, suggesting destabilisation of the redox status of cells. This destabilisation appears to have caused reductive stress rather than oxidative stress, with reduced GSH levels that may hinder detoxification of 4-OH-TAM. The reduced expression of ER-α reduces the proliferative capabilities of cells as less oestrogen will be able to bind and promote growth of breast cancer cells. *Solanum* constituents, like solasodine have been shown to dysregulate ER, which may be like what has been observed in the present study.

Conclusion: Combining 4-OH-TAM alongside the HWE increased the cytotoxicity in reference towards the MCF-7/TAMR-1 breast adenocarcinoma cell line via possible apoptotic changes, as well as destabilisation of redox status and dysregulated ER-α expression. Reduced GSH levels may hinder detoxification of 4-OH-TAM and contribute to reductive stress, impacting proliferation and cellular functions. Bioactivity is ascribed to steroidal alkaloids as seen in the extracts. Combinational treatment using *S. aculeastrum* needs to be further assessed to determine the potential for further development as an anticancer combination.

Keywords: Apoptosis**,** breast cancer, oestrogen receptor, *Solanum aculeastrum*, traditional medicine, tamoxifen resistance.

Study outputs

Poster presentation

Van Ballegooyen C, Mlambo-Bekoun Adja S, Flepisi B, Cordier W. Synergistic cytotoxicity of *Solanum aculeastrum* with 4-hydroxytamoxifen in tamoxifen-resistant MCF-7 breast adenocarcinoma cells. Health Sciences Faculty Day, University of Pretoria, South Africa, 23 and 24 August 2022.

South African Society for Basic and Clinical Pharmacology

Van Ballegooyen C, Mlambo-Bekoun Adja S, Flepisi B, Cordier W. Synergistic cytotoxicity of *Solanum aculeastrum* with 4-hydroxytamoxifen in tamoxifen-resistant MCF-7 breast adenocarcinoma cells. 57th Annual Conference of the South African Society for Basic and Clinical Pharmacology, Rhodes University, South Africa, 24 to 27 August 2023.

Eduvos Research Indaba

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Table of Contents

List of Figures

List of Tables

List of Equations

List of Symbols and Abbreviations

Symbols and numerical values

°C	Degrees centigrade
%	Percentage
%w/v	Percentage weight to volume
α	Alpha
β	Beta
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength
mg/mL	Milligram per millilitre
µg/mL	Microgram per millilitre
M	Molar
mM	Millimolar
µm	Micrometer
µM	Micromolar
µL	Microlitre
4-OH-TAM	4-hydroxytamoxifen

A

AAPH	2.2’-azibis-(2-amidinopropane dihydrochloride)
Akt	Protein kinase B

B

BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2

BSA	Bovine serum albumin
C	
CI	Combinational index
CYP450	Cytochrome P450 enzymes
D	
DCF	Dichlorofluoroscein
DICS	Ductal carcinoma *in situ*
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	
E2	Estradiol
ER	Oestrogen receptor
ER+	Oestrogen receptor-positive
ERE	Oestrogen receptor elements
ER-α	Oestrogen receptor-alpha
ER-β	Oestrogen receptor-beta
F	
FCS	Foetal calf serum
G	
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GATA-1	Erythroid transcription factor
GSH	Reduced glutathione

H

HER-2	Human epithelial growth factor hormone-2
HWE	Hot water extract

I

IARC	International Agency for Research on Cancer
IBC	Invasive breast cancer

M

MAPK	Mitogen-activated protein kinase
ME	Methanol extract
Mtor	Mammalian target of rapamycin
MTT	4,5-dimethyl-2yl-2,5-diphenyl-2H-tetrazolium bromide assay

N

NFM	Non-Fat Milk
NC	Negative control

P

PBS	Phosphate buffered saline
PC	Positive control
Pgp	P-glycoprotein
PI3K	Phosphoinositide-3 kinase
PPD	Potassium peroxydisulfate
PR	Progesterone receptor
Prx5	Peroxiredoxin-5
PVDF	Polyvinylidene difluoride

R

RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RZN	Resazurin

S

SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SRB	Sulforhodamine B
Src	Protooncogene tyrosine
SWR	Standard working reagent

T

TBST	Tris-buffered saline Tween-20
TGF-β	Transforming growth factor-beta
TP53	Tumour suppressor gene

V

VC	Vehicle control

Chapter 1: Introduction

1.1 Cancer, prevalence, and risk factors

Cancer, termed as the uncontrollable growth of cells, propagates due to various changes that occur to their physiological processes[1]. The hallmarks promote the escape of cells from cell death and allow for continuous proliferation of cancerous cells.[1, 2] Cancer is the leading cause of death worldwide, accounting for 10 million deaths in 2020[3] with prominent concern in low- and middle-income countries (Figure 1.1).[4] Risk factors associated with an increase in the prevalence of cancer include, but are not limited to; genetic predisposition, physical inactivity, smoking and excess body weight.[4] In addition to symptoms and side effects of treatment, cancer also exerts financial pressure on the patient, families of the diseases patients and the government when seeking public healthcare. Cost of treatment, premature death and loss of household income are some factors causing an increase in the economic burden of cancer.[4] An improvement in lifestyle choices, early detection, reduced cost of anti-cancer drugs and cheaper alternative therapies can decrease the burden.[5]

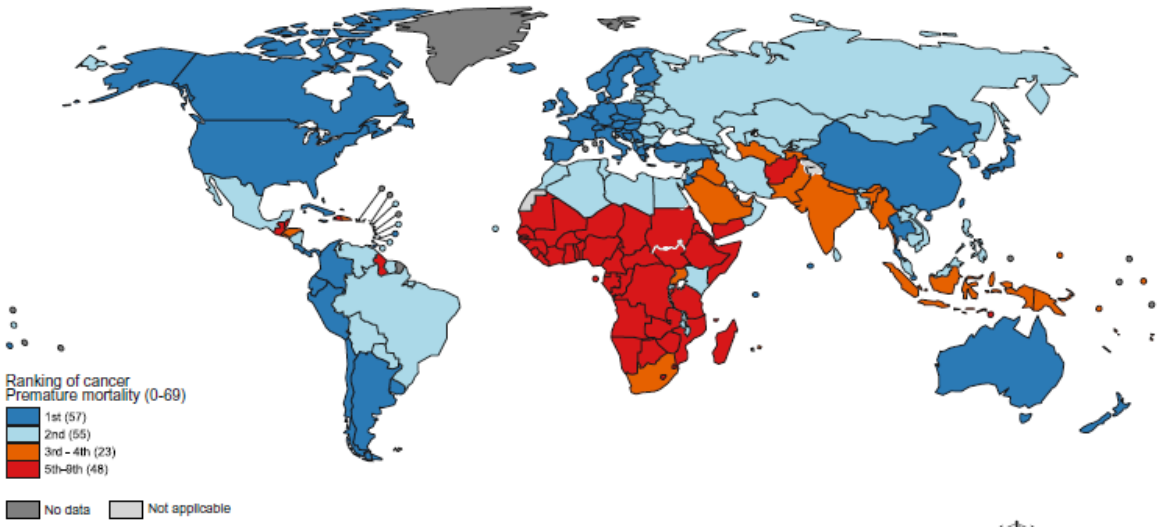


Figure 1.1: A global map showing the national ranking of cancer as causes of death in 2019.

Reproduced with permission from the International Agency for Research on Cancer (IARC).[6]

All cancers display unique characteristics, commonly known as hallmarks, which complicate treatment and promote cancer progression.[2] Tumorigenesis is a multi-step

process where healthy cells transform into cancerous cells through the acquisition of biological activities to sustain tumour progression.[7] This allows for more research of molecular targets and treatment options to improve the treatment thereof. These unique characteristics and biological acquisitions are known as the Hallmarks of Cancer (Figure 1.2).[2, 8] Different cancer types display several types of hallmarks, and thus not all hallmarks will be implicated for each cancer. As such, medical treatment of cancer is diverse and unique to the considerations thereof, invariably impacting the prognosis of some cancers.[7] A brief description of the various hallmarks contributing to the progression of breast cancer are listed in Table 1.1. The focus of this study is on breast cancer, specifically oestrogen receptor positive-alpha (ERα) breast cancer.

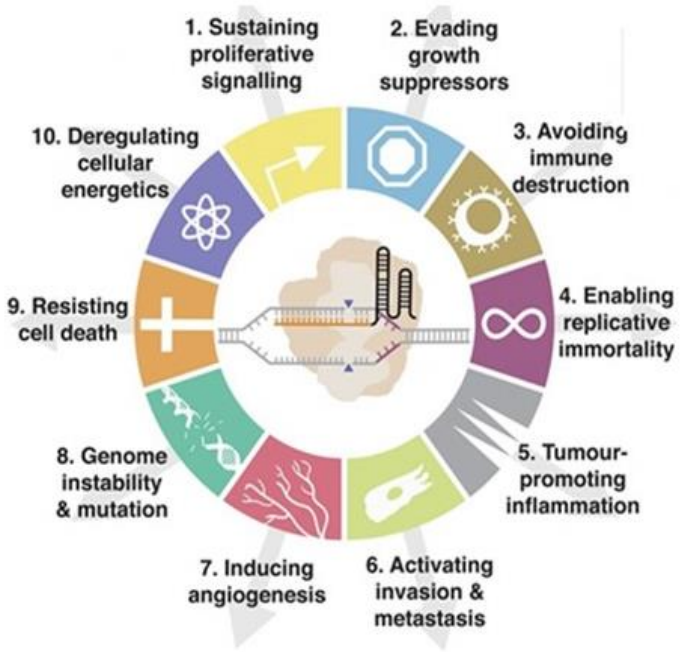


Figure 1.2: Hallmarks of cancer.[2] This image is created under an Attribution Non-Commercial- No Derivatives Creative Commons licence, https://creativecommons.org/licenses/by-nc-nd/4.0/.

Table 1.1: The eight hallmarks and two enabling factors with characteristics specifically related to breast cancer.

Hallmark	Description
Sustained proliferative signalling	Normal tissues can strictly control cellular growth and proliferation. Breast cancer cells can evade this control through receptor tyrosine kinases (RTKs) that initiate proliferative and cell survival pathways. Targeted therapies are used to target the specific RTKs involved in breast cancer, such as tamoxifen to treat oestrogen receptor-positive (ER+) subtypes of breast cancer.[8]
Evading growth suppressors	The tumour suppressor gene, *TP53*, play a vital role in governing apoptosis, gene expression and metabolism. If signalled, it will halt cell cycle progression. Mutations in the *TP53* gene account for 25 to 30% of breast cancer diagnoses, resulting in the inactivation of this tumour suppressor gene leading to the inability of the gene to identify mutations and deoxyribonucleic acid (DNA) repair.[8]
Avoiding immune destruction	Cancerous cells can inhibit certain components of the immune system,[2] such as natural killer cells that are inhibited through the secretion of transforming growth factor-β (TGF-β). TGF-β is an immunosuppressive agent secreted by cancerous cells.[9] Breast cancer cells use these checkpoint proteins mentioned above to avoid being invaded and attacked by immune cells.[8]
Enabling replicative immortality	Telomers are absent in normal chromosomes with their presence in all breast cancer cells, playing a vital role in preventing signalling for cellular apoptosis or senescence. The inability to undergo apoptosis or senescence contributes to the immortality of breast cancer cells.[8]
Tumour promoting inflammation	Promoting inflammation results in recruitment of enzymes that facilitate angiogenesis and metastasis leading to cellular proliferation.[8, 9]
Activating invasion and metastasis	Cancerous cells reduce the expression of E-cadherin (adhesion molecule responsible for maintaining quiescence) resulting in evasion of surrounding tissues.[2, 7] This is commonly referred to as the invasion metastasis cascade.[8]
Inducing angiogenesis	Tumours undergo an ‘angiogenic switch,’ allowing for angiogenesis to continuously take place, leading to the formation of new blood vessels around the cancerous tissue. This enabled a steady supply of nutrients and oxygen for the tumour to sustain growth and proliferation. Angiogenesis is a common characteristic in all cancers including breast cancer.[2, 8, 9]
Genome instability and mutation	High rates of genome mutations and instability contribute 10% towards breast cancer diagnoses. The most common genetic mutation resulting in the diagnoses and development of breast cancer is in the breast cancer susceptibility genes 1 and 2 (*BRCA1* and *BRCA 2*) responsible for DNA repair. The most common hormone related ER+ breast cancer, is a mutation of the BRCA2 gene.[8]

