

***Biological evaluation of Pheroid[®] formulated
plant bio-actives against oestrogen active breast
cancer***

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Dissertation accepted in fulfilment of the requirements for the
degree Master of Science in Pharmaceutical Science at the
North-West University

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Graduation: June 2022

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Preface

This study was done because of the promising results from a precursor Master's study," Co-formulation and therapeutic evaluation of bioactive plant compounds in Pheroid®" by Bekele *et al.* (2019). The study aims and objectives were conceptualised by the study leader to investigate the anticancer effects of these specific plant bio-actives on breast cancer, using an improved study design.

Before this study could commence, research ethics training and an animal handling course were completed. The experimental work for this study was predominantly done at the NWU/DSI Preclinical Drug Development Platform's facilities, other sections were completed at the NWU in other researcher's laboratories. The Soxhlet extraction of turmeric was conducted in the laboratory of Prof. Rialet Pieters at the Unit for Environmental Science and Management of the Faculty of Natural and Agricultural Sciences. The extraction was performed by myself with the assistance and training of Dr Wihan Pheiffer. The quantification of actives in both turmeric and ginger extract was performed by Dr Thrineshen Moodley by using an LC-MS/MS at the PCDDP. Pheroid® formulation training was received from Mr Bisrat Bekele and performed by me. After formulation, quality control was done by means of particle size distribution, zeta potential and vesicle morphology. Particle size distribution training was received from Mr Neil Barnard and formulation stability training via the zeta potential analysis was received from Mr Charl Olivier, both these tests were performed by me. The vesicle morphology assay was performed by Dr Thrineshen Moodley on the confocal microscope at the PCDDP. Training for all *in vitro* anti-proliferation assays was given by Mr Bisrat Bekele, Mr Charl Olivier, and Dr Wihan Pheiffer. Furthermore, these assays were done by myself. The apoptosis assay preparations were done by me and the flow cytometry analysis was performed at Pharmacen by Prof Lissinda Du Plessis. The oestrogen inhibition assay was performed in Prof Rialet Pieter's tissue culture lab with assistance and training from and Dr Suranie Horn and Miss Vicky-Luanne Harris.

The *in vivo* study was performed at the DSI/NWU Preclinical Drug Development Platform's Vivarium with technical assistance from this study's LAT, Mr. Cor Bester, and Mr Kobus Venter. All Chapters were written by myself and reviewed by my supervisors

Acknowledgements

I would like to extend my far most gratitude to the following who advised and supported me throughout my two years of post-graduate studies:

- First, my supervisor, Dr Wihan Pheiffer for your broad knowledge, constant guidance and support. Furthermore, thank you for your motivation, uplifting spirit, patience, and problem-solving skills when complications presented itself.
- My co-supervisor, Mr Bisrat Bekele for your exceptional skills and patience in the cell laboratory. For your invaluable input and support in-and outside of the laboratory.
- My co-supervisor, Dr Thrineshen Moodley, for your feedback, support and expertise, and technical assistance with the bio-analytical analysis using the LC-MS/MS.
- Prof Rialet Pieters, Dr Suranie Horn and Miss Vicky-Luanne Harris, thank you for the use of your facilities, assistance and help with both the Soxhlet extraction and oestrogen inhibition assay.
- Prof Sias Hamman and Mr Happiness Netshimbupfe for the use and assistance of the freeze dryer.
- Mr. Charl Olivier, for your assistance with assays when needed.
- Prof Lissinda Du Plessis, for your technical assistance and expertise in the apoptosis assay.
- To the PCDDP Vivarium LATs — Mr Cor Bester, Mr Kobus Venter and Mrs Antoinette Fick — thank you for your assistance and kind help in the animal study.
- To the staff members of the DSI/NWU Preclinical Drug Development Platform, for your assistance when needed.
- To the NWU postgraduate and DSI/NWU Preclinical Drug Development Platform bursaries for the financial support.

- To my parents, Wilhelm and Estelle Boers for your unconditional love and support. Thank you for your constant inspiration and motivation since the first day. To my brother, Cornel Boers for your never-ending support and care.
- To Aidan Higgs and Stephan van der Westhuizen, for the constant moral support and motivation when I most needed it. You are truly exemplary good friends.
- My colleagues, Charl Olivier, Shingai Yolanda Mutero, Nicholas Manqoba Mashego and Nicole Mathebula who became friends, thank you for your support, motivation and kindness.
- Thank you to my Heavenly Father for this opportunity, love, and grace. Thank You for the strength and guidance to finish this project.

ABSTRACT

Cancer is a serious public health complication worldwide. Annually 115 000 South Africans are diagnosed with cancer, where men have a lifetime risk of 1:7 and women a lifetime risk of 1:8. The most frequently diagnosed and common cause of cancer-associated deaths in women is breast cancer, of which the predominant form is oestrogen active breast cancer. For these cancers, antineoplastic drugs that specifically inhibit oestrogen binding — such as tamoxifen — are frequently used as prophylactic and chemo-therapy treatments. A frequent complication of these treatments are severe side effects, as well as drug resistance. Thus, there is a great need to explore alternative treatment options, including adjuvant or supplementary treatments. There are many known plant bio-active compounds and extracts that have shown potential as adjuvant anticancer treatments. The limitation of many of the plant-based remedies is their poor physico-chemical and biopharmaceutical properties. The research aim of this study was to evaluate the potential anticancer activity of turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*) extracts formulated in a Pheroid® drug delivery system. This was achieved using *in vitro* evaluations on the oestrogen active human breast cancer cell line (MCF-7), and an *in vivo* study using a MCF-7 tumour bearing rodent model. Unformulated concentrations of both extracts were used to determine potencies, which were incorporated into the formulations. The formulations were characterised in terms of particle size and electro-kinetic stability. The anti-proliferation effects of these formulations were gauged against the cancer line, while determining the cytotoxicity in a non-cancer cell line — to prove anticancer effects. The potential anticancer effects were supported by calculating the cancer selectivity index (CSI). Additionally, the ability of the formulations to induce apoptosis was measured using flow cytometry. To establish whether the induced apoptosis was due to oestrogen inhibition, the T47D-KBluc reporter gene bioassay was used. Based on the results of the formulation characterisation and *in vitro* assays there were multiple candidates as adjuvant treatments. The most suitable candidate as the final formulation was selected specifically based on the CSI and apoptotic induction. The anticancer effects *in vivo* was to be determined using MCF-7 xenografts. The effects of the final formulation treatment alone, and as adjuvant to a known anticancer drug (cisplatin) would have been assessed over a period of 15 days, compared to a control. However, after the inoculation of the animals no tumours formed after 21 days. This led to this section of the study to be prematurely concluded. Even so, the results from the *in vitro* study have shown that the Pheroid® formulated extracts are promising anticancer agents against oestrogen active breast cancer.

