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Combinational effects of crude garlic and 4-hydroxytamoxifen on the MCF-7 breast cancer cell line

by

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Acknowledgements

To my late son (2016-∞): Dearest, sweet Lithemba. "I have trouble accepting that the fact that you're gone, so I won't. It'll be like we went for a while without seeing each other, but I can understand why God would want you close to Him because you were truly an angel on earth. In a special way, I love you. I miss you." ❤️ – Earl "DMX" Simmons

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"Dreams delayed are not dreams denied" – R.L. White

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Abstract

Tamoxifen remains the preferred standard choice of treatment for oestrogen receptor- and/or progesterone-positive breast cancer among females. Unfortunately, approximately 30% of these patients develop resistance to tamoxifen (*de novo* or acquired), despite an initial positive response to the treatment. Tamoxifen-resistance presents an obstacle to hormone treatment since it has often been associated with poor survival rates. Due to such resistance, alternatives have been investigated either to replace treatment, or reverse resistance mechanisms. The use of complementary and alternative medicine has drastically increased among breast cancer patients. It is used either exclusively, or in addition to prescribed treatment (termed integrative medicine). Garlic (*Allium sativum* L.) is known to contain a variety of phytochemicals potentially beneficial in disease treatment due to anti-proliferative, anti-oxidative, and anti-inflammatory effects. However, sparse data is available on the combinational use of crude garlic extracts with tamoxifen in breast cancer treatment. The aim of the project was to determine the effects of crude garlic extracts in combination with tamoxifen in tamoxifen-sensitive breast adenocarcinoma cell lines.

Ethnomedicinal (hot-water) and pharmaceutical-representative (methanol) extracts of the garlic bulb were prepared by brewing and ultrasonic maceration, respectively. Preliminary cytotoxicity evaluation of the crude garlic extracts and 4-hydroxytamoxifen on MCF-7 breast adenocarcinoma cells were performed using the sulforhodamine B (SRB) assay. Thereafter, the synergistic cytotoxic evaluation of the combination of the crude garlic extracts and 4-hydroxytamoxifen were performed in a checkerboard manner using the SRB assay. The mechanism of action of the crude garlic extract, 4-hydroxytamoxifen, and combination thereof, was assessed by determining the alterations to the cell cycle, levels of nitric oxide and lipid peroxidation, and the activity of caspase-3/7.

Crude hot-water and methanol extracts were prepared successfully; however, the extracts showed minimal inherent cytotoxicity as a half-maximal inhibitory (IC_{50}) concentration could not be calculated for either extract at the highest concentration tested (100 µg/mL). The cytotoxicity of 4-hydroxytamoxifen was determined with an IC_{50} of 10.99 µM. Combining the extracts (7.5, 15 and 30 µg/mL) with 4-hydroxytamoxifen (¼IC_{50}, ½IC_{50} and IC_{50}) yielded a range of compound interactions including synergistic, additive, and antagonistic effects. The combination with the highest synergistic activity was 7.5 µg/mL of the hot-water extract and 10.99 µM of 4-hydroxytamoxifen (combination index = 0.624). The hot-water extract (7.5 µg/mL) potentially induced cell cycle arrest in MCF-7 cells at G_0/G_1 phase. The cells treated

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with 4-hydroxytamoxifen (10.99 µM) indicated cell death as most cells were in the sub-G_1 phase. The combination of the hot-water extract and 4-hydroxytamoxifen arrested more MCF-7 cells in the G_0/G_1-phase similarly to the solo treatment with the hot-water extract. The hot-water extract (7.5 µg/mL; $p < 0.05$) and 4-hydroxytamoxifen (10.99 µM; $p < 0.001$) alone significantly increased nitric oxide levels. Furthermore, the combination of the of hot-water extract and 4-hydroxytamoxifen increased nitric oxide levels significantly ($p < 0.001$). Treatment with the hot-water extract (7.5 µg/mL) significantly ($p < 0.001$) decreased lipid peroxidation suggestive thereof that the hot-water extracts possess intrinsic anti-oxidant properties, while 4-hydroxytamoxifen (10.99 µM) significantly ($p < 0.001$) increased lipid peroxidation. Though, the combination of hot-water extract and 4-hydroxytamoxifen increased lipid peroxidation significantly ($p < 0.001$), the amplified nitric oxide levels is also associated with nitrosative stress resulting in lipid peroxidation. The hot water extract (7.5 µg/mL) did not significantly decrease nor increase caspase-3/7 activity after 24 or 48 h, respectively. However, 4-hydroxytamoxifen did increase caspase-3/7 activity significantly after 24 ($p < 0.01$) and 48 h ($p < 0.001$). The combination of the hot-water extract and 4-hydroxytamoxifen increased caspase-3/7 activity significantly ($p < 0.001$; for both) after 24 and 48 h incubation. The mechanism of cell death induced by the combination of the hot-water extract and 4-hydroxytamoxifen appears to be mediated by the synergism of the individual treatments. The use of a synergistic combination in tamoxifen-resistant breast cancer cells have the potential to re-sensitise resistant cells to tamoxifen. This could be a step towards overcoming resistance in breast cancer patients, thereby improving prognosis.

Keywords: 4-hydroxytamoxifen, breast cancer, combinational treatment, garlic, MCF- cells

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Abbreviations

Ac-DEVD-AMC	N-Acetyl-Asp-Glu-Val-Asp-7-amindo-4-methylcoumarin
AF-2	Activation factor 2
AI	Aromatase inhibitor
AKT	Protein kinase B
Bax	Bcl-2 associated protein x
Bcl-2	B cell leukaemia/lymphoma 2
BMI	Body mass index
BRCA	Breast cancer susceptibility gene
CAM	Complementary and alternative medicine
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CI	Combinational index
CK	Cytokeratin
COVID-19	Coronavirus disease 2019
CYP450	Cytochrome P450
DADS	Diallyl disulphide
DAS	Diallyl sulphide
DATS	Diallyl trisulphide
DCIS	Ductal carcinoma *in situ*
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
ErbB2	v-Erb-b2 avian erythroblastic leukaemia viral oncogene homolog 2

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	receptor tyrosine kinase 2
ERE	Oestrogen-response element
FCS	Foetal calf serum
H12	Helix 12
HER2	Human epidermal growth factor receptor-2
HR	Hormone receptor
IC_{50}	Concentration of a drug required to inhibit the response by 50%
IGF	Insulin-like growth factors
MAPK	Mitogen-activated protein kinase
PBS	Phosphate-buffered saline
P-gp	Permeability glycoprotein
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-biphosphate 3-kinase
PMSF	Phenylmethanesulfonyl fluoride
PR	Progesterone receptor
ROS	Reactive oxygen species
SERD	Selective oestrogen receptor degrader
SERM	Selective oestrogen receptor modulator
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TNBC	Triple-negative breast cancer
TP53	Tumour protein 53 gene
UGT	Uridine diphosphoglucuronosyl transferase

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Measurements

^{o}C	Degrees Celsius
%	Percent
cells/mL	Number of cells per millilitre
cells/well	Number of cells per well
g	Grams
g	Relative centrifugal force
kg/m^{2}	Kilogram per square meter
mg/day	Milligram per day
mg/mL	Milligram per millilitre
mg/µL	Milligram per microlitre
M	Molar
mL	Millilitre
mM	Millimolar
µM	Micromolar
µL	Microlitre
µg/mL	Microgram per millilitre
v/v	Percentage of volume of solution to total volume
w/v	Percentage of weight per volume

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Chapter 1: Literature review

1.1. Introduction to breast cancer

Breast cancer is the uncontrolled proliferation of cancerous mammary epithelial cells that line the lobules and ducts,[1-4] and in more rare occurrences, the stromal portion of the breast.[3,5] Breast cancer is characterised as a highly heterogeneous spectrum of diseases clustered into numerous subtypes according to its aetiology, and distinctive histological and clinical presentations.[6] It is the leading malignancy diagnosed in females worldwide.[7] An estimated 2.31 million new cases of breast cancer and approximately 665,684 breast cancer-related deaths were reported in 2022.[8] Furthermore, it was estimated that the incidence rate of breast cancer will reach 3.2 million new cases by 2050.[9]

Breast carcinogenesis originates from hyperproliferation[10] and gradually evolves to morphologically distinct precursor lesions, to carcinomas *in situ,* sarcomas,[5] and invasive breast cancer, resulting in metastatic breast cancer.[10,11] The precursor lesions and varied histologic morphologies of breast tumours are the phenotypic manifestations of multifaceted genetic and epigenetic variations that drive breast carcinogenesis.[12] The putative precursor lesions range from atypical ductal hyperplasia, columnar cell lesions, ductal carcinoma *in situ*, flat epithelial atypia and lobular neoplasia.[11,13] The lesions are associated with low nuclear grade breast cancer carcinogenesis, implying that neoplastic developments occurs earlier than *in situ* carcinomas.[11,13] There is increasing evidence that stem cells are considered to be the origin and driving force in initiation, progression, metastasis, drug resistance and recurrence of all types of breast cancers.[13,14]

Initiation and promotion of breast cancer is a consequence of complex cumulative genetic mutations that favour activation of proto-oncogenes and silencing of tumour suppressor genes.[15] The disruption of regulated cell function including deoxyribonucleic acid (DNA) repair, unregulated proliferation, and suppressed apoptosis follow the genetic alterations.[15,16] The underlying aetiology of breast cancer is associated with multiple factors, such as, exogenous oestrogen, environmental factors, and viruses. Previously, it was considered that spontaneous mutations or exposures to carcinogens were the sole driving force in carcinogenesis.[17] Once the development of breast cancer is initiated in mammary cells by a leading mutation, there are three foremost genetic pathways of carcinogenesis (**Figure 1**).[12]

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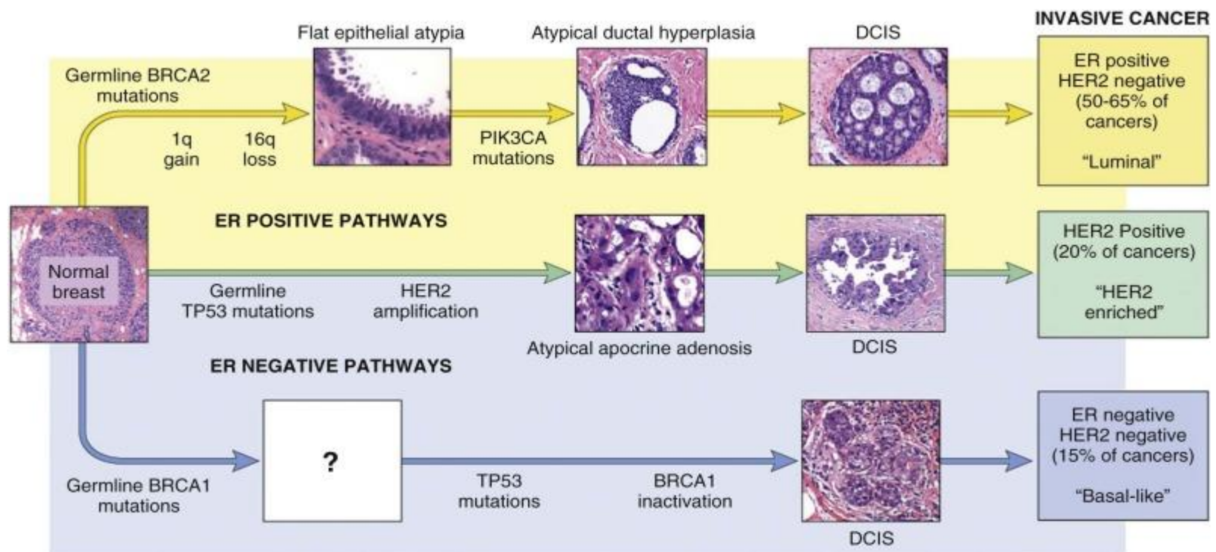

Figure 1. The three main genetic pathways of breast carcinogenesis. Breast cancer susceptibility type 1 (BRCA1) gene, ductal carcinoma in situ (DCIS), human epidermal growth factor 2 (HER2), phosphatidylinositol-4,5-biphosphate 3-kinase CA gene (PIK3CA), oestrogen receptor (ER), tumour protein 53 (TP53) gene, and unknown mutations following germline BRCA1 mutations (?).[12] [Image published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

The most common germline mutations implicated in breast cancer are breast cancer susceptibility gene 1 (*BRCA1*), breast cancer susceptibility gene 2 (*BRCA2*), and tumour protein P53 gene (*TP53*).[12,18] Germline mutations in the *BRCA1* and *BRCA2* genes predispose females to a high risk of breast cancer and remains the prominent explanations for developing breast cancer, with minimal influence of the population and statistical method used to determine the estimates.[19] It is estimated that the lifetime risk is 40-87% for carriers with *BRCA1* mutations[20,21] and 18-88% for carriers with *BRCA2* mutations.[20,22] Additionally, germline mutations in *TP53* pose a high risk of breast cancer, and is estimated to be 80-90%.[19,23]

The first pathway of breast cancer, initiated by the allelic loss of *BRCA2*, leads to the development of oestrogen receptor (ER)-positive and human epidermal growth factor 2 (HER2; syn. ErbB2 receptor tyrosine kinase [ErbB2])-negative tumours, and is considered the dominant pathway.[12,24,25] The loss at *BRCA2* locus is associated with chromosome 1q-gains and 16-losses, and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) catalytic subunit α mutations, leading to oestrogen- and progesterone receptor (PR)-positive breast tumours.[12] Endogenous oestrogens, which are steroid hormones, have attracted attention in the tumorigenesis of breast cancer.[26,27] Biosynthesis of endogenous oestrogens originates primarily

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in the ovaries from dietary cholesterol, particularly low-density lipoprotein cholesterol, in a process termed steroidogenesis.[26,27] The physiological function of the oestrogens is largely regulated by ER alpha (ERα) and beta (ERβ), which are ubiquitous in many cells and tissues.[28] The ERα and ERβ receptors are a part of a superfamily of transcription factors and are an expression of two independent genes located on different chromosomes.[29,30] Structurally, the two receptors bind to similar DNA response elements as the DNA-binding domain is 97% similar.[29,30] However, they exhibit different ligand selectivity as the ligand-binding domain is 55% similar between the two receptors.[29,30] The activation of ERα by oestrogens in breast cancer is considered responsible for the amplified proliferation, whereas the presence and activation of ERβ is described to exert an anti-proliferative effect.[28]

Therefore, it is theorised that the development of ER-positive breast cancer is further perpetuated by the amplified activation of ERα as a result its proliferative effect.[28] Endogenous oestrogens may initiate breast cancer through ER-dependent and -independent pathways.[31,32] The presence of oestrogen initiates either the nuclear (genomic ER) or non-nuclear (non-genomic ER) signalling pathways (**Figure 2**).[24]

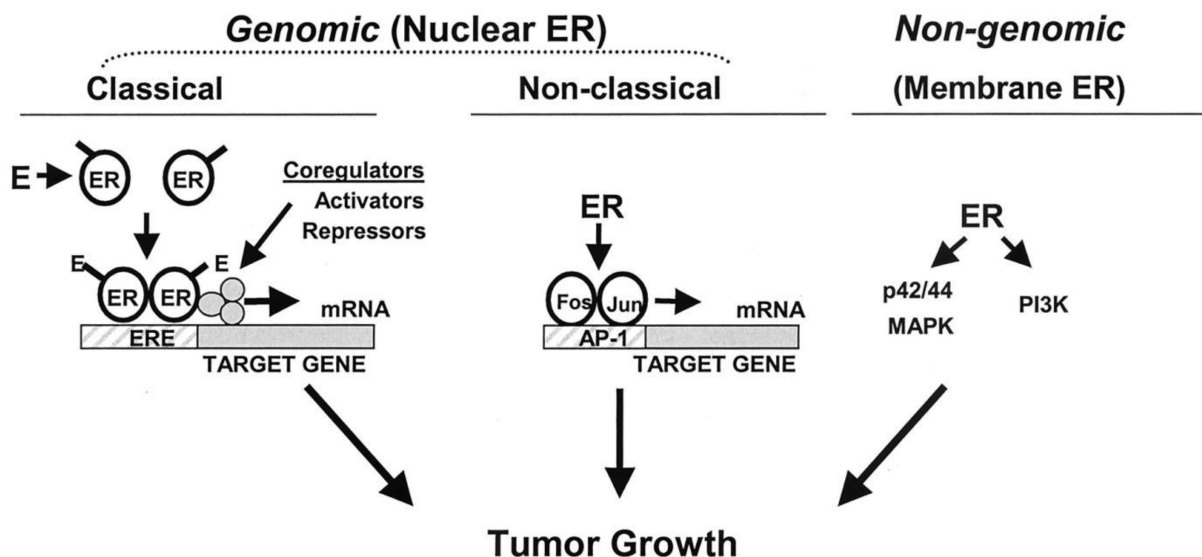

Figure 2. Schematic diagram of the nuclear and non-nuclear oestrogen receptor (ER) pathway. Protein kinase B (AKT), activating protein-1 (AP-1), oestrogen (E), oestrogen receptor (ER), oestrogen-response elements (ERE), messenger ribonucleic acid (mRNA), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K).[33] [Image published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

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During the first nuclear pathway (often referred as the classical pathway), oestrogen enters the cells in the breast through passive diffusion to bind to the ER at high affinity.[34,35] The complexation allows for receptor dissociation from inhibitory heat shock proteins,[34,35] inducing phosphorylation and conformational change.[34,36] The conformational changes lead to self-homodimerisation, allowing helix 12 (H12) to seal the ligand into the binding pocket.[24,36] The action of H12 results in the activation factor 2 (AF-2) cleft opening for the associated co-factors to bind to the cleft at the LXXML motif.[36] Thereafter, the homodimer translocates to the nucleus, forming a transcriptional complex with co-regulators at the DNA region of oestrogen-response elements (ERE), 3'-untranslated regions of target genes, and/or within regions in close proximity to promoters.[26,34,36] The transcriptional complex activates the transcription of the targeted genes, and their products promote the transcription of secondary, and/or tertiary genes, thereby influencing gene expression of ER targets.[37] The second nuclear (non-classical) pathway indirectly influences gene expression using other transcription factors (e.g. activating protein 1/Fos/Jun complex).[34] The activation of these pathways leads to increased cell growth, proliferation and division with a concomitant increased risk of DNA mutations.[31,32]

In the non-nuclear ER pathway, oestrogen binds directly to membrane ER to phosphorylate and activate tyrosine kinase receptor and insulin-like growth factor 1 (IGF-1). The activation of IGF-1 induces the activation of mitogen-activated protein kinase (MAPK)/extracellular activated kinase pathway. Moreover, the membrane ERα activates the G-proteins, matrix metalloproteinases, and tyrosine sarcoma kinase, thereby activating epidermal growth factor receptor (EGFR) and PI3K, leading to the induction of the protein kinase B (AKT) signalling pathway. The induction of the EGFR/PI3K pathways promote proliferation and cell survival[.24,38] Lastly, the membrane ERα may lead to changes in second messengers and cytoplasmic concentrations of calcium, and ultimately activation of additional signalling pathways.[38]

The ER-independent pathway involves the metabolism of 17β-oestradiol and oestrone by the cytochrome P450 (CYP450) enzymes, particularly CYP1A2, CYP1A1, CYP1A2 and CYP1B1 isoforms.[2,17] The oxidative metabolism of these oestrogens generates catechols followed by genotoxic quinones.[31,32] The quinones then form mutagenic adenine-guanine adducts.[31,32] The mutagenic adducts are released from the backbone of the DNA leaving depurinated sites that undergo erroneous DNA repair, leading to mutations.[31,32] Additionally, reactive oxygen species (ROS) are formed as a result of the reduction-oxidation (redox) cycling of 4-hydroxyestradiol

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to 3-4-oestradiol quinone and contribute to oxidative DNA damage (e.g., 8-oxo-deoxyguanine).[31,32]

The development of HER2-enriched tumours arise through the second pathway whereby non-*BRCA1/2*, and *TP53* mutations induce *HER2* overexpression on chromosome 17q12-21 (**Figure 1**).[12,39,40] Research has shown that the transformation potential of HER2 is conferred by a V664E point mutation on the chromosome, and the mutated protein is named neuT.[40] While the point mutation promotes increased tyrosine kinase activity and receptor dimerisation, it is unlikely that the mutation occurs spontaneously. Many HER2-enriched tumours are a result of gene amplification, but some are derived from transcriptional dysregulation, such as the induction of prostaglandin synthase cyclooxygenase-2, chemokine receptor CXCR4, and E26 transformation specific transcription factors.[40] Overexpression of HER2, either through transcriptional dysregulation or gene amplification, is an early event in breast cancer pathogenesis, but HER2-enriched subtypes are molecularly different to the other subtypes.[40] Moreover, germline *TP53* mutations primarily cause Li-Fraumeni Syndrome, a cancer predisposition syndrome, commonly associated with HER2-enriched sub-types.[12,41] The development of the HER2-enriched sub-type is less common than the ER-positive carcinomas, and may express or lack ER and PR.[12]

The least common type of breast cancer lacks ER, PR and HER2, and is termed triple-negative breast cancer (TNBC).[12] Several theories exist regarding the pathogenesis of this breast cancer sub-type (**Figure 1**).[42] Nevertheless, it is widely accepted that a germline dysfunction of *BRCA1* plays an important role in the development of this breast cancer sub-type.[12,42] Protein BRCA1 has a multifactorial role in transcriptional regulation of ER, and DNA damage response.[42] Apart from the role in the conversion of ER-negative breast cells to ER-positive breast cells, BRCA1 is implicated in DNA repair functions by means of homologous recombination.[12,42] Thus, cells with mutated *BRCA1* genes resort to DNA repair mechanisms that are non-conservative and are genomically unstable.[12,42] The instability should activate checkpoint mechanisms that would favour cell cycle arrest or apoptosis.[12,42] Thus, tumours with germline *BRCA1* mutations tend to develop mutations that regulate these checkpoints, e.g., inactivation of *TP53*.[12,42] Consequently, the majority of TNBCs are classified as "basal-like" carcinomas by gene expression profiling.[12,42]

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1.2. Molecular classification of breast cancer

Clinically, breast cancer is characterised according to the histological grade and morphological characteristics of the tumours (**Figure 3**).[43,44] These methods are considered imperative diagnostic and prognostic tools for breast cancer management.[43] Based on the histology, various subtypes exist, including invasive ductal carcinoma, invasive lobular carcinoma, mucinous carcinoma, medullary carcinoma, and tubular carcinoma.[43]

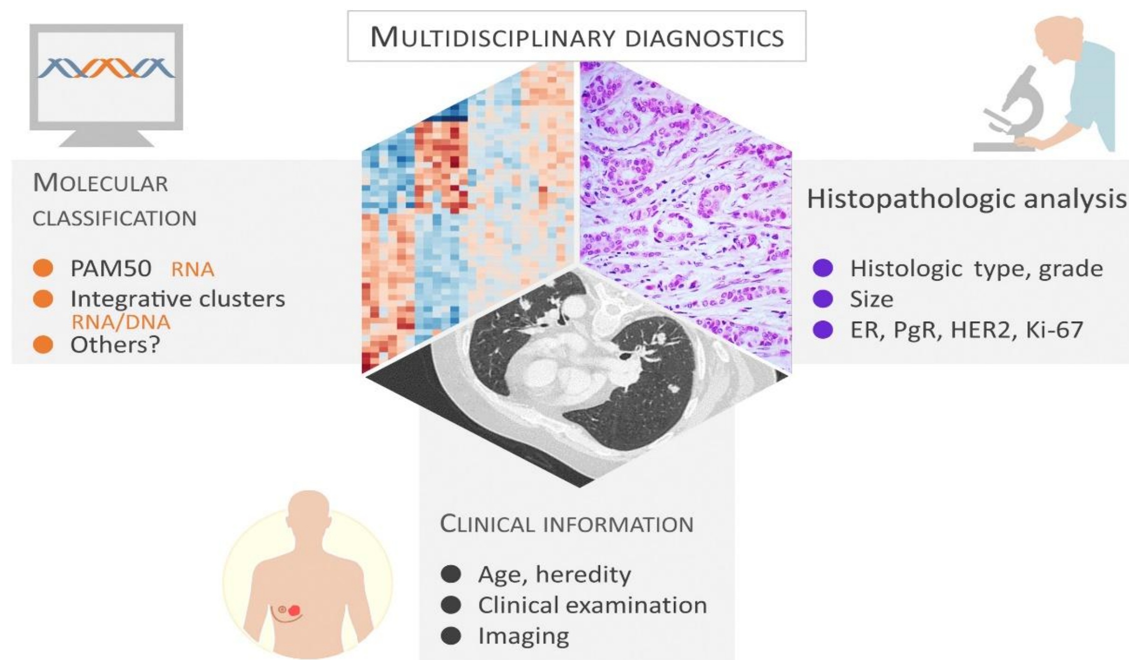

Figure 3. Schematic diagram of a multidisciplinary diagnostic approach for breast cancer. Deoxyribonucleic acid (DNA), oestrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), prediction analysis of microarray 50 (PAM50), progesterone receptor (PgR), and ribonucleic acid (RNA).[45] [Image published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

Invasive ductal carcinomas constitute a heterogeneous group of tumours that lack adequate characteristics of a unique differentiation, and represent 70-80% of invasive tumours.[43,45] Macroscopically, the tumours are typically hard with stellar outlines, rarely with sharp outlines and are of a soft nature.[43] The histological grading (three grades) of malignancy takes into consideration mitoses, nuclear atypia, and formation of glandular tissue.[43] Invasive lobular carcinomas represent 5-15% of invasive tumours.[43,45] Macroscopically, the tumours consist of abrasions that are more tangible than observable.[43] The intrusion of the tumours often conforms to the pre-existing structure of the breast.[43] The lack of E-cadherin expression, a type of intercellular adhesion molecule, is a definitive marker of invasive lobular carcinomas that are no longer cohesive.[43] The malignant cells are small in size, remote or in a single file, invading

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fat tissue links without reaction stroma or surrounding canals.[43] This partially explains the challenges in detecting these type of carcinomas.[43] The term, mucinous carcinomas, is set aside for the pure morphology, and represents 2% of all invasive carcinomas.[43,45] The carcinomas appear round with huge areas of extracellular mucus filled with floating cancerous cells.[43] Medullary carcinomas also represent 2% of all invasive carcinomas, and are often associated with *BRCA1* mutations.[43,45] Classically, these carcinomas have distinctive outlines containing poorly differentiated cells with a moderate to discernible lymphoid infiltration.[43] Tubular carcinomas were seldomly observed (representing 1% of invasive carcinomas), but since screening methods improved, an increase in these carcinomas have been noted.[43] These tumours typically appear hard with star-shaped abrasions formed by normal cells deposited in tubules, enclosed by a large quantity of fibrous stroma.[43] On imaging of these carcinomas, they usually appear as a star-shaped mass with a minute centre that is dense or morphologically distorted, seldom with amorphous micro-calcifications or a round mass.[43] There are other infrequent carcinoma sub-types, and the World Health Organization has identified and characterised twenty-one of them including inflammatory and papillary carcinomas.[43,45]