Hallmark	Description
Resisting cell death	Reduced TP53 expression results in mutagenic cells to continuously divide and surpass activation for apoptosis.[2, 9]
Deregulating cellular genetics	Deregulated cellular genetics enable the cancerous cells to grow and divide uncontrollably,[2] resulting in increased energy for cell growth due to the increase in glucose consumption of tumour cells, shifting energy consumption from aerobic mitochondria-mediated oxidative phosphorylation to anaerobic glycolysis.[8]

1.2 Breast cancer

1.2.1. Breast cancer, prevalence and risk factors

Breast cancer is the most common cancer worldwide, characterised by cancerous growth of the breast cells of biological female individuals (although it may develop in biological males as well).[6] From 2018 to 2020, breast cancer incidence has increased from 2.1 million to 2.26 million new cases (morbidity) and 627 000 to 685 000 deaths (mortality).[10] In South Africa in 2018, 13.1% of all new cancer cases (107 467) and 8.2% of deaths (57 373) were breast cancer-related (Figure 1.3).[10, 11]

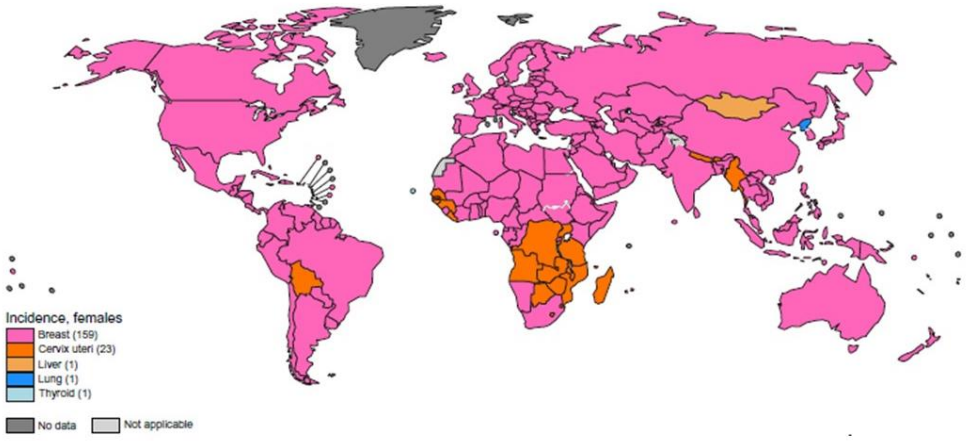


Figure 1.3: Global map showing the ranking of breast cancer as the most diagnosed cancer in females per country in 2020. Reproduced with permission from the IARC.[6]

Developing countries, such as South Africa, have a higher prevalence of breast cancer and poorer survival rate.[12] Risk factors include, but are not limited to age, westernised diets (increased fat and preservative intake), obesity, race, genetic predisposition, lack of screening, and inadequate healthcare facilities.[4] Survival rates of cancer also differ between developing and developed countries. Developed countries have an 80% survival rate, in comparison to developing countries, with survival rates of 40%, which may be

due to late diagnosis and lack of healthcare in some areas.[12] Many factors contribute to the high prevalence, incidence, and poor prognostic outcomes in developing countries, such as limited screening and treatment resources and lack of diagnostic screening. Inadequate healthcare facilities in rural areas lead to difficulties in maintaining treatment regimens, thus reducing the efficacy thereof.[11] The risk factors and increased incidence of breast cancer emphasises the need for new treatment strategies to aid in the prevention and treatment of breast cancer. Although most cases reported are for woman between ages 45 and 65, breast cancer may arise in younger individuals as well, with individuals of African or Caucasian descent are also more at risk than those of Hispanic lineage.[13] Genetic susceptibility also increases the risk of breast cancer, for example, mutations in the breast cancer tumour suppressor genes (*BRCA1* and *BRCA2*) are associated with an increase in hereditary predispositions, which leads to familial breast cancer.[12-14]

1.2.2. Characterisation of breast cancer according to cell and receptor and receptor type

Breast cancer is characterised by cell type. Many breast cancers originate in the duct or lobules, with ductal carcinomas being the most common type of breast cancer represented in Figure 1.4.[15] Ductal carcinoma *in situ* (DCIS) refers to the growth and proliferation of epithelial cells within the underlying basement membrane, thus the growth remains within a specific area which may lead to breast cancer.[15] As DCIS progresses, it may proliferate beyond the basement membrane, resulting in invasive breast cancer (IBC). Both DCIS and IBC may eventually spread outside the breast tissue and throughout the body, indicative of metastasis.[16] Metastatic cancers are difficult to treat as targeted therapy is no longer an option.[17]

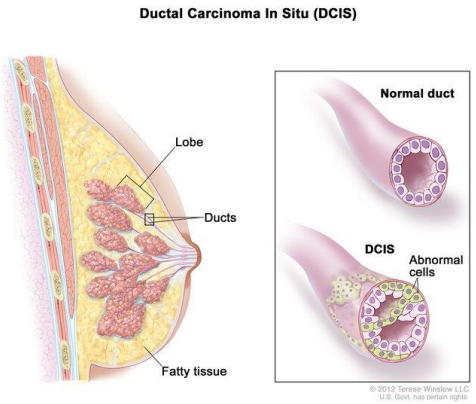


Figure 1.4: Illustration and description of the development and progression of breast cancer from ductal carcinoma *in situ* through to invasive breast cancer. © (2016) Terese Winslow LLC, U.S. Govt. has certain rights.

Breast cancer can also be classified according to the receptor profile that is expressed. Relevant receptors include the oestrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER-2).[18] The level of receptor expression may fluctuate between cancer types; some may express all three receptors, while others may be poorly expressed.[16] The ER is the most expressed receptor, accounting for up to 70% of breast cancer, and thus referred to as ER-positive breast cancer.[16] The ER occurs as two subtypes, namely ER-alpha (ERα) and ER-beta (ERβ).[19] These receptors mediate oestrogen signalling via ligand-dependent transcription factors and are necessary to maintain cellular proliferation.[19] The ERα-subtype is mostly expressed in mammary glands, ovaries, uterus, and adipose tissues, and maintains cellular growth and proliferation.[16] 70% of breast cancer cases are ERα-positive,[20] making it an attractive target in pharmacotherapy. The ERβ-subtype is mostly expressed in the prostate, ovaries, immune system, and mammary gland, and is responsible for preventing hyperproliferation in the uterus and breast tissues.[16] Both subtypes function together in regulating the function of the cardiovascular system and ovaries.[16] ER-α activation is closely related to increased cellular proliferation whereas ER-β exerts the opposite, antiproliferative effect. Together, ER-α and ER-β function to maintain homeostasis of cellular proliferation.[16] The over-expression of ERα disrupts the antiproliferative control afforded by the ERβ, and thus a higher frequency of oestrogen-ERα binding occurs to promote growth and proliferation.[19, 21]

Oestrogen, the endogenous ligand of the ER, is a steroidal hormone synthesised from cholesterol.[16, 22] Oestrogen mediates several physiological processes, including reproductive control, secondary sexual characteristic development, cardiovascular function, mediation of inflammation, metabolism, stem cell survival, regulation of body temperature, memory and libido, menstruation, and breast tissue growth.[23] The most usual form of oestrogen is oestradiol (E2).[22]

In breast tissue, oestrogen is responsible for cell growth and proliferation via the ER.[16] The E2/ERα-signalling pathway, either activated genomically or extranuclearly, is responsible for regulating cell proliferation and apoptosis.[22] Within the genomic pathway, upon binding of E2 to ERα, ER is displaced and enters the nucleus to bind to oestrogen receptor elements (ERE). Binding to ERE promotes target genes' expression with subsequent cellular proliferation (Figure 1.5).[22] The extranuclear pathways may occur by one of two ways: the mitogen-activated protein kinase (MAPK) and the proto-oncogene tyrosine/phosphoinositide-3 kinase/protein kinase B/mammalian target of rapamycin (Src/PI3K/Akt/Mtor) pathway. The MAPK pathway is responsible for the regulation of the Src/PI3K/Akt/Mtor pathway. The main extranuclear pathway is the E2/ERα binding which activates the Src/PI3K/Akt/Mtor pathway to regulate transcription and translation of proliferative genes.[22]

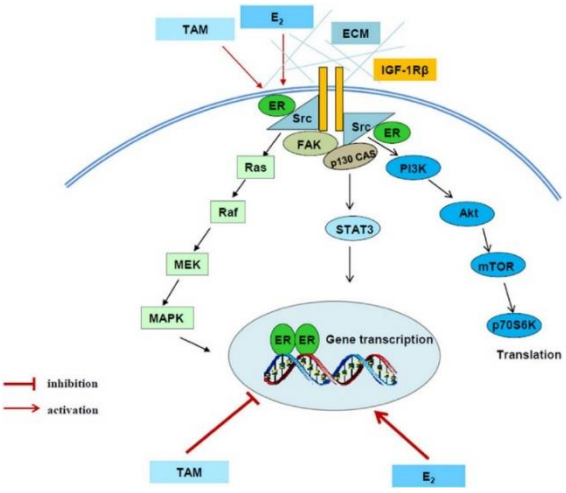


Figure 1.5: E2/ERα signalling pathways responsible for cellular proliferation, cell cycle regulation and apoptosis. E2: Oestradiol, ER: Oestrogen receptor alpha, ERE: Oestrogen receptor elements. Src: Proto-oncogene tyrosine receptor kinase, PI3K: Phophatidylinositol-4,5-bisphosphate 3-kinase, Akt: Protein Kinase B, mTOR: Mammalian target of rapamycin gene.[22] This image is created under an Attribution Creative Commons Licence, https://creativecommons.org/licenses/by/4.0/.

Modulation of these receptor types have been used to prevent, among others, breast cancer and prostate cancer.[19] Activation of this pathway leads to resistance to therapies that target this pathway, resulting in tumour invasion and metastasis in breast cancer, notably the most common hallmark associated with breast cancer.[24, 25] Additionally, genetic mutations alter the metabolism of oestrogen, such as those facilitated by cytochrome P450 (CYP) enzymes, specifically CYP1B1.[21, 26] The CYP1B1 enzyme is responsible for 4-hydroxylation of E2 into quinone metabolites. Mutations of the CYP1B1 enzyme result in the production of pro-oxidant quinone metabolites that damage DNA through alkylation or oxidation of proteins. Damage to DNA results in deletion mutations contributing to uncontrollable growth and proliferation epithelial breast cells.[19, 26] Culmination of such effects may lead to breast cancer.[19, 27]

1.2.3. Treatment options for breast cancer

Depending on the severity and stage of breast cancer, different treatment options are considered. Surgery (the complete resection of the cancer if not metastasised), chemotherapy and radiation are commonly used in breast cancer.[12] Chemotherapy is usually used in conjunction with other treatment options. Use of chemotherapeutics before or after surgery is common, depending on the intention of the medicine such as complete eradication of remnants of tumour cells to prevent re-initiation of growth of the tumour cell.[12, 28]

Targeted therapy (such as hormonal treatment and monoclonal antibodies) is more selective and beneficial treatment options but rely on the breast cancer presenting with amenable receptor profiles.[12] Late-stage cancer is often diagnosed as such due to the spread of cancerous tissues throughout the body resulting in metastasis.[29] The treatment approach for late-stage cancer is to provide palliative care to improve the patient's quality of life and to extend life expectancy A targeted approach, such as hormonal therapy, displays the highest efficacy against cancer. Targeted therapy is dependent on the receptor expressed.[12, 30] Tamoxifen is the 'gold standard' of treatment for ER-positive breast cancer.[12]

1.3. Treatment of oestrogen receptor-positive breast cancer

Tamoxifen is a selective oestrogen receptor modulator where it targets breast cancer cells that express ER.[18] Breast cancer cells that are ER-negative will not respond to

tamoxifen treatment due to its selectivity, and thus it will not work effectively as an anticancer agent.[12]

Given the biological variation in breast cancer, treatment not only depends on the patient characteristics, but also on the classification of the cancer itself. Cancers that are positive for ER, PR or HER2 expression are typically treated via targeted therapy, while those lacking them present with more complexity as generalised cytotoxic mechanisms are needed.[18, 31] Tamoxifen is the most common targeted therapy used for ER-positive breast cancer.[30]