Keywords: Breast cancer; Cisplatin; Drug delivery system; Ginger extract; Oestrogen receptor; Turmeric extract; Xenograft model

TABLE OF CONTENTS

Preface	i
Acknowledgements	ii
Abstract	iii
Table of Contents	iv
List of Figures	viii
List of Tables	xi
List of Equations	xii
List of Abbreviations	xiii

CHAPTER 1: STUDY INTRODUCTION

1.1	Background	1
1.2	Research problem	2
1.2.1	Aims and objectives	3
1.2.1.1	<i>Research aim</i>	3
1.2.1.2	<i>Research objectives</i>	3
1.3	Study design	3
1.4	Animal study	5
1.5	Ethical considerations	6
1.6	Dissertation outline	7

CHAPTER 2: LITERATURE REVIEW

2.1	Introduction to cancer	10
2.2	Breast cancer	10
2.3	Epidemiology	12
2.4	Etiology	13
2.5	Current breast cancer treatments	14

2.5.1	Breast cancer chemotherapy	15
2.5.2	Natural remedies for breast cancer	16
2.6	Natural adjuvant treatments for breast cancer	17
2.6.1	Turmeric extract	18
2.6.2	Ginger extract	18
2.7	The effect of natural turmeric and ginger in the body	19
2.8	Drug delivery systems	20

CHAPTER 3: MATERIALS AND METHODS

3.1	Materials	30
3.2	Research methods	30
3.2.1	Soxhlet extraction of curcumin	31
3.2.2	Liquid Chromatography tandem mass spectrometry (LC–MS/MS): The quantification of curcumin and [6]-shogaol	32
3.2.3	Formulation of plant bio-actives in Pheroid® delivery systems	33
3.2.3.1	<i>Extract concentration screening</i>	33
3.2.3.2	<i>Pheroid® formulation</i>	34
3.3	Particle size distribution	34
3.4	Electro-kinetic potential analysis	34
3.5	Vesicle morphology	35
3.6	Cell culturing and assay preparation	35
3.7	Cell viability	36
3.7.1	Neutral red cellular uptake assay	36
3.8	Calculation of inhibition concentration	37
3.9	Flow cytometric analysis for apoptotic phase detection	37
3.10	Cancer selectivity index (CSI)	38
3.11	Oestrogen inhibition assay	38
3.12	Final formulation conclusion	39

3.13	Animal study	39
3.13.1	Experimental animals and husbandry	39
3.13.2	Sample size	40
3.13.3	Cell culture and inoculation in athymic nude mice	40
3.13.4	Experimental design	40
3.13.5	Monitoring, humane endpoints, euthanasia and sample collection	41
3.14	Statistical analysis	42

CHAPTER 4: RESULTS AND DISCUSSION

4.1	The extraction of curcumin	45
4.2	Liquid chromatography tandem mass spectrometry (LC-MS/MS): The quantification of curcumin and [6]-shogaol	45
4.3	Antiproliferation effects and potencies of turmeric and ginger extracts on non-cancer Vero cell line	47
4.3.1	Solvent and carrier control	48
4.3.2	Extract concentration determination for formulation	49
4.3.3	Inhibitory concentration (IC) determination	50
4.4	Formulation characterisation	52
4.4.1	Particle size distribution and zeta potential	52
4.4.2	Vesicle morphology	55
4.5	Anticancer effects of turmeric and ginger combination formulations against MCF-7 breast cancer cells, relative to non-cancer Vero cells	55
4.6	Cancer Selectivity Index (CSI)	57
4.7	Apoptotic effects of turmeric and ginger combination formulations on MCF-7 breast cancer cells	57
4.8	Potential oestrogen receptor inhibition by turmeric and ginger combination formulations	61
4.9	Determination of the final formulation to be administered during <i>in vivo</i> study	62
4.9.1	Formulation and characterisation of turmeric-ginger final formulation for animal study	62

4.9.1.1	<i>Particle size distribution and zeta potential</i>	62
4.9.1.2	<i>Vesicle morphology</i>	63
4.10	Animal study	64
4.11	The promise of turmeric and ginger extract as adjuvant breast cancer therapy	65

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1	Study outcomes and conclusions	73
5.2	Recommendations for future studies	75

APPENDICES

Appendix 1	The optimisation of MCF-7 and Vero cell seeding densities for use in bioassays	76
Appendix 2	Animal welfare monitoring sheets	78
Appendix 3	Analysis report of the quantification of curcumin and [6]-shogaol from turmeric and ginger extracts	87
Appendix 4	Confocal micrographs obtained during the vesicle morphology quality control for formulation characterisation	96
Appendix 5	AnimCare research ethics approval letter	97

LIST FIGURES

Chapter 1:

Figure 1.1: Study design and procedure for the biological evaluation of Pheroid® formulated plant bio-actives against oestrogen active breast cancer (pg. 4)

Figure 1.2: Experimental design evaluating the reduction of in vivo MCF-7 induced tumours as a result of phytochemical combination in Pheroid®. Figure generated using BioRender.com (pg. 5)

Chapter 2:

Figure 2.1: The signalling pathways associated with the activation of ER α leading to tumour growth. Figure adapted from Liu *et al.* (2020). Image generated using Biorender.com (pg. 11)

Figure 2.2: Development of metastasis from a primary breast tumour. Figure generated using BioRender.com (pg. 12)

Chapter 3

Figure 3.1: Soxhlet extraction setup for the extraction of curcumin from turmeric. This image was generated using Biorender.com (pg. 31)

Chapter 4

Figure 4.1: Mass chromatograms reporting elution times (min) for curcumin and [6]-shogaol in the curcumin standard solution (A), [6]-shogaol standard solution (B), turmeric extract (C), and ginger extract (D) (pg. 46)

Figure 4.2: Percentage cell viability of Vero cells after a 24h treatment of the carrier (Pheroid®) at serial dilution concentration range (0.00625 – 0.1% v/v), and solvent (0.6% v/v DMSO) using the neutral red cellular uptake assay. All results are given in terms of the control. Asterisk (*) indicates significant differences ($p < 0.05$) compared to the control. The solid red line indicates 70% cell viability (threshold for cytotoxicity), and the dashed line indicates 50% cell viability (threshold for severe cytotoxicity) (pg. 48)

Figure 4.3: Percentage cell viability of Vero cells after a 24h treatment of unformulated concentrations of turmeric (A) and ginger (B) extract on Vero cells via the neutral red cellular

uptake assay. All results are given in terms of the control. Asterisk (*) indicates significant differences ($p < 0.05$) compared to the control. The solid red line indicates 70% cell viability (threshold for cytotoxicity) and the dashed line indicates 50% cell viability (threshold for severe cytotoxicity) (pg. 49)

Figure 4.4: Vero cell viability log graphs for turmeric extract (4.3 A) and ginger extract (4.3 B) with their linear equations, respectively, which was used to determine the different inhibitory concentrations (IC₂₅, IC₅₀ and IC₇₅). The cell viability assays were done via the neutral red cellular uptake assay after 24h exposure to different treatments (pg. 50)

Figure 4.5: Particle size distribution for Pheroid® formulations A-I using the Malvern 2000 Mastersizer. Measurements were calculated as an average of triplicate readings for each formulation (pg. 54)

Figure 4.6: Confocal (A) and light micrograph (B) of Pheroid® formulations (representing image used of Formulation I) obtained by using Nikon D–Eclipse C1 Confocal inverted microscope TE 2000-fitted with a Helium–Neon (543 nm) polarized laser. Nile red was used as the fluorescence marker (excitation: 515–560 nm, emission: >590 nm). The Scale bar represents 10 μm of micrograph A, and 12.5 μm of micrograph B. All arrows in light micrograph B indicates the entrapment of phytochemicals in Pheroid® (pg. 55)

Figure 4.7: Percentage cell viability of Pheroid® formulations tested against the non-cancer cell line, Vero cells (A) and the breast cancer cell line, MCF-7 (B) using the neutral red cellular uptake assay after cells were treated for 24h. All results are given in terms of the control. Asterisk (*) indicates significant differences ($p < 0.05$) compared to the control. The solid red line indicates 70% cell viability (threshold for cytotoxicity), and the dashed line indicates 50% cell viability (threshold for severe cytotoxicity). Formulation A-I were formulated based on their GE and TE potencies. All formulations were formulated in their respective inhibitory concentration combinations (GE:TE). Formulation A (75:25), B (50:25), C (25:25), D (75:50), E (50:50), F (25:50), G (75:75), H (50:75), I (25:75) (pg. 56)