The grade of the tumours is categorised as grade I (low grade; with a growth rate that is fairly slow), grade II (intermediate grade) and grade III (high grade; most aggressive).[43] The classical criteria (i.e., histology, size and grade of the tumour, as well as status of axillary lymph nodes) are essential for the initial prognostic assessment.[46] Despite these criteria being well established for each grade of the tumour, the clinical presentation is not seen in reality.[45,46] A robust classification system is essential for optimal disease management and treatment.[45,47] The presence or absence of hormone receptors (HR) (i.e., receptors for oestrogens and progesterone) is visualized using histological imaging and affects prognosis.[43] Immunohistochemistry is used to detect HR in the nuclei of invasive malignant cells, whereas the surrounding tissue is regarded as the control.[43] The threshold for a positive diagnosis is set as detection in 10% of marked cells.[43] The presence of the receptors indicates hormone-sensitive tumours and is associated with a more favourable prognosis, whereas the absence of these receptors results in treatment with chemotherapy.[43] Expression of these receptors namely, ER, PR, and HER2 remains an essential part in diagnostics, as it determines suitability for hormone therapy.[43]

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Gene expression profiling has played a huge role in breast cancer biology.[48] There are five main molecular sub-types of breast cancer, which are based on molecular and genetic information: luminal A, luminal B, HER2-enriched, basal-like and normal breast-like (Figure 4).[43,48]

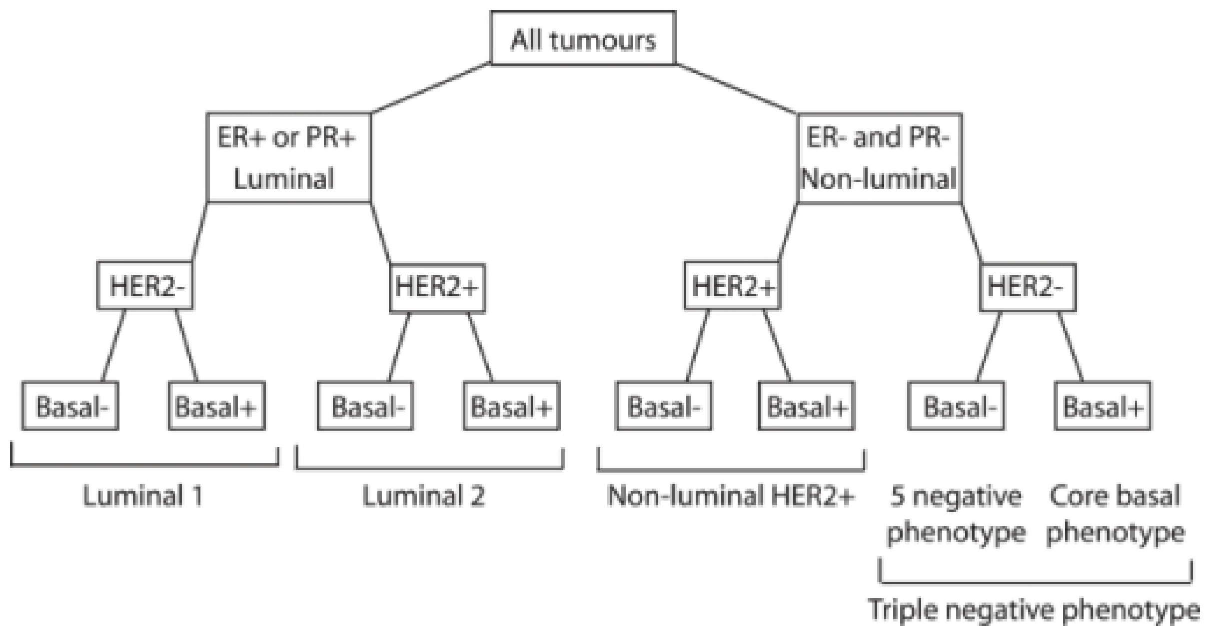

Figure 4. Classification of breast cancer according to gene profiling. Absence of surface markers (-), human epidermal growth factor 2 (HER2), oestrogen receptor (ER), presence of surface markers (+), and progesterone receptor (PR).[49] [Image published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

Luminal type tumours have cell protein expression at the lumen of the ducts hence the term 'luminal'.[50] Luminal A type tumours represent 50-60% of breast cancers and are characterised by the high expression of ER genes and ER regulatory genes (forkhead box A1 gene, GATA binding protein 3 gene), low proliferation genes (cell division cycle 6 gene, cell cycle-related protein B1 gene), and the absence of HER2 expression.[50-52] An estimated 13% of these luminal A subtypes have *TP53* gene mutations.[43] Phenotypically, luminal A types correspond to grade I or II invasive ductal carcinomas or invasive lobular carcinomas and tubular carcinomas, express ER and PR, and have low expression of the proliferation marker (Ki-67 < 14%).[43,50,52] Luminal A breast cancer tends to have a good prognosis, with a 94.3% five-year survival rate.[53]

Luminal B tumours are more aggressive phenotypically than luminal A,[50,52] and represent 15-20% of breast cancer cases.[43,52] In contrast to the luminal A types, luminal B types have a lower expression of ER regulatory genes and a higher expression of proliferative genes.[50,52] Approximately 66% of luminal B types have *TP53* mutations.[43] In clinical practice, luminal B

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tumours correspond to grade II or III invasive ductal carcinomas or invasive lobular carcinomas, express ER, have no amplified HER2, and possess a high expression of the proliferation marker (Ki-67 >14%).[43,50,52] Carcinomas with *BRCA2* mutations fall within the luminal B category.[43] Luminal B breast cancer tends to have a good prognosis, with a 90.5% five-year survival rate.[53]

Approximately 10-20% of the breast cancers are HER2 type.[43,52] This breast cancer subtype is characterised by the absence of ER genes and ER regulatory genes, overexpression of HER2 amplicon genes (growth factor receptor-bound protein 7 gene) and genes related to proliferation.[50,52] Furthermore, an estimated 71% of these HER2 subtypes have *TP53* mutations.[43] With regards to the phenotype, this subtype corresponds to grade II invasive carcinomas, does not possess ER and PR, is HER2 enriched, and the expression of Ki-67 does not have an influence on the tumours.[43] Historically, HER2 subtypes were associated with a poor prognosis, but this has since improved to 82.7-94.7% five-year survival rate.[54,55]

Basal-like tumours are a distinctive molecular subtype and account for 10% of all tumours,[43,50] with 56-85% of TNBC classified as this tumour type.[43] Basal-like tumours have contrasting histo-clinical features with luminal subtypes.[43,50,52] Core basal-like types express basal cytokeratins (CKs) in the basal cells of healthy milk ducts or CKs with large molecular weights (fibrous intracellular polypeptides).[50,52] They are characterised by the high expression of basal CKs (CK5/6, CK14, and CK17),[50,52] FAB7,[43] laminin[43,52] and genes related to proliferation, particularly *EGFR*.[43] Mutations in *TP53* has been observed in 82% of basal-like types[43] and there is evidence of an inactive retinoblastoma protein pathway in these types.[52] Clinically, this type of tumour correlates to grade III invasive ductal carcinomas, there is the absence of all three receptors, poor differentiation, and the presence of central necrotic or fibrotic zones, genomic instability, and highly proliferative and mitotic indices.[52] The deregulated expression of integrin has also been observed and explains the aggressiveness of cell behaviours and progression of this tumour type.[43,52] It is estimated that TNBCs represent 7-16% of all breast tumours[43] and the deletion in chromosome 5q in basal-like TNBCs is associated with 71% of breast cancers with *BRCA1* mutations, while no correlation between TNBC and *BRCA2* mutations have been identified.[43,56,57] Similarly to core basal-like types, TNBCs are characterised by the high expression of CK5/6 and CK14, EGFR, and the complete lack of expression of ER, PR, and HER2, hence the term ‘triple-negative’.[43,56] The phenotype of TNBCs corresponds with grade II or most likely grade III poorly differentiated invasive carcinomas.[43] Tumours characterised as TNBCs are heterogeneous clusters consisting of 85%

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of medullary, metaplastic, apocrine, secretory, and *BRCA1* related cancers.[43,58] This subtype is known to be aggressive, and the lack of receptors restricts treatment to chemotherapy. Furthermore, TNBCs have been associated with the worst overall survival and poor clinical outcomes (70.2-77% five-year survival rate).[59,60] Approximately 4-20% of patients with metastatic TNBCs have a five-year survival rate.[61] Blows *et al* have sub-classified another basal-like phenotype, which is negative for ER, PR, HER2, EGFR, and CK5/6.[49]

It is assumed that the subtype of breast cancer considered non-cancerous-like might not comprise a true subtype, but rather is an overrepresentation of normal breast cells.[56] Luminal A and non-cancerous-like share similar immunohistochemical characteristics, i.e., ER- and PR-positive, HER2-negative, and Ki-67 negative. However, they differ in the pattern of expression, with non-cancerous-like sub-types being a reflection of normal breast cells rather than cancerous cells[56,62,63]

1.3. Epidemiology of breast cancer

The incidence of breast cancer varies between countries and within countries.[64,65] Incidence is influenced by income status and socioeconomic status, with higher incidence rates seen in high income countries and used to be low in low- and middle-income countries but is on the increase.[9,64,66] The incidence rates range between 13.5 and 44 per 100 000 women in eastern, central and southern parts of Asia, Europe, sub-Saharan Africa, Latin America and the Caribbean.[9,67,68] In parts of the Middle East, North America, and Australia, the incidence rate is 90 to 120 per 100 000 women .[9,67,68] Within Africa, incidence rates vary, with northern Africa reported to have the highest incidence (53.2 per 100 000 women), followed by southern Africa (46.2), western Africa (45.4), eastern Africa (31.9), and lastly central Africa (26.7).[69] Data from GLOBOCAN in 2022 recorded 14,712 new cases of breast cancer and 5,232 breast cancer-related deaths in South Africa.[8] The burden of the disease is projected to reach more than 3 million new cases and more than 1 million breast cancer-related deaths by 2040.[70] The projected increase in incidence rate of breast cancer in Africa is thought to be due to increased urbanisation, more African females adopting Westernised lifestyles that favour delayed age at first pregnancy, reduced parity, and increased postmenopausal weight.[64,67]

Conversely, mortality rates are higher in less developed countries;[9,71,72] with the highest being reported for Africa (20 per 100 000 women).[9,69] The high mortality rate in Africa is the result of several factors (e.g., delayed health-seeking behaviour, limited or no access to healthcare

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centres for early detection, less effective diagnostic and therapeutic methods, poor standards of diagnostic infrastructures, and lack of breast cancer awareness).[9,64]

The emergence of Coronavirus disease (COVID-19) led to an unprecedented prioritisation of healthcare facilities shifting the focus on the management of other treatable conditions, including breast cancer.[73-75] Breast cancer patients are considered high-risk patients and prioritisation of COVID-19 negatively affected the early diagnosis and surgical treatments of these patients, ultimately affecting their survival rate.[74]

Genetics, the environment, and hormonal risk factors all play a role in breast cancer.[76] It is well known that African Americans and females who live in low- and middle-income counties in Africa present with tumours that are at an advanced staged and which are larger, more aggressive, and lack HR compared to those in high-income countries, thereby decreasing survival outcomes.[58,67,68,72] An estimated 89.6%, 72.8%, and 50-55% of females with breast cancer present with advanced stage tumours, in Kenya, Nigeria and South Africa, respectively.[68,71,72] Though, high-quality population based cancer registries encompass only 2% of the population in Africa.[77]

1.4. Risk factors for breast cancer

Breast cancer risk is mostly associated with sex, age, genetics, reproduction (e.g., menarche, parity, pregnancy, duration of breastfeeding, and use of contraceptives), lifestyle (e.g., diet and obesity), and environmental factors (e.g., low-dose radiation, pollutants, and consumer products), some of which are modifiable.[78,79] These factors are discussed below.

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1.4.1. Sex

Globally, male breast cancer represents less than 1% of all cancer-related cases.[9,46,78] Similar to female breast cancer, the incidence of male breast cancer varies significantly with region and ethnicity, with the largest number of cases reported in Africa.[80] The risk factors for male breast cancer are not different to that of female breast cancer (i.e., age, genetics, and hormone imbalance).[9,81] The most prevalent subtype of male breast cancer is luminal.[46,50,52]

1.4.2. Age

The risk of breast cancer developing in females increases with older age reaching its peak at the onset of menopause and then steadily declines or remains constant.[9,79] Furthermore, advancements in screening and diagnosis of breast cancer have led to a greater number of the population being detected early with breast cancer.[82] Age and genetic profile of females play an influential role in the molecular subtype presented.[82] It is estimated that females under the age of 70 with *BRCA1* and *BRCA2* mutation face a 57% and 49% risk of developing breast cancer, respectively.[82] Furthermore, TNBC is the most likely subtype to develop in females under the age of 40, followed by the HER2-enriched subtype.[82] This tendency is said to continue until the age of 60, where both luminal A and B subtypes become the most prevalent subtype, whereas TNBC has the lowest incidence rate.[82]

1.4.3. Genetics

Many breast cancer tumours result from accumulation of somatic mutations, but it is well-established that there is a considerable amount of mutations that predispose individuals to breast cancer, apart from the inherent risks of age and sex.[5,83,84] The assessment of breast cancer risk takes into account the number of family members, subtype of breast cancer, and age at first diagnosis of the relatives.[85] An estimated 20-25% of breast cancer patients are genetically predisposed to breast cancer, but only 5-10% of these cases exhibit an autosomal dominant inheritance (i.e., hereditary).[5,79,83] It is postulated that the risk of developing breast cancer almost doubles if a female has a first-degree relative (i.e., daughter, son, sister, brother, mother, or father) who has been diagnosed with breast cancer (i.e., familial).[5,86] The risk further increases 2.93- or 3.90-fold in females with two or three diagnosed relatives, respectively.[86] Genetic mutations and aberrant expressions of oncogenes and tumour suppressor genes play crucial roles in the development and progression of breast cancer.[87] Numerous genes have been identified in relation to breast cancer, but the main genes identified to influence the risk of breast cancer is mutations in *BRCA2*, *TP53* and *BRCA1*, leading to three main genetic pathways.[12,82,88] As previously mentioned, the pathways initiate the development of HR-

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positive,[12,25] HER2-enriched,[12,39,40] and HR- and HER2-negative “basal-like”[12,42] breast tumours, respectively.

1.4.4. Reproduction

Exposure to exogenous and endogenous oestrogens, (e.g., hormone replacement therapy) used by postmenopausal females and oral contraceptive use, is considered a risk factor for developing breast cancer for a various reasons.[89-91] Epidemiological studies associate the production and/or the overexposure to oestrogens in postmenopausal females as a factor that may enhance the risk of developing breast cancer.[31] A pooled analysis of nine prospective studies found a correlation between concentrations of circulating endogenous oestrogen (i.e., oestrone and oestradiol) with breast cancer risk in postmenopausal females.[92] Postmenopausal females with elevated levels of oestrogens in the serum were twice more likely to develop breast cancer compared to those with lower serum concentrations.[27,92] A large prospective study also found that females with higher concentrations of oestrogens in urine were significantly at risk of developing breast cancer.[93] There are fewer studies published on the correlation of oestrogen levels and the risk of breast cancer in premenopausal females. However, one study that assessed elevated levels of free oestrogen in premenopausal found a correlation with an increase in breast cancer risk.[94] A common limitation reported in assessing the correlation of oestrogen levels and breast cancer risk in premenopausal females, is the complex method to adjust for the hormone concentration variation during the menstrual cycle.[95] Menopausal females are strongly associated with the risk of developing luminal and HER2-enriched tumours.[79,96] [96]

Reproductive factors, such as young age at the onset of menarche, is considered a risk factor among both pre- and postmenopausal females for developing breast cancer.[97] Delayed onset of menarche has consistently been associated with moderate reduction in the risk of TNBC and luminal A breast tumours.[97] Furthermore, menarche delay by two years is linked with a corresponding risk reduction of 10%.[79] It is hypothesised that high consumption of meat and obesity may be the causative factors that lead to early onset of menarche,[78] thus implicating nutrition in cancer progression as well.[79] Nulliparous females are at a greater risk of developing breast cancer in comparison to parous females.[79] Early age at first birth has a general protective effect, whereas relatively older age at first birth confers a risk of breast cancer higher than that of a nulliparous female.[79] The cumulative risk of breast cancer in females bearing their first child at age 20, 25, and 35 years was 20% lower, 10% lower and 5% higher, respectively, compared to nulliparous females.[79]

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Lactation is also proposed to play a role in preventing breast cancer development by reducing levels of circulatory endogenous sex hormone and delay return of ovulatory cycles.[79] Approximately, a risk of 4.3% of breast is reduced for every year of breastfeeding.[79] Furthermore, longer duration of lactation was found to be inversely associated with basal-like subtypes.[96] There is conflicting data about the correlation of natural or self-induced abortions with breast cancer, though a study has demonstrated that a higher incidence rate of abortion was linked with increased risks of breast cancer.[79] Late onset of menopause has also been linked with higher risks of breast cancer, conferring a 3% risk increase every year, and 17% for every five years.[79] The correlation between these reproductive risk factors and breast cancer is based on sex hormones (i.e., androgen, oestrogen, and progesterone) that begin at puberty, continuing during the menstrual cycles, and eventually declining in menopause.[9,98] These sex hormones are also affected by parity as parity is said to have a protective effect against breast carcinogenesis due to the decreased circulatory oestrogen levels.[99] Due to the crucial role of oestrogens in breast carcinogenesis, it is proposed that these risks factors are positively linked to HR-positive breast cancer subtypes.[96]

1.4.5. Lifestyle

1.4.5.1. Diet

Diet may play a role in the development of breast cancer, and an association between fat intake and breast cancer has been reported.[100,101] High dietary fat intake leads to a build-up of adipose tissue, a major site for androstenedione conversion.[100,101] A metabolite of polyunsaturated fatty acids, arachidonic acid, induces the activity of aromatase, which converts androstenedione.[101] The fatty acid metabolite may also reduce the binding of oestrogen with albumin and globulin, therefore, increasing the systemic concentration of oestrogen.[101] It has been hypothesised that intake of saturated fat may promote breast tumorigenesis by reducing induction of apoptosis, increasing cholesterol and low-density lipoproteins, and promoting an inflammatory response.[100] The consumption of meat has also been associated as a risk factor for breast cancer in postmenopausal females.[102] Apart from heterocyclic amines and polycyclic amino hydrocarbons, meat contains 40% heme iron, an organic form of iron, and is said to be significantly associated with an increased risk of breast cancer.[102-104] A high consumption of dietary meat, food fortified with iron, and dietary supplements containing iron may lead to lipid peroxidation,[101,105] as well as DNA damage, and oxidative stress owing to its pro-oxidant properties.[101] Iron is thought to be involved in breast carcinogenesis through its interaction with oestrogen and other cellular pathways (i.e., upregulation of interleukin-6/janus kinase 2/signal

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transducer and activator of transcription pathway 3).[103,106]

1.4.5.2. Obesity

Obesity is often associated with the increased risk of various conditions, including breast cancer.[90,107,108] Obese postmenopausal females are at a higher risk for breast cancer, with the HR-positive subtype being most prevalent.[108,109] It is estimated that for every 1 kg/m^2 increase in BMI, the risk for breast cancer increases by 3.4%,[110] or a 5 kg/m^2 increase in BMI requires a 2% increase in the risk of breast cancer in postmenopausal females.[108,111] It has been suggested that the association between obesity and the increased risk of breast cancer may be due to hyper-activation of insulin and IGF, increase in adipokine and oestrogen levels, and overexpression of pro-inflammatory cytokines, which result in hypercholesterolemia.[112,113] Another reason for the increased risk of breast cancer in postmenopausal females may be a result of unregulated hormones and uninhibited expression of adipokines in the adipose tissue, causing obese or overweight postmenopausal females.[108,110] Most circulatory oestrogen in postmenopausal females is derived from the conversion of androstenedione by adipose aromatase.[108,110] Consequently, this perpetuates a prominent rise in oestradiol, free oestradiol, and oestrone in these individuals, and these sex hormones control adipocyte differentiation and fat distribution leading to elevated adipose tissue.[108] Subsequently, the breast tissue is exposed to elevated levels of oestrogen, a condition that is known to be the main cause of breast carcinogenesis.[108] However, it is proposed that a higher BMI in premenopausal females may be associated with a decreased risk of developing luminal A breast cancer subtype, with the exception of those who have a family history of this disease.[97,108] The mechanism of the protective effect remains unclear, but there may be lower levels of oestrogen and progesterone, and a combination of longer anovulatory cycles.[108,111] However, such individuals do remain at an increased risk of developing TNBCs.[97]

1.4.6. Environment

Lifestyle and environmental factors contribute approximately 70-95% in the risk of developing cancer.[114] Numerous environmental carcinogens have been identified by the International Agency for Research on Cancer, and the prominent factors are low-dose ionising radiation and environmental oestrogens.[114,115]

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1.5. Treatment

Treatment of breast cancer involves a multidisciplinary approach that includes local (i.e., surgery and radiation therapy) and systemic therapy (i.e., chemotherapy drugs, hormonal drugs, and immunotherapy drugs).[116,117] For patients with invasive non-metastatic breast cancer in stages I to III, local therapy comprises mastectomy or breast-conserving surgery, which may be followed up with post-operative radiation therapy.[117] Systemic therapy may be administered pre-operatively (neo-adjuvant), post-operatively (adjuvant), or both.[117] The breast cancer subtype guides the systemic therapy administered.[117] These methods have led to successful clinical response and outcome in patients diagnosed with early-stage breast cancer.[117] For decades, hormone- and chemotherapy have been used to treat breast cancer.[118] Currently, the use of biologic agents is steadily increasing in breast cancer treatment, with trastuzumab emtansine being the first biologic agent developed.[118,119] These agents are designed to target different aspects known to contribute to breast carcinogenesis, such as mammalian target of rapamycin (mTOR) pathway (e.g., everolimus), poly-(ADP)-ribose polymerase (e.g., olapirib), HER2 (e.g., trastuzumab emtansine), and IGF receptors (e.g., ganitumab).[119-122] Unfortunately, resistance to treatment develops in a subset of patients, where the cancer aggressively progresses to more advanced stages.[123,124] However, for patients with metastatic breast cancer, the therapeutic goals are palliative. Currently, metastatic breast cancer remains incurable in the majority of patients that are affected. Local therapy modalities are usually used for palliative care, while the same guidelines for non-metastatic breast cancer are applied.[117]

1.5.1. Surgery

The surgical treatment of breast cancer has undergone a profound and continuous evolution in the past decades, with developments focused on minimising the long-term aesthetic and functional consequence of local therapy.[117,125] Surgery remains the mainstay therapy for breast cancer, and continues to play a pivotal role in the control of the disease.[126,127] There are two main types of breast cancer surgery: mastectomy and breast-conserving surgery, with subclasses within each type.[126]

Mastectomy procedures (e.g., total or simple mastectomy, Halsted's radical mastectomy, modified radical mastectomy, skin-sparing mastectomy, and nipple-sparing mastectomy) aims at surgically removing as much breast tissue as possible, with glandular tissue most likely to remain at the inframammary fold.[128,129] Mastectomy remains the backbone of breast cancer treatment for patients with *BRCA1* mutations as they are most likely to have an increased risk of experiencing local recurrence.[129]

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Breast-conservation surgery (also called a lumpectomy or quadrantectomy) is the complete removal of the cancerous tumour with a negative margin (defined as the margin of the normal tissue surrounding the cancer) conducted in an aesthetically-acceptable manner.[125,130] It is generally followed by adjuvant radiation therapy to the entire breast as it has proven to be an effective alternative to mastectomy in early stage invasive breast cancer.[125,130,131] Mammographic screening, diagnostic ultrasound, and physical examination are the standard imaging modalities used to screen patients for breast-conservation surgery.[132] The aim of the surgery is to provide a clinical survival rate equivalent to mastectomy, yet maintain the aesthetic appeal acceptable to the patient.[130,131,133,134]

1.5.2. Radiation therapy

Radiation therapy (or radiotherapy) is a multidisciplinary treatment of breast cancer and an indispensable tool for the treatment of all breast cancer stages.[135,136] Similar to chemotherapy, radiation therapy can be administered prior to surgery to reduce the size of breast tumours, allowing resection of the tumour, and has become standard adjuvant treatment following breast-conservation surgery and mastectomy.[137-139] The goal of radiation therapy is to reduce the risk of local recurrence within the treated region.[140] There are two main types of radiation therapy used to treat breast cancer: external beam radiation and internal radiation (or brachytherapy). External beam radiation utilises high-energy rays from a machine outside the body for six weeks in fractioned courses.[141] Conventional external radiation therapy has been replaced by newer techniques such as three-dimensional-conformal radiation therapy and intensity-modulated radiation therapy.[138,142] Internal radiation therapy can be completed in four to five days, and therefore there is an increased interest in using this therapy as the only modality following breast-conservation surgery.[143] Radiation therapy is painless, though not devoid of adverse effects.[139] As such, patients, especially children, are administered anaesthetics (e.g., propofol, barbiturates, and ketamine) to ensure complete immobility; however, problems could arise following chronic exposure to the anaesthetics.[139] Patients could develop tachyphylaxis; and daily intubations may damage the trachea causing stenosis, also, poor nutritional status amongst children due to the daily fasting may prolong recovery from the anaesthetics.[139] With internal radiation, a radioactive source is placed inside the body for a limited time.[139,142] Both types of radiation therapy requires the physician to identify the malignancy, the healthy cells, and recommend an appropriate radiotherapy dose based on the size of the tumour and its histology.[138] It is important to note the tolerance level of the tissue affected by the radiation and the level of the tissues between the skin and the tumour.[139] The

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duration of the treatment is over a number of days (lasting a couple of minutes) for several weeks. [138,139]

1.5.3. Chemotherapy

Chemotherapy involves the use of chemical compounds to treat various types of cancer, including breast cancer.[144] These drugs are specifically designed to target essential processes involved in cell division in actively-reproducing cells.[145,146] Chemotherapeutic drugs are typically classified, among others, as alkylating agents (e.g., mechlorethamine, a nitrogen mustard), anti-metabolites (e.g. methotrexate, a folic acid analogue), synthetic congeners (e.g., polychlorinated biphenyls) and natural products (e.g., doxorubicin, an antibiotic), as well as miscellaneous agents (e.g., cisplatin, a platinum co-ordinating complex).[144,145,147] The aim of chemotherapy is to eradicate all potential micro-metastases, thereby reducing risk of recurrence and breast cancer-related death.[123] However, the use of chemotherapy is dependent upon consideration of the potential benefits and risks.[123] It is generally offered prior to or following surgery, with a similar clinical outcome and survival rate.[123] Localised breast cancer occurs in 61% of cases and is theoretically curable with local and/or systemic therapy.[148] Chemotherapy drugs are used as part of neo-adjuvant and adjuvant chemotherapy, including anthracyclines (epirubicin, doxorubicin) and taxanes (docetaxel, paclitaxel), and can reduce the risk of recurrence.[148] Neo-adjuvant chemotherapy offers several advantages over adjuvant chemotherapy, and has been used successfully to reduce the size of breast carcinomas allowing for breast-conservation surgeries.[149] Breast-conservation surgery following neo-adjuvant chemotherapy may, however, result in higher rates of recurrence.[150] The international breast cancer guidelines recommend the use of neo-adjuvant chemotherapy compared to adjuvant chemotherapy for patients under the age of 70 years with locally advanced grade III tumours.[123] Neo-adjuvant chemotherapy can also be considered for patients with grade II tumours, but with a clear intention for the use of adjuvant chemotherapy.[123] Thus, predictors considered significant for considering using neo-adjuvant chemotherapy appear to be a young age, large tumours, and absence of the three HRs.[123] Despite its well-established therapeutic effects, chemotherapy drugs also negatively affect the non-cancerous actively reproducing cells.[145] The cells involved in the reproduction system are affected as they rapidly multiply. The key to effectiveness of chemotherapy is the ability to balance the death of the healthy cells along with the cancerous cells.[145]