1.3.1. Tamoxifen

Tamoxifen is a pro-drug requiring bioactivation after oral administration through metabolism into two active metabolites: N-desmethyltamoxifen and trans-4-hydroxytamoxifen (Figure 1.6). The trans-4-hydroxytamoxifen metabolite (4-OH-TAM) has the most potent anti-oestrogen activity, and thus is considered the biological active molecule against ER-positive breast cancer.[19, 32] Tamoxifen induces a conformational change in the ER by binding to the receptor. This conformational change results in the blockage or alteration of expression of oestrogen dependent genes.[32] In breast tissue, tamoxifen acts as a competitive antagonist of oestrogens by binding to the ER (Figure 1.6),[19] inhibiting cell growth and proliferation, and reducing tumour growth and size.[33]

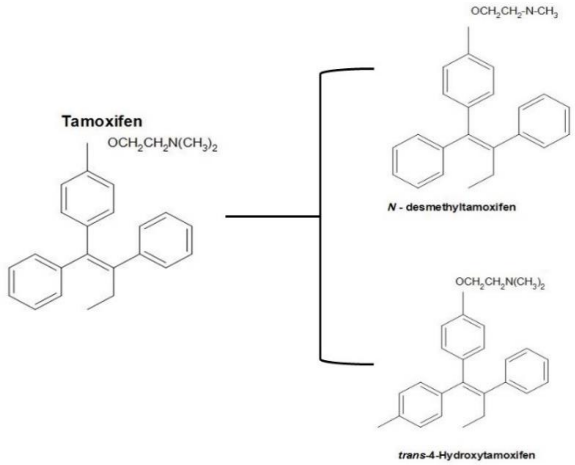


Figure 1.6: Tamoxifen is metabolised into two metabolites namely N-desmethyltamoxifen and trans-4-hydroxytamoxifen. Trans-4-hydroxytamoxifen is the active metabolite which exerts is anti-estrogenic activity at the oestrogen receptor as a selective oestrogen receptor modulator. Figure created by the author.

Tamoxifen is also prescribed as a preventative agent for woman with an elevated risk of developing breast cancer. The use of tamoxifen is extremely popular as it does not present with the damaging side effects of alternative treatments, such as aromatase inhibitors' increased risk of osteoporotic fractures.[34] As tamoxifen is the "gold standard" of treatment, most patients that are prescribed tamoxifen, use it as a long-term treatment regimen for up to five years, which affords patients 18% reduced death rate when compared to patients on two-year treatment.[35] There is a 46.5% increase in the recurrence of breast cancer in patients on long-term treatment of tamoxifen. The recurrence occurs after 15 years of treatment (25.1%), with 21.4% recurrence starting as early as five years after treatment.[35]

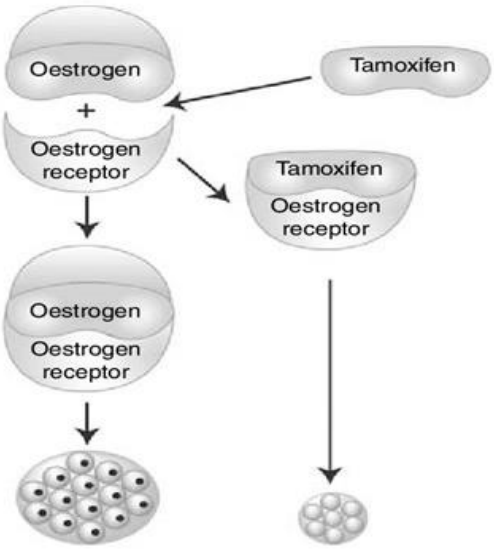


Figure 1.7: Function of tamoxifen as a selective oestrogen receptor modulator. Image permission using an attribution Creative Commons licence, http://creativecommons.org/licenses/by-nc-sa/3.0/.

1.3.2. Tamoxifen-resistant phenotypes and mechanisms

Drug-resistance during breast cancer treatment remains a challenge.[36] With targeted therapy being the number one treatment option, there stands a substantial risk of resistance, leading to patient relapse.[36] In recent years, resistance to tamoxifen has become a growing problem,[35, 37] with approximately 50% of breast cancer patients relapsing and becoming resistant to further treatment.[38] This resistance may be present from the start of treatment, or only develop after prolonged exposure. A cancer phenotype

is referred to as a physical observed characteristic of a cancer cell, specifically looking into the behavioural, biochemical and physical states of the cell.[35, 38]

The exact nature of tamoxifen-resistance is unknown; however, several mechanisms have been proposed (Figure 1.8). Although these mechanisms of resistance are singular, they may contribute to one another in the development of resistance, thus creating difficulty in combatting exacerbations.[31, 39, 40]

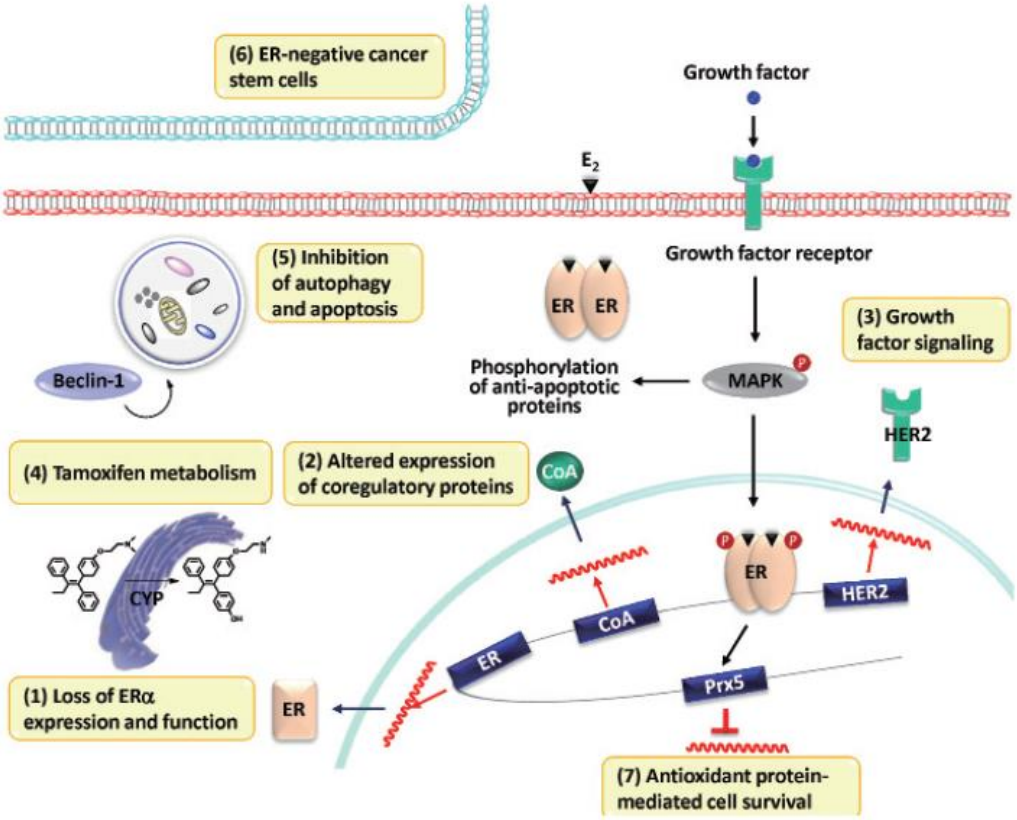


Figure 1.8: Proposed mechanisms of tamoxifen-resistance. ER - Oestrogen receptor, E2 - Oestradiol, MAPK – Mitogen activated protein kinase, HER2 – Human epithelial growth receptor 2, CoA - Acetyl CoA enzyme, Prx5 - Peroxiredoxin 5.[39] Permission granted with the use of an Attribution Non-Commercial Creative Commons licence, https://creativecommons.org/licenses/by-nc/3.0/.

1.3.2.1. Loss of oestrogen receptor expression or function

The loss of ER expression or function is a form of *de novo* resistance which changes ER-positive cells to ER-negative.[39] The loss of expression occurs due to reduction in ER transcription, and thus the receptor is not maintained. Remodelling of the receptor may also take place, leading to a higher expression of ER-negative cells. [39] The ER gene is inactivated by deacetylation or hypermethylation through a naturally occurring mutation

in transcription factors, leading to a mixed population of ER-positive and -negative tumour cells.[31] Alterations in the function of the ER receptor occur due to gene mutations, however, not much is known on the significance of the mutations as a definite contributing factor to resistance (Figure 1.8, mechanism 1). [31]

1.3.2.2. Alterations in the signaling between the oestrogen receptor and growth factors

The ER is involved in the phosphorylation of mitogen-activated protein kinases (MAPK) and protein kinase B (Akt/PTB) which form part of the HER-2 signalling pathway.[40] The MAPK and Akt pathways lead to the ligand-independent activation of the ER.[31] This crosstalk that is seen by HER-2 and ER, and the increased expression thereof, leads to the increased expression of a co-factor Src, which causes the ER to change its tamoxifen bound state from an antagonistic function to an agonistic function.[31] The overexpression of these components, specifically HER-2 and MAPK, can also lead to loss of ER function and further resistance (Figure 1.8, mechanisms 2 and 3).

1.3.2.3. Change in the pharmacokinetic and pharmacodynamic profile of tamoxifen

Alterations in the pharmacological profile of tamoxifen is a result of increased efflux of the drug by the cancerous cell. Increased efflux of tamoxifen is controlled by a multi-drug resistance (MDR)-P-glycoprotein pump in the cell membrane.[39] Altered uptake of tamoxifen into the cell may result in low intracellular concentrations, thus failing to reach a therapeutic threshold.[31] Tamoxifen may bind to microsomal anti-oestrogen binding proteins, inducing a conformational change that reduces the affinity for ER (Figure 1.8 mechanism 4).[31]

1.3.2.4. Response to oxidative stress

Tamoxifen is not only metabolised by CYP450 enzymes, but also undergoes oxidative metabolism leading to reactive oxygen species generation.[31] The hydroxyl radicals formed in oxidative metabolism damage guanidine, and acid in DNA, forming 8-hydroxydeoxyguanosine which contributes to enhanced tumour growth and proliferation.[39] The erythroid transcription factor (GATA1) and peroxiredoxin-5 (Prx5) play a key role in tamoxifen resistance. GATA1 serves as a suppressor on the Prx5 gene when bound to the ER. When tamoxifen binds to the ER in tamoxifen-resistant cells, the

bound complex serves an agonistic function.[31] The bound GATA1-ER inhibits the functioning of Prx5, thus preventing its antioxidant function in breast cancer cells. Decreased antioxidant activity due to a decrease in Prx5 expression leads to decreased apoptosis and increased tumour growth[31] (Figure 1.8 mechanisms 5 and 7). One of the mechanisms of tamoxifen is triggering autophagy, a response that results in the removal of damaged proteins and organelles, suppressing tumour growth. Tamoxifen-resistance is acquired and increases autophagy, protecting the lysosomes of the cell from tamoxifen-induced damage, increasing tumour cell growth.[41, 42]

1.3.2.5. The presence of oestrogen-receptor negative cancer stem cells

Anti-oestrogen treatment, like that of tamoxifen, will initially arrest cell growth of ER-positive breast cancer cells, allowing ER-negative breast cancer cells to proliferate and outnumber ER-positive cells.[39] Tumours switch to an ER-negative phenotype containing ER-negative stem cells which undergo differentiation to express the ER, derived from a mutated version of normally ER-negative cells.[40] Due to the mutation, the cells can differentiate into luminal cancer cells, leading to relapse in breast cancer[39] (Figure 1.8, mechanism 6).

1.4. Mechanisms for overcoming tamoxifen-resistance

Given the increase in tamoxifen-resistance, it is necessary to elucidate potential mechanisms to overcome treatment inefficacy. Many cancers may develop multi-drug resistance to conventional treatment, which increases the need to find alternative treatments.

By sensitising cells to compounds that they were previously resistant to, resistant phenotypes may gain renewed chemosensitivity.[43] As such, tamoxifen-resistant phenotypes may become susceptible to tamoxifen's cytotoxicity under sensitisation conditions. One avenue of sensitisation involves combinational treatment, thus creating a synergistic effect between compounds.[38] In such a case, the additional treatment may re-sensitise tamoxifen-resistant cells or increase their current susceptibility, thus improving tamoxifen's anti-cancer effect. The inherent cytotoxicity of this additional treatment may also yield further anticancer effects.[38] The advantages of synergism are a reduction in dose of medication, and potential reduction of the associated side effects.[44] One advantageous synergistic combination that has spiked the interest of many

researchers is the use of herbal medicines in combination with standard therapy in the treatment of many diseases including breast cancer.