Figure 4.8: Representative scatter graph for the negative control group, displaying the percentage of MCF-7 cell population in various apoptotic phases after 24h culturing in plain DMEM. The flow cytometry analysis was measured with acridine orange (AO) and ethidium bromide (EtBr). The emission of AO and EtBr was detected with a 525/20-nm filter (FL1) and a 635/20-nm filter (FL3), respectively, after excitation at 488 nm. The lower left quadrant (Q1),

represents the percentage viable cells, the upper left quadrant (Q2) represents the percentage early apoptosis, the upper right quadrant (Q3) represents the percentage apoptotic cells and the lower right quadrant (Q4), the percentage late apoptotic/necrotic cells

(pg. 58)

Figure 4.9: Representative scatter graphs displaying the percentage of the MCF-7 cell population in various apoptotic phases after a 24h exposure to formulation A–I. The flow cytometry analysis was done with acridine orange (AO) and ethidium bromide (EtBr). The emission of AO and EtBr was detected with a 525/20-nm filter (FL1) and a 635/20-nm filter (FL3), respectively, after excitation at 488 nm. The lower left quadrant (Q1), represents the percentage viable cells, the upper left quadrant (Q2) represents the percentage early apoptosis, the upper right quadrant (Q3) represents the percentage apoptotic cells and the lower right quadrant (Q4), the percentage late apoptotic/necrotic cells

(pg. 59)

Figure 4.10: Percentage composition of different induced apoptotic phases in MCF-7 cells after a 24h exposure to Formulation A–I. The flow cytometry analysis was done with acridine orange and ethidium bromide

(pg. 60)

Figure 4.11: Oestrogen receptor inhibition in TK47-KBluc cells after 24h exposure to candidate formulations, where GE represents ginger extract, TE represents turmeric extract. Formulations were formulated in their respective inhibitory concentration combinations (GE:TE). Formulation D (75:50), E (50:50), F (25:50)

(pg. 61)

Figure 4.12: Average particle size distribution for TGFF using the Malvern 2000 Mastersizer. Measurements were calculated as an average of triplicate readings for each formulation

(pg. 63)

Figure 4.13: Confocal (A) and light micrograph (B) of TGFF obtained by using Nikon D–Eclipse C1 Confocal inverted microscope TE2000-fitted with Helium–Neon (543 nm) polarized laser. Nile red was used as the fluorescence marker (excitation: 515–560 nm, emission: >590 nm). Scale bar represents 15 μm of micrograph A, and 18.75 μm of micrograph B

(pg. 64)

Appendices

Figure A1: Seeding cell densities of MCF-7 and Vero cells after 24hr (A and C) and 48 hrs (B and D), respectively. The solid red line indicates 100% confluency

(pg.77)

LIST OF TABLES

Chapter 3

Table 3.1: Pheroid® formulation concentrations based on the combination of the inhibitory concentration values of the respective extracts. Formulations represent as F_{A-I} (pg. 33)

Chapter 4

Table 4.1: Calculated concentration and accuracy of curcumin and [6]-Shogaol. Standard calibrators used for quantification (pg. 47)

Table 4.2: Calculated inhibitory concentration (IC₂₅, IC₅₀ and IC₇₅) using predetermined x-and y values. The final concentration (µg/mL) of the active ingredient in turmeric extract (curcumin) and ginger extract ([6]-shogaol are reported (pg. 51)

Table 4.3: Determining Formulations with various combined concentrations of ginger (GE) and turmeric (TE) extracts (pg. 52)

Table 4.4: Particle size distribution, span and zeta potential of Pheroid® formulations (pg. 53)

Table 4.5: The cancer selectivity index of the formulations (Formulations A–I) of turmeric extract and ginger extract in Pheroid® calculated from the inhibitory effect in MCF-7 breast cancer cells and Vero cells (non-cancer cells). CSI values greater than 1 given in bold (pg. 57)

Table 4.6: Particle size distribution, span and zeta potential of TGFF (pg. 62)

Table 4.7: Various cell densities of MCF-7 breast cancer cells used for xenograft studies. The onset days and palpable tumour size are also reported (pg. 65)

LIST OF EQUATIONS

Equation 1:

$$\text{Percentage yield} = \frac{\text{Total mass of extract powder}}{\text{Total mass of raw material}} \times 100$$

(pg. 31)

Equation 2:

$$T = C \left(\frac{V}{M} \right)$$

(pg. 32)

Equation 3:

$$\text{Span} = \frac{(d_{0.9} - d_{0.1})}{d_{0.5}}$$

(pg. 34)

Equation 4:

$$\% \text{Cell viability} = \frac{A_{\text{Experiment}} - A_{\text{Blank}}}{A_{\text{Control}} - A_{\text{Blank}}} \times 100$$

(pg. 37)

Equation 5:

$$\text{Cancer selectivity index} = \frac{\% \text{Cell viability}_n \text{ (Normal cells)}}{\% \text{Cell viability}_n \text{ (Cancer cells)}}$$

(pg. 38)

Equation 6:

$$\text{Tumour volume} = W^2 \times \frac{L}{2}$$

(pg. 41)

Equation 7:

$$\text{RTV} = \frac{V_n}{V_0}$$

(pg 41)

Equation 8:

$$\text{TIR} = \frac{T_{\text{RTV}}}{C_{\text{RTV}}}$$

(pg. 41)

LIST OF ABBREVIATIONS

A

A549	Human non-small cell lung cancer cell line
ABCIR	Annual breast cancer incidence rate
AIDS	Acquired immunodeficiency syndrome
AKT	Ak strain transforming
AO	Acridine orange
ATP	Adenosine triphosphate

B

BCL-2	B-cell lymphoma-2
BCS	Breast conserving surgery

C

Ca ²⁺	Calcium ion
cAMP	Adenosine monophosphate
Cdk	Cyclin-dependent kinase
CDT	Charcoal/dextran-treated
cIAP-1	Cellular Inhibitor of Apoptosis 1
CO ₂	Carbon dioxide
COX-2	Cyclooxygenase-2
CSI	Cancer selectivity index

D

DMEM	Dulbecco's Modified Eagle Medium
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DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
<i>E</i>	
E2	Oestradiol/17 β -oestradiol
ECM	Extracellular matrix
EGFR	Growth factor receptor
EGFR1/2	Growth factor receptor one and two
EMA	European Medicine Agency
EMT	Epithelial-to-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ER	Oestrogen receptors
ERK1/2	Extracellular signal-regulated kinase one and two
ERM	Endoplasmic reticulum
ER α	Oestrogen receptor alpha
ER β	Oestrogen receptor beta
ESI	Electrospray ionization
EtBr	Ethidium bromide
<i>F</i>	
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor

G

GE Ginger extract

GF1R Growth factor-I receptor

H

HCT116 Human colon cancer cell line

HepG2 Hepatocarcinoma cell line

HER2 Human epidermal growth factor receptor 2

HIV Human immunodeficiency virus

HPLC-VWD High performance liquid chromatograph with a variable wavelength detector

HPTLC High-performance thin layer chromatography

hr Hour

HT29 Human Colorectal Adenocarcinoma Cell Line

I

IC Inhibitory concentration

IGF Insulin-like growth factor

IGF-IR Insulin-like growth factor-I receptor

IP Intraperitoneal

IVC Individual ventilated cage

K

KBM-5 Human chronic myeloid leukaemia

L

LAT	Laboratory animal technologists
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LG	Lauryl gallate