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1.5.4. Hormone therapy

Currently, an estimated 75% of documented breast cancer cases are HR-positive.[36,38,52] Hormone therapy (also referred to as endocrine therapy) targeting the ER pathway is a highly effective choice of treatment for ER-positive patients (**Figure 5**).[36]

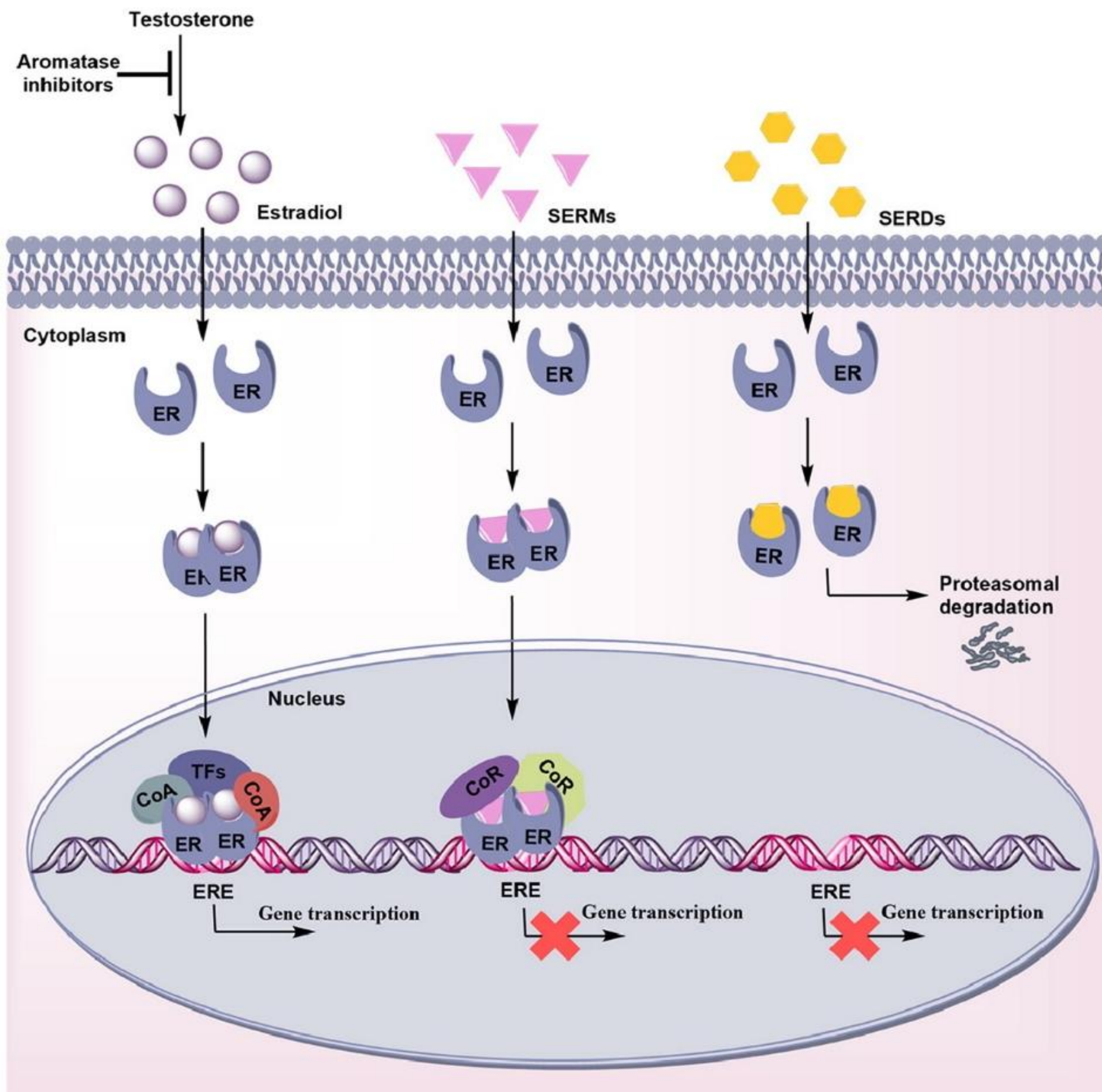

Figure 5. The mechanism of action of the hormone therapies on the nuclear ER pathway in breast cancer cells. Co-activators (CoA), co-repressors (CoR), oestrogen-responsive elements (ERE), selective oestrogen receptor degraders (SERDs), selective/ oestrogen receptors (SERMs), and transcription factors (TFs). [36] [Image published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

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Endocrine therapy's efficacy is largely due to the ER pathway's driving force of development, proliferation, progression, and metastasis of HR-positive breast cancers.[38] Existing hormone therapies target the ER pathway and are grouped according to their mechanism of action: i) inhibition of oestrogen production (i.e., aromatase inhibitors [AI]), ii) degradation of ER expression (i.e., selective oestrogen receptor degraders [SERD]), and iii) modulation of ER activity (i.e., selective oestrogen receptor modulators [SERM]).[36,38] The use of different hormonal drugs is regulated by guidelines that factor the stage of the tumour, age of the patient, and previous medical history.[36,38]

1.5.4.1. Aromatase inhibitors (AI)

As previously mentioned, postmenopausal females who are obese or overweight, are often diagnosed with ER-positive breast cancer as a result of increased adiposity.[108,110] The majority of the circulatory oestrogen in postmenopausal females is synthesised from the conversion of androstenedione by adipose aromatase in peripheral tissues such as the ovaries, breast, endometrium, bone, and skeletal muscle.[108,110,151] Consequently, a high BMI in postmenopausal females results in an obesity-induced hyper-activation of the aromatase enzyme resulting in breast tissue being continuously exposed to elevated levels of oestrogen.[108,111,152] Therefore, the blocking of oestrogen production, particularly in this subset of females, is an essential therapeutic target.[153]

Aromatase, belongs to the CYP450 superfamily of enzymes and is involved in oestrogen biosynthesis by converting androgens (e.g., androstenedione and testosterone) into oestradiol and oestrone in a process termed aromatisation.[153] Unlike the other naturally occurring oestrogens, oestrone is predominant in postmenopausal females.[151] Thus, the overexpression of aromatase in ER-positive postmenopausal females results in the breast cells becoming the primary source of oestrogen production in the neoplastic cells.[151] As part of first-line treatment for postmenopausal females with ER-positive breast cancer, AIs are included in the armamentarium, as evidence has shown that AIs are superior to tamoxifen, a first-line drug for ER-positive breast cancer in premenopausal females.[153-155] Molecularly, AIs can be classified into two types: steroidal (e.g., exemestane and fornestane) and non-steroidal (e.g., letrozole and anastrozole).[153] However, the use of both types of AIs have shown unforeseen problems such as non-responsive, acquired resistance, and CYP450 enzyme inhibition, which results in relapses in patients.[151,153,154]

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1.5.4.2. Selective oestrogen receptor modulators (SERMs)

The SERMs are used for breast cancer in premenopausal treatment, and rely on their tissue-specific interactions with the ER, suggesting a certain level of complex molecular and functional drug properties.[156] As such, SERMs are termed 'selective', as the modulation activity and downstream signalling pathway is dependent on the type of tissue that expresses the receptors.[36] An example is tamoxifen, a drug used to treat breast cancer, that presents with ER antagonism in breast tissue, while exerting agonist or partial ER agonism in the heart, uterus, and the bone.[36]

These non-steroidal compounds are designed to compete with oestrogen for binding to the active site on the ER.[36] The binding of the pendant side-chain of SERMs initiates a physiological change between helices 11 and 12, preventing H12 from covering the ligand-binding pocket, forcing the helix to reach the stationary region of the AF-2 surface.[36] Once H12 reaches the region, it mimics the action of the co-factors by binding to AF-2 using its own LXXML motif, thereby blocking the binding of co-factors. Ultimately, this results in the receptor being inhibited and inactivated.[36] The SERMs are categorised according to their chemical makeup as either triphenylethylenes (e.g., tamoxifen and 'tamoxifen-like), phenylindoles (e.g., bazedoxifene and pipindoxifene), benzothiophenes (e.g., raloxifene and arzoxifene), or tetrahydronaphthalenes (e.g., lasoxifene).[36]

1.5.4.3. Selective oestrogen receptor degraders (SERDs)

Both SERMs and AIs are considered part of first-line treatment for ER-positive patients; however, within five years, nearly 50% of patients fail to respond or develop resistance to treatment.[157] In these instances, patients are treated with SERDs, since the SERDs possess the potential to inhibit oestrogen-dependent and oestrogen-independent signalling pathways by degrading the ER.[36,38] Furthermore, they are accepted as therapeutic treatment for ER-positive breast cancer in both early and advanced stages of treatment resistance.[157]

Both SERMs and SERDs are considered anti-oestrogens due to the effect on receptors of the ER signalling pathway; however, their mechanistic actions differ.[36] While both SERMs and SERDs are anti-oestrogens, SERDs are described as pure anti-oestrogens due to their lack of agonistic effect in all ER-positive cancer cells.[36] There are two common examples of SERDs, i.e., fulvestrant and elacestrant, that are used in treatment of breast cancer.[35,38,157] Fulvestrant,

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a first generation SERD, is recommended for postmenopausal females who experience treatment failure with anti-oestrogen therapy.[38,157]

1.5.4.4. Targeted therapy for HER2 overexpressed tumours

As part of the HER family of tyrosine kinase receptors, HER2 is a major driver of tumour growth in 20% of breast cancers.[158] Unlike ER and/or PR-positive tumours, HER2 enriched tumours do not have a ligand and therefore, depend on hetero-dimerisation with other HER receptors or homo-dimerisation when expressed at extremely high levels.[159] These HER2 receptors are favoured dimerisation partners for other HER receptors, and these hetero-dimers have the highest mitogenic potential compared to all of the HER hetero-dimer complexes; thus, the overexpression of HER2 in the breast tumours results in constant receptor activation and uncontrolled cell proliferation.[159,160] Consequently, there is unpredictable and exaggerated cell survival, migration, invasion, and adhesion with increased disease recurrence, aneuploidies, and characteristics strongly associated with poor survival rates.[120,161]

Monoclonal antibodies are a class of drugs that are used to treat HER2-enriched breast cancer because they are designed to target the extracellular domain of HER2 receptors, and include trastuzumab emtansine.[120,159,162] These drugs suppress the homo- and hetero-dimerisation of HER2, averting the activation of the intracellular domain, and delaying the onset of the downstream cascades accordingly.[160] Chronic exposure to trastuzumab has led to acquired resistance to the drug.[160,162] Pertuzumab is another recombinant humanised monoclonal antibody therapy used for the treatment of HER2, and has mechanistic actions complementary to trastuzumab.[160,163] It was designed specifically to bind to the dimerisation domain of HER2 to sterically inhibit HER2 dimerisation, while trastuzumab binds near the transmembrane subdomain of the receptor.[160,163] The second class of drugs used in treatment for HER2-enriched tumours are the intracellular tyrosine kinase inhibitors (e.g., lapatinib).[162]

1.6. Use of tamoxifen in breast cancer treatment

Tamoxifen has been considered a pioneering drug in medical oncology, and has been used for over forty years for the treatment of ER-positive breast cancer in pre- and postmenopausal females.[36,164,165] Furthermore, tamoxifen remains the preferred choice for treatment of male breast cancer, node-negative premenopausal females, and can be used either alone or in conjunction with AIs in node-positive postmenopausal females.[155,165,166] Following its discovery, tamoxifen has been well-received as an inexpensive drug which is easily accessible for under-developed health care systems.[155] In females with moderate to high risk of

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developing cancer, tamoxifen or raloxifene, is documented to decrease the risk of breast cancer by 33-47%.[167,168]

Tamoxifen is administered orally at doses of 10 mg twice daily or as a 20 mg drug taken daily for five years.[165,169,170] As a prophylaxis for high-risk patients, the administered dose is 20 mg daily for five years, and it is recommended that tamoxifen be taken on the second to fifth day of the menstrual cycle.[165] It is readily absorbed in the gastrointestinal tract and systemic concentrations peak after four to seven hours.[165] Once absorbed, tamoxifen is bound to protein, predominately albumin, and is distributed in the circulatory system to various sites such as the liver.[165] In the liver, tamoxifen is extensively metabolised by CYP450 enzymes, particularly CYP2D6 and CYP3A4/3A5 isoforms, to yield its active metabolites *N*-desmethyltamoxifen (major metabolite) and 4-hydroxytamoxifen (

Figure 6).[36,155,165] These metabolites are further metabolised to yield 4-hydroxy-*N*-desmethyltamoxifen (endoxifen).[36,155,165] Tamoxifen is a prodrug as these two metabolites present with a 100-fold greater affinity towards ER than tamoxifen and exert greater anti-oestrogen potency in breast cancer cells, particularly 4-hydroxytamoxifen and endoxifen.[166,171]

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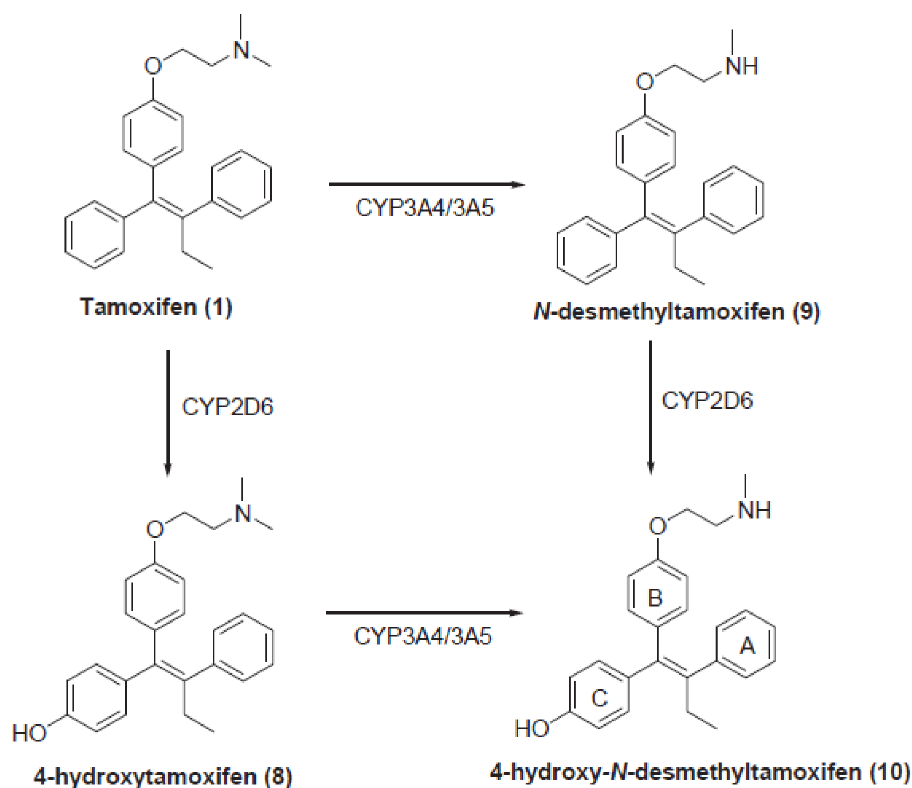

Figure 6. The metabolism of tamoxifen by CYP450 enzymes to yield its active metabolites.[155] [Image published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

The metabolites bind to the ligand-binding site of ERα with the side chain extending out of the ligand-binding down pocket between helices 3 and 11.[36] This side chain blocks H12 from covering the domain, thereby antagonising the ER.[36] Thereafter, co-repressors are recruited and a silenced ER transcriptional complex forms at the ERE region.[36] The interaction between the ligand and receptor is a biomarker of ER conformation and function, but the cellular response to a ligand-receptor interaction is not determined exclusively by it.[36] The biological composition of the cell is also influential in determining the agonistic and antagonistic response of ER signalling.[36] Differential expression of co-regulators, depending on the type of tissue, could be one of the reasons why 4-hydroxytamoxifen is a partial agonist in certain tissues (e.g., endometrium), while functioning as potent antagonist in malignant breast cells.[36] The majority of the phase II reactions involved in the conversion of the metabolites into excretable forms are catalysed by uridine diphosphoglucuronosyl transferases (UGT, mainly isoforms 1A8, 1A10, 2B7, and 2B15) that add glucuronide moieties to 4-hydroxytamoxifen and endoxifen,

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negating their anti-oestrogenic ability, or sulphated by sulfotransferases (mainly sulfotransferase 1A1) .[166,172]

Tamoxifen, and its metabolites, can induce cell death in breast cancer cells outside the ER genomic and nongenomic signalling pathway.[173,174] One mechanism of cell death is via nitric oxide-dependent pathways.[173,174] Tamoxifen increases the intra-mitochondrial calcium concentrations, thereby increasing the activity of mitochondrial nitric oxide synthase activity in breast cancer cells.[173,174] The elevated activity of mitochondrial nitric oxide potentiates the generation of nitric oxide via its nitric oxide synthase, which is known to inhibit cytochrome *c* oxidase, thereby initiating apoptosis.[173,175] Tamoxifen, and its metabolites, also act as an antagonist in breast cancer cells by binding to ERβ located in the mitochondria.[176,177] The binding with mitochondrial ERβ increases concentrations of ROS to cytotoxic levels.[176] The excessive nitric oxide and ROS react to form peroxynitrite anions resulting in nitrosative stress and peroxynitrite-induced damage, such as lipid peroxidation.[173,174] The effects of tamoxifen,[178,179] and 4-hydroxytamoxifen,[180,181] on cellular kinetics of breast cancer cells have also been assessed and extensively described to inhibit the cell cycle at G_0/G_1 phase while influencing the expression of proteins associated with cellular kinetics (i.e., p21, p53, and cyclin D).

1.7. Resistance towards tamoxifen in breast cancer treatment

Although the initial response to tamoxifen is positive, approximately 20-30% of hormone-sensitive tumours have *de novo* (i.e., present prior to treatment) or develop acquired (i.e., caused by long-term exposure to tamoxifen) resistance towards tamoxifen, possibly within the first two years of treatment.[7,182,183]

All of the enzymes involved in the metabolism of tamoxifen are encoded by polymorphic genes, each with unique allele frequencies and significance, and these polymorphisms may be responsible for the *de novo* resistance.[166] The gene that encodes for CYP2D6 is highly polymorphic and has more than a hundred documented different variations, and gene duplications and deletions are recurrent.[166] Single nucleotide polymorphisms are the most prevalent form of genetic polymorphisms.[184] Many of these variants can result in the formation of CYP2D6 enzymes with little to no enzymatic activity, and the effectiveness of tamoxifen may be dependent on the individual's CYP2D6 gene variation.[155,166] Based on the variations, individuals can be categorised as either: a) poor metabolisers, b) intermediate metabolisers, c) extensive metabolisers, or d) ultra-rapid metabolisers. Poor metabolisers are defined as

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individuals with little to no CYP2D6 activity since they possess two inactive alleles.[166] Intermediate metabolisers are categorised as individuals with reduced enzymatic activity due to one active and one inactive allele, or two decreased activity alleles.[166] Extensive metabolisers have typical CYP2D6 activity owing to no gene variants or possessing a single decreased activity allele.[166] Lastly, ultra-rapid metabolisers either do not have any inactive variants or have duplicated genes resulting in abnormally high activity.[166] Polymorphisms have been identified in genes encoding UGT and may play a role in tamoxifen-resistance.[184]

Several theories explaining the mechanism of acquired tamoxifen resistance have been put forward, many of which are centred around the activation, function, and structure of ERα and ERβ, along with the cross-talk between its signalling pathway and other signalling pathways (such as PI3K/AKT/mTOR pathway).[7,185] Expression of ERα has been the main target of hormone therapy and patients lacking the receptor usually do not experience any therapeutic benefits, although a small portion (5-10%)[186] of ERα-negative patients are tamoxifen-sensitive.[186,187] Conflicting evidence exist regarding the role of ERβ and response to hormone therapy.[187-190] It has been postulated that low levels of ERβ are associated with resistance,[187-190] while other studies have documented that the presence of ERβ in ERα-positive tumours are a biomarker for tamoxifen-resistance.[34,187,191] The loss of ER expression is often alluded to epigenetic changes such as abnormal methylation of cytosine-phosphate-guanine islands and histone deacetylation, which results in a compact nucleosome with restricted transcription.[34,187] Additionally, due to the crosstalk between the HER2 pathway and ERα, HER2 overexpression in tamoxifen-resistant cells reinforces the nuclear and non-nuclear signalling of ERα.[187,192] Pre-clinical and clinical studies have proposed various multifaceted pathways that lead to the development of tamoxifen resistance.[38] These mechanistic pathways include the activation and crosstalk between ER and receptor tyrosine cell signalling pathways (i.e., EGFR, IGF-1 receptor, and PI3K/AKT/mTOR pathways), abnormal expression of proteins (such as breast cancer anti-oestrogen resistance 1), and upregulation of the nuclear factor kappa-light-chain-enhancer of activated B cell signalling.[38,189,193] The development of acquired resistance to tamoxifen is unique because the growth of tamoxifen-resistant tumours is dependent on the presence of tamoxifen.[194] Thus, it seems that acquired resistance to tamoxifen exploits either, oestrogen or tamoxifen, as the growth stimulus in the ER-positive tamoxifen-resistant breast tumours.[194] Altered cellular and biochemical features of the tumours are often observed with acquired resistance.[183,195] This presents an important obstacle in hormone therapy as tamoxifen resistance is often correlated with decreased survival rates.[183,194] Additionally, there

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is increasing evidence suggesting that resistance to tamoxifen is accompanied by the change in the morphology of the cells akin to invasive and metastatic phenotypes, as there is loss of cell-to-cell junctions and the cells develop aggressive behaviours such as epithelial-to-mesenchymal transition, increased migratory capacity, and enhanced self-renewal abilities.[7,195]

1.8. Integrative medicine

1.8.1. Introduction to complementary and alternative medicine

Complementary and alternative medicine (CAM) refers to "a panoply of diverse healthcare and medical interventions, products, disciplines, or practices that are not considered part of mainstream conventional medicine" as defined by the National Centre for Complementary and Alternative Medicine.[196,197] The Centre later reclassified this definition, in 2017, under three titles: mind and body practices (e.g., meditation, chiropractic and yoga), natural products (e.g., probiotics, vitamins, and herbs), and other complementary methods (e.g., homeopathy, traditional Chinese medicine, and Ayurveda medicine).[197,198]

The prevalent use of CAM has garnered worldwide acceptance in recent years, varying according to geographic region, based on cultural influence.[197,199] It is estimated that 60-80% of the population in America,[199,200] and more than 80% of the Asian and African population use herbal remedies.[200] The acceptance of CAM can be attributed to the belief that they are effective, provides value for money, are not subjected to technology or complex scientific processing, and harnesses the body's natural ability to heal itself.[201,202] The term "alternative" in CAM refers to therapies that are used in place of the conventional medicine, while "complementary" refers to therapies that compliment conventional medicine.[203] Thus, it is increasingly becoming more common for patients to use a combination of complementary and conventional medicine, and this combination is termed integrative medicine.[203,204] Globally, the prevalence of integrative medicine among cancer patients has steadily increased, with breast cancer patients being the major potential users followed by prostate and melanoma cancer patients.[205] It is estimated that 80% of cancer patients worldwide pursue integrative medicine following diagnosis.[205,206]

Despite its popularity, concerns have been raised.[203] Research into CAM is expanding, however, the assessment of the clinical impact of CAM remains a challenge.[203] The reason being that CAM therapy lacks standardised manufacturing processes, contains varied amounts of bioactive compounds, are contamination prone, result in severe herb-drug interactions, and are usually not subjected to clinical trials.[203,207,208] As a result, herbal remedy preparations may

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yield products with differing pharmaceutical properties and chemical composition with differing safety, bioavailability, and pharmaceutical characteristics.[207] Therefore, little is known regarding the potential benefits, risks, adverse effects, and its interaction with conventional medicine.[203]

1.8.2. Phytochemistry of complementary and alternative medicine

Plants have long served as the major source of medicine, and are used as a part of CAM.[208] However, bioactive compounds vary in concentration and composition according to geographic distribution, harvest season, and processing.[203] Approximately 30-50% of all conventional drugs on the market are derived from plants.[209] Chemotherapy drugs derived from plants include paclitaxel (*Taxus brevifolia* and/or *Taxus baccata*),[210,211] vincristine (*Catharanthus roseus*),[212] vinblastine (*Catharanthus roseus*),[212] irinotecan (*Camptotheca acuminate*),[211] and topotecan (*Camptotheca acuminate*).[203,211] Thus, the potential to unearth prevalent anti-cancer compounds has been the driving force behind developing novel chemotherapy drugs.[144,203] Traditional healers use crude or whole-plant extracts to preserve the delicate chemical composition for effective treatment.[203,209] Conversely, pharmaceutical companies isolate specific bioactive compounds required for treatment.[203,209] Many studies have been carried out to identify the bioactive compounds of plants with anti-cancer activity.[203,209] Phytochemicals in cancer research with anti-cancer potential have been shown to inhibit carcinogenesis using various mechanisms; anti-proliferative, anti-oxidative, and anti-inflammatory effects by manipulating relevant cell signalling pathways.[203]

Recently, the correlation between diet and nutritional health has become accepted in the scientific community.[213] Phytoestrogens are polyphenolic compounds that naturally occur in plants and structurally resemble 17β-oestradiol.[214,215] Phytoestrogens are present in a variety of fruits (e.g., grapes, pears, and plums), vegetables (e.g., garlic, beans, and onions), tea, and wine.[213] They exist as inactive glycosides containing carbohydrate or sugar moieties.[216,217] Phytoestrogens consist of hydroxyl groups on the phenolic ring that are similar to the hydroxyl groups on the aromatic rings of 17β-oestradiol, and these structures are important in their abilities to bind to ERα and ERβ.[215,216,218] There are four major classes of phytoestrogens: stilbenes (e.g., resveratrol), lignans (e.g., matairesinol), isoflavones (e.g., genistein and daidzein), and coumestans, which are predominately found in legumes, wine, tea, vegetables, and fruits.[215,216,218] Garlic is one of the dietary sources of phytoestrogens, particularly lignans (i.e., enterolactone, secoisolariciresinol, matairesinol, and enterodiol).[219,220]

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Phytoestrogens are considered SERMs as they induce oestrogenic or anti-oestrogenic effects, dependent on the phytochemical-specific and tissue-specific elements.[216-218] Studies that have investigated the mechanistic action of phytoestrogens in breast cancer have observed that the analogues are more potent than the parent compounds in inhibiting the proliferation of breast cancer cells.[216] A frequently used CAM among cancer patients is garlic.[221] High concentrations of enterolactone, a lignan in garlic, has an affinity for ERα in HR-positive breast cancer cells, antagonistically competing with endogenous oestrogen for the receptor.[222] Thus, it is considered to have primarily anti-oestrogenic effects.[222] However, a weak proliferative effect on HR-positive breast cancer cells at high concentrations has also been observed.[222] Once the phytoestrogens in garlic bind to the receptors in breast cells, preferentially ERβ, the receptors translocate from the cytoplasm to the nucleus where they bind and influence the DNA transcription regions, eventually inhibiting cell signalling pathways via ER-dependent genomic and non-genomic cascades in ER-positive breast cancer.[216,218] Furthermore, the phytoestrogens have actions beyond its oestrogenic and anti-oestrogenic effect, since it may regulate the cell cycle, potentiate free radicals and/or anti-oxidants, induce autophagy or apoptosis, regulate oestrogen receptors, alter the epigenetics, and modulate signalling pathways.[216-218] Notably, the phytoestrogens and other phytochemicals at large are said to cause less cytotoxicity in healthy cells and present a low-cost, widely available epigenetic therapy.[218] Though, the herb-drug interactions may result in synergistic or additive effects such as hepatotoxicity[223] and nephrotoxicity[224] further scientific assessment of herbal products at a pharmacodynamic level is warranted.[224-226]