1.5. The use of herbal medicine in the treatment of breast cancer

Herbal remedies are a popular addition to treatment modalities,[45] and thus could be an ideal source of synergistic treatment options. Many Western medicines have been synthesised from plant species which highlight their importance in drug discovery and treatment modalities.[46] Antimitotic agents used in the treatment of cancer, like vincristine and vinblastine (alkaloids), are derived from Catharanthus roseus, commonly referred to as vinca plants.[41] Etoposide and teniposide (podophyllotoxin), used for their antileukemic properties, are semi-synthetic derivatives derived from the root of the Indian podophyllum plant.[42] Docetaxel and paclitaxel (alkaloids), commonly referred to as taxanes, show various anticancer activity from the bark of the Western yew plant.[47] Additionally, the diversity of chemical entities offers diverse ways in which biomolecular processes can be targeted.[45] An example of synergism can be seen with *in vitro* combinational treatment of phytochemical, genistein, and doxorubicin in the treatment of breast cancer.[45] By sensitising the breast cancer cells to tamoxifen treatment using herbal remedies, therapeutic effect may be promoted, thus overcoming resistance through re-sensitisation to treatment.[45] This can be seen through combinational treatments showing much higher apoptosis, antiproliferative and suppression of angiogenesis.[48] Various phytochemicals from a number of plants have been isolated and studied on MCF-7 breast cancer cells to evaluate the anti-cancer potential. Ginsenoside Rh2 (present in red ginseng) displays dose-dependent cytotoxicity in the MCF-7 breast cancer cells line through the induction of tumorigenesis.[49] Combinational treatment of ginseng and paclitaxel has shown a synergistic effect by enhancing the bioavailability of paclitaxel, increasing the anti-cancer potential of paclitaxel. This synergistic combination allows for a reduction in dosage of paclitaxel, potentially reducing adverse effects, along with the additional benefit of ginseng showing low toxicity, reducing the potential of side effects from ginseng.[49] Diallyl disulfide, a phytochemical in garlic, has also shown to decrease tumour growth and promote apoptosis by inducing caspase-3 expression. Garlic has the potential to activate various enzymes involved in carcinogen detoxification.[49] Table 1.2 displays the various drugs used in the treatment of breast cancer, along with major risks and problems

associated with treatment. These risks and problems call for a more sustainable, reduced risk treatment strategies.

Table 1.2: Current regimens used in the treatment of breast cancer, along with problems associated with treatment.

Drug Class[50]	**Drug examples[50]**	**Problems associated with current treatment**
Antimetabolites	5-flourouracil	Increased risk of bleeding, severe diarrhoea, bruising, anaemia.[51]
Alkylating agent	Triethylenethiophosphoramide	Increased risk of developing lung tumours as well as cancer in the mammary glands and uterus of females.[52]
Anthracyclines	Doxorubicin, epirubicin,mitoxantrone	Cardiotoxicity[53]
Taxanes	Paclitaxel	Increased risk of hypersensitivity reactions with 422 known drug interactions[54]
Vinca alkaloids	Vinorelbine	Breathlessness and loss of reflexes continue to be an issue[55]

The use of herbal remedies in everyday life has increased, due to, among other reasons, their accessibility, reduced costs, and incorrect belief of greater safety and efficacy profiles due to their natural origins.[56] Although herbal remedies are often thought of as safer, there is still a risk of toxicity, especially when used in excessive doses or in polypharmacy.[57] Herbal medicines are also more widely accepted in spiritual and cultural belief systems compared to Western medicine.[56] Herbal remedies are popular as primary therapy in developing countries,[57, 58] where they are prescribed by traditional healers, general practitioners, and homeopaths. The use of herbal medicines serves as a holistic approach to the treatment of diseases.[57]

Traditional healers use a variety of plant species to prepare remedies,[57] and numerous preparation methods, such as teas, tinctures, and oils.[56] Common families of plant species used by traditional healers are from the Fabaceae, Euphorbiaceae, Asteraceae, and Sapindaceae families. Common herbs used are turmeric, garlic, and ginger, known to treat various diseases and ailments like the common cold, nausea, inflammation and diseases of the cardiovascular system.[57] By adjusting the extraction method, a different profile of phytochemicals can be extracted, thus altering the biological activity thereof.[58] Common metabolites, also referred to as the secondary metabolites of extractions that

can be found in herbal remedies are polyphenols, carotenoids, flavonoids and tannins. Phytochemicals are responsible for the biological effects that herbal remedies may exert, which may produce either a positive or negative allelochemical effect.[45] It is therefore important to identify the phytochemical matrix in the plant to determine the type of extraction method to use.[59] Hot water extracts may not extract a high, pure concentration of the active phytochemical, but it is a straightforward process used by traditional practitioners and users to simulate the preparation of a tea.[59]

1.6. *Solanum aculeastrum*

Solanum aculeastrum, belonging to the Solanaceae family, also known as the soda apple or Mtuma, is a medicinal plant found in the tropical regions of Africa and South Africa. It is a thorny perennial plant widely used by local traditional healers.[60] The most common part of the plant used by the healers are the berries for the treatment of, among others, gonorrhoea, cancer, and wounds.[60] The most ordinary form of preparation of the berries is through boiling the berries until they burst, releasing the juices within the fruit, and then filtered.[34] From a sustainability point of view, using the fruit does not damage the plant, further benefiting it as an herbal remedy.[61]

S. aculeastrum is used and ethnomedicinal treatment of breast cancer in South Africa.[62] A previous study has shown its main mechanistic property of the plant in the treatment of breast cancer is through the initiation of anti-proliferation pathways resulting in cell death in a dose-dependent manner.[63] Steroidal glycosides isolated directly from the berries, tomatidine and solasodine, have been reported to induce apoptosis in colonic (HT-29), cervical (HeLa) and breast adenocarcinoma (MCF-7) cell lines.[60] The steroidal alkaloids present in *S. aculeastrum*, solanine and solamargine, have displayed anticancer activity in several cell lines and has shown to induce apoptosis and re-sensitize breast cancer cells to cisplatin in epithelial (HB-100), HER2 positive breast cancer (SK-BR) and mammary epithelial (ZR-75) cells, respectively, indicating its high potency.[64] Solanine induces apoptosis in pancreatic cancer cells through mitochondrial-mediated pathways,[64] and reduces ERα-expression in ER-positive endometrial cancer.[65] [66] Previous in-house experiments and studies conducted by Burger *et al.* displayed dose-dependent P-glycoprotein (Pgp) inhibition in aqueous extracts of *S. aculeastrum*, with the aqueous extract showing the most cytotoxicity. The phytochemical shown to display this cytotoxicity was solamargine.[67]

Given the cytotoxic properties and suggested potential of altering several pathways, this research aims to further investigate the properties *in vitro* for the potential use as combinational therapy for the treatment of ER-positive breast cancer.

1.7. Aims and objectives.

The study aimed to evaluate the potential synergistic effects of methanol and hot water extracts of the fruits of *S. aculeastrum* alongside 4-OH-TAM in tamoxifen-resistant MCF-7 adenocarcinoma cells (MCF-7/TAMR-1).

The objectives of the study were to determine the:

- Inherent cytotoxicity and selectivity of the crude extracts and 4-OH-TAM in the MCF-7 and MCF-7/TAMR-1 cell lines using the sulforhodamine B (SRB) staining and resazurin (RZN) conversion assays.
- Combinational effects of the crude extracts and 4-OH-TAM using a checkerboard assay; *and*
- Cytotoxicity of combinational treatments in relation to morphology (Hoechst 33258, propidium iodide and acridine orange fluorescence microscopy), oxidative stress (ROS and GSH fluorometry) and ER expression (Western blotting).

Chapter 2: Materials and methods

Chapter 3: Results and discussion

Table 3.1 Chromatographic fingerprint of phytochemicals identified in the extracts of *Solanum aculeastrum* fruits using the electron spray ionisation (ESI) technique.

Identified phytochemicals	Extract		Empirical formula	Monoisotopic calculated mass (Da)	Monoisotopic observed mass (Da)	ESI mode	Fragment ions observed (Da)
	Hot water	Methanol					
3-Caffeoylquinic acid	X	X	$C_{16}H_{18}O_9$	354,0951	353,0873	Negative	191.0493; 179.0276; 135.0416
4-Caffeoylquinic acid	X	X	$C_{16}H_{18}O_9$	354,0951	353,0873	Negative	191.0509; 179.0304; 173.0391; 135.0412
5-Caffeoylquinic acid	X	X	$C_{16}H_{18}O_9$	354,0951	353,0873	Negative	191.0532; 179.0313
5-Feruloylquinic acid		X	$C_{17}H_{20}O_9$	368,1107	367,1009	Negative	191.0518; 173.0406
Solasonine	X	X	$C_{45}H_{73}NO1_6$	883,4929	884,5005	Positive	738.4463; 592.3817; 430.3212
Solamargine	X	X	$C_{45}H_{73}NO_{15}$	867,498	868,5071	Positive	722.4603; 576.3885; 414.3287
Solasodine	X	X	$C_{27}H_{43}NO_2$	413,3294	414,335	Positive	397.3268; 253.1897; 173.1265; 147.1108
Solaculine A	X	X	$C_{50}H_{81}NO_{19}$	999,5403	1000,5481	Positive	926.5157; 722.4464; 576.3897; 558.3787
Novel (Solasonine-related)		X	$C_{45}H_{71}NO_{16}$	881,4773	882,4851	Positive	858.5078; 736.4304; 590.3645; 428.3141

3.2. Inherent cytotoxicity via the sulforhodamine B assay

There are several cell enumeration assays that exist for the evaluation of toxicity of compounds on cultured cells.[80] Each assay comes with its advantages and shortfalls. Linear range, reproducibility and reliability are characteristics that need to be considered when selecting the appropriate assay. The SRB assay is the preferred assay to use for screening lead compounds at the National Cancer Institute in the United States of America.[80] The SRB assay not only shows high reproducibility, but also shows low variability in comparison to other commonly used assays like that of the 4,5-dimethylthiazol-2-yl-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, therefore minimal repeats need to be conducted to obtain reliable results. The SRB assay was chosen to determine the cytotoxicity of the samples, however, was conducted in a multiplex format with RZN to determine cellular viability due to the metabolic considerations of the assay.[80] The inherent cytotoxicity of the HWE, ME, cisplatin (positive control) and 4-OH-TAM was performed in both cell lines to determine if the TAMR-1 cell line displayed chemoresistance. Cytotoxicity was measured over a period of 24 h, 48 h and 72 h to best represent kinetics and sensitivity between the cell line. The samples showed dose-dependent cytotoxicity at 24 h, 48 h and 72 h in MCF-7 cells when treated with various concentrations of 4-OH-TAM, HWE, ME and cisplatin, however, at 48 h and 72 h cisplatin reached a plateau (Figure 3.1). Cisplatin (positive control) did show a difference in cytotoxicity. At 24 h, the IC_{50} values obtained for 4-OH-TAM, HWE and ME was 5.63 µg/mL (15 µM), 39.69 µg/mL and 10.06 µg/mL, respectively. At 48 h, a decrease in IC_{50} values was observed, indicative of a time-dependent cytotoxicity between 24 h and 48 h. The IC_{50} values obtained for the MCF-7 cell line (Table 3.2) for cisplatin, 4-OH-TAM, HWE and ME was 2.21 µg/mL (7.35 µM), 4.03 µg/mL (10.4 µM), 33.65 and 12.54 µg/mL, respectively. When comparing the cytotoxicity at 48 and 72 h, there was no significant difference in the IC_{50} values, indicating that time-dependent cytotoxicity only occurred between 24 h and 48 h. The IC_{50}'s for 72 h for cisplatin, 4-OH-TAM, HWE and ME was 2.19 µg/mL (7.26 µM), 3.78 µg/mL (9.7 µM), 29.29 µg/mL and 11.72 µg/mL.