M

MAPK	Mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation-7
Mg(CO ₃) ₄	Magnesium carbonate
Mg(OH) ₂	Magnesium hydroxide
Mg ²⁺	Magnesium ion
MgSO ₄ ·7H ₂ O	Hydrous magnesium sulfate
MMP-9	Matrix Metalloproteinase-9
MRM	Multiple reaction monitoring
mRNA	Messenger Ribonucleic Acid
MS/MS	Tandem mass spectrometry
mTOR	Mammalian target of rapamycin

N

N ₂ O	Nitrous oxide
NEAA	Non-essential amino acids
NF-KB	Nuclear factor kappa light chain enhancer of activated B cells

P

p53	Tumour protein p53
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PBS	Phosphate-buffered saline
PI3K/AKT	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLGA-PEG-PLGA	Poly (dl-lactide-co-glycolide-b-ethylene glycol-b-dl-lactide-co-glycolide)
PPAR	Peroxisomal proliferator activated receptor
PPAR γ	Peroxisome proliferator activated receptor gamma
PR	Progesterone receptors
PVC	Polyvinyl chloride
<i>R</i>	
R ²	Coefficient of determination
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RTV	Relative tumour volume
<i>S</i>	
SA	South African
SD	Standard deviation
SERM	Selective oestrogen receptor modulator
SIRT-1	Sirtuin 1
SMEDDS	Self-micro emulsifying drug delivery System
STAT3	Signal transducer and activator of transcription 3
<i>T</i>	
TB	Tuberculosis

TE	Turmeric extract
TGFF	Turmeric-Ginger final formulation
TIR	Tumour inhibition rate
TNBC	Triple-negative breast cancer
TNF- α	Tumour Necrosis Factor alpha
<i>U</i>	
UPLC	Ultra-performance liquid chromatography
<i>V</i>	
VEGF	Vascular endothelial growth factor
<i>X</i>	
XIAP	X-linked inhibitor of apoptosis protein

CHAPTER 1: STUDY INTRODUCTION

1.1 Background

Cancer is a serious public health complication worldwide (Siegel *et al.*, 2020). In 2002, 6.7 million people died of cancer in the world (Parkin *et al.*, 2005). This is more than the combined deaths recorded from malaria, TB, and HIV and AIDS (Lingwood *et al.*, 2008). In 2016, a similar increase in the trend was observed (Jong, 2016) and it is expected that there will be 16 million global new cancer cases in 2020 (CANSA, 2017). In developing countries, such as Nigeria and South Africa, national cancer control plans may exist but challenges such as adequate funding and scarcity of resources are present (Ndlovu, 2019). In these countries, as well as in other African countries such as Rwanda, Nigeria and Egypt, patients' survival is crippled by different factors such as late presentation of cancer, as well as the lack of treatment as it is very costly and resource-intensive (Stefan *et al.*, 2013). In 2019, 115 000 South Africans were diagnosed with cancer where men had a lifetime risk of 1:7 and women a lifetime risk of 1:8. In men the most threatening cancers include prostate, colorectal and lung cancer and as for women, breast and cervical cancer are the most threatening (CANSA, 2019).

The most frequently diagnosed and common cause of cancer-associated deaths in women is breast cancer (Key, Verkasalo and Banks, 2001). An estimated 70% of all breast cancers are driven by oestrogen receptor α (ER α) (Tecalco-Cruz and Ramírez-Jarquín, 2018). Two intracellular receptors, ER α and oestrogen receptor beta (ER β) are used to mediate some of oestrogen's functions, namely cellular growth and differentiation (Ascenzi *et al.*, 2006). Estrogen has proliferative effects on breast cancer cells mediated by the ER α in ER α -positive breast cancer cell lines, which are in autocrine mode (Liao *et al.*, 2014). Recent studies have also shown that MCF-7 breast cancer cells are progesterone (PR)-negative, thus these cells won't be activated by progesterone (Yu *et al.*, 2017). 17- β -oestradiol has a great binding affinity to ER α . It was found that this activation of ER α increases S-phase entry of the human breast adenocarcinoma cell line, MCF-7 cells, as well as cell proliferation (Liao *et al.*, 2014).

Anti-estrogen medicine, tamoxifen, is frequently being used as a prophylactic and treatment against oestrogen-positive receptor breast cancer (Lazarus *et al.*, 2009). A problem with this treatment occurred as a great number of patients developed severe side effects, as well as tamoxifen resistance (Lazarus *et al.*, 2009). Cisplatin, an anticancer drug, has shown to be efficacious in the treatment of many forms of cancer (Manohar and Leung, 2018). Cisplatin binds to the DNA in the cancer cell nucleus, disrupting ordinary transcription and DNA

replication. These actions will cause a cytotoxic effect, killing the cancer cell (Fuentes *et al.*, 2003). Unfortunately, cancer cells become more resistant to cisplatin-based chemotherapy.

These drugs do not only have a cytotoxicity effect on cancer cells but on various kinds of dividing cells as well as, and lead to tremendous side effects such as alopecia, anaemia, diarrhoea, fatigue and nausea (Demaria *et al.*, 2017). By combining both herbal and synthetic drugs, bioavailability of one of the drugs could be improved by aiding the transport of the drug—often herbal extracts and phytochemicals, like *Aloe vera* (Haasbroek *et al.*, 2019) capsaicin and piperine (Gerber *et al.*, 2019) have absorption enhancer capabilities. This will lead to a reduction in dose, side effects, as well as overcoming the barrier of multi-drug resistance mechanisms in cancer cells (Mangla and Kohli, 2018). Curcumin (Diferuloylmethane) and ginger (*Zingiber officinale*) are most commonly used for culinary purposes. These natural remedies have shown to have profound healing properties. It is proven in a study that curcumin has prophylactic properties in diseases such as diabetes, cancer, cardiovascular diseases, as well as other biological active pathosis (Rahmani *et al.*, 2018). It has also been reported that ginger possesses antibacterial, anti-inflammatory, antidiarrheal properties and hypolipidemic and hypoglycaemic effects. Ginger also assists in the management of nausea, vomiting and weight reduction (Prakash, 2019).

Despite both curcumin and ginger's potential, there are factors that limit the usage of these ingredients for cancer therapy. Curcumin for instance has low bioavailability and solubility, weak tissue absorption, accelerated degradation in physiological pH, as well as systemic elimination following oral administration (Elbially *et al.*, 2020). These barriers could be overcome by nano-particulate delivery systems, as they improve drug bioavailability, pharmacokinetics and decrease biological degradation. The Pheroid® drug delivery system is a dynamic yet highly stable lipid bilayer colloidal system. It contains a plant and essential fatty acid dispersed phase, and nitrous oxide saturated water continuous phase (Slabbert, Du Plessis and Kotzé, 2011), which improves the chemical and physical properties of the drug delivery system (Date *et al.*, 2007).