At low concentrations, phytoestrogens have been shown to act via ER-dependent genomic and non-genomic cascades; however, it is also speculated that phytoestrogens may exploit different mechanism of inhibition in both ER-negative and ER-positive breast cancer,[216] and its derivatives also inhibit uncontrolled cell growth by inducing apoptosis in breast cancer cells via the intrinsic and extrinsic pathways.[216-218] The phytoestrogens generate ROS, which increases pro-apoptotic proteins and decreases anti-apoptotic proteins thereby decreasing the B cell leukaemia/lymphoma 2 (Bcl-2)/Bcl-2 associated protein x (Bax) ratio and increasing the Bax/Bcl-2 ratio.[216-218,227] Phytoestrogens (e.g., resveratrol, coumesterol, daidzein, and genistein) and their analogues/derivatives have been shown to inhibit the progression of the cell cycle in breast cancer cells by reducing the expression of cyclins (i.e., D1 and E) and cyclin-dependent kinases (i.e., 1, 2, 4 and 6), and increasing the expression of cyclin-dependent kinase inhibitors (i.e., p21, p27 and p53).[216,217]

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1.9. Garlic

1.9.1. Introduction to garlic

Garlic (*Allium sativum* L.) is a perennial plant, with the aromatic bulb being widely consumed and used as a culinary spice.[228] There are diverse sub-types of garlic with hard-neck and soft-neck garlic being the most common species.[229] For centuries, it has been well-regarded as a therapeutic panacea for a plethora of illnesses as its many medicinal properties and biological functions have been established, such as anti-bacterial, anti-cancer, anti-fungal, anti-oxidant, and immunomodulatory activities.[228,230]

The Egyptians described 22 formulations for garlic to treat cardiovascular problems, and snake bites, while ancient Greeks consumed garlic for treatment of pulmonary and intestinal disorder.[229] Garlic has a highly selective cytotoxic effect without causing significant cytotoxicity in non-cancerous cells.[231] Fresh garlic bulbs contain water (65%), digestible and non-digestible carbohydrates (28%), 33 oil- [e.g., ajoene, diallyl disulfide (DADS), diallyl trisulfide (DATS)] and water-soluble organosulfur compounds (e.g., γ-glutamyl-*S*-allyl-L-cysteine and *N*-acetyl-*S*-allyl-L-cysteine), 17 free amino acids (e.g., arginine), protein, and dietary fibre (1.5%).[232,233] The bulbs also contain therapeutic phytochemicals including; polyphenols (i.e., *p*-coumaric acid, quercetin, rutin, and ferulic acid), minerals (i.e., zinc, calcium, and magnesium), vitamins (i.e., ascorbic acid, folate, and vitamin C), and terpenoids (i.e., β-carotene). [234,235] When the bulbs are chopped or macerated, the parenchyma of the clove's membrane is disrupted, and alliinase converts alliin (or S-allylcysteine sulfoxide) to 2-propenesulfenic acid (**Figure 7**). This acid self-condenses to form allicin, a diallyl thiosulfinate.[229,236] This diallyl thiosulfinate is responsible for the distinct odour and taste, but is chemically unstable in nature and readily degrades to form stable second-generation organosulfur compounds, such as diallyl sulphide (DAS), DADS and DATS.[229,236] Further biotransformation of the organosulfur compounds may ensue if they interact with free sulfhydryl groups, predominately found in proteins, glutathione, or cysteine.[237]

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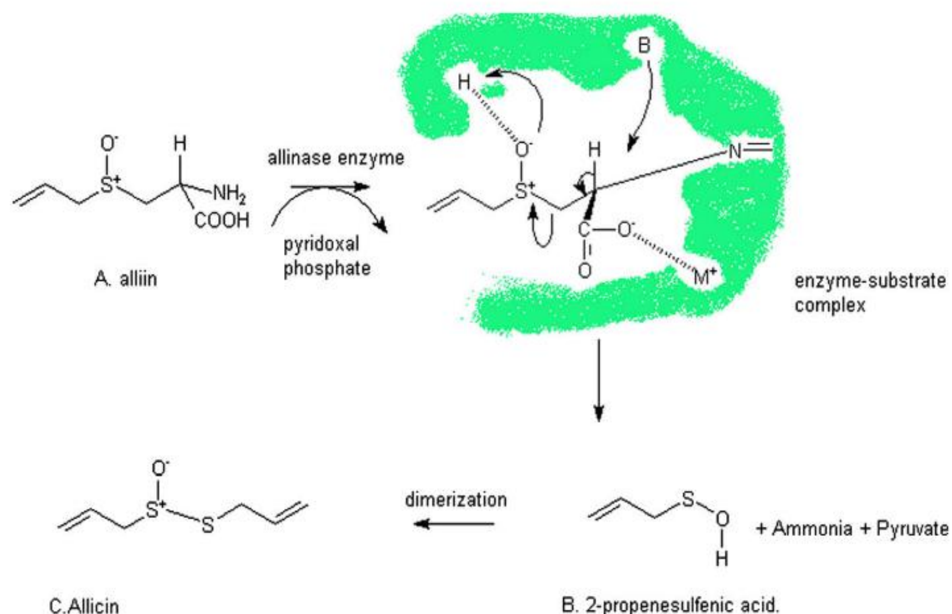

Figure 7. Diagram depicting the conversion of alliin to allicin.[238] [Image published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

The anti-cancer properties of the garlic-derived organosulfur compounds are widely reported in many cancers, but few have investigated the crude extract of these compounds, warranting research of new treatment strategies.[239] Recent literature suggests that these organosulfur compounds disrupt cell signalling by arresting the cell cycle at G_0/G_1 or G_2/M phases.[228,240] It is described that S-allyl cysteine inhibits the progression of the cell cycle into the S phase at the G_0/G_1 checkpoint, allicin inhibits the cell cycle at the S phase checkpoint, and DATS and S-propargyl-L-cysteine inhibit the cell cycle at the G_2 checkpoint.[228] Additionally, garlic is known to affect nitric oxide synthase activity, and promote the mitochondrial-dependent pathway for apoptosis and lipid peroxidation in cancer,[239,240] possibly in the same manner as tamoxifen.

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1.9.2. Combinational use of garlic and tamoxifen

Despite burgeoning research into the anti-cancer and transporter-enzyme modulation properties of garlic, few studies have investigated the impact of garlic in combination with standard therapeutic drugs, including tamoxifen.[241] Herb-drug interactions highlight a special interest in cancer patients as they are often treated with several co-administered chemotherapeutic medication aside from anti-neoplastic drugs.[226,241] The anti-cancer medication usually displays narrow therapeutic indices with inherent cytotoxicity at the recommended doses.[226] Herb-drug interactions occur when herbs are used in combination with conventional treatment to produce an outcome that is clinically or pharmacologically different than expected when conventional treatment is administered alone.[242] This combinational use has the potential to produce detrimental, therapeutic, or neutral effects at a pharmacodynamic, or -kinetic level (**Figure 8**).[205,209]

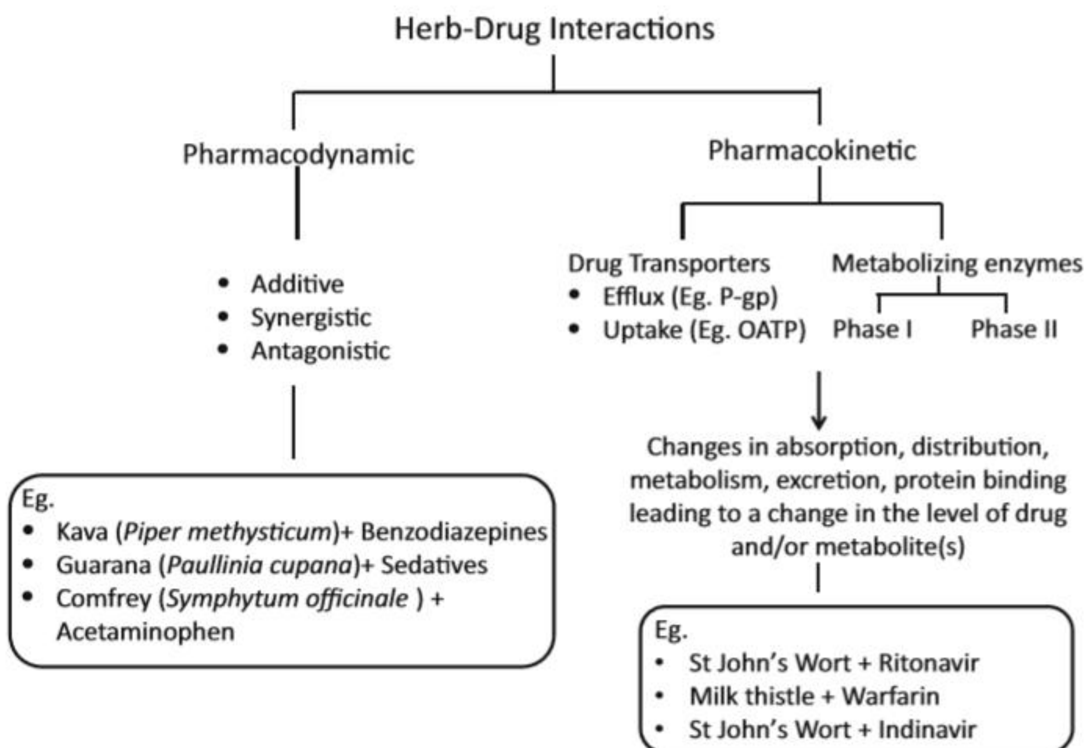

Figure 8. Illustration of the mechanisms of action of herb-drug interactions. Permeability glycoprotein (P-gp) and organic anion transporting polypeptides (OATP).[209] [Image published under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

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Herb-drug interactions are more likely to occur at pharmacokinetic level since this involves changes in the absorption, distribution, metabolism, and excretion of chemotherapeutic drugs.[243] From a pharmacokinetic perspective, certain herbal remedies have been shown to induce phase I and II enzymes of the CYP450 superfamily of enzymes and UGT.[243,244] At a pharmacodynamic level, herb-drug interactions may result in synergistic or additive effects when both herb and drug exert similar effects at their shared targets, thereby increasing the toxicity or biological response.[224,226] A common example of the synergistic or additive effects of integrative medicine at a pharmacodynamic viewpoint is the potentiation of the effects of warfarin in combination with garlic resulting in bleeding due to their anticoagulation properties.[244]

Garlic supplements may inhibit the activity of CYP3A4 in human hepatocytes.[245] This would suggest that the consumption of garlic may inhibit the metabolism of tamoxifen into *N*-desmethyltamoxifen and 4-hydroxy-*N*-desmethyltamoxifen.[36,155,165] However, the conversion of tamoxifen to 4-hydroxytamoxifen would remain unaffected as conversion is mediated by CYP2D6.[36,155,165] The inhibition of these enzymes and other metabolising enzymes is typically competitive with instantaneous and inhibitor concentration-dependent effects.[244] As a consequence of the inhibition of anticipated pre-systemic abdominal and hepatic drug metabolism, supra-therapeutic concentrations are often experienced.[244] Ultimately, a clinically toxic manifestation may be observed.[244] However, not all herb-drug interactions are negative.[224] Therapeutic herb-drug interactions include the enhancement of drug bioavailability (i.e., by improving absorption and/or inhibiting metabolism), and prolongation of the systemic concentration of the drug within its therapeutic index (i.e., by reducing excretion).[224] This potentially beneficial outcome is achieved through the improvement of the membrane permeation (e.g., opening of junctions and physiological changes in cell membranes), modulation of drug carrier proteins (e.g., induction of cellular uptake transporters and inhibition of efflux pumps), and inhibition of drug-metabolising enzymes.[224]

In summary, numerous studies have demonstrated the therapeutic effects of phytochemicals for the treatment of cancer, including breast cancer. Thus, it can be rationalised that the utilising the anticancer properties of commonly ingested food, e.g., garlic, may be beneficial in the treatment of cancer. Phytochemicals are often used by breast cancer patients in combination with conventional medicine, such as tamoxifen, termed integrative medicine. However, there is little information on the effects of integrative medicine in breast cancer cells.

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1.10. Aim and objectives

1.10.1. Aim

The aim of the study was to determine the effects of crude garlic extracts in conjunction with 4-hydroxytamoxifen on MCF-7 tamoxifen-sensitive breast adenocarcinoma cells.

1.10.2. Objectives

The objectives of the study were to:

- Evaluate the effect of the crude extracts and 4-hydroxytamoxifen on MCF-7 cell density using the sulforhodamine B assay;
- Determine the combinational effect of crude extracts and 4-hydroxytamoxifen on MCF-7 cells using a checkerboard sulforhodamine B assay; and
- Determine the mechanism of cytotoxicity of the most synergistic combination and its individual components on MCF-7 cells with regards to alterations of
    - i) the cell cycle (propidium iodide [PI] staining assay),
    - ii) caspase-3/7 activity (N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin [Ac-DEVD-AMC] cleavage assay),
    - iii) lipid peroxidation (thiobarbituric acid reactive species [TBARS]), and
    - iv) nitric oxide production (Griess assay).

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Chapter 2: Materials and methods

2.1. Ethical clearance

Ethical clearance to carry out the study was obtained from the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (REC 329/2019).

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2.2. Reagents

A list of the reagents that were used in this study and the preparation thereof is provided

List of reagents

The reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The GlutaMAX™ Supplement was purchased from ThermoFisher Scientific (Waltham, MA, USA). The n-hexane solvent was be purchased from Merck Millipore (Darmstadt, Germany).

Assessment of caspase-3/7 activity

Caspase-3/7 assay buffer

The buffer containing 4.3 mM β-mercaptoethanol, 5 µM Ac-DEVD-AMC, 2 mM EDTA, 20 mM HEPES and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) was prepared by dissolving 58.4 mg of EDTA and 476.6 mg of HEPES into 100 mL of deionised water. The incomplete buffer was stored at 4°C, and 3 µL of 14.3 mM β-mercaptoethanol, 10 µL of 5 mM Ac-DEVD-AMC, and 50 µL of 100 mM PMSF was added thirty minutes prior to use.

Lysis buffer

The buffer containing 4.3 mM β-mercaptoethanol, 2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 5 mM EDTA, 10 mM HEPES and 0.5 mM PMSF was prepared by dissolving 58.4 mg EDTA and 715.6 mg in 100 mL of deionised water. The buffer was stored at 4°C, and 3 µL of β-mercaptoethanol (14.3 mM) and 50 µL of PMSF (100 mM) was added thirty minutes prior to use.

N-Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin caspase 3 substrate

A stock solution (5 mM) was prepared by dissolving 3.378 mg of Ac-DEVD-AMC per 1 mL of dimethyl sulfoxide (DMSO) and stored in aliquots of 10 µL at -80°C.

Phenylmethanesulfonyl fluoride

A stock concentration (100 mM) was prepared by dissolving 87.1 mg of per 5 mL DMSO and stored in aliquots of 50 µL at -80°C.

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Assessment to cell cycle alterations

Curcumin

A 40 mM stock solution was prepared by dissolving 7.3 mg of curcumin per 5 mL of DMSO and stored in aliquots of 20 µL at -80ºC. A working solution of 40 µM was diluted in media prior to use and the final concentration in-reaction was 20 µM.

Methotrexate

A 20 mM stock solution was prepared by dissolving 4.5 mg of methotrexate per 5 mL of DMSO and aliquots of 20 µL was stored at -80ºC. A working solution of 20 µM was diluted in media prior to use and the final concentration in-reaction was 10 µM.

Propidium iodide staining buffer

A solution containing 40 µg/mL propidium iodide (PI), 0.1% Triton™ X-100 and 100 µg/ml DNA-free RNase was prepared by dissolving 0.4 mg PI per 10 mL of deionised water and 100 µL Triton™ X-100 per 100 mL in deionised water. The incomplete buffer was stored at 4ºC. DNA-free RNase (1 mg per 10 mL) was added five minutes prior to use.

Assessment of cytotoxicity

4-Hydroxytamoxifen

A stock concentration of 40 mM was prepared by dissolving 77.5 mg of 4-hydroxytamoxifen per 5 mL of DMSO and stored in aliquots of 20 µL protected from light. A working solution of 40 µM was diluted in media prior to use and the final concentrations in-reaction was 1, 5, 10, 15 and 20 µM.

Acetic acid

Acetic acid (1% v/v) was prepared by diluting 10 mL of acetic acid per 1 L of deionised water.

Sodium hydroxide

A 1 M solution of sodium hydroxide was prepared by dissolving 3.99 g per 100 mL of deionised water.

Sulforhodamine B dye

A 0.057% w/v solution was prepared by dissolving 0.285 g of sulforhodamine B powder per 500 mL 1% acetic acid.

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Trichloroacetic acid

A 50% w/v solution was prepared by dissolving 250 g trichloroacetic acid powder per 500 mL deionised water.

Tris-base

A 10 mM solution was prepared by dissolving 72.7 mg of Tris per 60 mL of deionised water and the pH was adjusted with 1 M sodium hydroxide to 10.5.

<u>Cell culture reagents</u>

Dulbecco's Modified Eagle's Medium culture medium

A working solution of media containing 1% (v/v) penicillin-streptomycin (10 000 U/mL) was prepared by adding 5 mL of penicillin-streptomycin into 500 mL. Where appropriate, a 10% (v/v) foetal calf serum (FCS) solution was prepared by diluting 50 mL foetal calf serum in 450 mL medium.

Phosphate-buffered saline

A working solution of phosphate buffered saline (FTA hemagglutination buffer, pH 7.2) was prepared by dissolving 9.23 g of powder per 1 L of deionised water.

Trypan blue

A 0.1% (w/v) trypan blue solution was prepared by dissolving 0.1 g of trypan blue powder in 100 mL of deionised water.

<u>Assessment of nitric oxide</u>

***N*-(1-napthyl)ethylenediamine dihydrochloride**

A 0.1% (w/v) solution was prepared by dissolving 500 mg of *N*-(1-napthyl)ethylenediamine dihydrochloride powder per 500 mL distilled water.

Sulphanilamide

A 1% (w/v) solution was prepared by dissolving 5 g of sulphanilamide powder per 500 mL of 5% phosphoric acid.

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Griess reagent

Equal volumes of 0.1% *N*-(1-napthyl)ethylenediamine dihydrochloride and 1% sulphanilamide were mixed to form the Griess reagent and protected from light.

Assessment of lipid peroxidation

Ascorbic acid

A 0.3% w/v solution was prepared by dissolving 1.5 g of ascorbic acid power per 500 mL distilled water.

Solvents

Acetic acid, dimethyl sulfoxide, methanol, and Triton™ X-100 were used undiluted.

Thiobarbituric acid

A 2.5% w/v solution was prepared by dissolving 12.5 g of thiobarbituric acid powder per 500 mL distilled water containing 0.3 mg EDTA and 80 mg sodium hydroxide.

Trichloroacetic acid

A 12.5% w/v solution was prepared by dissolving 62.5 g trichloroacetic acid powder per 500 mL deionised water.

2.3. Crude extraction

Garlic bulbs were collected from, and identity confirmed by the South African Garlic Growers Association. Bulbs were cleaned of dirt and allowed to air-dry at room temperature. Bulbs were ground using a Yellowline grinder (IKA, Staufen, Germany) until a yellow-orange paste was obtained. The crude extract was prepared by extraction with either n-hexane followed by methanol, methanol only, or hot-water. The sequential extraction using n-hexane and then methanol was prepared by macerating 10 g of plant material per 100 mL of n-hexane for 30 min in an ultrasonic bath (Bransonic Ultrasonics, Connecticut, United States of America). The plant material was agitated on a shaker for 2 h and incubated overnight at 4°C. The n-hexane garlic extract was centrifuged while the marc was left to dry underneath the Nederman arm (Nederman, Sweden). The n-hexane extracts were filter sterilised and the n-hexane was evaporated. The n-hexane extracts and its marc were not used in further analysis.

The methanol only extracts were prepared by macerating 10 g of plant material per 100 mL of methanol for 30 min in an ultrasonic bath (Bransonic Ultrasonics, Connecticut, United States

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of America), whereafter, it was agitated on a shaker for 2 h and incubated at 4°C overnight. The hot water extract was prepared by boiling 10 g of plant material per 100 mL of boiling water for 10 min to simulate tea preparation. The tea was allowed to cool to room temperature before further processing. All extracts were collected through centrifugation at 200 *x g* for 5 min and filtered through a 0.22 µm syringe filter. Extracts were evaporated using rotary evaporation (Büchi Rotavapor, St. Gallen, Switzerland) or lyophilisation (Freezone® Freeze Dry System, Labconco, Missouri, United States of America) for the methanol and hot water extracts, respectively.

The resultant crystals or powder were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL, and aliquots stored at -80°C. Prior to experimentation, aliquots were diluted in foetal calf serum (FCS)-free Dulbecco's Modified Eagle Medium (DMEM).

2.4. Cell culture maintenance

The MCF-7 cell line (ATCC® HTB-22) was purchased from the American Type Culture Collection (Virginia, United States of America). The MCF-7 cell line was maintained in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin (referred to as complete culture media). Cells were cultured to 80% confluence in 75 cm^2 culture flasks in a humidified incubator at 37°C and 5% carbon dioxide. Cells were rinsed two to three times with phosphate-buffered saline (PBS) and harvested with 0.25% trypsin- ethylenediaminetetraacetic acid (EDTA) solution until the cells started to detach from the flask (5 min). Harvested cells were centrifuged at 200 *x g* for 5 min, the supernatant was discarded, and the cell pellets were reconstituted with 1 mL complete culture media.

Viability of the cells was determined using the trypan blue (0.1%) exclusion method by pipetting 20 µL of the cell suspension and 180 µL of trypan blue into a 1.5 mL tube. Cells were counted using a microscope at 10x magnification and a haemocytometer; thereafter, the cell suspensions were diluted to the appropriate concentration for the assay to be conducted.

2.5. Assessment of cytotoxicity of crude extracts and 4-hydroxytamoxifen

Cells (100 µL; 5×10^4 cells/well) were seeded into a sterile 96-well plate and incubated overnight to allow for adherence to the plate surface. After attachment, cells were exposed to 100 µL FCS-free media (negative control), 100 µL DMSO (0.5%; vehicle control; in-reaction), 100 µL crude extracts (0.00001-100 µg/mL; in-reaction), 100 µL 4-hydroxytamoxifen (1-100 µM; in-reaction), or 100 µL cisplatin (1-100 µM; positive control; in-reaction) for 48 h. A blank control (100 µL; 5% FCS-supplemented media only; in-reaction) was used.

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The effect of the crude extracts and 4-hydroxytamoxifen on the MCF-7 cell line were evaluated using the sulforhodamine B (SRB) assay as described by Vichai and Kirtikara[246] with minor modifications. The assay is based on the binding of the bright pink aminoxanthene SRB dye to amino acid residues of cells fixed to cell culture plates using trichloroacetic acid (TCA).[246] After the incubation period, the cells were fixed with 50 µL cold TCA (50% v/v) overnight at 4°C. Fixed cells were washed four times with slow running water and stained with 100 µL SRB solution (0.057% w/v in 1% acetic acid) for 30 min in the dark. Stained cells were washed three times with 100 µL acetic acid (1% v/v) to remove the excess dye and air-dried. The dye was solubilised with 200 µL Tris-based solution (10 mM, pH 10.5) on a shaker for 1 h. Absorbance was measured at 540 nm (with a reference wavelength of 630 nm) using a BioTek ELx800 plate reader (BioTek Instruments, Vermont, United States of America), and the background absorbance adjusted by subtracting the medium blank. The percentage of cell density relative to the negative control was calculated as follows:

$$Cell\ density\ (\%\ relative\ to\ negative\ control) = \frac{absorbance\ of\ sample}{average\ absorbance\ of\ negative\ control}\, x\ 100$$

The half-maximal inhibitory concentration (IC_{50}) of samples was determined for 4-hydroxytamoxifen using GraphPad Prism v5.0 (San Diego, California, United States of America); however, both crude extracts failed to yield an IC_{50}.

2.6.Assessment of combinational effect of crude extracts and 4-hydroxytamoxifen

As IC_{50}'s could not be determined for the crude extracts at the highest concentrations tested, guidelines of the National Cancer Institute were used.[247-249] A concentration of 30 µg/mL was selected as the maximum concentration for both crude extracts to be used (¼IC_{50}, ½IC_{50} or IC_{50}) in the combinational assessment with 4-hydroxytamoxifen (at the ¼IC_{50}, ½IC_{50} or IC_{50}) in a checkerboard assay (**Figure 9**).

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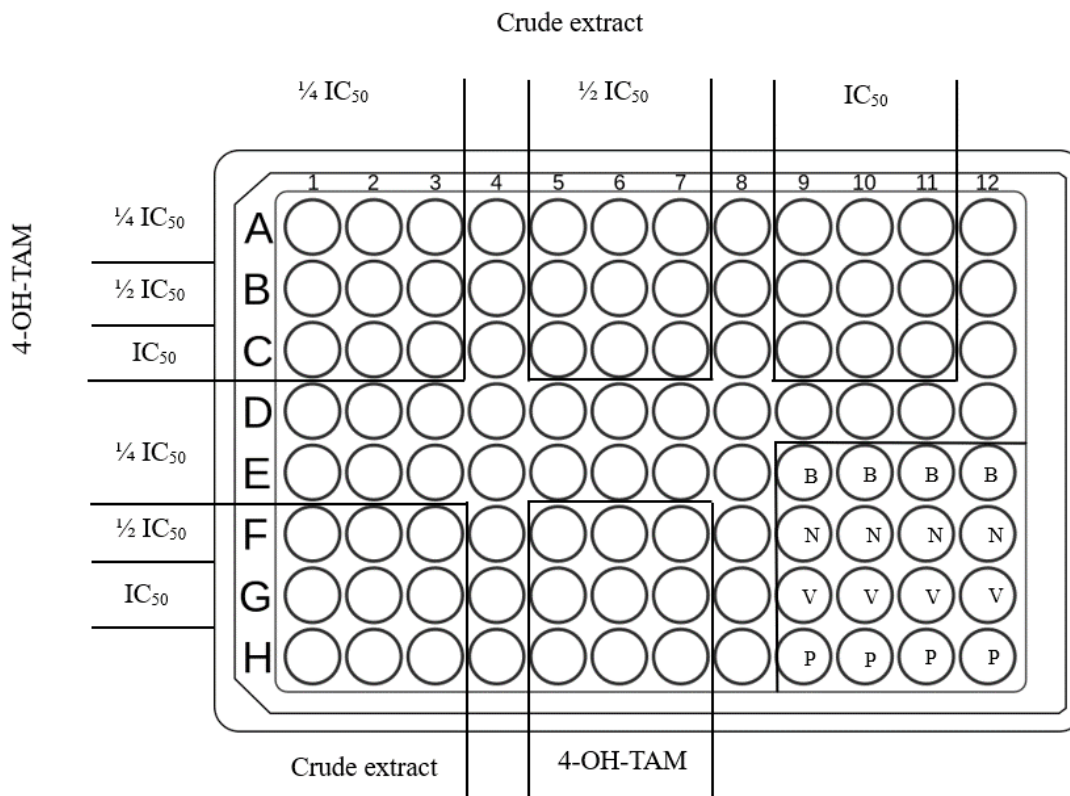

Figure 9. An overview of the checkerboard layout for the different combinations of crude extract and 4-hydroxytamoxifen. 4-Hydroxytamoxifen (4-OH-TAM), blank (B), negative control (N), positive control (P), and vehicle control (V).