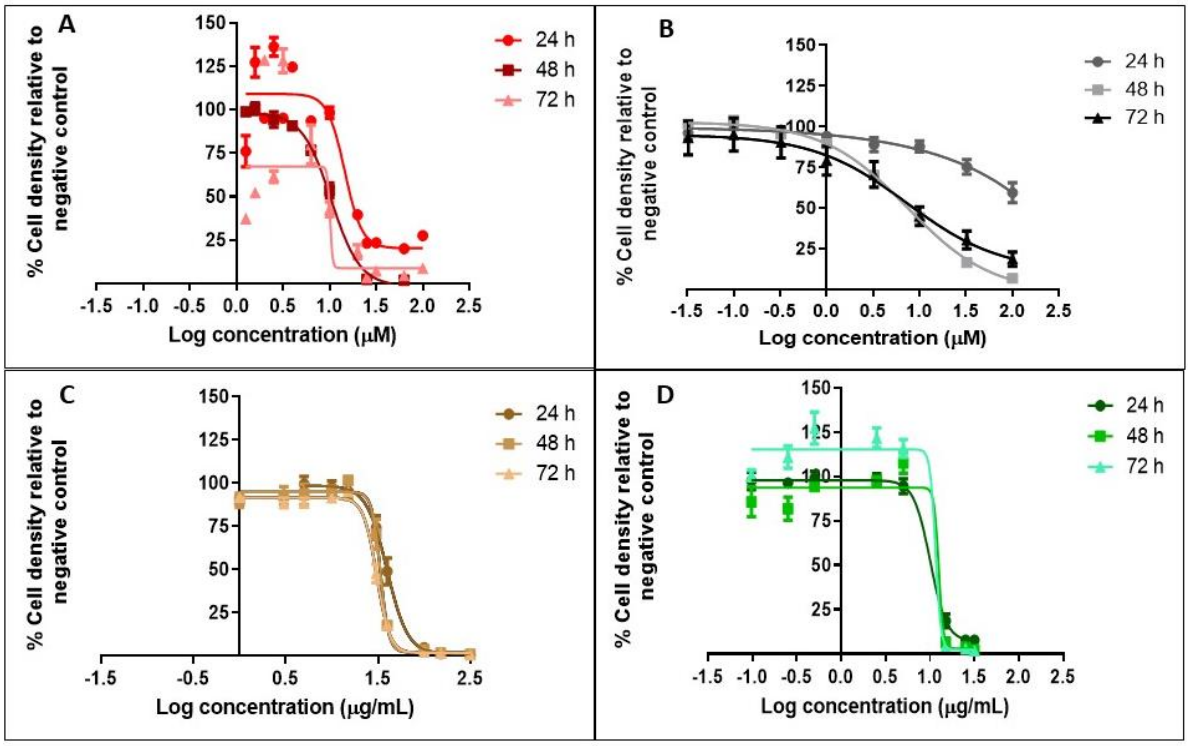


Figure 3.1: Inherent dose-dependent cytotoxicity, using the SRB assay, of 4-hydroxytamoxfen (A; 4-OH-TAM), cisplatin (B; positive control), hot water extract (C; HWE) and methanol extract (D; ME), in MCF-7 breast adenocarcinoma cells over a 24 h, 48 h and 72 h period.

The inherent cytotoxicity in the TAMR-1 cells lines displayed similar cytotoxicity as seen in the MCF-7 cell line (Figure 3.1). At 24, 48 and 72 h, dose-dependent cytotoxicity was observed, however, time-dependency was only displayed between 24 and 48 h. The IC_{50}'s for 4-OH-TAM, HWE and ME was 5.95 (5 µM), 40.21 and 5.09 µg/mL, respectively. When comparing the 24 h treatment in both cell lines, the TAMR-1 cell line was more sensitive to HWE, ME and 4-OH-TAM as the 50% decrease in cell density was observed at lower concentrations in comparison to the MCF-7 cells. Cisplatin did not show any significant difference in the TAMR-1 cell line at 48 h in the TAMR-1 cell line, dose-dependent cytotoxicity was seen in 4-OH-TAM, HWE and ME, yielding IC_{50}'s of 3.925 (10.13 µM), 34.89 and 11.9 µg/mL, respectively. The IC_{50} for 4-OH-TAM was slightly higher in the MCF-7 (10.4 µM) than the TAMR-1 (10.13 µM). Dose-dependent cytotoxicity was also seen at 72 h in 4-OH-TAM, HWE and ME displaying IC_{50}'s of 3.36 (11.16 µM), 1.95 (5.03 µM), 32.24 and 13.25 µg/mL, respectively.

The differences noted between the MCF-7 and TAMR-1 cell lines, at 24 h, 48 h and 72 h, showed increased sensitivity toward the TAMR-1 cell line in the SRB assay.

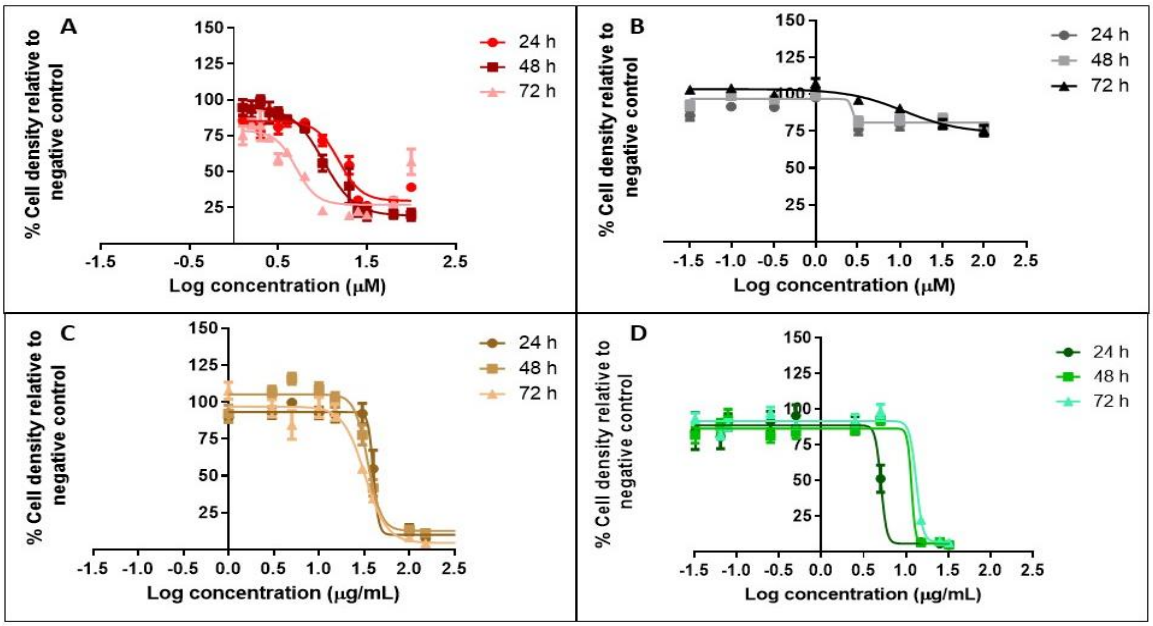


Figure 3.2: Inherent dose-dependent cytotoxicity, using the SRB assay, of 4-hydroxytamoxifen (A; 4-OH-TAM), cisplatin (B; positive controls), hot water extract (C; HWE) and methanol extract (D; ME) in TAMR-1 breast adenocarcinoma cells over a 24 h, 48 h and 72 h period.

When comparing Figures 3.1 and 3.2, the TAMR-1 cell line showed minimal chemoresistance to 4-OH-TAM, HWE and the ME. The minimal chemoresistance observed could be due to a change in the cell's phenotype following the addition of 1 µM 4-OH-TAM. Unfortunately, this does create an interpretative irregularity towards the selectivity of the samples towards the MCF-7 cell line that could not be accounted for at the stage of the experimental procedures. Research has shown, to obtain a resistant cell line, the cell density should have a fold-difference between two and eight in comparison to the parent cell line.[81] The difference in cell density values between both cell lines shown by the IC_{50}'s in Tables 3.2 and 3.3 do not display a two-to-eight-fold difference, indicating that the resistant cell line was not completely attained but some resistance is present. Previous studies developing TAMR-1 cell lines display the opposite effect, whereby the reduction in cell density is highest in MCF-7 cells and not the TAMR-1 cells.[82] This confirms that a sensitive, rather than a resistant cell line of TAMR-1 was developed in this study. The differences seen in research and in this study could be due to the methodology on how the resistant cell line was obtained as well as the concentration of 4-OH-TAM and duration of exposure to 4-OH-TAM in the process of developing the resistant cell line.[82].

No time-dependent cytotoxicity was seen between 24 h, 48 h and 72 h. It was therefore decided that the 24 h TAMR-1 IC_{50}'s would be studied further for combinational testing. Consistent with the current findings, Cuevas *et al.* showed cytotoxicity of 4-OH-TAM in the region of 10 µM. The IC_{50} values obtained at 48 h (10.4 µM) in the MCF-7 cell line were comparable to endometrial cancerous cell lines (HEC-1B) of around 10 µM.[83] The IC_{50} findings in this current study are consistent with Koduru *et al.* showing antiproliferative activity of HWE and ME of *S. aculeastrum* against various cancer cell lines (MCF-7, HT29 and HeLa) over a 48 h period. Environmental and experimental factors such as geographical location, extraction methods and seasonal variation in harvest may contribute to a slightly higher IC_{50} (HWE; 33.65 µg/mL and ME; 12.54 µg/mL) obtained in this study.[84] The ME was more cytotoxic than that of the HWE extract and can also be seen in the study conducted by Koduru *et al.*[85] According to the National Cancer Institute, any crude extracts obtaining an IC_{50} of <30 µg/mL is considered as favourable for further investigation as an anti-cancer agent.[86] Both the HWE and ME fit this criterion after 72 h of exposure in the MCF-7 cell line (Table 3.2). In the MCF-7/TAMR-1 cell line, only the ME fell within the favourable range at 24, 48 and 72 h (Table 3.2). Similar dose-dependent cytotoxicity was observed in Hep2G hepatocarcinoma cells over 48 h. [87] Similar cytotoxicity was observed for *S. aculeastrum* in IGROV1 ovarian cells[88] to that of the present study. Previous studies have shown that bioactive glycoalkaloids like that of solasonine, solamargine and solasodine extracted from *S. aculeastrum* and *S. nigrim* inhibit growth in cancerous cells,[89] supporting their potential as the bioactive cytotoxic constituents in the extracts.

3.3. Inherent cytotoxicity via the resazurin assay

To further understand the way in which cytotoxicity was induced, the RZN assay was performed as a measure of cell viability. Reduced metabolism of RZN to resorufin can be related to increased cell death.[90] Although SRB focusses on cellular proliferation, the RZN assay assesses metabolic activity. A similar dose-dependent conversion of resazurin to resorufin was seen in both MCF-7 and TAMR-1 cell lines at 24 h, 48 h, and 72 h, for the 4-OH-TAM (Figures 3.3A and 3.4A), cisplatin (Figures 3.3B and 3.4B), and HWE (Figures 3.3C and 3.4C). This displays the expected trend of reduced cell density and metabolism of cells, directly relating to reduction in viable cells. There is, however, a notable difference in cell density (SRB assay) and enzymatic conversion (RZN assay) for both cell lines in the ME at 24 h and 72 h (Figures 3.3D and 3.4D).