1.2 Research problem

Chemotherapy is quite common but is compromised by their side effects. Some of these adverse effects include nausea, vomiting, lack of appetite and diarrhoea (Decker-Baumann, 1999), in addition, this therapy does not only affect tumour cells, it is also toxic for normal tissue (Chen *et al.*, 2000). This is a very limiting factor for the treatment of cancer. For this reason, supplementary treatments are very much needed. Curcumin and ginger have shown great potential in treating breast cancer as these natural remedies have a great variety of

mechanisms to induce apoptosis, alter tumour growth regulatory proteins, thus inhibiting tumour growth. However, to harness the use of these ingredients several biopharmaceutical barriers must first be overcome such as low solubility and bioavailability. These barriers, as well as a few others can be overcome by utilising nano particulate delivery systems such as Pheroid®. The Bekele *et al.* (2019) study will be used as a reference point for the formulations and *in vitro* anticancer effects. In the current study, the anticancer effects of ginger and curcumin extracts, will be tested against the oestrogen active MCF-7 breast cancer cell line. Our study will focus on the anticancer effects of the formulations *in vivo* (improved formulations), after confirming quality control in *in vitro* methods.

1.2.1 Aim and objectives

1.2.1.1 Research aim

The research aim of this study is to evaluate the potential anticancer efficacy of a Pheroid® formulation of combined curcumin and ginger extracts, against the human breast cancer cell line (MCF-7) *in vitro* and *in vivo* and finally promote adjuvant anticancer therapy.

1.2.1.2 Research objectives

The objectives of the study are:

- Formulating combinations of curcumin and ginger extracts in Pheroid®.
- Investigating the *in vitro* anticancer potential of Pheroid® formulated plant extracts against the MCF-7 cell line via cytotoxicity, apoptosis, and oestrogen receptor inhibition.
- Evaluating the *in vivo* anticancer activity of Pheroid® formulated plant extracts in a xenograft mouse model through tumour regression.

1.3 Study design

The study design (Figure 1.1) consists of four stages: formulation, formulation characterization, *in vitro* study and *in vivo* study. Formulations will include pro-Pheroid® only, individual and combinations of ginger and curcumin formulations in Pheroid® and dimethyl sulfoxide (DMSO). The Pheroid® formulations will be characterised in terms of vesicle morphology and size distribution, particle size analysis, and electrostatic repulsion and attraction analysis (Zeta potential), and compatibility study. Furthermore, determination of phytochemical content in their respective extracts will be performed.

In the *in vitro* study, cell viability, apoptosis and oestrogen inhibition assays will be measured to investigate the anticancer potential of curcumin and ginger extract combination formulations. The safety of the formulations will be determined by measuring oxidative stress markers. All assays will be performed using MCF-7 cells except for the oestrogen receptor inhibition assay, which will be conducted with T47D-KBluc cells — an oestrogen active cell line with a stable luciferase reporter gene.

For the *in vivo* study, xenografts of athymic nude mice will be used and anticancer activity will be measured by performing tumour inhibition studies. Tumour inhibition will be done to evaluate if the formulation prohibits tumour growth over the treatment period.

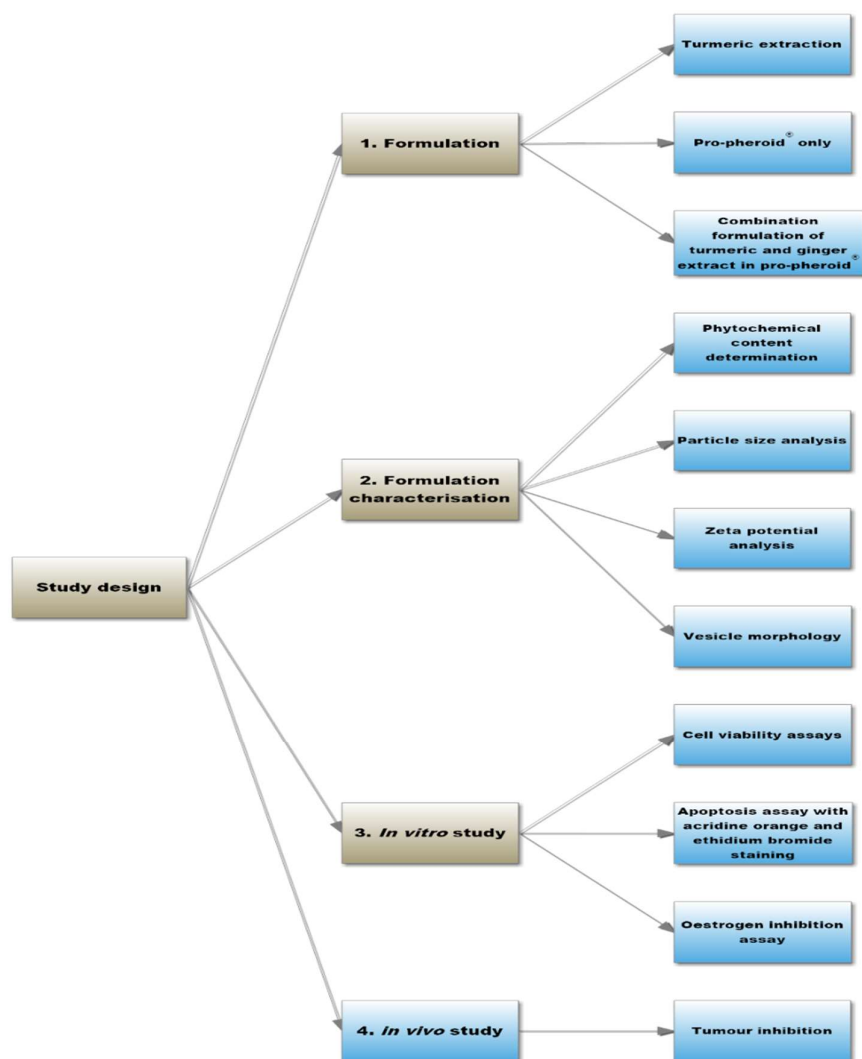


Figure 1.1: Study design and procedure for the biological evaluation of Pheroid® formulated plant bio-actives against oestrogen active breast cancer

1.4 Animal study

The study design and treatment groups are given in Figure 1.2. To avoid any possible biases to occur, after inoculation, the mice will be randomly and equally divided into five groups, so that each group will contain five female mice. The study will be performed over 15 days. Only after the tumour has reached a size of 100 mm³, different combinations of Pheroid® formulated phytochemical and cisplatin combination treatment therapy will commence. When the tumour reaches a palpable size, that specific day will be assigned as day zero. Group one will serve as a negative control group and group two will serve as a vehicle control group, receiving 0.9% saline and a blank Pheroid® formulation, respectively (Figure 1.2). The most effective Pheroid® formulated phytochemical combination (from *in vitro* data) will be administered orally to group three, which acts as a treatment group (Figure 1.2). Group four will serve as the positive control group and will receive a known anticancer drug, cisplatin (3 mg/kg), which will be administered intraperitoneally (IP) (Figure 1.2). Group five will receive a combination; full dose cisplatin full dose of the phytochemical combination therapy (ginger and curcumin), which will be orally administered (Figure 1.2). Oral administration will be done daily, and cisplatin will be administered once a week.