Cells were seeded as per Section 2.5. After attachment, three combinational algorithms were used: i) 24 h pre-treatment with the crude extract followed by treatment with 4-hydroxytamoxifen for 48 h, ii) 1 h pre-treatment with the crude extracts followed by treatment with 4-hydroxytamoxifen for 48 h, or iii) dual-exposure to the crude extracts in conjunction with 4-hydroxytamoxifen for 48 h. Cells were pre-treated with 50 µL of 30, 60, and 120 µg/mL of garlic extracts for 24 or 1 h. Thereafter, the pre-treated cells were exposed to 50 µL of 11, 22 and 43.96 µM of 4-hydroxytamoxifen and 100 µL of FCS-free media for 48 h. For the dual-exposed setting, cells were treated with 50 µL crude extracts and 50 µL 4-hydroxytamoxifen simultaneously; thereafter 100 µL of FCS-free media for 48 h. Controls were exposed to 100 µL of FCS-free media (negative control), DMSO (0.5%; in-reaction, vehicle control), cisplatin (3.2, 10, 32, and 100 µM; in-reaction, positive control) for 48 h. Controls for the crude extracts were exposed to 100 µL different concentrations of the crude extract in FCS-free media for 72 (24 h pre-treatment), 49 (1 h pre-treatment), and 48 h (dual-exposure). Additionally, controls for 4-hydroxytamoxifen were exposed to different concentrations of 4-hydroxytamoxifen in

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FCS-free media for 48 h. A blank control (5% FCS-supplemented media only) was used. The SRB assay was conducted as described in Section 2.5. The combinational index (CI) of the drug combinations of the crude extract and 4-hydroxytamoxifen was calculated with CompuSyn v1.0 (ComboSyn Inc, Parmaus, New Jersey, United States of America) using the calculated fraction affected. The formula for CI is:

$$CI = \frac{Fa1}{Fu1} + \frac{Fa2}{Fu2}$$

where, CI = combinational index, F_{a1} = fraction affected by drug 1, F_{u1} = fraction unaffected by drug 1, F_{a2} = fraction affected by drug 2, F_{u1} = fraction unaffected by drug 2; Fu is calculated as $1 - F_a$.

The resulting CI provides a quantitative definition for synergism (CI < 1), addition (CI = 1), or antagonism (CI > 1).[250]

2.7. Evaluation of cytotoxicity

2.7.1. Exposure to cytotoxic compounds

Mechanistic evaluation was done for only the bioactive combination determined in Section 2.6. Cells (100 µL; 5 x 10^4 cells/well) were seeded into a sterile 96-well plate and incubated overnight to allow for adherence to the plate surface. All experiments were exposed to FCS-free media (negative control), DMSO (0.5%; in-reaction, vehicle control), positive control, crude extracts (7.5, 15, and 30 µg/mL), 4-hydroxytamoxifen (2.75, 5.5, and 10.99 µM) or the most synergistic crude extract-4-hydroxytamoxifen combination for 48 h, which was dual-exposure to 7.5 µg/mL of hot-water crude extract and 10.99 µM of 4-hydroxytamoxifen. The blank control was 5% FCS-supplemented media.

2.7.2. Assessment of cell cycle kinetics

Cell cycle kinetics refers to the pattern of growth and rate of division of cells.[251] The effect of the crude extracts, 4-hydroxytamoxifen, and the combination thereof on the cellular kinetics of the MCF-7 cell line were assessed using the PI staining assay as described by Cordier and Steenkamp[252] with slight modifications. The principle of the assay is based on the ability to differentiate cells in the varying cell cycle stages due to the staining of DNA by PI in their haploid, diploid, or tetraploid state. The cells are permeabilised and treated with PI, a stoichiometric DNA-binding fluorescent dye.[252] Cells (5 mL; 2.5x10^5 cells/flask) were seeded into a 25 cm^2 culture flask and allowed to attach overnight. Cells were washed twice with PBS

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and cultured for a further 32 h in serum-deprived medium to synchronise cells at the G_0/G_1 phase. Synchronised cells were exposed to treatment as described in Section 2.7.1. Cells were exposed to the following positive controls in-reaction: 10 µM methotrexate (a S-phase blocker) and 20 µM curcumin (a G_2/M phase blocker) for 18 h.

After the incubation period, the media was collected, and the cells were rinsed once with 3 mL of PBS. The wash-off was added to the collected supernatant in a 15 mL tube. Cells were harvested using 2 mL trypsin and added to the collected supernatant-wash off mixture. Furthermore, cells were rinsed once with 3 mL of complete growth media, and the wash-off added to the collected supernatant. Cells in the 15 mL tube were centrifuged at 200 *x g* for 5 min and washed with 1 mL of PBS containing 0.1% FCS. The supernatant from the wash step was discarded, and the pellet was resuspended with 200 µL of PBS containing 0.1% FCS. Thereafter, the cells were fixed with cold 70% ethanol in a drop-wise fashion under vortex-agitation and incubated overnight at 4°C. Fixed cells were centrifuged at 200 *x g* for 5 min, and the supernatant discarded. The pellet was replenished with 500 µL staining solution containing 40 µg/mL PI, 0.1% Triton X-100 and 100 µg/mL DNA-free RNase (added prior to use) at 37°C for 40 min. The fluorescence was analysed flow cytometrically using the FL-3 excitation filter (CytoFLEX, Beckman Coulter, California, United States of America). Cells were counted up to 20,000 events. The data was expressed as percentages of cells in the following cell cycle phases: sub-G_1, G_0/G_1-, S-, and G_2/M-phase using the CytExpert software.

2.7.3. Assessment of nitric oxide levels

The amount of nitrite formed following exposure to 7.5, 15, and 30 µg/mL hot-water crude extracts, 2.75, 5.5 and 10.99 µM 4-hydroxytamoxifen, and the most synergistic combination (dual-exposure to 7.5 µg/mL hot-water crude extract and 10.99 µM 4-hydroxytamoxifen) was determined using the Griess assay as described by Heo *et al*[253] with minor modifications to the volumes used. Nitrate and nitrite are by-products of nitric oxide production[254] and used as biomarkers of nitrosative stress.[255]

Cells (100 µL; 5×10^4 cells/well) were seeded exposed to treatment as described in Section 2.7.1. Sodium hypochlorite (0.5;% in-reaction), a highly reactive oxidant,[256] was used as the positive control. After exposure, 100 µL of the supernatant and 100 µL of cells harvested with 0.25% trypsin-EDTA were collected, invert-mixed and centrifuged at 200 *x g* for 5 min. An aliquot of 100 µL of the supernatant was mixed with 100 µL of Griess reagent acid in a 96-well plate. The plate was incubated at room temperature for 10 min in the dark. The absorbance was

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measured at 540 nm using the BioTek ELx800 microplate reader (BioTek Instruments, Vermont, United States of America). The background absorbance was adjusted by subtracting the blank. The fold-change of nitric oxide levels relative to the negative control was calculated as follow:

$$Nitric\ oxide\ fold - change\ (relative\ to\ negative\ control) = \frac{Absorbance\ of\ sample}{Absorbance\ of\ negative\ control}$$

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2.7.4. Assessment of lipid peroxidation levels

The ability of 7.5, 15, and 30 µg/mL hot-water crude extracts, 2.75, 5.5 and 10.99 µM 4-hydroxytamoxifen, and the most synergistic combination (dual-exposure to 7.5 µg/mL hot-water crude extract and 10.99 µM 4-hydroxytamoxifen) to induce lipid peroxidation was assessed using the TBARS as described by Cordier *et al*[257] with minor modifications. Malondialdehyde is a by-product of lipid peroxidation of polyunsaturated fatty acids and was used as a biomarker of oxidative stress.[258]

Cells (100 µL; 5×10^4 cells/well) were seeded into a sterile, 96-well plate and exposed to treatment as described in Section 2.7.1. Sodium hypochlorite (0.5;% in-reaction), a highly reactive oxidant,[256] was used as the positive control. After exposure, 200 µL of the supernatant and 100 µL of cells harvested with 0.25% trypsin-EDTA were collected into 5 mL test tubes and centrifuged 200 *x g*. The 300 µL supernatant was mixed with 12.5% TCA (100 µL), 2.5% 2-thiobarbituric acid (100 µL) and 0.3% ascorbic acid (400 µL) solution. The mixture was vortex-mixed and incubated in a hot-water bath (95°C, 15 min). After adding butan-1-ol (1 mL), the mixture was vortex-mixed and allowed to separate into an organic and aqueous phase. Aliquots (100 µL) of the organic butan-1-ol phase were transferred into a sterile, white 96-well plate. The fluorescence was measured at 532 nm (λ_{ex}) and 590 nm (λ_{em}) using BioTek Synergy 2 plate reader (BioTek Instruments, Vermont, United States of America). The background fluorescence was adjusted by subtracting the blank. The fold-change of lipid peroxidation relative to the negative control was calculated as follow:

$$Lipid\ peroxidation\ fold - change\ (relative\ to\ negative\ control) = \frac{FIs}{FIc}$$

where, FIs denotes fluorescent intensity of the sample and FIc is average fluorescent intensity of negative control.

2.7.5. Assessment of caspase-3/7 activity

The activity of caspase-3/7 was evaluated using the Ac-DEVD-AMC cleavage assay as described by Cordier and Steenkamp.[252] The hydrolysis or enzymatic cleavage of Ac-DEVD-AMC by activated caspase-3/7 releases the fluorescent AMC moiety.[259] Detection of the fluorescent moiety indicates caspase-mediated cell death most likely due to programmed cell death.[252] Cisplatin, a well-known chemotherapeutic drug,[260] was used as the positive control at 10 µM in-reaction.

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Cells (100 µL; $5x10^4$ cells/well) were seeded into a sterile, white 96-well plate and exposed to treatment as described in Section 2.7.1. The plates were centrifuged (200 *x g*, 5 min), media exchanged for 25 µL cold lysis buffer, and incubated on ice for 15 min. Then, 100 µL of caspase-3 substrate buffer containing Ac-DEVD-AMC was added to the plates and incubated at 37°C for 4 h. The fluorescence was measured at 355 nm (λ_{ex}) and 460 nm (λ_{em}) using a BioTek Synergy 2 plate reader (BioTek Instruments, Vermont, United States of America). The background fluorescence was adjusted by subtracting the medium blank. The caspase-3/7 activity was calculated as follow:

$$Caspase - \frac{3}{7}\ activity\ fold - change\ \ (relative\ to\ negative\ control) = \frac{FIs}{FIc}$$

where, FIs denotes fluorescent intensity of the sample and FIc is average fluorescent intensity of the negative control.

2.8. Statistics

Data obtained from the study was stored online in the Institutional Repository of the University of Pretoria and in the Department of Pharmacology, University of Pretoria, and will be kept for a minimum of 15 years. Raw data was captured and analysed using Microsoft Excel (Microsoft, Washington, United States of America). Each experiment contained a minimum of three internal and three external replicates, thus, producing a minimum of nine data sets for each experimental condition. All results were expressed as the mean ± standard error of mean (SEM). The statistical analyses of the data were performed using GraphPad v5.0 (GraphPad Software Inc., California, United States of America).

Cytotoxicity data over a range of concentrations was used to generate dose-response curves via bell-shaped and non-linear regression models to determine the IC_{50} values. The CI was determined using CompuSyn (ComboSyn Inc., New Jersey, United States of America). Comparisons between the negative control and samples were determined via Kruskal-Wallis testing followed by a post-hoc Dunn's test. All mechanistic assays were assessed for statistical significance between the negative control and samples via the Kruskal-Wallis test followed by a post-hoc Dunn's test. A p value < 0.05 was considered statistically significant. Flow cytometry data was analysed with FCS Express 7 software (De Novo Software, California, United States of America).

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Chapter 3: Results

3.1. Inherent cytotoxicity of crude extracts and 4-hydroxytamoxifen

Exposure to the vehicle control (0.5% DMSO; in-reaction) significantly ($p < 0.001$) decreased cell density of MCF-7 cells to 92% (**Table 1**). Cisplatin induced a significant ($p < 0.001$) dose-dependent reduction in cell density with a calculated IC_{50} of 8.11 µM (**Table 1**).

Table 1. Half-maximal inhibitory concentrations (IC_{50}) of crude extracts, 4-hydroxytamoxifen, and cisplatin on MCF-7 cells after 48 h.

Treatment	IC_{50} ± SEM	Cell density at highest concentration (100 µg/mL[a] or 100 µM[b])
Methanol crude extract[a]	>100	62.4%
Hot-water crude extract[a]	>100	105.6%
4-Hydroxytamoxifen[b]	10.99 ± 0.01	1.5%
Cisplatin[b]	8.11 ± 0.03	6.9%

[a.] Crude extracts were measured per µg/mL.

[b.] 4-Hydroxytamoxifen and cisplatin were measured per µM.

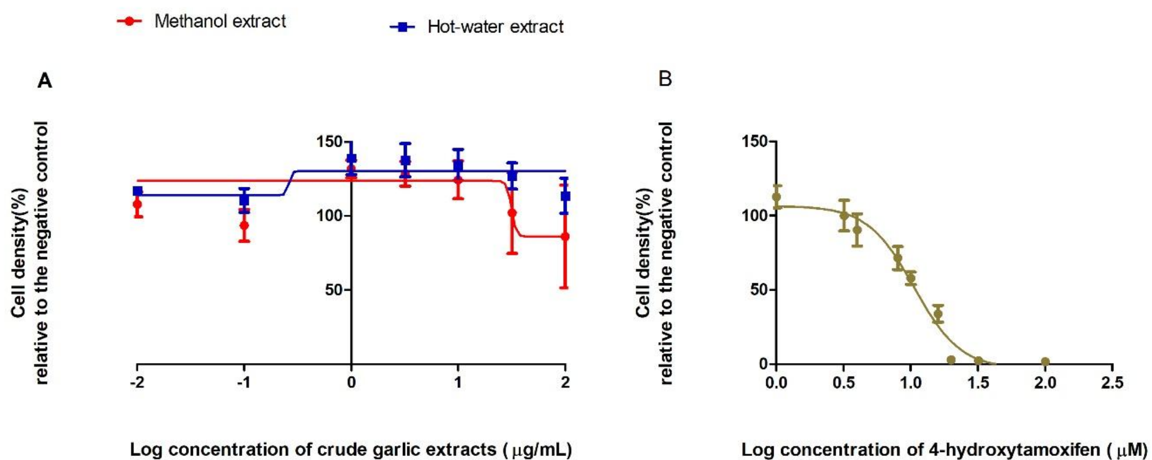

Figure 10. The effect of crude extracts and 4-hydroxytamoxifen on MCF-7 cell density after a 48 h exposure.

The methanol extract (0.01 and 0.1 µg/mL, and 1 µg/mL) resulted in cell densities between 101 and 90% (**Figure 10A**). The cell density increased to 129% at 1 µg/mL, remained increased at 122% at 10 µg/mL, and thereafter, decreased to 62% at 100 µg/mL. At 48 h, lower

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concentrations of the hot-water extract (0.01 and 0.1 µg/mL, and 1 µg/mL) increased cell density to between 110% and 133%. The cell density remained increased at 130% at 10 µg/mL. The cell density remained above 100% at 100 µg/mL, the highest concentration tested, with a cell density of 106% (**Figure 10A**). Therefore, an IC_{50} could not be calculated for either of the extracts because the cell density was above 50% at the highest concentration. In addition, 4-hydroxytamoxifen decreased cell density dose-dependently (**Figure 10B**). The cell density increased to 109% at 1 µM, decreased to 58% at 10 µM, and decreased to 1% at 100 µM (Figure 10**B**). The calculated IC_{50} of 4-hydroxytamoxifen was 10.99 µM (**Table 1**).

3.2. Combinational cytotoxicity of crude extracts and 4-hydroxytamoxifen

Cells were exposed to a combination of the crude extracts and 4-hydroxytamoxifen in three different experimental conditions. Each experimental setting assessed the prolonged (24 h pre-treatment with garlic then 48 h exposure to 4-hydroxytamoxifen), intermediate (1 h pre-treatment with garlic then 48 h exposure to 4-hydroxytamoxifen), or immediate (dual-exposure to garlic and 4-hydroxydroxytamoxifen for 48 h) combinational effects. Concentrations of 7.5, 15, and 30 µg/mL were selected for combinational assessment as IC_{50} values were not obtained. The selection of 30 µg/mL as the IC_{50} was based on the definition of the National Cancer Institute. A crude extract is considered cytotoxic if the IC_{50} was ≤30 µg/mL after a 48 h incubation. Furthermore, it was not physiologically feasible to increase the concentration. A mixture of antagonistic, additive, and synergistic combinations was observed depending on the concentrations and incubation period.

Table 2. Combinational indices of the crude extracts and 4-hydroxytamoxifen in the three experimental conditions.

	Combinational Indices[a]	
Concentration	Hot-water extract	Methanol extract
24 h pre-treatment		
7.5 µg extract + 2.75 µM 4-hydroxytamoxifen	1.660	1.587
15 µg extract + 2.75 µM 4-hydroxytamoxifen	2.526	2.381
30 µg extract + 2.75 µM 4-hydroxytamoxifen	4.259	3.969
7.5 µg extract + 5.5 µM 4-hydroxytamoxifen	2.453	2.381

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15 µg extract + 5.5 µM 4-hydroxytamoxifen	3.320	3.175
30 µg extract + 5.5 µM 4-hydroxytamoxifen	5.053	4.762
7.5 µg extract + 10.99 µM 4-hydroxytamoxifen	0.887	0.767
15 µg extract + 10.99 µM 4-hydroxytamoxifen	1.165	0.861
30 µg extract + 10.99 µM 4-hydroxytamoxifen	1.109	0.798
1-hour pre-treatment[b]		
7.5 µg extract + 2.75 .µM 4-hydroxytamoxifen	3.089×10^{5}	0.366
15 µg extract + 2.75 µM 4-hydroxytamoxifen	3.76×10^{5}	2.424
30 µg extract + 2.75 µM 4-hydroxytamoxifen	4.556	1.201
7.5 µg extract + 5.5 µM 4-hydroxytamoxifen	1.052×10^{6}	0.866
15 µg extract + 5.5 µM 4-hydroxytamoxifen	1.51×10^{6}	1.219
30 µg extract + 5.5 µM 4-hydroxytamoxifen	1.631	2.795
7.5 µg extract + 10.99 µM 4-hydroxytamoxifen	1.31×10^{7}	0.810
15 µg extract + 10.99 µM 4-hydroxytamoxifen	2.933	0.869
30 µg extract + 10.99 µM 4-hydroxytamoxifen	1.04×10^{5}	1.093
Dual-exposure		
7.5 µg extract + 2.75 µM 4-hydroxytamoxifen	0.739	0.497
15 µg extract + 2.75 µM 4-hydroxytamoxifen	3.323	0.901
30 µg extract + 2.75 µM 4-hydroxytamoxifen	4.688	1.544
7.5 µg extract + 5.5 µM 4-hydroxytamoxifen	0.907	0.693
15 µg extract + 5.5 µM 4-hydroxytamoxifen	1.152	1.003
30 µg extract + 5.5 µM 4-hydroxytamoxifen	1.796	1.173
7.5 µg extract + 10.99 µM 4-hydroxytamoxifen	0.624	1.166

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15 µg extract + 10.99 µM 4-hydroxytamoxifen	1.360	1.371
30 µg extract + 10.99 µM 4-hydroxytamoxifen	2.181	1.496

[a.] The combinational indices are highlighted in different colours. Green represents synergism, orange represents additive, and red represents antagonism.

[b.] There were large combination index values far greater than 2 calculated for the cells pre-treated with hot-water garlic extract for an hour then exposed to 4-hydroxytamoxifen due to hyper-antagonism, where they had opposing effects.

Cells pre-treated with hot-water or methanol extracts for 24 h resulted in synergistic, additive, or antagonistic combinations (**Figure 11; Table 2**). Treatment with 2.75 and 5.5 µM of 4-hydroxytamoxifen alone increased cell density to 158% and 145%, respectively, which decreased to 10% when treated with 10.99 µM of 4-hydroxytamoxifen (**Figure 11**). Cellular density reached 104%, 108%, and 92% for cells treated with 7.5, 15, and 30 µg/mL of methanol extract alone, respectively (**Figure 11A**). The different combinations of the methanol extract (7.5, 15 and 30 µg/mL) and 4-hydroxytamoxifen (2.75 and 5.5 µM) resulted in increased cell density between 116% and 133%. These cell densities of the combinations were above the single treatment for the methanol extract alone. The combinations were additive or antagonistic depending on the concentrations of either compound. The IC_{50} concentration, 10.99 µM, of 4-hydroxytamoxifen decreased cell density of the 24 h pre-treated methanol extract cells to 10%, 13%, and 9% (7.5, 15 and 30 µg/mL of methanol extract, respectively). The addition of 10.99 µM of 4-hydroxytamoxifen to cells pre-treated with 7.5 or 15 µg/mL of methanol extract decreased cell density lower than the methanol extract alone, but densities were higher than the 4-hydroxytamoxifen only treatment. Though, the combinations were found to be synergistic [combination indices (CI) = 0.797 and 0.861] (**Table 2**), the addition of 10.99 µM of 4-hydroxytamoxifen to cells pre-treated with 30 µg/mL of methanol extract for 24 h resulted in a cell density lower than its individual counterparts. The combination was also found to be synergistic (CI = 0.798) (**Table 2**).

Solo treatment with 7.5, 15 and 30 µg/mL of hot-water extract resulted in cell densities of 107%, 98%, and 81%, respectively (**Figure 11B**). The different combinations of the hot-water extract (7.5, 15 and 30 µg/mL) and 4-hydroxytamoxifen (2.75 and 5.5 µM) resulted in increased cell density between 118% and 133%. The addition of 10.99 µM of 4-hydroxytamoxifen to the pre-treated hot-water cells resulted in cell densities of 13%, 28% and 17% for its combination with 7.5, 15 and 30 µg/mL of hot-water extract. The combination of

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cells pre-treated with 7.5 μg/mL of hot-water extract for 24 h followed by a 48-h exposure to 10.99 μM of 4-hydroxytamoxifen was the only synergistic combination within this treatment setting (CI = 0.887) (**Table 2**) noted.

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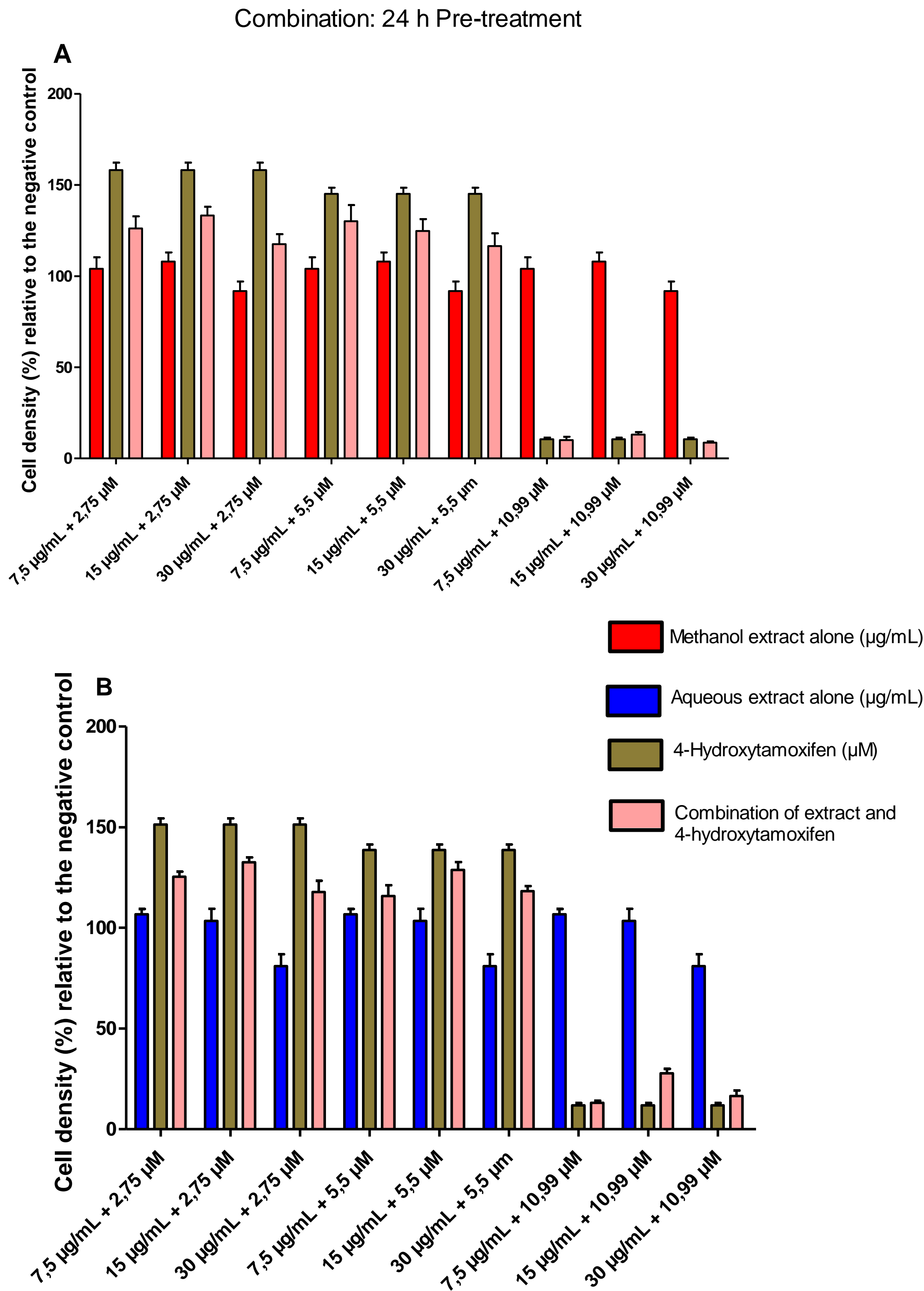

Figure 11. The cell density of MCF-7 cells pre-treated with crude a) methanol and b) hot-water extracts for 24 h, followed by an exposure to 4-hydroxytamoxifen for 48 h.

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Pre-treatment with methanol extract for an hour resulted in a mixture of synergistic, additive, and antagonistic activity (**Figure 12A; Table 2**). Pretreatment with hot-water garlic extract for an hour largely yielded antagonistic activity (**Figure 12B; Table 2**). Many hyper-antagonistic effects were observed in this treatment setting. This would suggest that the addition of 4-hydroxytamoxifen opposes the effect that the 1 h pretreatment of hot-water extract treatment initiated. The combination of 30 µg/mL of hot-water extract and 5.5 µM of 4-hydroxytamoxifen was additive. Treatment with 4-hydroxytamoxifen only (2.75, 5.5 and 10.99 µM) yielded cell densities of 107%, 99%, and 59%, respectively (**Figure 12**).