Although the SRB assay shows a reduction in cell density, indicative of cell death, the RZN assay shows the same percentage metabolism of around 96.7% for all concentrations and time periods for the ME. It is possible for cells to reduce proliferation but increase enzymatic activity as an adaptive response.[90] An example being cells under stress, which in this study are due to the exposure to the samples such as HWE, 4-OH-TAM and ME that may prioritise enzyme production over cellular proliferation to combat cellular assault.[90, 91] A study conducted by Walzl *et al.* showed similar results in colorectal cancer cells whereby viable cells under stress for a long period of time tend to combat the stress by increasing enzymatic activity, specifically in this case, resazurin to resorufin.[91] Consistent with the present study, the RZN results in studies conducted by Ursaki *et al.* and Balbaied *et al.* suggest that cellular stress, increased initial cell density and duration of stress to the cells contribute to the changes observed in the current study.[90, 92] Another study conducted on an organic ME of *S. aculeastrum* in MCF-7 breast adenocarcinoma cells increased metabolic activity upon a reduction in cell density, conclusive that this may be due to an adaptive metabolic response toward the stress of exposure to the ME.[68]

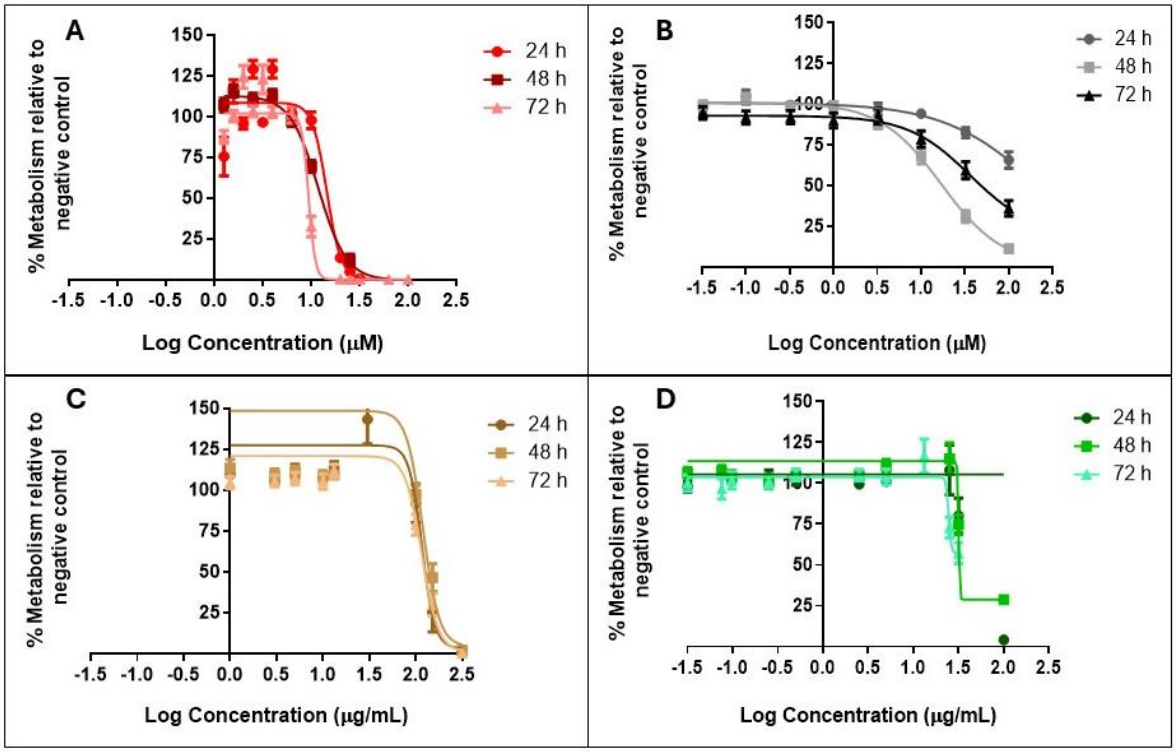


Figure 3.3 Inherent dose-dependent cytotoxicity, using the RZN assay, of 4-hydroxytamoxfen (A; 4-OH-TAM), cisplatin (B; positive control), hot water extract (C; HWE) and methanol extract (D; ME), in MCF-7 breast adenocarcinoma cells over a 24 h, 48 h and 72 h period.

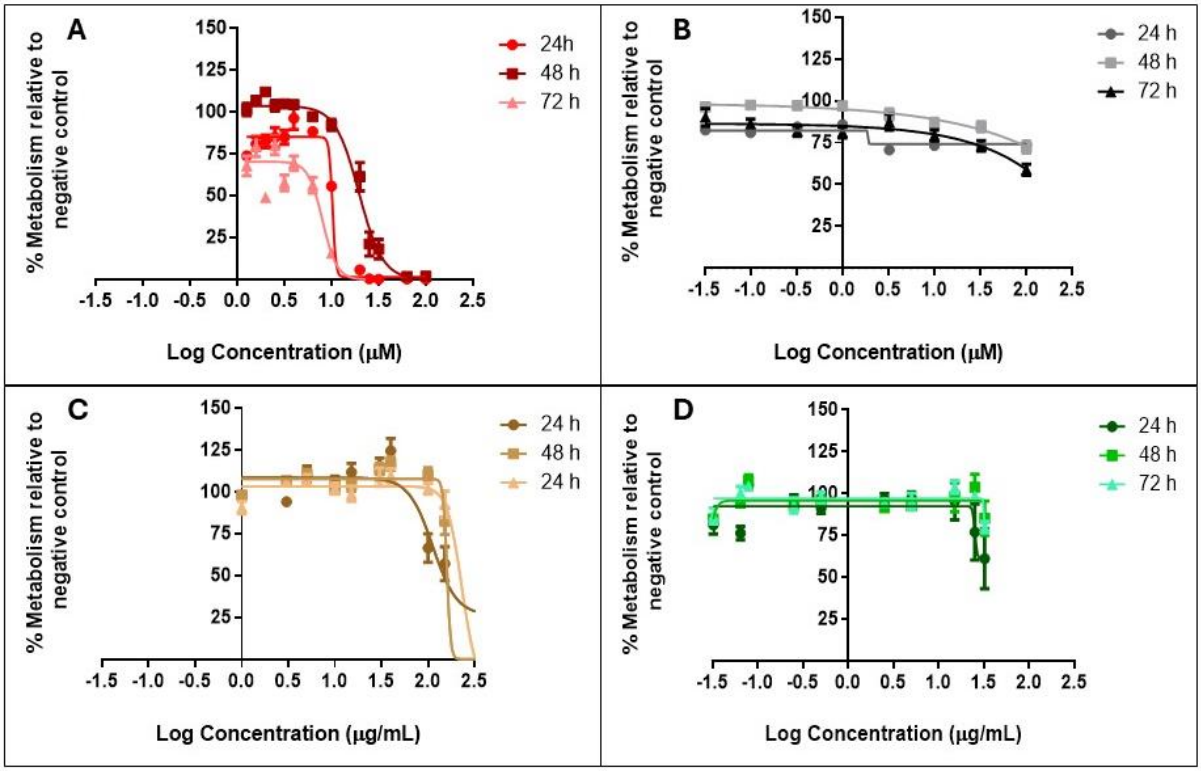


Figure 3.4 Inherent dose-dependent cytotoxicity, using the RZN assay, of 4-hydroxytamoxifen (A; 4-OH-TAM), cisplatin (B; positive controls), hot water extract (C; HWE) and methanol extract (D; ME) in TMA-1 breast adenocarcinoma cells over a 24 h, 48 h.

To remedy the inefficacy towards cancer treatment, synergistic combinational treatment with standard treatment therapies along with herbal remedies is being widely and more commonly studied today.[29] Combinational cytotoxicity was assessed for all samples alongside 4-OH-TAM using the SRB assay after a 24 h and 48 h exposure period in the TAMR-1 cell line.

Table 3.2: The 24 h, 48 h and 72 h cytotoxicity (expressed as IC50's) of 4-hydroxytamoxifen (4-OH-TAM), hot water extract (HWE), methanol extract (ME) and cisplatin (positive control) as determined by the sulforhodamine B (SRB) and resazurin (RZN).

	MCF-7 ± SEM						MCF-7/TAMR-1 ± SEM					
	24 h		48 h		72 h		24 h		48 h		72 h	
	SRB	RZN	SRB	RZN	SRB	RZN	SRB	RZN	SRB	RZN	SRB	RZN
4-OH-TAM (µM)	14.54 ±1.13	14.51 ±1.11	10.40 ±1.05	11.85 ±1.06	9.74*	9.44 ±1.07	15.33 ±1.08	12.15 ±1.05	10.28 ±1.10	19.41 ±1.04	5.03 ±1.09	8.07 ±1.04
HWE (µg/mL)	39.69 ±1.05	>100	33.65 ±1.02	>100	29.29 ±1.03	>100	40.21 ±1.02	>100 ±1.13	34.89 ±1.03	>100	32.24 ±1.06	>100
ME (µg/mL)	10.06 ±1.11	>100	12.54*	31.69*	11.72*	24.76*	5.09 ±5.44	26.41 ±2.34	11.90*	>100	13.25 ±8.99	27.39*
Cisplatin (µM)	>100	>100	7.40 ±1.12	16.71 ±1.21	7.26 ±1.98	86.15 ±1.82	>100	2.09*	>100	>100	11.16 ±1.54	>100

*The SEM could not be determined for these extracts due to the results presenting with an ambiguous case.

3.4. Combination effects of the extracts and 4-hydroxytamoxifen

3.4.1. Cytotoxicity of combinations

Research into the synergistic combination between western medicine and traditional medicine is an avenue being continuously studied. Combinational treatment may increase sensitivity of TAMR-1 cells to 4-OH-TAM leading to reduced chemotherapeutic doses to elicit the same therapeutic effect.[56] Dose reduction is advantageous as this may lead to reduced side effects while maintaining therapeutic efficacy.[56] In the current study, the CI of different combinations of the extract and 4-OH-TAM at IC_{50}, ½IC_{50} and ¼IC_{50} at 24 and 48 h using cell density measurement, suggests that most of the combinations are not synergistic (Tables 3.3 and 3.4). The

CI values were calculated, where a synergistic (CI<1), additive (CI=1) and antagonistic response (CI>1) can be identified.[93] The majority of CI obtained indicated antagonistic responses with the most antagonistic combination (CI = 2.48) being between the ½IC_{50} 4-OH-TAM (2.54 µg/mL) and ¼IC_{50} ME extract (1.45 µg/mL). The most synergistic combination was determined to be ¼IC_{50} HWE and ½IC_{50} 4-OH-TAM. Antagonistic mechanisms may be due to competition between the phytochemicals of interest and 4-OH-TAM, thus reducing the efficacy of the compounds in combination as one another reducing the binding possibilities at the drug targets,[94] such as the ER. The effect of combination may differ ratiometrically due to alterations of the interaction, suggesting a concentration-dependent efficacy.[95] The most synergistic combination (10.05 µg/mL HWE; 5.83 µg/mL 4-OH-TAM) that was chosen for further mechanistic evaluation yielded a CI of 0.78. Molecules may also interact with different drug targets in several ways, resulting in modulation of several pathways that have the same therapeutic effect,[95, 96] but alter their biological efficacy depending on the strength at which the pathway is modulated.

Table 3.3: Combination indices obtained for the combinations of HWE and 4-OH-TAM at ¼IC_{50}, ½IC_{50} and ½IC_{50} over 24 and 48 h, respectively. The synergistic combination has been highlighted in **bold**.

		4-OH-TAM (µg/mL)					
		1.45		**2.91**		**5.83**	
		24 h	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
HWE (µg/mL)	**10.05**	1.07	2.03	1.80	1.52	**0.78**	1.00
	20.11	1.70	1.92	2.29	1.64	1.20	1.20
	40.21	1.52	1.20	1.93	1.43	1.30	1.53

Table 3.4: Combination indices obtained for the combinations of ME and 4-OH-TAM at ¼IC_{50}, ½IC_{50} and ½IC_{50} over 24 and 48 h, respectively. NaN indicates an exceptionally low fraction of cells affected; hence no index could be obtained. The synergistic combinations have been highlighted in **bold.**

		4-OH-TAM (µg/mL)					
		1.45		**2.91**		**5.83**	
		24 h	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
ME (µg/mL)	**1.27**	1.08	NaN	1.43	NaN	1.17	**0.89**
	2.54	2.48	NaN	2.46	1.08	1.30	**0.82**
	5.08	1.81	NaN	1.93	NaN	1.19	**0.86**

The most synergistic combination at 24 h (10.05 µg/mL HWE; 5.83 4-OH-TAM; combinational index 0.78289) significantly ($p<0.001$) reduced cell density (76.65%) in comparison to the HWE (27.15%) and alone (Figure 3.5).