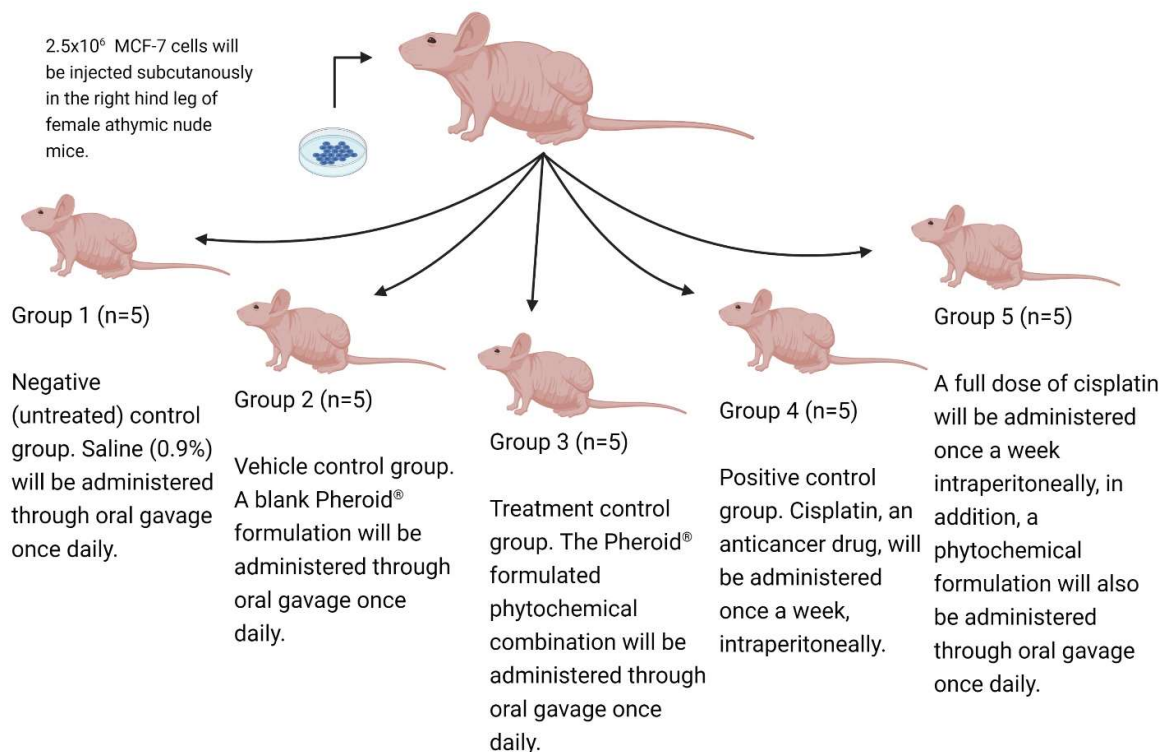


Figure 1.2: Experimental design evaluating the reduction of *in vivo* MCF-7 induced tumours as a result of phytochemical combination in Pheroid®

Addendum 1(A): Key for assignment of health check, as the scoring tool, will be used to monitor the mice's health, as well as to record it. Addendum 1 (B): Log sheet for assignment of health check scores, will be used to determine pain and distress levels throughout the study. According to SOP_Viv_Anim 27 these levels will be monitored every second day. Addendum 2: Tumour monitoring data collection sheet, will be used to record the adjustment of the daily dose of the phytochemical combination after the animals were weighed.

1.5 Ethical considerations

This study was approved by the NWU AnimCare Research Ethics Committee (NWU-00533-20-A5).

The Guidelines for the welfare and use of animals in cancer research will be used to conduct the *in vivo* studies in such a way that the least harm possible will be conveyed onto the animals (Workman *et al.*, 2010). The principle of the 4R's will be followed namely: **Replace**, **Reduce**, **Refine** and **Responsibility** (Kilkenny *et al.*, 2010).

Replace: Tests such as cytotoxicity and apoptosis, as well as oestrogen inhibition were done *in vitro*, thus replacing *in vivo* methods. However, no other alternatives other than mice can be used *in vivo* that will act as tumour-bearing models to observe the anticancer activity of natural medicines.

Reduce: The number of animals that were used to accomplish the scientific goal of the study is acquired from previous published papers (Li *et al.*, 2009; Kang *et al.*, 2010, Yin *et al.*, 2013, Lv *et al.*, 2016, Lu *et al.*, 2017, Bekele 2019).

Refine: To reduce the tumour burden, as well as the distress of the animals, humane end point has been established. To refine the experiment even more the health of all the animal will also be monitored.

Responsibility: This will be achieved by improving the social life of experimental animals. The social life of these animals will be enhanced by containing them in groups, as mice need social interaction with each other. Furthermore, environmental enrichment will be added for these animals to interact with.

1.6 Dissertation outline

Chapter 1: This chapter will set the basis for the dissertation. Some background information stating why research is done on the specific topic, describing modern day problems and possible solutions. Furthermore, the study design and animal study are also described in this chapter, explaining how this study will proceed.

Chapter 2: The literature review is an in-depth discussion of cancer but more specific breast cancer. Current chemotherapy and associated problems are also discussed in this chapter, as well as the potential of natural adjuvant therapy in various drug delivery systems.

Chapter 3: The materials that were used and the methods, which were followed are described in this chapter. The materials and methods, which were used to formulate, characterisation of these formulations and the *in vitro* and *in vivo* studies are explained.

Chapter 4: In this chapter the final results was reported and discussed. The efficacy of the extraction, characterisation of the different combination formulations and the effects of these formulations in the *in vitro* study will be discussed. Finally, a final formulation will be chosen and characterised based on the *in vitro* results, which will be used in the *n vivo* study.

Chapter 5: This chapter will conclude the study. A summary of the final results will be given and some recommendations for future studies on this topic will be discussed.

Appendices: This section contains supplementary material and information not included in the main dissertation. These include the relevant animal welfare log sheets, SOPs, LC-MS/MS reports, confocal images, and ethics approval letter.

Referencing: References were done according to the NWU referencing guidelines of 2020. Each chapter has its own reference list.

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CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to cancer

Cancer is a continuously rising health threat with increasing prevalence and mortality rates around the world. Recent global cancer statistics indicated that there were 18.1 million new cancer cases and 9.6 million deaths in 2018 (Bray *et al.*, 2018). Although countries across the world suffer from cancer, sub-Saharan African countries are at a greater risk as these countries do not always have adequate resources to treat cancer as effectively, thus leading to 1.5 to 4-fold the mortality rates of high-income countries (Rebbeck, 2020). The most common cancers in African men are prostate, lung and liver cancer (Rebbeck, 2020), while breast cancer is the major malignancy for African women (Sharma, 2020).

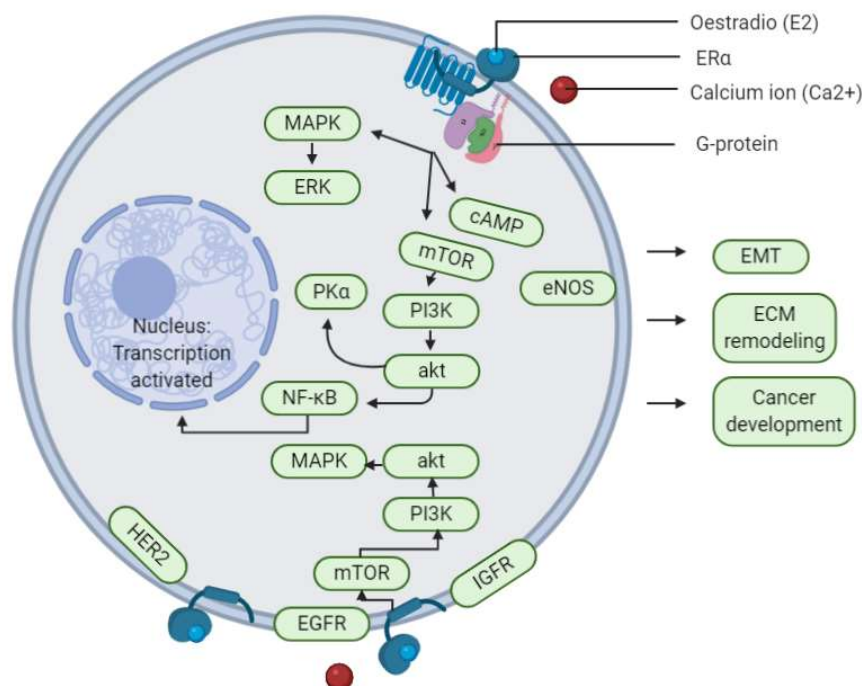
In this chapter the signalling pathways of breast cancer will be addressed. Secondly, the epidemiology and etiology of breast cancer will be discussed to emphasise the impact of this disease in the world. Thirdly, a discussion of current breast cancer treatments, as well as natural and adjuvant breast cancer therapy will be described. Furthermore, the anticancer activities of turmeric- and ginger extracts will be briefly addressed. Finally, the different drug delivery systems will be discussed in order to address possible solutions for some of the solubility and absorption issues related to turmeric and ginger extracts in the physiological system.