Methanol extract (7.5, 15 and 30 µg/mL) alone decreased cell densities to 87%, 92%, 77%, respectively (**Figure 12A**). The different combinations of the methanol extract (7.5, 15 and 30 µg/mL) and 4-hydroxytamoxifen (2.75, 5.5 and 10.99 µM) resulted in range of cell densities between 57% and 90%. The synergistic combinations in this treatment setting were 7.5 µg/mL of methanol extract and 2.75 (CI = 0.366) (**Table 2**), 5.5 (CI = 0.866) (**Table 2**) and 10.99 (CI = 0.810) µM of 4-hydroxytamoxifen (**Table 2**), and 15 µg/mL of methanol extract and 10.99 µM of 4-hydroxytamoxifen (CI = 0.869) (**Table 2**).

Exposure to 7.5, 15, and 30 µg/mL of hot-water extract only yielded cell densities to 95%, 102%, and 96% **(Figure 12B**). The different combinations of the methanol extract (7.5, 15 and 30 µg/mL) and 4-hydroxytamoxifen (2.75, 5.5 and 10.99 µM) resulted in cell densities between 62 and 102%. While there were various combinations of hot-water extract and 4-hydroxytamoxifen that decreased cell density, they were found to be antagonistic. The combination of 5.5 µM of 4-hydroxytamoxifen with 30 µg/mL of hot-water extract was additive, the only non-antagonistic combination (**Table 2**).

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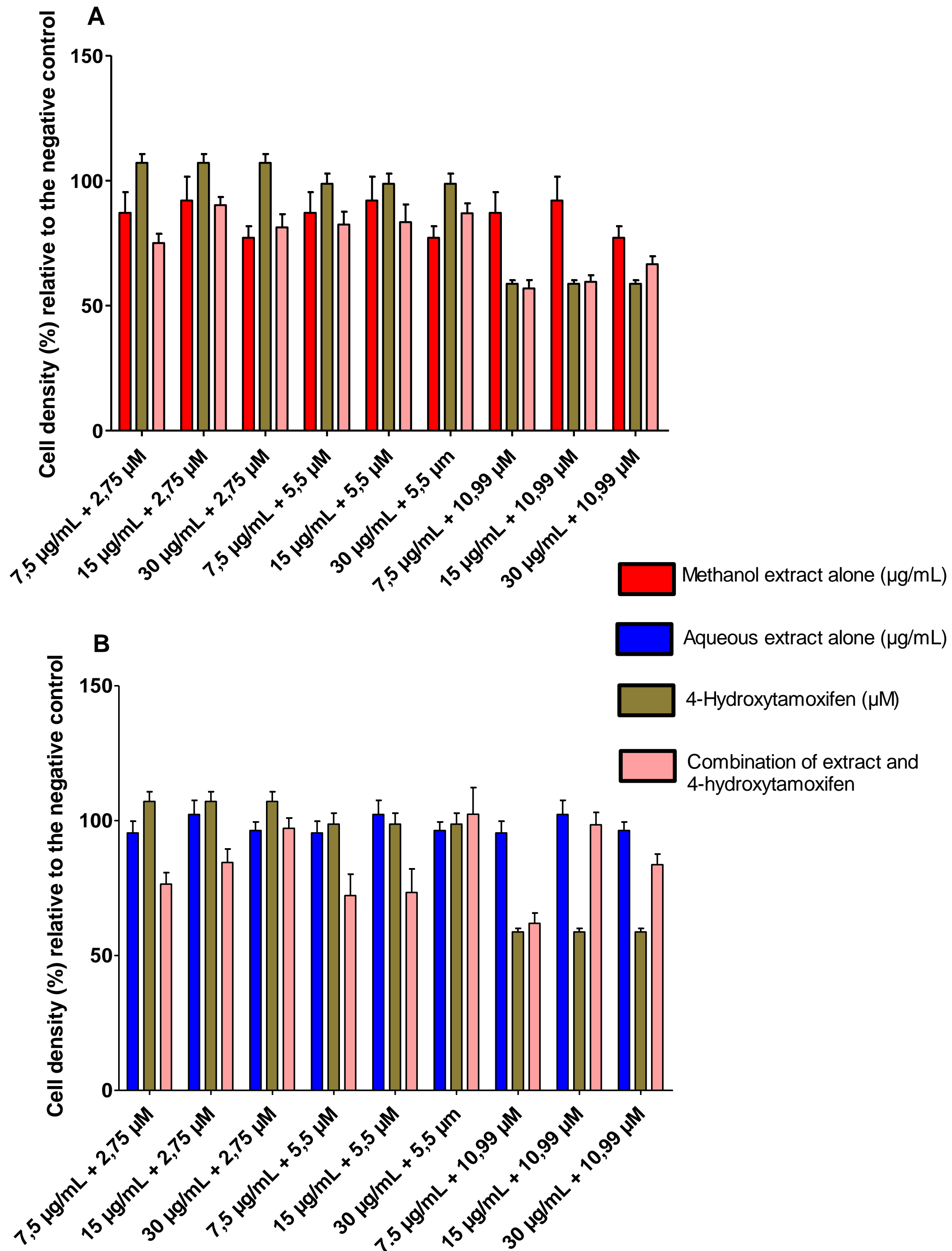

Figure 12. The cell density of MCF-7 cells pre-treated with a) methanol and b) hot-water extracts for 1 h followed by an exposure to 4-hydroxytamoxifen for 48 h.

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Combinatorial-exposure to the crude extract and 4-hydroxytamoxifen resulted in synergistic, additive, or antagonistic combinations (**Figure 13**; **Table 2**). Exposure to only 4-hydroxytamoxifen (2.75, 5.5 and 10.99 µM) resulted in cell densities of 122%, 86%, and 60%, respectively (**Figure 13**). Solo exposure to 7.5, 15, and 30 µg/mL of methanol extract resulted in cell densities to 96, 98, and 87%, respectively (**Figure 13A**). The different combinations of methanol extract (7.5, 15 and 30 µg/mL) and 4-hydroxytamoxifen (2.75, 5.5 and 10.99 µM) resulted in cell densities between 63-91% (**Figure 13A**). The combinations of 7.5 µg/mL of methanol extract and 2.75 µM of 4-hydroxytamoxifen (CI = 0.497) (**Table 2**) and 7.5 µg/mL of methanol extract and 5.5 µM of 4-hydroxytamoxifen (CI = 0.693) (**Table 2**) were synergistic in this treatment setting. Combination of 15 µg/mL of methanol extract and 2.75 of 4-hydroxytamoxifen (CI = 0.901) (**Table 2**) was also synergistic in this treatment setting.

Exposure only to 7.5, 15, and 30 µg/mL of hot-water extract resulted in cell densities of 100, 94%, and 88%, respectively (**Figure 13B**). The different combinations of hot-water extract (7.5, 15 and 30 µg/mL) and 4-hydroxytamoxifen (2.75, 5.5 and 10.99 µM) resulted in cell densities between 20% and 107% (**Figure 13**). Simultaneous exposure to 7.5 µg/mL of hot-water extract and 2.75 µM (CI = 0.739) (**Table 2**), 5.5 µM (CI = 0.907) (**Table 2**), or 10.99 µM of 4-hydroxytamoxifen (CI = 0.624) (**Table 2**) were synergistic. The combination of 7.5 µg/mL of hot-water extract and 10.99 µM of 4-hydroxytamoxifen was chosen as the most synergistic combination based on its index and cell density. The combination was used to assess the synergistic mechanism of cell death.

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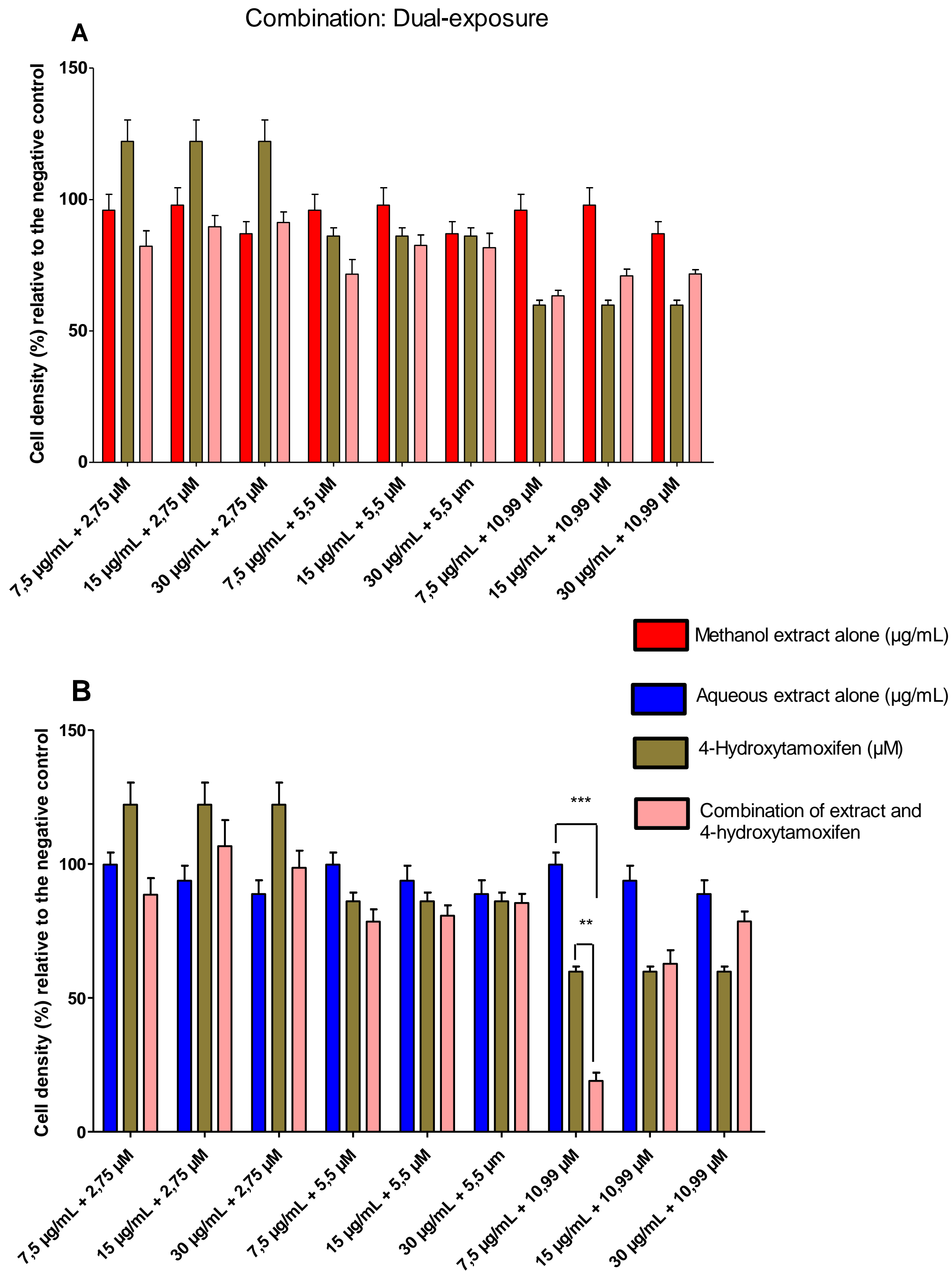

Figure 13. The cell density of MCF-7 cells treated with a) methanol and b) hot-water extracts and 4-hydroxytamoxifen simultaneously for 48 h. ** $p < 0.01$ and *** $p < 0.001$.

A cross-comparison between solo treatment of methanol extract across the three experimental conditions demonstrates that there was greater cell density in MCF-7 cells pre-treated for 24 h (92-108%) compared to 1 h pre-treatment (77-92%), and dual-exposure (87-98%). Conversely,

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the 1 h pre-treatment had the lowest cell density across the three conditions following treatment with methanol extract. Similar pattern was observed for the hot-water extract where the 24 h pre-treatment showed greater cell density (81-107%) compared to the 1 h pre-treatment (95-102%) and dual-exposure (88-100%). Additionally, this trend was seen for 4-hydroxytamoxifen where the greater cell density in the experimental condition was in the 24 h pre-treatment (10-158%), 1 h pre-treatment (59-107%), and dual-exposure (60-122%). Though, it is noted that the

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3.3. Assessment of synergistic mechanism of cell death

3.3.1. Synergistic alterations to cell cycle

The negative and vehicle controls showed similar cellular kinetics, but the difference was not significant (**Figure 14A**). Cells were exposed to 20 µM curcumin (a G_2/M phase positive control) and 10 µM methotrexate (an S phase positive control) for 18 h (**Figure 14A**). The curcumin positive controls blocked MCF-7 cells in its respective cell cycle phases, while methotrexate did not, though the effects of the positive controls were not significant compared to the negative control.

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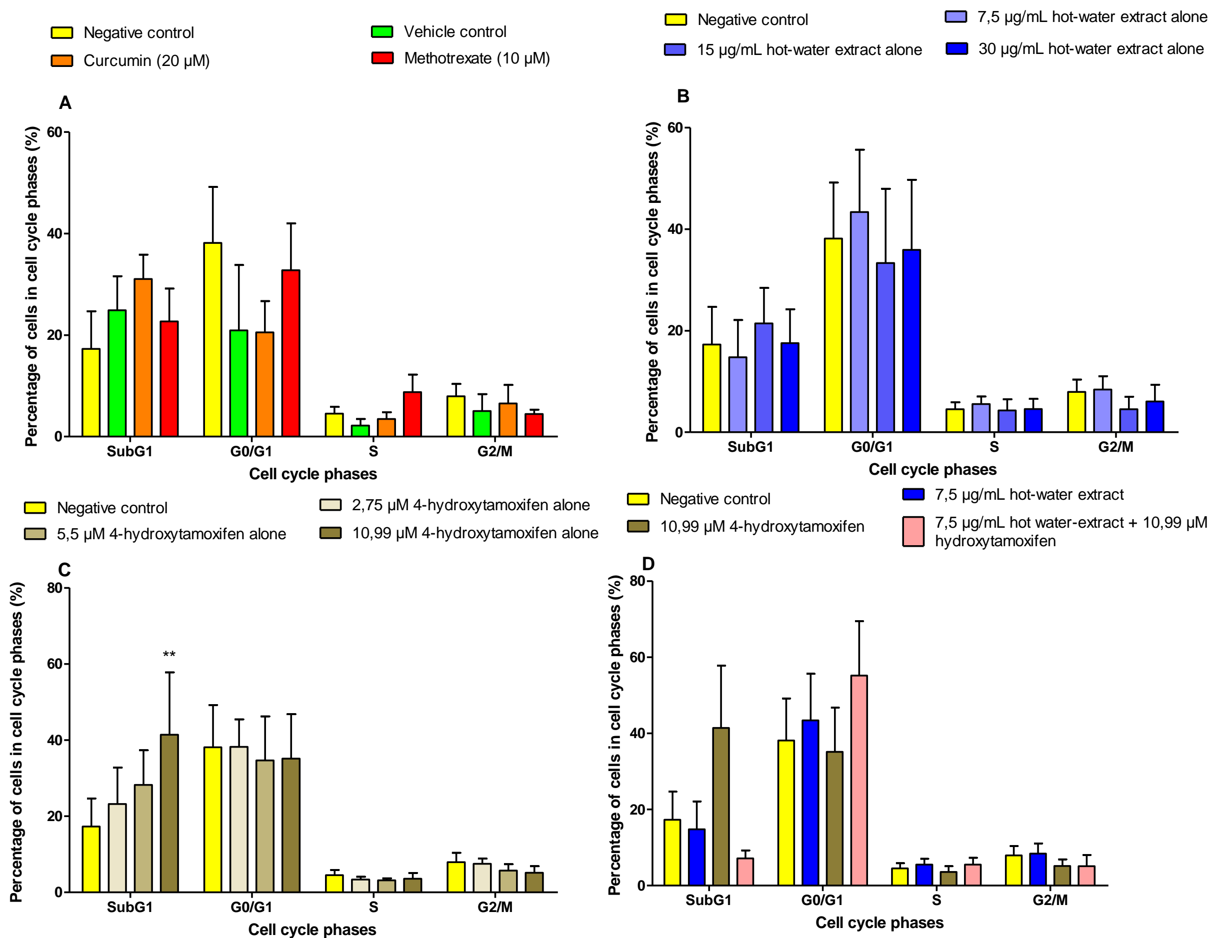

Figure 14. Cellular kinetics of MCF-7 cells exposed to a) controls, b) concentrations of hot-water extract, c) concentrations of 4-hydroxtamoxifen and d) the combination thereof for 48 h. The combination of the hot-water and 4-hydroxytamoxifen arrested on average 17.04% more cells in G_0/G_1 phase relative to the negative control

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Exposure to all concentrations of the hot-water extract, at all phases had no significant effect. The 7.5 µg/mL hot-water extract decreased 2.51% of MCF-7 cells in the sub-G_1 phase compared to the negative control. The MCF-7 cells occupying the G_0/G_1 phase increased by 5.23% compared to the negative control (**Figure 14B; Table 3**). The MCF-7 cells occupying the S and G_2/M phases also increased by 0.98% and 0.49%, respectively (**Figure 14B; Table 3**). The 15 µg/mL hot-water extract increased the percentage of MCF-7 cells in the sub-G_1 phase by 4.15%. However, decreased the percentage of MCF-7 cells in the G_0/G_1, S, and G_2/M phases by 4.83%, 0.26%, and 3.42% , respectively (**Figure 14B; Table 3**). The 30 µg/mL hot-water extract increased 9.46% of MCF-7 cells in sub-G_1 phase and decreased 2.23% of MCF-7 cells and G_0/G_1 phase, respectively. It increased 0.06% of MCF-7 cells in the S phase and decreased 1.88% of MCF-7 cells in the G_2/M phase (**Figure 14B**; **Table 3**).

Table 3. Effect of hot-water extract, 4-hydroxytamoxifen and the combination thereof on cellular kinetics in MCF-7 cells for 48 h compared to the negative control. The percentages represent the average difference of cells in the different phases of the cell cycle compared to the negative control.

	Hot-water extract[a]			4-Hydroxytamoxifen[b]			Combination[c]
Phase of cell cycle	7.5	15	30	2.75	5.5	10.99	7.5 and 10.99
Sub-G_1	2.51% ↓	4.15% ↑	0.25% ↑	5.89% ↑	10.93% ↑	24.12% ↑	10.17% ↓
G_0/G_1	5.23% ↑	4.83% ↓	2.23% ↓	0.08% ↑	3.48% ↓	3.03% ↓	17.04% ↑
S	0.98% ↑	0.26% ↓	0.06% ↑	1.13% ↓	1.38% ↓	0.95% ↓	0.99% ↑
G_2/M	0.49% ↑	3.42% ↓	1.88% ↓	0.43% ↓	2.20% ↓	2.79% ↓	2.82% ↓

[a.] Hot-water extract was measured per µg/mL.

[b.] 4-Hydroxytamoxifen was measured per µM.

[c.] Combination of hot-water extract and 4-hydroxytamoxifen was measured per µg/mL and µM.

Exposure to all concentrations of 4-hydroxytamoxifen at all phases had no significant effect except for the MCF7- cells treated with 10.99 µM of 4-hydroxytamoxifen ($p < 0.01$) in sub-G_1 phase (**Figure 14**). The 2.75 µM of 4-hydroxytamoxifen increased 5.89% of MCF-7 cells in the sub-G_1 phase. It increased 0.08% of MCF-7 cells in G_0/G_1 phase and decreased 1.13% and 0.43% of MCF-7 cells in the S and G_2/M phase, respectively (**Figure 14C; Table 3**). The 5.5

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μM of 4-hydroxytamoxifen increased 10.93% of MCF-7 cells in sub-G_1 phase. It decreased 3.48%, 1.38%, 2.20% in the G_0/G_1, S, and G_2/M phases, respectively (**Figure 14C; Table 3**). The 10.99 μM of 4-hydroxytamoxifen increased 24.12% of MCF-7 cells in sub-G_1 phase ($p < 0.01$), while decreasing 3.03%, 0.95%, 2.79% of MCF-7 cells in the G_0/G_1, S and G_2/M phases, respectively (**Figure 14C; Table 3**).

The dual exposure of 7.5 μg/mL of hot-water extract and 10.99 μM of 4-hydroxytamoxifen decreased 10.17% of MCF-7 cells in the sub-G_1 phase with no significant effect. The combination increased 17.04% and 0.99% of MCF-7 cells in the G_0/G_1 and S phases, respectively, while decreasing 2.82% of MCF-7 cells in the G_2/M phase with no significant effect (**Figure 14D; Table 3**).

3.3.2. Synergistic increase in nitric oxide levels

The vehicle control (0.5% DMSO; in-reaction) had no significant effect on nitric oxide levels for 48 h compared to the negative control (**Figure 15**). Sodium hypochlorite (0.5%; in-reaction) increased nitric oxide levels significantly ($p < 0.001$) by 5.6-fold more than the negative control (**Figure 15**).

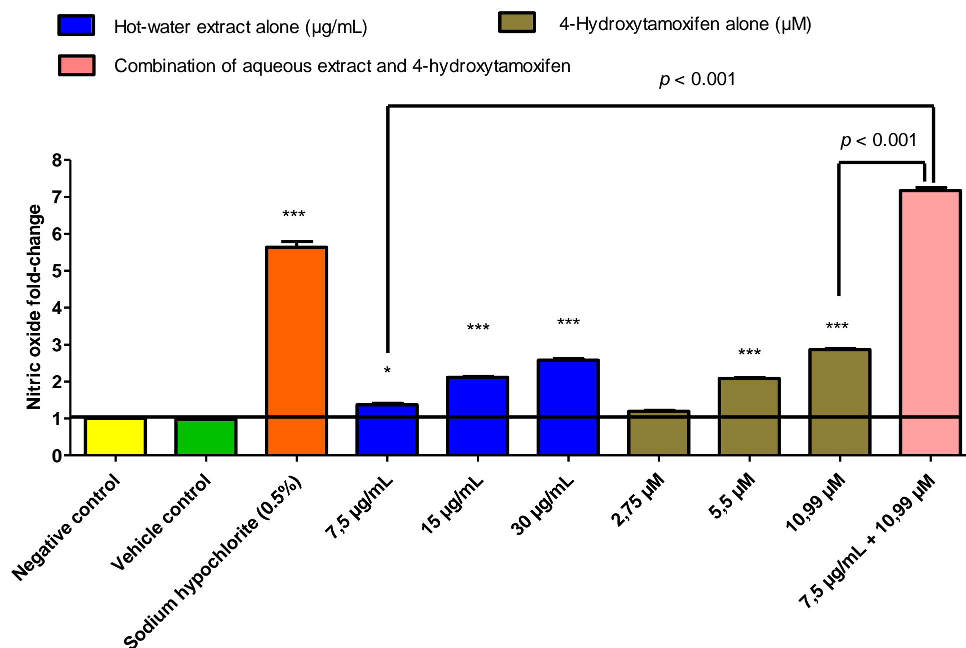

Figure 15. The effect of the combination of hot-water extract and 4-hydroxytamoxifen on nitric oxide levels in MCF-7 cells induced a 7.2-fold increase. The 7.2-fold increase in nitric oxide levels induced by the combination of 7.5 μg/mL hot-water extract ($p < 0.001$) and 10.99 μM of 4-hydroxytamoxifen (p < 0.001) was significant. *$p < 0.05$ and ***$p < 0.001$ relative to the negative control.

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Hot-water extract-treated cells increased the levels of nitric oxide in MCF-7 cells in a dose-dependent manner relative to the negative control (**Figure 15**). The hot-water extract increased nitric oxide levels by 1.4- ($p < 0.05$), 2.1- ($p < 0.001$), and 2.6-fold ($p < 0.001$) at 7.5, 15 and 30 µg/mL, respectively (**Figure 15**). The 4-hydroxytamoxifen increased nitric oxide levels in MCF-7 cells in a dose-dependent manner as well compared to the negative control (**Figure 15**). The 4-hydroxytamoxifen increased nitric oxide levels by 1.2- (no significant effect), 2.1- ($p < 0.001$), and 2.9-fold ($p < 0.001$) at 2.75, 5.5 and 10.99 µM, respectively (**Figure 15**). The combination of 7.5 µg/mL hot-water extract and 10.99 µM of 4-hydroxytamoxifen significantly increased nitric oxide levels by 7.2-fold relative to the negative control (**Figure 15**). The 7.2-fold increase in nitric oxide levels induced by the combination of 7.5 µg/mL hot-water extract ($p < 0.001$) and 10.99 µM of 4-hydroxytamoxifen ($p < 0.001$) was up to 5.1-fold greater than its individual counterparts.

3.3.3. Synergistic increase in lipid peroxidation levels

The vehicle control (0.5% DMSO; in-reaction) had no significant effect on lipid peroxidation for 48 h compared to the negative control (**Figure 16**). Sodium hypochlorite (0.5%; in-reaction) increased lipid peroxidation significantly ($p < 0.001$) by 5.0-fold compared to the negative control (**Figure 16**).

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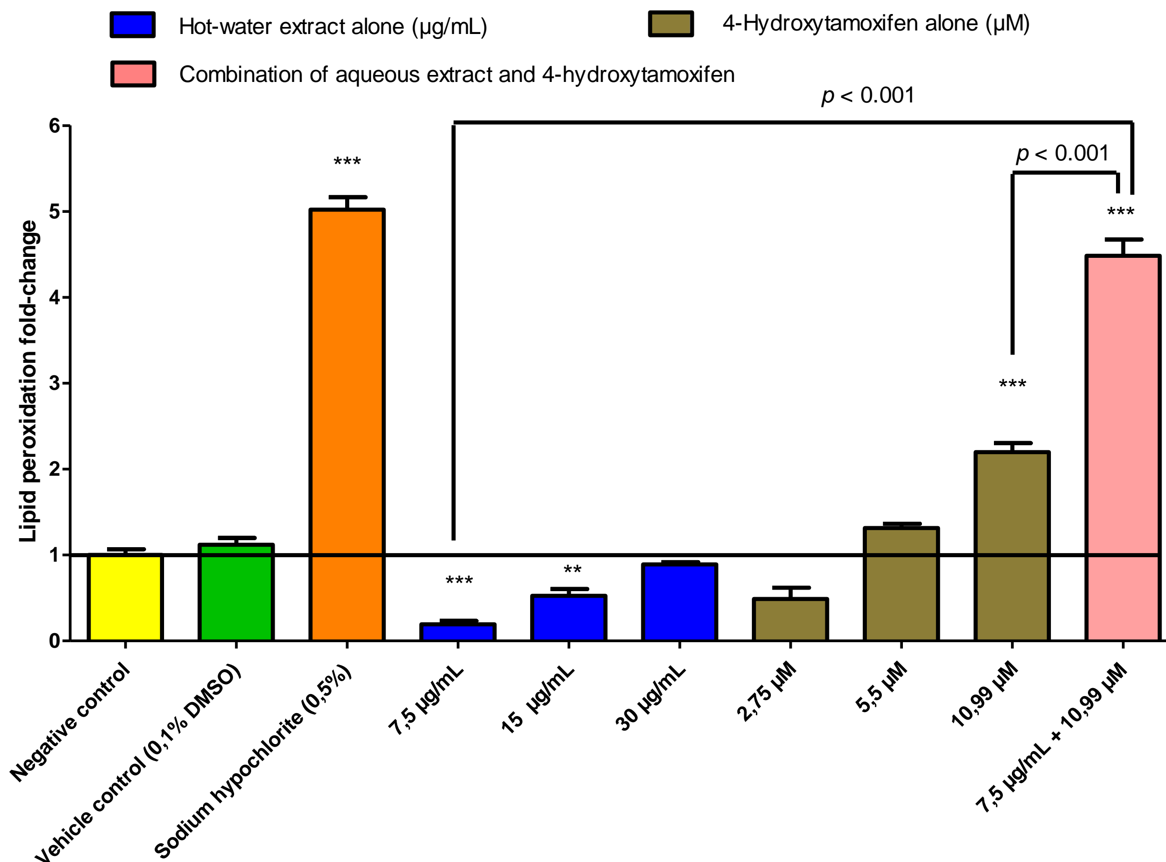

Figure 16. The effect of the combination of hot-water extract and 4-hydroxytamoxifen on MCF-7 cells induced a 4.3-fold increase in lipid peroxidation. The 4.3-fold increase in lipid peroxidation induced by the combination of 7.5 µg/mL hot-water extract ($p < 0.001$) and 10.99 µM of 4-hydroxytamoxifen ($p < 0.001$) was significant. ** $p < 0.01$ and *** $p < 0.001$ relative to the negative control.