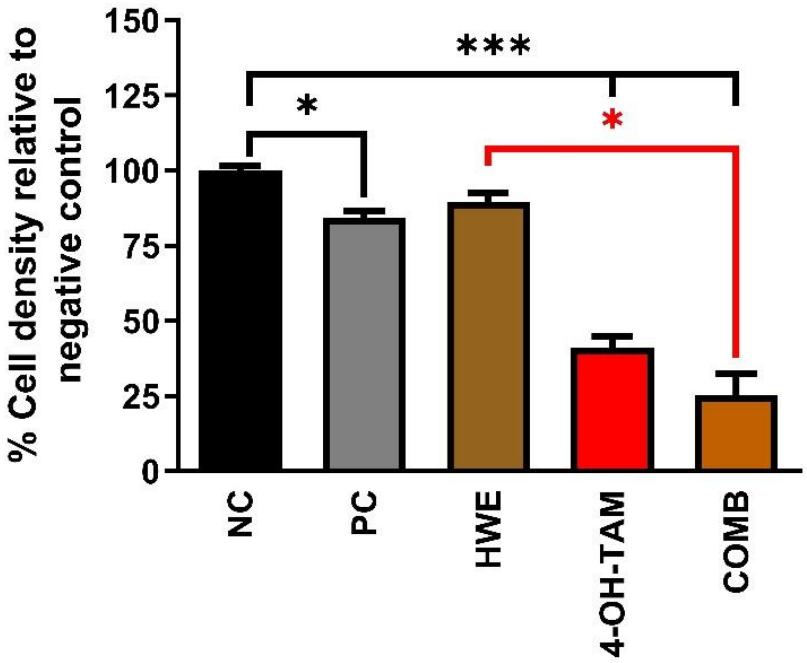


Figure 3.5 Cell density (%) of selected combinational treatment of negative control (NC), positive control (PC; cisplatin), hot water extract (10,05 µg/mL; HWE) and 4-hydroxytamoxifen (5,83 µg/mL; 4-OH-TAM) alone and in combination (COMB) ($P<0.0001$).

Research shows that combinational treatments results in increased sensitivity of breast cancer cells to 4-OH-TAM when used in combination with 3-caffeoylquinic acid,[97] a phytochemical commonly found in HWE and ME of *S. aculeastrum*. As seen in this study, the combinational treatment of 4-OH-TAM and the HWE of *S. aculeastrum* displayed the same increase in sensitivity, supporting the literature and research conducted by Lyvia *et al.*[97] This provides a promising inclusion of the COMB treatment as a possible option in the treatment of tamoxifen-resistant ER + breast cancer.

The most synergistic combination was further analysed to identify mechanistic methods by which it exerts its potential synergistic effect.

3.4.2. Mechanistic evaluation of combinations

To better understand the mechanistic changes that occur in the synergistic combination chosen, further experiments investigating morphological changes, intracellular ROS, GSH and ER-α expression alterations were conducted.

3.4.2.1. Morphological changes

Morphological changes were identified using Hoechst, acridine orange and propidium iodide. Healthy cells and cells undergoing apoptosis are easily distinguishable based on the amount of dye that the cells take in and consequently the amount of fluorescence that illuminates. Healthy cells are spherical, with the DNA evenly distributed among the cells.[98, 99]

Hoechst 33258, acridine orange and propidium iodide are fluorescent dyes, that upon excitation at specific wavelengths fluoresce cellular components of a cell blue (DNA in the nucleus), green (DNA strands and acidic vacuoles) and red (membrane-compromised cells). Figure 3.6 depicts the cellular components as well as change in cellular morphology in the MCF-7/TAMR-1 cells upon exposure to different treatments. Cell density was reduced, with observed cellular rounding and blebbing after exposure to the HWE, which had less than 4-OH-TAM and a combination thereof. The negative control (Figure 3.6A) displayed healthy cells where consistent DNA staining with Hoechst and acridine orange displays an intact nucleus, representative of viable cells. The positive control (Figure 3.6B) showed a slight reduction in cell density, confirming previous findings during combinational treatments. Treatment with the HWE alone (Figure 3.6C) showed a reduction in cell density in comparison to the positive control, however, no cellular blebbing was noted. The HWE (Figure 3.6D) displays reduction in cellular density, directly correlating to the 60% reduction displayed in Figure 3.3. Additionally, increased nuclear staining and chromatin condensation is visible in the HWE (Figure 3.6D). A larger reduction in cell density, increase in chromatin condensation and cellular blebbing is observed with the COMB treatment, like the significant reduction in cell density of COMB Figure 3.5.

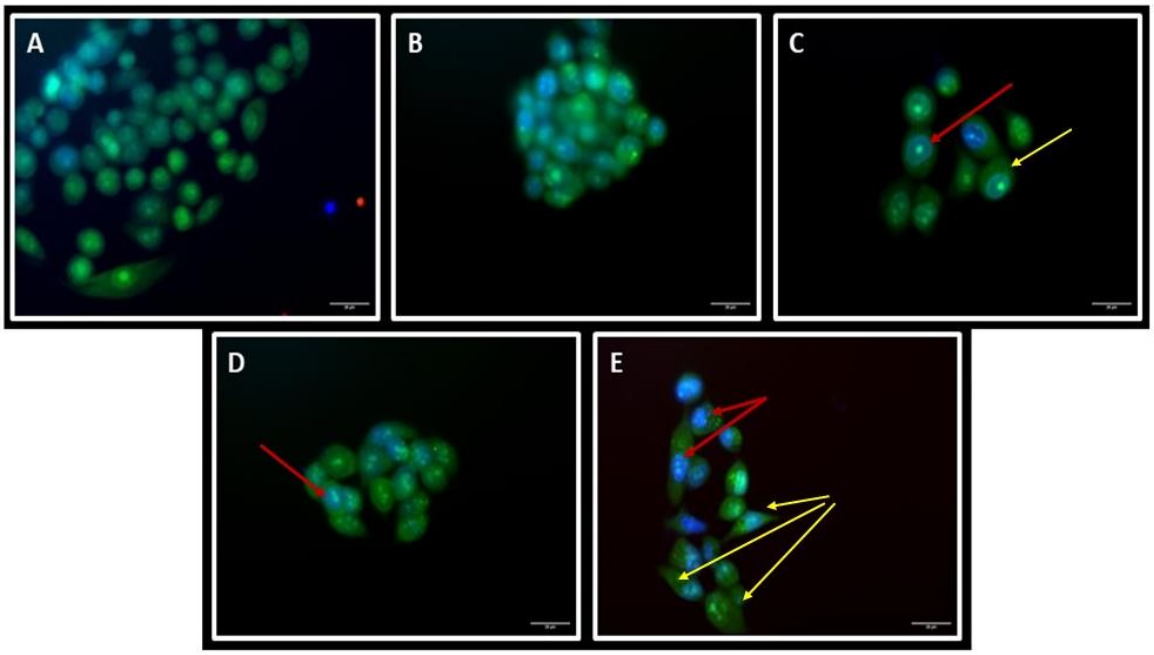


Figure 3.6 Fluorescence microscopy of the negative control (A), positive control cisplatin (B), 4-hydroxytamoxifen (C), hot water extract (D) and combinational treatment (E). Blue stains are indicative of Hoechst staining of the DNA, green is indicative of acridine orange staining od dsDNA and red propidium iodide staining indicative of cells death. Cellular blebbing (yellow arrow) and chromatin condensation (red arrow) is evident in the 4-OH-TAM and COMB.

The morphological changes of chromatin condensation and cellular blebbing are characteristics of cellular apoptosis.[100] Similar research conducted by Sezer *et al*. has shown the clear association between cell viability and apoptosis in various cancer cells including breast cancer.[100, 101] As seen in Figure 3.5, the COMB shows the most reduced cell number in comparison to the individual treatment of 4-OH-TAM and HWE. The reduction in cell density is directly correlated to a decrease in cell viability. Studies on the treatment of tamoxifen on primary breast cancer cells and breast cancer cell lines displayed similar morphological changes of chromatin condensation and cellular blebbing when in combination with 4-OH-TAM and calmodulin inhibitors.[100] Studies have shown comparable results in Hoechst staining in the treatment of hepatocellular carcinoma cells,[102] human glioma cells[102, 103] and lung cancer cells[104] and chromatin condensation indicative of apoptosis.[102] DNA fragmentation can also be seen in Figure 3.6E, another characteristic of apoptosis. Previous studies on the treatment of *S. nigrim* in MCF-7 and AU565 HER-2 positive and non-small lung cancer cells also displayed DNA fragmentation.[105, 106]

Acridine orange is a fluorescent dye used to identify lysosomal accumulation, which can infer further organelle changes that support cell death. Cell death is said to be mediated by the control of various pathways namely, apoptosis, autophagy, necrosis, necroptosis, ferroptosis and cuproptosis. Increased lysosomal accumulation result in organelle changes and signals the release of proteolytic enzymes, leading to apoptosis.[107] Studies in the treatments of 4-OH-TAM on tamoxifen resistance have displayed a similar trend in the increased intensity of green fluorescence as seen in this study, indicative of lysosomal accumulation.[108] Increased chromatin condensation and lysosomal accumulation is characteristic of increased apoptosis.[100, 107]

All three caffeoylquinic acids are known esters of caffeic acid, also found in teas and wines. Studies have shown that these caffeic acid esters mediate apoptosis in various cancers, including breast cancer cells. Additionally, lysosomal accumulation is present, disrupting cell growth and proliferation.[109] Consistent with this study, Tarek *et al.* showed a synergistic combination between caffeic acid and 4-OH-TAM in MCF-7 breast cancer cells.[110] The same reduction in cell viability was seen after exposure to COMB. The COMB induced apoptosis as can be seen in Figure 3.6E showing increased chromatin condensation whereby the blue DNA staining is brighter and shows separate particles instead of one full intact blue circle. This is indicative of DNA damage which results in apoptosis. Cellular blebbing is also more predominant in the COMB showing increased degradation of the cancerous cells, which leads to apoptosis, reducing the cancer cell's ability to grow and proliferate.

The cytotoxicity of solasonine, solamargine and solaculine A in breast cancer has been observed. Huang *et al.* showed an increased sensitivity of cells to a HWE of *Solanum nigrim* Linn, containing the phytochemical along with its esters of caffeic acid.[105] A study carried out by Tian-Chuan *et al* shows that solamargine can induce apoptosis via the endoplasmic reticulum stress pathway and mitochondrial pathway.[79] Cellular features of chromatin condensation, reduced cell size, and membrane blebbing, as in the present study, was observed in HepG2 and MCF-7 cells after exposure to solamargine.[87] Reduced cell number and chromatin condensation was also seen by Zhuang *et al*. in MCF-7 as well as colorectal cells (HT-29) after exposure to solasodine.[111]

3.4.2.2. Intracellular reactive oxygen species and reduced glutathione levels

Homeostasis of ROS is important for cell functioning, where a shift in the equilibrium thereof will increase the risk of disease. Reactive oxygen species may cause reactive toxicity to macromolecules, particularly DNA, carbohydrates, proteins, and lipids, which imbalances the redox status and impacts biological functions.[112] Oxidative stress is defined as the imbalance between the production of ROS and the ability of the cells in the body to readily detoxify macromolecules, resulting in damage.[113] Antioxidants are molecules that can prevent oxidation of macromolecules by blocking the production of free radicals, oxidant scavenging, conversion of toxic radicals to non-toxic as well as the initiation and enhancement of the endogenous antioxidant system.[114] Any defects in the above function may results in oxidative stress.

Although free radicals are needed for cellular signalling, the depletion of them can also destabilise the redox system and thus impact the health of the cell.[113] Intracellular ROS generation and determination is an important mechanistic evaluation in finding the cause of apoptosis.[115] The MAPK pathways, involved in the activation of many cellular pathways including apoptosis and survival, are modulated by ROS. [101] An increase in cellular ROS can be related to the subsequent production of free radicals which may increase cell death through apoptosis, a characteristic that all cancer studies capitalise on.[116, 117]

The effect of HWE, 4-OH-TAM and the combination thereof on ROS formation was determined through DCF fluorescence. The positive control, PPD, showed a significant 2-fold increase in ROS generation relative to the negative control ($p = 0.0037$) (Figure 3.7A). The HWE showed a reduction in ROS, whereas the 4-OH-TAM treatment displayed increased ROS levels to above the baseline after 24 h, with the combinational treatment showing reduced ROS generation.