2.2 Breast cancer

Breast cancer is known to be hormone dependent where 70–80% of these tumours express oestrogen (ER) and/or progesterone receptors (PR) (Hilton, Clarke and Graham, 2018). Both oestrogen and progesterone are used in breast development during puberty (Javed and Lteif, 2013). Oestrogen is furthermore involved in the regulation of physiological systems such as cardiovascular, central nervous, and reproductive (Begam *et al.*, 2017), while progesterone primarily functions in the menstrual cycle; peri- and post-ovulatory phases, as well as in pregnancy (Trabert *et al.*, 2020). Two types of oestrogen receptors are expressed in cancer cells. The alpha oestrogen receptor (ER α) is predominantly expressed in the breast, uterus and the ovarian theca cells while the beta oestrogen receptor (ER β) is expressed in the brain, prostate epithelium and testes (Begam, Jubie and Nanjan, 2017).

Seventy percent of all breast cancers are ER α mediated (Xiao *et al.*, 2019). Studies have shown that cell proliferation is the result of environmental oestrogen binding to ER α receptor site of ER α positive breast cancer cells (Lei *et al.*, 2017). The ER α receptor is associated with

G proteins (Figure 2.1), which allows cells to receive and transmit signals (Dohlman, 2015). When these proteins are stimulated by oestradiol (E2), a series of signalling pathways catalysed by endothelial nitric oxide synthase (eNOS), phosphatidylinositol 3-kinase (PI3K/AKT) (Chambliss *et al.*, 2010) and adenosine monophosphate (cAMP) are activated (Aronica *et al.*, 1994). This leads to a rise in cAMP levels, and the assemblage of Ca^{2+} , which in turn activates the C-terminal of the $\text{ER}\alpha$ receptor (Divekar *et al.*, 2011), mediating the stimulation of oestrogen signalling (Divekar *et al.*, 2011). By activating $\text{ER}\alpha$ with PI3K, mitogen-activated protein kinase (MAPK), AKT, p21ras and PKC signalling molecules, a rapid activation of a series of signalling molecules will be the result (Piperigkou and Karamanos, 2020). This includes the activation of human epidermal growth factor receptor one and two (EGFR1/2). Subsequently this will cause the up regulation of mTOR/PI3K/AKT/MAPK pathways (Márquez *et al.*, 2006). The stimulation of $\text{ER}\alpha$ also leads to the binding of IGF-IR (insulin-like growth factor-I receptor), which in turn leads to the activation of the IGF (insulin-like growth factor) signalling pathway and in result forms part of cancer development (Yu *et al.*, 2013).



Breast cancer in women that has not undergone metastasis and is still localised is usually diagnosed by screening mammography and is easily treated with local treatments. Unfortunately, subclinical metastases can occur in most women with primary breast cancer (Hortobagyi, 1998).

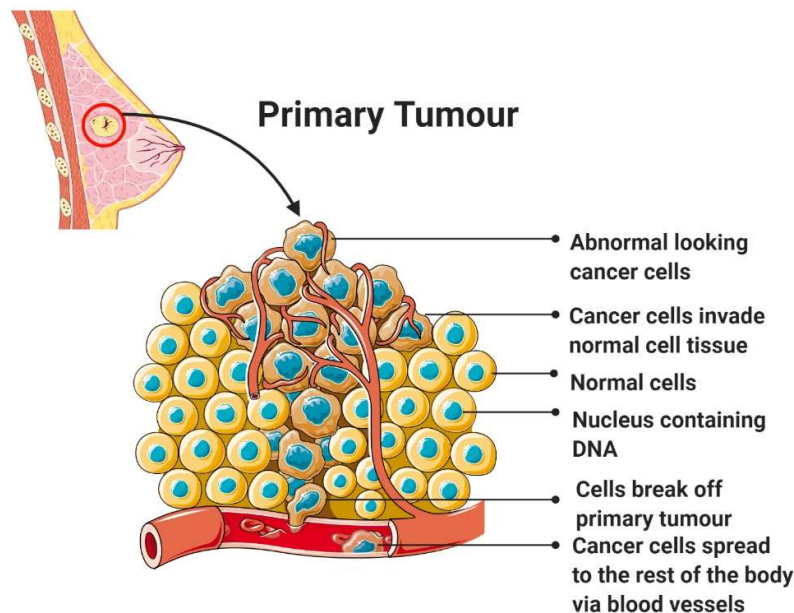


Figure 2.2: Development of metastasis from a primary breast tumour. Figure generated using BioRender.com

Metastasis occurs when cells of the primary tumour rupture and ends up in surrounding blood or lymph vessels (Figure 2.2). The cancer cells are transported via these vessels to outlying organs. Finally, the cancer cells undertake cell cycle arrest and attach to the capillaries of the designated organ, where secondary tumour growth may commence (Scully *et al.*, 2012).

2.3 Epidemiology

Breast cancer continues to disrupt women's lives impacting 2.1 million lives annually, and the approximate mortality of breast cancer among women was estimated at 627 000 in the world (WHO, 2020). Even though breast cancer is recognisable worldwide, different parts of the world experience it differently in cases of prevalence, mortality and survivability (Momenimovahed and Salehiniya, 2019). This is due to factors such as socio-economic status and ethnicity (Akinyemiju *et al.*, 2016).

A study has shown evidence that the life expectancy of low- and middle-income countries are roughly 30 years lower than high income countries (Newman, 2017). For instance, developing

countries in Africa have the lowest incidence rates, but also the highest mortality rates, which displays a lower survivability (Joko-Fru *et al.*, 2020).

When comparing race as a risk factor and keeping the stage of breast cancer, the size of the tumour, age, histology, nodal status and the hormone receptor status in consideration; it has been reported in the USA that black women have a higher mortality rate than white women (Walsh *et al.*, 2019). According to Desantis *et al.* (2017), black women are 39% more likely to die from breast cancer compared to white women. Possible reasons for this outcome include inadequate access to health care (Tao *et al.*, 2015), prescriptions of suitable adjuvant therapy (Freedman *et al.*, 2013), as well as non-compliance to treatment (Reeder-Hayes *et al.*, 2014).

Breast cancer survival rates of Africans (black and non-black populations) are still much lower when compared to black and white American populations. This is due to existing regional, sub-regional racial and gender disparity (Ssentongo *et al.*, 2019).

In South Africa, the annual breast cancer incidence rate (ABCIR) of black South African (SA) women (1:3436) are much lower comparing to the ABCIR of other SA ethnical women (Mixed race, Asian and white with rates of 52.92–79.3/100000) (Phaswana-Mafuya and Peltzer, 2018). Regardless of race, ER+ breast cancer was diagnosed in more than 60% of SA women in 2014 (Dickens *et al.*, 2014).