The hot-water extract decreased lipid peroxidation to below the baseline peroxidation of the negative control after 48 h. (**Figure 16**). Concentrations of 7.5 ($p < 0.001$) and 15 µg/mL ($p < 0.01$) of hot-water extract significantly lowered lipid peroxidation to 0.19-fold and 0.5-fold compared to the negative control (**Figure 16**). The 30 µg/mL of the hot-water garlic lowered lipid peroxidation to 0.89-fold compared to the negative control, though the decrease was not significant (**Figure 16**). Cells exposed to 4-hydroxytamoxifen also increased lipid peroxidation in a dose-dependent manner after 48 h (**Figure 16**). Concentrations of 2.75 µM of 4-hydroxytamoxifen lowered lipid peroxidation 0.49-fold with no significant effect compared to the negative control (**Figure 16**). Treatment with 5.5 µM of 4-hydroxytamoxifen increased lipid peroxidation 1.3-fold more than the negative control, though it was not significant (**Figure 16).** At 10.99 µM of 4-hydroxytamoxifen, lipid peroxidation significantly increased by 2.2-fold relative to the negative control ($p < 0.001$) at 48 h (**Figure 16**). Combination of 7.5 µg/mL

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of hot-water garlic and 10.99 µM of 4-hydroxytamoxifen significantly increased lipid peroxidation 4.3-fold ($p < 0.001$) relative to the negative control (**Figure 16**). The fold change increase for the combination was also significantly higher ($p < 0.001$; for both) than for both 7.5 µg/mL of hot-water garlic and 10.99 µM of 4-hydroxytamoxifen.

3.3.4. Synergistic increase in caspase-3/7 activity

The vehicle control (0.5% DMSO; in-reaction) exerted no significant effect on caspase-3/7 activity after 24 or 48 h compared to the negative control (**Figure 17A and B**). Cisplatin (10 µM) increased caspase-3/7 activity 2-fold after 24 h compared to the negative control, though it was not significant (**Figure 17A**). Caspase-3/7 activity of cells treated with 10 µM cisplatin doubled at 48 h compared to the 24 h (**Figure 17B**). The 4-fold increase in caspase-3/7 activity at 48 h was significant ($p < 0.001$) compared to the negative control (**Figure 17B**).

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Hot-water extract alone (µg/mL) | 4-Hydroxytamoxifen alone (µM)

Combination of aqueous extract and 4-hydroxytamoxifen

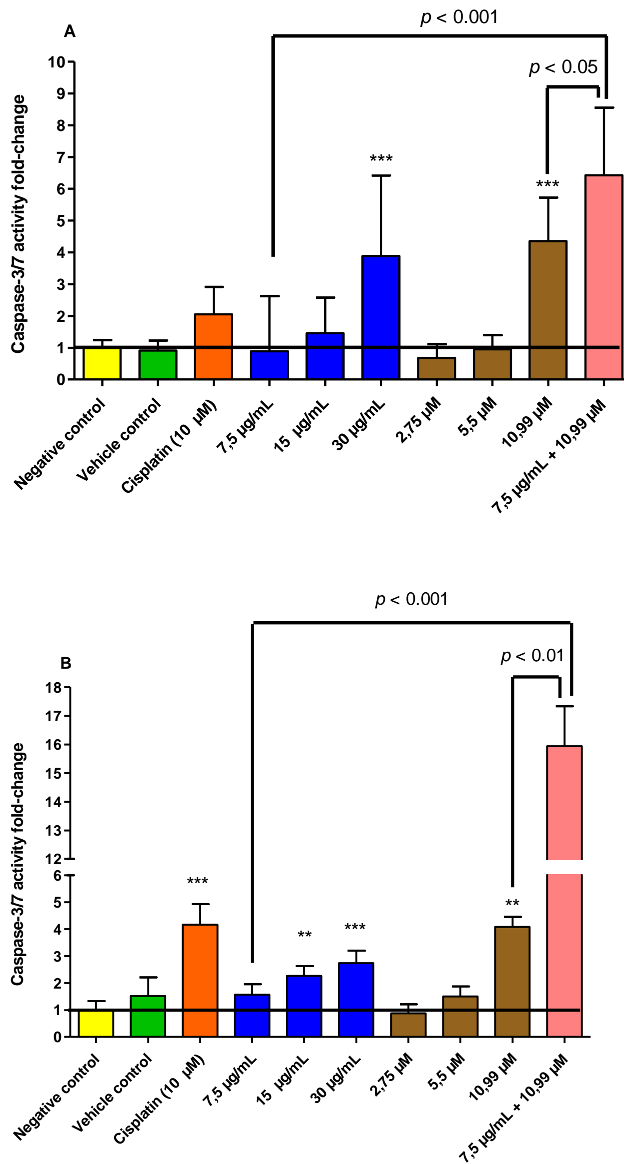

Figure 17. The effect of the combination of hot-water extract and 4-hydroxytamoxifen increased caspase-3/7 activity in MCF-7 cells a) 6-fold and B) 16-fold after 24 and 48 h, respectively. ***$p < 0.01$ and ***$p < 0.001$ relative to the negative control. The 6-fold increase in caspase-3/7 activity induced by the combination of 7.5 µg/mL hot-water extract ($p < 0.001$) and 10.99 µM of 4-hydroxytamoxifen ($p < 0.05$) was significant. The 16-fold increase in caspase-3/7 activity induced by the combination of 7.5 µg/mL hot-water extract ($p < 0.001$) and 10.99 µM of 4-hydroxytamoxifen ($p < 0.01$) was significant.

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Exposure to hot-water garlic resulted in a dose- and time-dependent increase in caspase-3/7 activity after 24 and 48 h incubation, except for treatment with 7.5 µg/mL of hot-water extract after 24 h, which was below the baseline (**Figure 17**). The 7.5 µg/mL of hot-water extract reduced caspase activity 0.9-fold, with no significant effect, compared to the negative control (**Figure 17A**). Caspase-3/7 activity of cells exposed to 15 µg/mL of hot-water garlic extract for 24 h increased to 1.1-fold; however, it was not significant compared to the negative control (**Figure 17A**). Caspase-3/7 activity increased significantly, reaching 3.9-fold after treatment with 30 µg/mL ($p < 0.001$) of hot-water garlic extract for 24 h (**Figure 17A**). The 48 h incubation with 7.5 µg/mL of hot-water garlic increased caspase-3/7 activity 1.6-fold, 1.8-fold more than the 24 h incubation, but the 1.6-fold increase was not significant compared to the 48 h negative control (**Figure 17B**). The difference between the 24 incubation and 48 h incubation was not significant. Incubation with 15 µg/mL for 48 h of hot-water garlic extract increased caspase-3/7 activity 2.3-fold, significantly ($p < 0.01$) compared to the negative control (**Figure 17B**). Additionally, treatment with 30 µg/mL of hot-water garlic extract for 48 h increased caspase-3/7 activity 2.8-fold relative to the negative control, significantly ($p < 0.001$) compared to the negative control (**Figure 17B**). The 48 h incubation with 15 µg/mL of hot-water garlic increased caspase-3/7 activity 1.5-fold more than the 24 h incubation, though it was not significant. While the 48 h incubation with 30 µg/mL of hot-water garlic reduced caspase-3/7 activity 0.7-fold less than the 24 h incubation, though it was not significant.

Concentrations of 2.75 and 5.5 µM of 4-hydroxytamoxifen decreased caspase-3/7 activity relative to the negative control after 24 h with a fold-change of 0.7 and 0.9, respectively, after 24 h (**Figure 17A**). The 10.99 µM increased caspase-3/7 activity significantly ($p < 0.01$) with a fold-change of 4.4 (**Figure 17A**). The 2.75 µM of 4-hydroxytamoxifen continued to decrease caspase-3/7 activity below baseline at 48 h with a fold-change of 0.9, though 5.5 µM of 4-hydroxytamoxifen increased caspase-3/7 activity with fold-changes of 1.5 at 48 h (**Figure 17B**). The 10.99 µM of 4-hydroxytamoxifen continued to increase caspase-3/7 ($p < 0.001$) activity with a fold-change of 4.1 at 48 h; however, the fold-change was more prominently observed at 24 h (**Figure 17B**).

The combination of 7.5 µg/mL of hot-water garlic extract and 10.99 µM of 4-hydroxytamoxifen increased caspase-3/7 activity 6.7-fold after 24 h, an increase that was significant ($p < 0.001$) compared to the negative control (**Figure 17A**). Moreover, the fold change increase in caspase-3/7 activity was up to 6-fold higher compared to its individual counterparts of 7.5 µg/mL of garlic ($p < 0.001$) and 10.99 µM of 4-hydroxytamoxifen ($p <$

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0.05). At 48 h, caspase-3/7 activity of cells treated with the combination increased 16-fold compared to the negative control ($p < 0.001$) (**Figure 17B**). Moreover, the fold change increase in caspase-3/7 activity was up to 16-fold higher than its individual counterparts of 7.5 µg/mL of hot-water extract ($p < 0.001$) and 10.99 µM of 4-hydroxytamoxifen ($p < 0.01$).

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Chapter 4: Discussion

4.1. Assessment of cytotoxicity in crude extracts and 4-hydroxytamoxifen

4.1.1. Inherent cytotoxicity

A significant difference ($p < 0.001$) was seen for cells exposed to the vehicle (0.5%; in-reaction) compared to the negative control. As a familiar bio-suitable solvent commonly used, DMSO dissolves various organic and inorganic compounds due to its physiochemical properties.[261] It has been documented that MCF-7 cells are susceptible to damage from exposure to concentrations of 0.5% ≥ of DMSO.[262-264] Cisplatin, used as the positive control, is a known chemotherapeutic drug used to treat several types of cancer.[260,265] Its cytotoxicity is mediated by its ability to form cisplatin-DNA adducts to inhibit DNA synthesis and induce apoptosis.[260,265] The study noted an 8.11±0.03 µM (**Table 1**). A study that explored the cytotoxic effects of cisplatin in MCF-7 cells reported an IC_{50} value of 11.9 µM after 48 h incubation,[266] which is comparable.

The hot-water and methanol extracts increased cell density up to 110% at 0.1 µg/mL of crude extract. Cell density further increased to 132% at 1 µg/mL of crude extract before decreasing in a dose-dependent manner. This well-known phenomenon is termed hormesis or preconditioning.[267] Low concentrations of dietary phytochemicals (i.e., allicin, curcumin, and quercetin) are not considered toxic and may activate a mild stress response pathway in cells, leading to a potential increase in cell survival factors and density.[267,268]

There are three classical pathways involved in hormesis that have been widely described.[269] The first pathway, termed oxidative conditioning hormesis, involves subjecting cells to an oxidant at low doses.[269] This pathway conditions the cell to adapt when re-exposed to the same oxidant.[269] The second pathway involves the nuclear factor erythroid 2-related factor (Nrf2)/antioxidant response element (ARE) pathway, which increases the expression of antioxidant enzymes (i.e., catalase, manganese superoxide dismutase, and glutathione peroxidase) to confer resistance to severe stress.[267,269,270] The last pathway, the “obesity paradox”, is more complex and is an *in vivo* response to oxidative stress and chronic inflammation,[269] which does not bear relevance in the current study.

Water- (i.e., γ-glutamyl-*S*-allyl-L-cysteine and *N*-acetyl-*S*-allyl-L-cysteine) and oil-soluble organosulfur compounds (i.e., DADS and DATS) found in garlic have been reported to activate

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the Nrf2/ARE pathway in MCF-7 cells,[267,271-273] which may have been responsible for the increased cell density observed in the current study.

The National Cancer Institute considers a crude plant extract with *in vitro* cytotoxic potential if the IC_{50} is ≤30 µg/mL after a 48 h incubation period.[248,274] The crude hot-water and methanol extracts in this study had minimal inherent cytotoxicity on MCF-7 cells at 48 h, as noted by the inability to obtain an IC_{50} value at the concentration range tested. Thus, the crude extracts used in the study are not considered cytotoxic. The cytotoxicity of garlic has been investigated in malignant (e.g., AGS, HT-29, MCF-7) and non-malignant cell lines (e.g., L929), though typically the studies are based on single garlic-derived compounds rather than crude extracts. The few studies that investigated cytotoxicity of crude garlic extracts in breast cancer, favoured hot-water extracts. Ghazanfari *et al* observed a lack of inherent cytotoxicity of their hot-water garlic extract on MCF-7 cells as their IC_{50} was 1821 µg/mL after 48 h.[275] Modem *et al* reported an IC_{50} value of 2500 µg/mL after exposing MCF-7 cells to a cold-water garlic extract for 24 h[276]. However, it was observed that the MCF-7 cells continued to proliferate in the presence of the hot-water extract and it was theorised that the heat may have destroyed the enzymatic activity of alliinase.[276] This appears to coincide with the hormesis seen in the present study. There is conflicting evidence on the impact of cold- and hot-water crude extracts on cytotoxicity. Bagul *et al* observed significant inhibition of MCF-7 cell proliferation ($p < 0.05$) after exposure to cold-water garlic extracts (0.25, 0.5 and 1 µg/mL) which was between 85-92% after a 72 h incubation period.[277] This is in contrast to the observation of El-khamissi *et al* who reported IC_{50} values of 33.2 and 33.4 µg/mL after 24 h for their cold- and hot-water garlic extracts on their MCF-7 cells, respectively.[278] The difference observed between the cytotoxicity of the hot-water extract in their study and the hot-water extract in this study may be explained by the type of garlic used, different quantities of bioactive compounds in the respective garlic extracts, and the incubation.

Literature is scarce on the effect of the methanol garlic extracts on breast cancer cell lines. Most of the literature available is focused on hot-water extracts as previously mentioned.[275,276,278] The methanol extract in this study displayed inherent cytotoxicity slighter greater than the hot-water extract. The ethanol extract in the El-khamissi study showed more prominent cytotoxicity (IC_{50} = 15.3 µg/mL after 24 h) compared to the cold- and hot-water extracts[278] which surpassed the cytotoxicity seen for the methanol extract in the present study. Though, the pattern of alcohol solvent-based extract showed greater cytotoxicity than its aqueous (cold or hot) counterpart, it was comparable to the findings in this study. Oil-soluble organosulfur

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compounds derived from garlic, i.e., allyl methylsulphide, DAS and DADS are more effective in suppressing breast cancer compared to water-soluble compounds.[240,279] It may be reasoned that these oil-soluble organosulfur compounds are volatile, and therefore more toxic than water-soluble organosulfur compounds which are non-volatile.[280,281]

Tamoxifen is the mainstay chemotherapeutic drug used to treat ER-positive breast cancer in pre- and postmenopausal women.[282] A number of studies have investigated the role of tamoxifen on breast cancer, however, rarely its metabolites. This study explored the inherent cytotoxicity of 4-hydroxytamoxifen in MCF-7 cells. Cell density of MCF-7 cells treated with 4-hydroxytamoxifen decreased in a dose-dependent manner over 48 h**,** with an IC_{50} of 10.99±0.01 µM. Several authors have assessed the inherent cytotoxicity of tamoxifen on MCF-7 cells and the reported IC_{50} values ranged from 1[283] to 11 µM after 24 h, 10 µM after 48 h,[284] 4.2[285] to 27 µM after 96 h,[286] and 1 µM after 144 h.[287] The IC_{50} of 10 µM after 48 h reported by Yaacob *et al* was comparable to this study. The observations are also contrary to those of Salami *et al* where tamoxifen-exposure to 0.1 µM was cytostatic, 0.5 and 1 ($p < 0.05$) µM were cytotoxic, and concentrations of 2.5, 5, 10, 15 and 20 µM exerted a mild proliferative effect on the MCF-7 cells after 48 h.[283] Though, a mild proliferative effect was seen at 1 µM in this study.

4.1.2. Combinational cytotoxicity of crude extracts and 4-hydroxytamoxifen

The imperative need to renew attention to indigenous knowledge systems and integrative medicine has been highlighted.[288,289] The use of CAM, in combination with conventional medicine, has drastically increased among breast cancer patients.[205] The combination of crude garlic extracts and 4-hydroxytamoxifen showed synergistic, additive, and antagonistic effects within the specific confines of the experimental setting with the most synergistic combination being 7.5 µg/mL of hot-water extract and 10.99 µM of 4-hydroxytamoxifen.

A study by Vemuri *et al* explored the crude hot-water combinational mixture of garlic, ginger and turmeric.[249] The herbal mixture (5, 10 and 20 µg/mL) and tamoxifen [10 (26.9 µM) and 20 µg/mL (53.8 µM)] were tested in three different breast cancer cell lines, including MCF-7 cells.[249] The authors did not evaluate the individual components of the herbal mixture, albeit exposure to the herbal mixture at 5, 10, 20 µg/mL did not show any indication of inherent cytotoxicity after 42 h. The lack of inherent cytotoxicity of the hot-water herbal mixture in MCF-7 cells is comparable to the present findings. The selection of 7.5 µg/mL of hot-water extract and 10.99 µM of 4-hydroxytamoxifen in this study was based on calculations which

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showed that it was a synergistic combination and the cell density of 19%. Vemuri *et al* used a combination of 10 µg/mL of the herbal mixture and 20 µg/mL (53.8 µM) of tamoxifen.[249] Cells treated with their combinational treatment of the herbal mixture and tamoxifen, indicated a more prominent decrease in cell density than the individual treatments of the herbal mixture and tamoxifen alone.[249] The reduced cell density was comparable to the observations in this current study. However, the combinational treatment of 7.5 µg/mL of hot-water garlic extract and 10.99 µM of 4-hydroxytamoxifen resulted in a significant difference compared to the single treatment with the hot-water garlic extract ($p < 0.001$) and 4-hydroxytamoxifen ($p < 0.05$). This would suggest that the combination of the hot-water extract and 4-hydroxytamoxifen exerted enhanced cytotoxicity. The combinational indices showed that concentrations of either compound is important in determining synergistic, additive, and antagonistic combinations, thereby impacting physiological functions.[290] The antagonistic combinations seen in this study were possibly a result of the combinations of crude extracts and 4-hydroxytamoxifen masking the effects of either compound in a complex nature. Thus, the combination of the crude extracts and 4-hydroxytamoxifen led to the cumulative effects that were less than the two individual components separately.[290,291] Alternatively, the combination of the two compounds led to a cumulative effect of the multiple stressors that were greater than the sum of effects of the two individual compounds combined, termed synergism.[291,292] The mechanistic evaluation of the most synergistic combination in this study showed that these two compounds target similar pathways in combination.

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4.2. Assessment of synergistic mechanism of cell death

4.2.1. Cell cycle

The percentage of MCF-7 cells in sub-G_1 phase decreased after exposure to 7.5 µg/mL of hot-water extract while increased when exposed to concentrations of 15 and 30 µg/mL hot-water extract. Vemuri *et al* also noted that 10 µg/mL of the natural extract of turmeric, ginger, and garlic mixture increased to 9.67% MCF-7 cells in the sub-G_1 phase.[249] Modem *et al* did not state the percentage increase in MCF-7 cells in sub-G_1 phase, but from the histogram it was evident that there was an increase in this phase after treatment with 2500 µg/mL cold-water extract.[276] The 15 and 30 µg/mL hot-water extract decreased the percentage of MCF-7 in the G_0/G_1 phase at 48 h, whereas the 7.5 µg/mL hot-water extract increased the percentage of MCF-7 cells in this phase. Modem *et al* observed that the 2500 µg/mL cold-water extract increased the percentage of MCF-7 cells in G_0/G_1 phase by 27.5% after an hour compared to their negative control.[276] On the contrary, similar to this study, Vemuri *et al* noted that treatment with 10 µg/mL of natural extract of turmeric, ginger, and garlic decreased the percentage of MCF-7 cells in the G_0/G_1 phase by 5% after 42 h.[249] Thus, data from the current study suggest that the cold- and hot-water extracts exert comparable effects on cellular kinetics by arresting MCF-7 cells in G_0/G_1 phase at low concentrations. The 7.5 and 30 µg/mL extracts increased percentage of MCF-7 cells in the S phase, whereas the 15 µg/mL extract decreased the percentage. Both Modem *et al* and Vemuri *et al* reported a decrease in cells in the S phase.[249,276] Modem *et al* had noticed their 2500 µg/mL cold-water extract decreased the percentage of MCF-7 cells in S phase by 19.5%.[276] Vemuri *et al* reported a 3% decrease in the S phase with their 10 µg/mL of natural extract of turmeric, ginger, and garlic.[249] The percentage of MCF-7 cells either increased (7.5 µg/mL), or decreased (15 and 30 µg/mL) in the G_2/M phase when treated with the hot-water extract. Modem *et al* and Vemuri *et al* observed a decrease in cells in the G_2/M phase, of 8.00%[276] and 5.00%, respectively.[249] The observations of the hot-water extracts suggests that low concentrations allow for a cell cycle arrest at G_0/G_1 phase and possibly promote cell death. As concentrations increase there is DNA fragmentation that is beyond repair, as indicated by the sub-G_1, leading to cell death. Nonetheless, the findings of this study cannot conclusively support this.

All three concentrations of 4-hydroxytamoxifen increased the percentage of cells in the sub-G_1 phase compared to the negative control at 48 h with the cells treated with 10.99 µM of 4-hydroxytamoxifen being significant ($p < 0.05$). This is in agreement with Abdallah *et al* where the authors treated MCF-7 cells with 100 nM for 24 h and noted a 8.2% increase of cells in the

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sub-G_1 phase.[178] Vemuri *et al* also noted an increased percentage of MCF-7 cells in the sub-G_1 phase, where 20 µg/mL (53.8 µM) increased 6.67% of MCF-7 cells in the sub-G_1 phase compared to the negative control.[249] Li *et* al noted a ~5% ($p < 0.05$), ~20% ($p < 0.01$), and ~80% ($p < 0.01$) increase in MCF-7 cells in sub-G_1 phase when treated with 1, 2, 4 µM of tamoxifen for 48 h, respectively.[293] The 2.75 µM of 4-hydroxytamoxifen in this study increased the percentage of MCF-7 cells in the G_0/G_1 phase at 48 h, whereas 5.5 and 10.99 µM decreased the percentage. Sutherland *et al* treated MCF-7 cells with 1, 5, and 10 µM of tamoxifen and found an 6%, 16.8%, and 26.1% increase in the G_0/G_1 phase after 36 h, respectively.[294] The increase at 1 µM is comparable to this study; however, the increase following treatment with 5, and 10 µM of tamoxifen is not line with this study. Though, treatment with 20 µM of tamoxifen for 24 h resulted in a 2.6% decrease in cells in the G_0/G_1 phase.[294] Additionally, Liu *et al* reported that 10 µM of tamoxifen significantly ($p < 0.01$) increased the percentage of MCF-7 cells in the G_0/G_1 phase compared to the control group at 48 h; however, the percentage is not stated.[295] This finding also does not support the observations of this study. Vemuri *et al* reported that treatment of MCF-7 cells for 42 h with 20 µg/mL (53.8 µM) tamoxifen resulted in a 1.33% decrease in the G_0/G_1 phase,[249] which is in agreement with this study. Contrastingly to the findings in this study, Moriai *et al* observed that 1 and 2.5 µM of tamoxifen decreased 0.7% and 1%, respectively, of MCF-7 cells in the G_0/G_1 phase after 24 h.[296] While, 5 and 10 µM of tamoxifen increased 4% and 13.4%, respectively, of MCF-7 cells in the G_0/G_1 phase after 24 h.[296] Both Sutherland *et al* and Vemuri *et al* noted a decrease in percentages, possibly due to the higher concentrations of tamoxifen used. Nevertheless, there seems to be more at play as Moriai *et al* observed a decrease at lower concentrations. All three concentrations of 4-hydroxytamoxifen in the current study decreased the percentage of cells in the S phase at 48 h. Vemuri *et al* treated MCF-7 cells with 20 µg/mL (53.8 µM) tamoxifen for 42 h and observed a 3% decrease in cells in the S phase.[249] Li *et* al also noted a decrease in MCF-7 cells in the S phase when treated with 1 µM (~30%), 2 µM (20%; $p < 0.05$), and 4 µM (0%; $p < 0.01$) tamoxifen at 48 h.[293] Sutherland *et al* described a dose-dependent decrease in percentage of MCF-7 cells treated with 1 (2.4%), 5 (12.3%), and 10 (23.2%).[294] All three concentrations of 4-hydroxytamoxifen in this study decreased the percentage of cells in the G_2/M phase at 48 h. Similarly, Vemuri *et al* described their treatment with 20 µg/mL (53.8 µM) tamoxifen decreased 6% of the MCF-7 cells in the G_2/M phase.[249] Li *et* al observed that treatment with 1 µM (~20%) decreased, 2 µM (20%; $p < 0.01$), and 4 µM (~40%; $p < 0.01$) tamoxifen increased MCF-7 cells in the G_2/M phase.[293] The findings of Vemuri *et al* support the findings of the current study. As previously mentioned, Li *et* al reported dose-dependent increase in MCF-7

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cells in the G_0/G_1 phase, indicating a tamoxifen-induced G_0/G_1 block. Corresponding with the hot-water extract, 4-hydroxytamoxifen potentially arrests the cell cycle arrest at G_0/G_1 phase at low concentrations. Again, this cannot be said definitively as the increase was similarly non-significant. This study observed the genotoxic effects of 4-hydroxytamoxifen, which is known to be more potent than tamoxifen.[166,171]

Dual-exposure to 7.5 µg/mL hot-water extract and 10.99 µM 4-hydroxytamoxifen arrested on average 17.04% more cells in the G_0/G_1 phase, with no significant effect, compared the negative control at 48 h. The percentage of cells arrested in G_0/G_1 was 11.81% (7.5 µg/mL hot-water extract) and 14.01% (10.99 µM 4-hydroxytamoxifen) more than the individual counterparts. This would suggest that the combination of the hot-water extract and 4-hydroxytamoxifen induces a cell cycle arrest, though this cannot be definitively concluded. Vemuri *et al* noted that their combination of 10 µg/mL of hot-water turmeric, ginger, and garlic mixture with 20 µg/mL (53.8 µM) of tamoxifen increased the percentage of MCF-7 cells arrested in the G_0/G_1 phase by 2.67% compared to the negative control.[249] The combination of the herbal mixture and tamoxifen was 7.67% and 4% higher than its herbal mixture and tamoxifen counterparts, respectively.[249] The findings of Vemuri *et al* support the combinational effects of a herbal extract and tamoxifen, and as such 4-hydroxytamoxifen, arrest the MCF-7 cells at G_0/G_1 phase. However, it is important to note that the separate treatment with 10 µg/mL herbal mixture and 20 µg/mL (53.8 µM) of tamoxifen both decreased the percentage cells in the G_0/G_1 phase compared to the negative control comparable to this study. Overall, in this study, there was genotoxicity and induction of cell death induced by the solo treatments of the hot-water extract and 4-hydroxytamoxifen. Though, there appears to be a complex dynamic that allowed for combinational synergism to indicate that the MCF-7 cells could be arrested the G_0/G_1 phase.