The endogenous antioxidant GSH is responsible for protecting cells against oxidative stress. By reducing GSH levels, anticancer compounds' prooxidant activity cannot be counteracted, thus increasing their cytotoxic effect, such as antiproliferation or apoptosis.[118] GSH also serves as a detoxification molecule, hence a reduction in GSH may perturb the detoxification of cytotoxins[119] such as 4-OH-TAM, thus increasing their potential for incurring cytotoxicity. Figure 3.7B shows that the PC, NEM, displayed a significant 0.25-fold reduction in GSH levels ($p = 0.0346$). The HWE increased GSH

by 1.25-fold, suggesting an increase in antioxidant potential of cells when exposed to HWE alone, which may correlate to the slight decreased ROS observed. The individual treatment of 4-OH-TAM decreased GSH levels, which directly correlates to the increase in ROS. The COMB decreased the GSH levels, although not significantly so, indicating a slight reduction in antioxidant potential. Such a decrease may impact the ability of the cell to detoxify xenobiotics and creating an increased change of susceptibility to apoptosis.[113, 114]

Cells have a natural cellular protection mechanism whereby an increase in GSH is related to increased antioxidant ability of the cells, increasing cells survival.[120] Studies have shown that aqueous extracts of the berries of *S. aculeastrum* display antioxidant activity.[62] The increased antioxidant potential of aqueous extracts may be due to the high number of polar constituents like tannins, anthocyanin, terpenoids, saponins, flavonoids, and alkaloids (solamargine, solasodine, solanine) in the berries of the *S. aculeastrum* plant.[85, 120, 121] The exogenous antioxidants present may have cleared ROS, however, did not impact GSH levels.

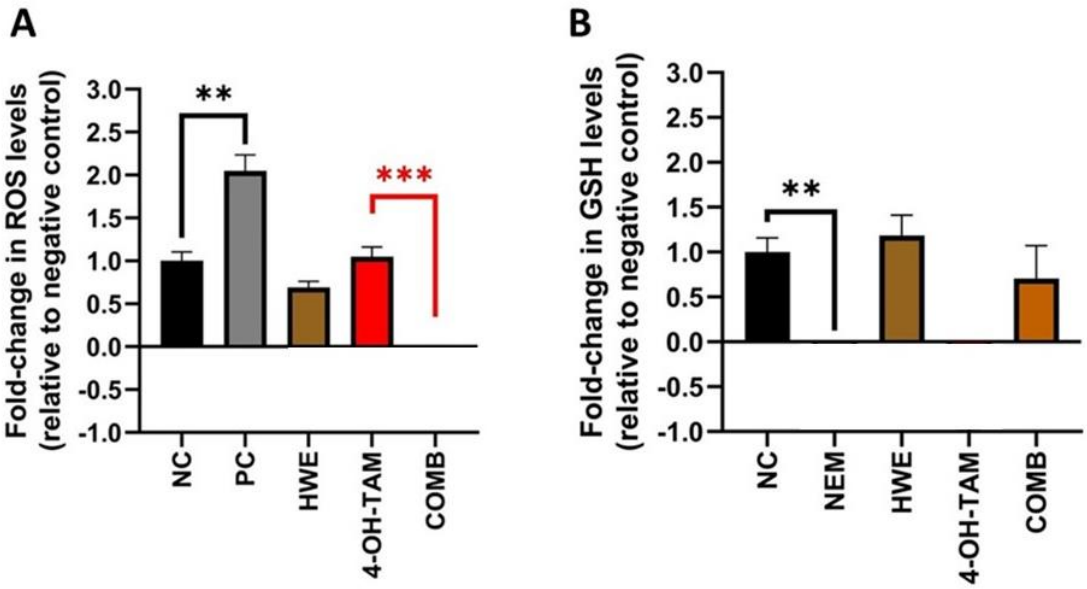


Figure 3.7 The effect of combination treatment on A) ROS, and B) GSH. Abbreviations: NC: negative control, PC: positive control, NEM: n-ethylmaleimide control, HWE: hot water extract, 4-OH-TAM: 4-hydroxytamoxifen and COMB: combination.

The generation of ROS contributes to apoptosis of breast cancer cells, alongside a reduction of GSH availability.[117] Although ROS generation was not prominently displayed, research has shown that several steroidal alkaloids, such as holamine and funtumine, may reduce ROS in MCF-7 cells.[122] There was a notable reduction in GSH levels ROS after 24 h, suggesting destabilisation of the redox status of cells which contribute to the perturbed growth and viability of breast cancer cells.[117] This destabilisation appears to have caused reductive stress, rather than oxidative stress, with reduced GSH hindering detoxification of 4-OH-TAM.[122] Reductive stress results in response to aspects that shift the redox balance to a more reducing state, rather than an oxidising state, which is the most common trend.[123] Overexpression of antioxidants results in excess redox particles over oxidative ones,[124] and given the reduced ROS after exposure to the HWE, may indicate an exogenous antioxidant potential without contribution to the endogenous levels of GSH. As a result of a surplus of redox particles cell growth, mitochondrial function and cellular metabolism may be reduced.[123] Steroidal alkaloids like that of holamine and funtumine have been shown to contribute to apoptosis due to redox stress.[122]

A similar trend in the increased antioxidant activity was seen by Peng *et al*., whereby caffeic acid and their derivatives and esters displayed an increased redox activity against cells that develop ROS.[125, 126]

To further analyse the COMB, identifying whether the COMB changes the expression of ER-α in TAMR-1 cells was further investigated to better understand the possible mechanism of increased apoptosis.

3.4.2.3. Western blotting

The expression of ER-α was determined through Western blotting. The loading control (GADPH 37 kDa) was used to confirm accurate protein loading during gel electrophoresis (Figure 3.8), while ER-α protein was confirmed at 68 kDa. A study conducted by Rago *et al.* identifies the parent ER-α molecular weight in MCF-7 breast adenocarcinoma cells to be at 67 to 68 kDa.[127] The 4-OH-TAM had no statistically significant effect on ER-α expression.

Figure 3.8 displays the effect of COMB treatment on ER-α expression. It can be seen, in comparison to the HWE with a fold-change of 1.5 (Figure 3.8), there is reduction in the expression of ER-α in 4-OH-TAM and COMB, as depicted by lighter band intensity

(Figure 3.8). There was an increase in fold-change of 1.3 and 0.4 in 4-OH-TAM and COMB, respectively (Figure 3.9).

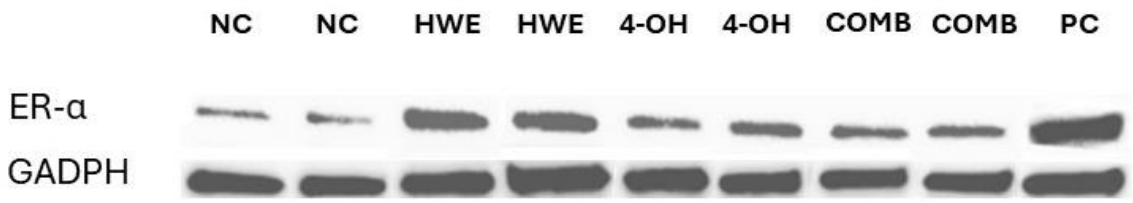


Figure 3.8 The effect of combination treatment on relative ER-alpha expression normalised to glyceraldehyde-3-phosphate dehydrogenase (GADPH). Abbreviations: NC: negative control, PC: 17-β Oestradiol, HWE: hot water extract, 4-OH-TAM: 4-hydroxytamoxifen and COMB: combination.

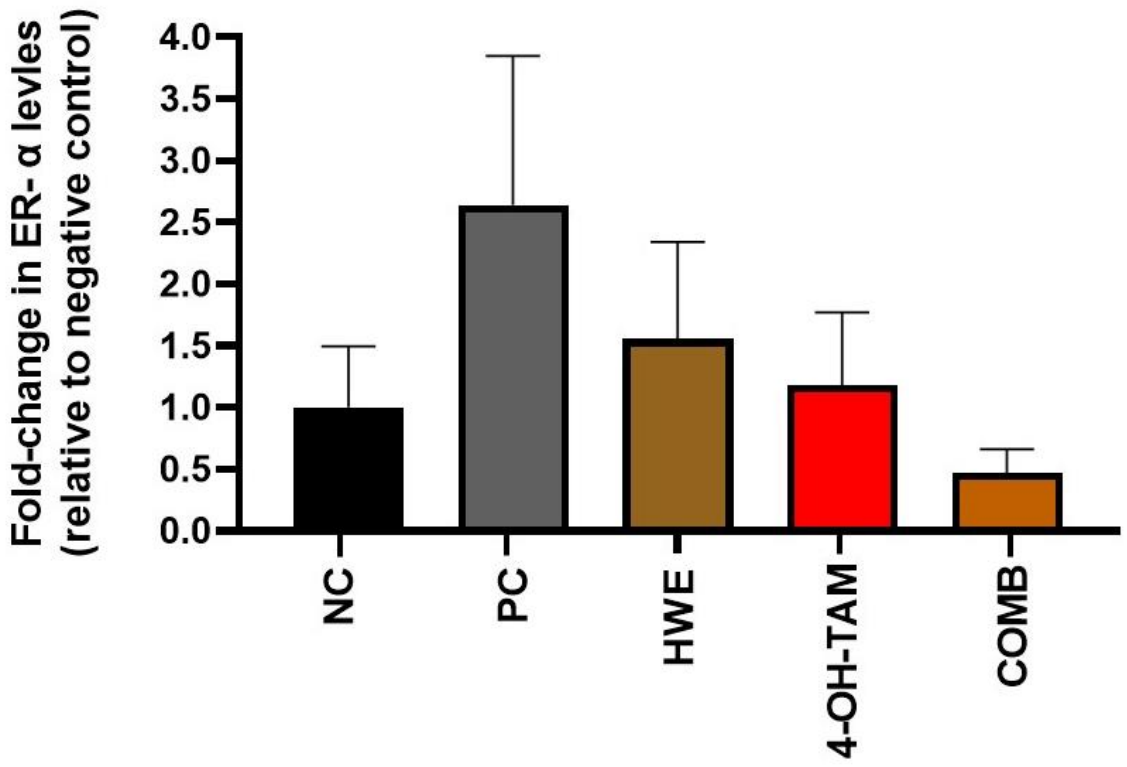


Figure 3.9 The effect of combination treatment on ER-α expression. Abbreviations: NC: negative control, PC: 17-β oestradiol control, HWE: hot water extract, 4-OH-TAM: 4-hydroxytamoxifen and COMB: combination.

Although the HWE increased ER-α expression, the combination reduced expression via possible nuclear dysregulation. Studies have shown that tamoxifen-induced apoptosis may be related to the recruitment of coregulators that do not normally

interact with ER-α, resulting in the dysregulation of ER-α.[128] More research into the coregulators is needed to confirm this. *Solanum* constituents, like solasodine, have been shown to dysregulate ER,[129] which may be similar to what has been observed in the present study. Literature on the dysregulation of ER-α outlines that this can be both advantageous and disadvantageous depending on the target and outcome thereof.[130] In this study, the advantage of dysregulation of ER-α may be due to the negative feedback of the COMB, whereby the receptor signalling is reduced. Decreased activation of the ER may result in decreased binding or oestrogen to ER-α, thus leading to reduced tumour growth and proliferation.[131] Consistent with the current findings, a study conducted by Milanesi and Boland showed that the extracts of the Solanaceae family of plants contain specific endogenous compounds that closely resemble 17β-oestradiol, a compound that binds to ER, which can bind to ER, competitively inhibiting the binding of oestrogen to the ER.[132] The reduced ER-α signalling with the COMB treatment directly corresponds with reduced cell density (Figures 3.3E and 3.4E), indicative of decreased cellular growth and proliferation. This provides evidence to state that *S. aculeastrum* in combination with 4-hydroxytamoxifen may be a beneficial synergistic mechanism for the treatment of ER-positive breast cancer.

Chapter 4: Conclusion

References

Appendix I: Ethics approval for the study

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through June 30, 2025 and Expires 07/28/2026.

Faculty of Health Sciences **Research Ethics Committee**

13 June 2024

Approval Certificate
Annual Renewal

Dear Miss CS Van Ballegooyen,

Ethics Reference No.: 441/2020 – Line 5
Title: Synergistic cytotoxicity of Solanum aculeastrum with 4-hydroxytamoxifen in the MCF-7/TAMR-1 breast adenocarcinoma cell line

The **Annual Renewal** as supported by documents received between 2024-05-15 and 2024-06-12 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-06-12 as resolved by its quorate meeting.

Please note the following about your ethics approval:

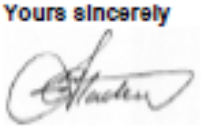
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2025-06-13.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *ad hoc* by the REC;
  c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (441/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely



On behalf of the FHS REC, Professor Werdie (CW) Van Staden
MBChB, MMed(Psych), MD, FCPsych(SA), FTCL, UPLM
***Chairperson**: Faculty of Health Sciences Research Ethics Committee*

Appendix II: Reagent preparation