4.2.2. Nitric oxide

The 7.5 ($p < 0.05$), 15 ($p < 0.001$), and 30 ($p < 0.001$) µg/mL hot-water extract alone significantly increased levels of nitric oxide in MCF-7 cells in a dose-dependent manner relative to the negative control. Most of the experimental assessment of the effect of garlic on nitric oxide levels is based on the cardiovascular system.[297,298] Nonetheless, it is thought that its ability to elevate the activity of intracellular nitric oxide synthase extends to many therapeutic applications.[297] Garlic has been associated with elevating cytosolic calcium; thereby increasing mitochondrial calcium in MCF-7 cells.[299,300] Elevated calcium levels in the mitochondria negatively affect the energy-dependent mechanisms.[301,302] Consequently, the

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mitochondrial membrane is depolarised and the activity of mitochondrial nitric oxide synthase in MCF-7 cells is upregulated as a response to the elevated calcium levels.[173,179] The upregulated mitochondrial nitric oxide synthase activity leads to elevated nitric oxide levels within MCF-7 cells.[173,179] An *et al* demonstrated that MCF-7 cells treated with 2- , 4 - and 8 g/100µL of a crude alcohol extract of elephant garlic for 24 h decreased the mitochondrial membrane potential of MCF-7 cells, increased depolarisation, in a dose-dependent manner, thereby activating the nitric oxide-dependent pathway.[299] In another study by Na *et al* it was shown that 50 µM of DATS for 24 h rapidly disrupted mitochondrial transmembrane of MCF-7 cells and the membrane became depolarised.[300] While different methods of assessment were used, this study saw an increase in nitric oxide levels as well. Nonetheless, the lack of minimal inherent cytotoxicity observed, despite the increased nitric oxide levels, suggests that there was initial protection from cell damage.[303]

There was also a dose-dependent increase in MCF-7 cells treated with 4-hydroxytamoxifen, with the elevation of nitric oxide levels significant at 5.5 ($p < 0.001$) and 10.99 µM ($p < 0.001$). As previously mentioned, tamoxifen can induce cell death in MCF-7 cells via a nitric oxide-dependent pathway.[173] Tamoxifen targets the mitochondrial nitric oxide synthase in MCF-7 cells, thereby elevating nitric oxide levels,[173] in a manner similar to garlic. Nazarewicz *et al* reported that concentrations of 0.1 ($p < 0.01$) and 0.5 µM ($p < 0.01$) of tamoxifen disrupted the calcium homeostasis by significantly increasing intra-mitochondrial calcium concentrations.[173] Additionally, the concentrations of 0.1 ($p < 0.05$) and 0.5 µM ($p < 0.01$) of tamoxifen increased the activity of mitochondrial nitric oxide synthase.[173] In the study conducted by Parihar *et al* 5 µM of tamoxifen significantly ($p < 0.05$) elevated nitric oxide levels after 20 min; though, the unit of measurement was 4,5-diaminofluorescein diacetate fluorescence (au), which is a different unit of measurement than was used in this study.[174] Although the abovementioned studies allude to nitric oxide levels by assessing intra-mitochondrial calcium levels and mitochondrial nitric oxide synthase activity, evaluations not conducted in this study, it provides insight to the fold-change increase in nitric oxide levels observed.

Dual-exposure to 7.5 µg/mL of the hot-water extract and 10.99 µM of 4-hydroxytamoxifen significantly ($p < 0.001$; for both) increased nitric oxide levels by 7.2-fold relative to the negative control. There are no current studies that have assessed the combination of an extract and tamoxifen, nor any of its metabolites, on nitric oxide levels proving comparison difficult. However, it is plausible to theorise that the hot-water extract and 4-hydroxytamoxifen synergistically targeted nitric oxide-dependent pathways in MCF-7 cells.

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4.2.3. Lipid peroxidation

Exposure to treatment with hot-water crude extract only displayed decreased lipid peroxidation in the MCF-7 cells at 48 h compared to the negative controls. Concentrations of 7.5 ($p < 0.001$) and 15 µg/mL ($p < 0.01$) of the hot-water extract significantly decreased lipid peroxidation below the baseline, whereas the decrease following exposure to 30 µg/mL garlic was not significant. Experimental studies that have focused on garlic, be it crude extracts or its derivates (e.g., allicin, DADS and DATS), have rarely explored its role on lipid peroxidation in cancer research; though, it is widely described as a free radical scavenging antioxidant in cholesterolemic studies.[304-306] This makes it difficult to make many comparisons with other experimental studies as these are not available.

Redox status is a crucial factor in determining anti-oxidant/pro-oxidant activity.[307] A balanced redox status influences nitric oxide to act as an anti-oxidant by rapidly scavenging lipid peroxyl radicals.[297,307] As mentioned earlier, garlic can stimulate the production of nitric oxide correlating with increased caspase-3/7 activity, suggesting one of the mechanisms of cell death is nitric oxide-dependant; however, there appears to be a nexus of co-existing pathways that protects the MCF-7 cells from lipid peroxidation that may arise from the increased nitric oxide production. Water-soluble S-allyl cysteine, an organosulfur compound derived from garlic, is known to scavenge peroxyl radicals and peroxynitrite radicals;[308] however, it remains unknown to what extent the role of S-allyl cysteine has an inhibiting nitrosative stress effect in this study. Alternatively, the rate of nitric oxide-induced lipid peroxidation induced by garlic in this study may be subtoxic, prompting cells to activate antioxidant pathways to remedy the lipid peroxidation whilst maintaining cell death.

The 4-hydroxytamoxifen-exposed cells (2.75 µM) decreased lipid peroxidation below baseline. Thereafter, the 5.5 and 10.99 µM ($p < 0.001$) of 4-hydroxytamoxifen demonstrated a dose-dependent increase in lipid peroxidation. This is in contrast with the findings of Nazarewicz *et al* as they found that sub-micromolar concentrations of tamoxifen (0.1 and 0.5 µM) significantly ($p < 0.05$) increased lipid peroxidation in MCF-7 cells.[173] Tamoxifen and hydroxytamoxifen have been described as antioxidants[309,310] and it is probable that 2.75 µM of 4-hydroxytamoxifen may have scavenged lipid peroxyl radicals in a similar fashion as the hot-water extracts. The only similarity for tamoxifen-reduced lipid peroxidation was described by Thangaraju *et al*; however, the authors assessed the serum concentration of malondialdehyde of tamoxifen-treated patients after three and six months of treatment.[311] It would need to be investigated further whether there is a relationship between cellular and serum levels of

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malondialdehyde, and the mechanism of action. Theodossiou *et al* denoted a similar pattern of increase in lipid concentration, of which 15 μM of 4-hydroxytamoxifen increased lipid peroxidation in MCF-7 cells relative to the negative control.[312]

Exposure to the 7.5 μg/mL of hot-water extract only may have reduced lipid peroxidation, but dual-exposure with 10.99 μM of 4-hydroxytamoxifen significantly ($p < 0.001$; for both) increased lipid peroxidation by 4.3-fold above the baseline. The combination increased nitric oxide levels as previously mentioned, probably due to elevated intracellular calcium levels. Sustained elevated levels of intracellular calcium result in an imbalance in redox status by causing oxidative stress whereby an increased production of hydroxyl and superoxide radicals was noted.[301,302] Sustained elevated levels of ROS further contributes to mitochondrial damage forming a vicious cycle.[302] Nitric oxide is a known promoter and inhibitor of lipid peroxidation.[313] It reacts with the free radicals and in its presence stimulates the production of peroxynitrite, an oxidant capable of lipid peroxidation.[173,313] Hydroxyl and superoxide radicals also promote induce lipid peroxidation via enzymatic and non-enzymatic pathways[314] and release further cytochrome *c* from the mitochondria.[173] Subsequently, these products led to additional lipid peroxidation and induction of apoptosis.[173]

4.2.4. Caspase-3/7 activity

The 7.5 μg/mL hot-water extract reduced caspase-3/7 activity with no significant effect, while 15 and 30 μg/mL significantly ($p < 0.05$) increased caspase-3/7 activity after 24 h. The hot-water extracts increased caspase-3/7 activity in MCF-7 cells in a dose-dependent manner at 48 h. Vemuri *et al* treated MCF-7 cells with 5 (10%; $p < 0.01$), 10 (12.5%; $p < 0.01$) and 20 μg/mL (15%; $p < 0.01$) of a hot-water herbal mixture of turmeric, ginger, and garlic noted a dose-dependent increase in the percentage of apoptotic cells after 42 h.[249] This contradicts the findings in this study where treatment with 7.5 μg/mL of the hot-water extract increased caspase-3/7 activity non-significantly relative to the negative control at 48 h. However, treatment with 15 and 30 μg/mL ($p < 0.001$; for both) increased caspase-3/7 activity similarly to their study. Garlic-derived compounds are known to induce caspase-dependent and caspase-independent pathways in MCF-7 cells.[315,316] Garlic has the capacity to depolarise the mitochondrial membrane as mentioned earlier.[299,300] The loss of the mitochondrial membrane potential leads to the release of cytochrome *c* which binds and activates procaspase-9 and apoptotic protease activating factor 1, leading to caspase-9 activation, and downstream activation of caspases-3 and -7.[300,316,317] The findings of Isbilen and Volkan showed that cold-water extracts [156.25, 312.5, 625, 1250, 2500 ($p < 0.05$), 5000 ($p < 0.05$), and 10,000 (p <

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0.05) µg/mL] increased caspase-9 activity in the MCF-7 cells in a dose-dependent manner.[318] This study did not assess caspase-9 activity, but the results of Isbilen and Volkan provides insight regarding caspase-3 activity. This is due to an activated caspase-9 that can directly activate caspase-3 and -7.[319] The hot-water extracts in this study increased caspase-3/7 activity in the MCF-7 cells at 48 h, with a significant increase at 15 and 30 µg/mL ($p < 0.001$; for both). However, Isbilen and Volken observed a reduction in caspase-3/7 activity after treatment with 156.25, 312.5, 625, and 5000 µg/mL of cold-water garlic extracts, though there was no significant effect possibly indicating the activation of a different cell death pathway, such as autophagy.[318,320] The latter authors found that 1250, 2500 and 10,000 µg/mL of the hot-water extract non-significantly increased caspase-3/7 activity.

The 2.75 and 5.5 µM concentrations of 4-hydroxytamoxifen decreased caspase-3/7 activity below baseline with no significant effect, whereas 10.99 µM of 4-hydroxytamoxifen activated caspase-3/7 activity significantly ($p < 0.01$) after 24 h. The 2.75 µM of 4-hydroxytamoxifen continued to reduce caspase-3/7 activity, while 5.5 and 10.99 ($p < 0.01$) µM of 4-hydroxytamoxifen activated caspase-3/7 activity at 48 h. Tamoxifen, and 4-hydroxytamoxifen, are known to induce apoptotic pathways in MCF-7 cells.[293,321] Mandlekar *et al* did not detect increased caspases-3 and -6 activity in the MCF-7 cells after treatment with 5 µM of tamoxifen for 24 and 48 h.[322] The finding at 24 h is comparable with what was observed in this study; however, this study noted a significant increase in caspase-3/7 activity at 48 h. Vemuri *et al* observed that 10 (26.9 µM; $p < 0.05$) and 20 µg/mL (53.8 µM; $p < 0.01$) of 4-hydroxytamoxifen increased the percentage of apoptotic cells to 10 and 12.5%, respectively, after 42 h.[249] Han *et al* also assessed the percentage of apoptotic cells and found that 20 µM of tamoxifen caused 20% apoptosis in MCF-7 cells,[323] similarly to this study. Li *et al* treated MCF-7 cells with 1, 2 and 4 µM of tamoxifen and reported that it was effective in inducing apoptosis in MCF-7 cells at 48 h.[293] Apoptotic cells increased from 20% (1 µM; $p<0.05$), 60% (2 µM; $p<0.01$) to 80% (4 µM; $p<0.001$).[293] This is in contrast to this study as 2.75 µM of 4-hydroxytamoxifen decreased caspase-3/7 activity. The authors may have assessed the percentage of apoptotic cells compared to caspase-3/7 activity, however a relative comparison between the studies exists as the result of caspase-3/7 activity precedes the apoptotic features. Interestingly, Han *et al* quantified the number of MCF-7 cells that expressed caspase-3 after treatment with 20 µM of tamoxifen and observed that 40% of MCF-7 cells ($p < 0.01$) expressed caspase-3.[323]

Combinational treatment in this study significantly ($p < 0.001$) elevated caspase-3/7 activity up to 6.4-fold after 24 h and 15.9-fold ($p < 0.001$) at 48 h. The combination of 10 µg/mL of the

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hot-water mixture and 20 µg/mL (53.8 µM) of tamoxifen in the study of Vemuri *et al* resulted in 20% of apoptotic cell death .[249] Comparable with this study, the authors noted a dose-dependent increase in apoptotic MCF-7 cells following treatment with the crude extract, tamoxifen, and the combination thereof.

Downstream consequences of significantly elevated nitric oxide levels may possibly result in aberrant cell cycle kinetics by arresting the cell cycle mainly at the G_1/S checkpoint in breast cancer cells.[324,325] The negatively affected mitochondrial energy-dependent mechanism generates excessive ROS, which can react with the nitric oxide to form nitrosative stress.[301,302] The nitrosative stress can potentiate protein tyrosine nitration, promoting lipid peroxidation and DNA fragmentation.[326] Lipid peroxidation and its products, such as 4-hydroxynonenal, initiate apoptosis by activating the FAS and tumour necrosis factor -related apoptosis-inducing ligand pathways; thereby, initiating caspase activation.[327] Moreover, lipid peroxidation damages the mitochondria, the dysfunctional mitochondria release cytochrome *c* leading to a further caspase-dependent activation of apoptosis.[300,316,327]

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Chapter 5: Conclusion

The aim of this study was to investigate the combinational effects of crude garlic extracts and 4-hydroxytamoxifen in adenocarcinoma breast cancer cells. Breast cancer is the leading malignancy diagnosed in females worldwide and there is increased use of CAM, among these patients in combination with conventional medicine, termed integrative medicine. However, literature on the mechanism of integrative medicine in breast cancer patients is minimal.

Ethnomedicinal (hot-water) and pharmaceutical-representative (methanol) extracts were prepared. The crude hot-water and methanol extracts displayed minimal inherent cytotoxicity as an IC_{50} for either extract could not be calculated at the highest concentration tested, being 100 µg/mL. The minimal inherent cytotoxicity may be attributed to hormesis where exposure to phytochemicals may stimulate a mild stress response pathway in cells; thereby increasing the cell density. The inherent IC_{50} and dose-dependent decrease in MCF-7 cell density following exposure to 4-hydroxytamoxifen was comparable to that available in literature. The cytotoxicity of the combination of various concentrations of the crude extract and 4-hydroxytamoxifen were assessed at different time points using a checkerboard method. There were a variety of synergistic, additive, and antagonistic combinations observed, with the most synergistic combination being a dual-exposure to 7.5 µg/mL hot-water extract and 10.99 µM 4-hydroxytamoxifen, which was selected for further mechanistic evaluation.

Synergism between the combination of the hot-water extract and 4-hydroxytamoxifen was seen throughout the mechanistic evaluations. The combination increased nitric oxide levels in MCF-7 cells. It has been described previously how garlic and tamoxifen share a pathway that disrupts the mitochondria to promote the generation of nitric oxide. The elevated nitric oxide has various downstream consequences, such as cell cycle arrest at G_0/G_1 phase. It was observed that the combination potentially arrested the cell cycle at the G_0/G_1 phase, possibly due to the increased nitric oxide levels, coupled with other known factors involved in the cell cycle arrest. Additionally, elevated nitric oxide levels stimulated by the combination may extend to the formation of lipid peroxidation, which is a by-product of nitrosative stress. The cell cycle arrest induced by the combination allowed for the MCF-7 cells to undergo DNA repair or apoptosis. The prolonged nitrosative stress stimulated by the combination may have damaged the DNA beyond repair. Moreover, the increase in nitric oxide suggests that the mitochondria are negatively affected, whereby the cytochrome *c* are released from the mitochondria. These factors may have led to a cell cycle exit at the G_0/G_1 phase and undergo apoptosis as seen with the elevated caspase-3/7 activity at 24 and 48 h (**Figure 18**).

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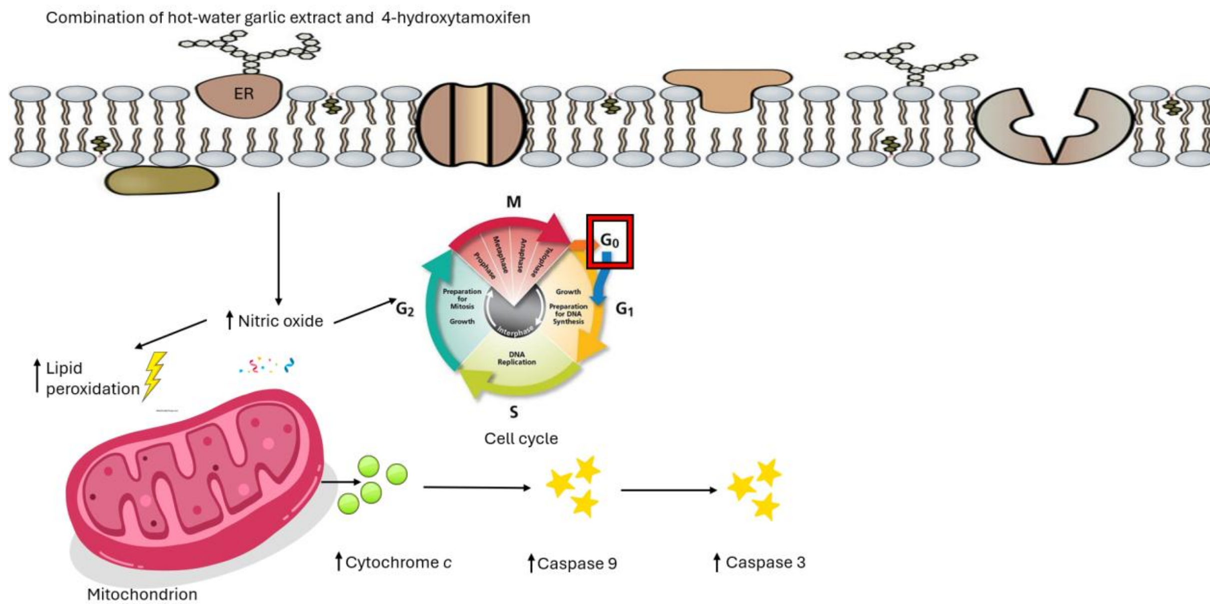

Figure 18. Diagram depicting the proposed mechanism of synergism of the combination of hot-water extract and 4-hydroxytamoxifen observed in the study. Oestrogen receptor (ER). Diagram was constructed using Microsoft PowerPoint. Images used to construct the diagram were obtained under the terms of the Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND)].

5.1. Limitations of the study and recommendations

The assessment of the combination of herbal remedies with conventional medicine in cancer patients and its synergistic mechanisms was chosen to further understand and promote research in this combinational therapy. Moreover, the use of herbal remedies aimed to bring forth research into Indigenous Knowledge Systems. Nonetheless, there were limitations. The single and combinational treatments were assessed in an adenocarcinoma cell line, but the non-malignant human mammary epithelial cell line, such as the MCF-10A, could have been included as a control group. These non-malignant mammary cells are often used as a model for normal breast cells; thereby their inclusion would have built a better safety profile of the combinational therapy. The study aimed to assess cytotoxicity of the combination of herbal remedies with conventional medicine in treatment-sensitive breast cancer cells, but the inclusion of treatment-resistant breast cancer cells could shed light on whether the combination can re-sensitise the resistant cells to treatment.

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Crude extracts contain various phytochemicals with varying concentrations depending on geography and season among other factors. A phytochemical screening of the crude extracts would have provided a clearer picture of the different constituents and quantities that were present in the extracts. Furthermore, the screening would have given an indication of the phytochemicals that played a predominate role in the solo and combinational treatments. The exposure to the crude extracts increased cell density within the MCF-7 cells resulting in their minimal inherent cytotoxicity. Additionally, it was demonstrated that the presence of the crude hot-water extract influenced the effect 4-hydroxytamoxifen had on cellular kinetics in MCF-7 cells compared to the single treatment of 4-hydroxytamoxifen. Therefore, the evaluation of protein expression using the Western blotting technique would have given an indication of which proteins were expressed and the signalling pathways involved, e.g., the EGFR pathway. This pathway is mediated by reactive species, where high concentrations of reactive species during cellular stress leads to cell cycle arrest and apoptosis.[328] Another pathway that could be assessed would be the p38 MAPK pathway. The pathway is known to be activated by EGFR and nitrosative stress, also leading to cell cycle arrest and apoptosis.[329]

Overall, there is potential for the use of synergistic combinational therapy in treatment of breast cancer. More interestingly, the use of the synergistic combination could be assessed in tamoxifen-resistant breast cancer cells to evaluate whether the combination could re-sensitise the cells to tamoxifen, and by extension, 4-hydroxytamoxifen.

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Appendix I

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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**

17 May 2024

Approval Certificate
Annual Renewal

Dear Miss NNS Nzama,

Ethics Reference No.: 329/2019 – Line 5
Title: Combinational effects of crude garlic and 4-hydroxytamoxifen on the MCF-7 breast cancer cell line

The **Annual Renewal** as supported by documents received between 2024-04-10 and 2024-05-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-05-15 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 2025-05-17.
- 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 (329/2019) 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, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
***Deputy Chairperson** of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

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Appendix II

Turnitin Originality Report

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Nzama
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Combinational effects of crude garlic and 4-hydroxytamoxifen on the MCF-7 breast cancer cell line by Nomfundo Nicolette Sildget Nzama u18207482 A dissertation submitted in fulfilment of the requirements for the degree Magister Scientiae in Pharmacology in the Faculty of Health Sciences University of Pretoria August 2024 Supervisor: Prof W Cordier (Department of Pharmacology, University of Pretoria) Co-supervisor: Prof V Steenkamp (Department of Pharmacology, University of Pretoria) Co-supervisor: Dr MH Visagie (Department of Physiology, University of Pretoria) i Plagiarism declaration Full names Nomfundo Nicolette Sildget Nzama Student number u18207482 Topic of work Combinational effects of crude garlic and 4-hydroxytamoxifen on the MCF-7 breast cancer cell line Declaration 1. I understand what plagiarism is and am aware of the University's policy in this regard. 2. I declare that this dissertation is my own original work. Where other people's work has been used (either from a printed source, internet, or any other source), this has been properly acknowledged and referenced in accordance with the requirements as stated in the University's plagiarism prevention policy. 3. I have not used another student's past written work to hand in as my own. 4. I have not allowed and will not allow anyone to copy my work with the intention of passing it off as his or her own work. ______________________ Signature 01 August 2024 ______________ Date ii Acknowledgements To my late son (2016-∞): Dearest, sweet Lithemba. "I have trouble accepting that the fact that you're gone, so I won't. It'll be like we went for a while without seeing each other, but I can understand why God would want you close to Him because you were truly an angel on earth. In a special way, I love you. I miss you." ♥ – Earl "DMX" Simmons To my mother and grandmother: Your prayers sustained me through moments where I felt overwhelmed, and I could not see the light at the end of the tunnel. To my supervisor Prof Cordier: There is so much I could say, but I would like to say thank you. Thank you for your unwavering support throughout the highs and lows. I could never thank you enough for all that you have done for me. I'll always be proud to have been one of your students. To my co-supervisors Prof Steenkamp and Dr Visagie: Your wisdom and guidance has played a great role in moulding me into being a better scientist. To my best friends: I appreciate you picking up my calls late in the night when I was stressed, sharing memes to remind me what it feels like to laugh, and the gift packages to help me relax. You have played a massive role in keeping me mentally sane. To every student at the Department of Pharmacology (past and present): All the assistance, tips, and guidance contributed greatly towards my journey. To the Institute of Cellular and Molecular Medicine: Thank you for allowing me to use your flow cytometer. To every organisation that has funded my research: The financial assistance is greatly appreciated. To every person that has played a role in my completion: Thank you, your kindness has not gone unnoticed. "Dreams delayed are not dreams denied" – R.L. White iii Abstract Tamoxifen remains the preferred standard choice of treatment for oestrogen receptor- and/or progesterone-positive breast cancer among females. Unfortunately, approximately 30% of these patients develop resistance to tamoxifen (de novo or acquired), despite an initial positive response to the treatment. Tamoxifen-resistance presents an obstacle to hormone treatment since it has often been associated with poor survival rates. Due to such resistance, alternatives have been investigated either to replace treatment, or reverse resistance mechanisms. The use of complementary and alternative medicine has drastically increased among breast cancer patients. It is used either exclusively, or in addition to prescribed treatment (termed integrative medicine). Garlic (Allium sativum L.) is known to contain a variety of phytochemicals potentially beneficial in disease treatment due to anti-proliferative, anti-oxidative, and anti- inflammatory effects. However, sparse data is available on the combinational use of crude garlic extracts with tamoxifen in breast cancer treatment. The aim of the project was to determine the effects of crude garlic extracts in combination with tamoxifen in tamoxifen-sensitive breast adenocarcinoma cell lines. Ethnomedicinal (hot-water) and pharmaceutical-representative (methanol) extracts of the garlic bulb were prepared by brewing and ultrasonic maceration, respectively. Preliminary cytotoxicity evaluation of the crude garlic extracts and 4-hydroxytamoxifen on MCF-7 breast adenocarcinoma cells were performed using the sulforhodamine B (SRB) assay. Thereafter, the synergistic cytotoxic evaluation of the combination of the crude garlic extracts and 4- hydroxytamoxifen were performed in a checkerboard manner using the SRB assay. The mechanism of action of the crude garlic extract, 4-hydroxytamoxifen, and combination thereof, was assessed by determining the alterations to the cell cycle, levels of nitric oxide and lipid peroxidation, and the activity of caspase-3/7. Crude hot-water and methanol extracts were prepared successfully; however, the extracts showed minimal inherent cytotoxicity as a half-maximal inhibitory (IC50) concentration could not be calculated for either extract at the highest concentration tested (100 µg/mL). The cytotoxicity of 4-hydroxytamoxifen was determined with an IC50 of 10.99 µM. Combining the extracts (7.5, 15 and 30 µg/mL) with 4-hydroxytamoxifen (¼IC50, ½IC50 and IC50) yielded a range of compound interactions including synergistic, additive, and antagonistic effects. The combination with the highest synergistic activity was 7.5 µg/mL of the hot-water extract and 10.99 µM of 4-hydroxytamoxifen (combination index = 0.624). The hot-water extract (7.5 µg/mL) potentially induced cell cycle arrest in MCF-7 cells at G0/G1 phase. The cells treated iv with 4-hydroxytamoxifen (10.99 µM) indicated cell death as most cells were in the sub-G1 phase. The combination of the hot-water extract and 4-hydroxytamoxifen arrested more MCF- 7 cells in the G0/G1-phase similarly to the solo treatment with the hot-water extract. The hot- water extract (7.5 µg/mL; $p < 0.05$) and 4-hydroxytamoxifen (10.99 µM; $p < 0.001$) alone significantly increased nitric oxide levels. Furthermore, the combination of the of hot-water extract and 4-hydroxytamoxifen increased nitric oxide

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