2.4 Etiology

Breast cancer occurrence is much higher in woman than in men but other risk factors for obtaining breast cancer includes unhealthy lifestyle, family history, aging, oestrogen, reproductive factors and gene mutations (Sun *et al.*, 2017).

Unhealthy lifestyles consisting of excessive alcohol and fat consumption may contribute to the development of breast cancer by increasing the levels of oestrogen-related hormones, which further interacts with oestrogen receptors (Jung *et al.*, 2016). Smoking, particularly at a younger age also increases the risk of breast cancer occurrence (Kispert and McHowat, 2017). Breast cancer has been found to be a hereditary disease (Brewer *et al.*, 2017). Research showed that the risk of developing cancer for women with one first-degree relative increases 1.75-fold while the risk with women having two or more first-degree relatives increases by 2.5-fold when compared to women with no family history of breast cancer (Brewer *et al.*, 2017).

Oestrogen is related to an increasing risk of breast cancer. Oestrogen can be obtained endogenously and exogenously. Endogenous oestrogen is produced by the premenopausal women's ovaries (Hormones and Breast Cancer Collaborative Group, 2013). Exogenous

oestrogen is usually obtained via oral contraceptives and hormone replacement therapy (Stewart *et al.*, 2020).

Although numerous studies have proven that hormone replacement therapy increases the risk for breast cancer (Million Women Study Collaborators, 2003, Liu *et al.*, 2016), it was found that the risk for obtaining breast cancer greatly decreases after two years of ending therapy (Narod, 2011). Low parity, late menopause, older age at initial pregnancy, early menarche are all reproductive factors contributing to high risk for breast cancer. For every year menopause is delayed, the risk of breast cancer increases by 3% (Dall and Britt, 2017).

2.5 Current breast cancer treatments

Different approaches to breast cancer therapy have been used, which include surgery, radiation, and targeted gene therapy. Surgery is currently the primary treatment for a wide range of cancers (Eriksson *et al.*, 2018). The two types of surgeries available for the treatment of breast cancer are breast conserving surgery (BCS) (tumour- and circumambient tissue removal) combined with radiation therapy and a mastectomy (removal of the breast). Recent studies have shown that patients who have undergone BCS have a better survival rate than patients who have had a mastectomy thus BSC is the preferred method of surgery (Christiansen *et al.*, 2018).

If it is not suitable to operate on tumours, radiation therapy is the last resort (Durante and Loeffler, 2010). The concept of radiation resides in destroying cancer cells using radiation from ionising electromagnetic sources (McKelvey *et al.*, 2018). Furthermore, ionising radiation kills cells directly by targeting DNA, subsequently leading to a decrease in cell density, causing a decrease in functionality, increased apoptosis and necrosis (Wang, Wang and Qian, 2018). Indirect cell destruction has also been observed, as free radicals are produced when the water component of cells are ionised (Baskar *et al.*, 2014).

Another potentially therapeutic response includes targeted gene therapy (Zhang *et al.*, 2016). A study has shown that the alteration and expressing of unique genetic material influence the maturation of different breast cancer subtypes (Bottai *et al.*, 2017). This can be achieved via *in vivo* and *ex vivo* methods where the genetic material of targeted cells can be modified by relocating genes, components of genes or even oligonucleotides (Yin *et al.*, 2014). Unfortunately, this therapy has numerous limitations around delivery systems, alterations of genetic information in adequate amount of cancer cells, as well as diversity in tumours (Nastiuk and Krolewski, 2016).

2.5.1 Breast cancer chemotherapy

Chemotherapy is based on the principle of using natural or synthetic substances and hormones to treat various types of cancer (Tatli Seven *et al.*, 2020).

There are various approaches to treating breast cancer using chemotherapy (Waks and Winer, 2019). Endocrine therapy is the initial systemic therapy for hormonal receptors /ERBB2–breast cancer and functions by countering oestrogen-promoted tumour growth (Waks and Winer, 2019). Tamoxifen is the most established drug used for oestrogen receptor positive breast cancer (Ahmad, 2018). Tamoxifen, once known as an oestrogen antagonist but now classified as a selective oestrogen receptor modulator (SERM), displays ER agonist or antagonist activity depending on the specific tissue. Tamoxifen inhibits tumour growth via inhibiting 17-beta-oestradiol dependent gene description and cell proliferation (Maximov *et al.*, 2014). In addition, tamoxifen disrupts cell proliferation by initiating cell cycle arrest in the G0/G1 phase of the cell growth cycle (Rondón-Lagos *et al.*, 2016).

Despite the promising anticancer evidence that tamoxifen presents, some concerning implications have risen in the use of this drug such as drug resistance. Studies have shown that ER signalling pathways, including the NF-KB signalling, PI3k-PTEN/ V-Akt, AKT, mTOR pathway, as well as receptor tyrosine kinases signal transduction pathways such as IGF1R, HER2, EGFR, FGFR (fibroblast growth factor receptor) are involved (Ojo *et al.*, 2015). Changing levels of bioavailability of tamoxifen may also contribute to drug resistance (Nass and Kalinski, 2015).

Another efficacious strategy in treating various forms of cancer is the use of anticancer drugs like cisplatin (Manohar and Leung, 2018). The generalised mechanism of action includes the binding of the drug to the genomic DNA or the mitochondrial DNA, which causes DNA lacerations to develop. Furthermore, cisplatin will terminate the synthesis of DNA, mRNA and corresponding proteins (Ghosh, 2019). DNA replication will also discontinue and finally necrosis or apoptosis will be the result of drug-activation of certain transduction pathways (Achkar *et al.*, 2018). Unfortunately, cancer cells become more resistant to cisplatin-based chemotherapy. This is done by four recognisable mechanisms of resistance namely: mechanisms of pre-target, on-target, post-target resistance, and off-target resistance. Pre-target resistance refers to the inhibition of cisplatin binding to DNA, while on-target resistance describes the occurrence of drug resistance after cisplatin has bonded with the cell DNA. Furthermore, post-target resistance is caused by cisplatin inducing damage to DNA via activation of lethal signalling pathways. Finally, off-target resistance describes the effects in

molecular systems which has an unclear relation with signals activated by cisplatin (Galluzzi *et al.*, 2012).

Besides the threat of resistance compromising the efficacy of these treatments, these drugs have shown to have serious side effects. Studies have shown that patients using cisplatin showed an increase in nausea and vomiting, cardiotoxicity, neurotoxicity, nephrotoxicity, neurotoxicity and in higher doses hepatotoxicity (Aldossary, 2019). Tamoxifen has also shown to increase the risk of pulmonary embolism, stroke and deep vein thrombosis. Some less severe side effects such as nausea, diarrhoea, hot flashes and an increase in vaginal discharge have also been reported (Flynn, Heale, and Alisaraie 2017). These adverse effects occasionally cause patient non-compliance leading to further complications treating cancer (Moon *et al.*, 2017).

2.5.2 Natural remedies for breast cancer

There is evidence of natural medicines being used dating as far back as 5000 years in the Nagpur area in Iran (Giannenas *et al.*, 2020). The use of traditional and natural medicines has shown to have great potential in treating breast cancer and improving the quality of the patient's life (Kumar and Jaitak, 2019). For instance, cardamom (*Elettaria cardamomum*) or better known as the "Queen of Spices" has shown anticancer and chemopreventative properties (Kareparamban *et al.*, 2012). Cardamom reduces cancerous activity of both the MCF-7 (breast) and HEP-G2 (liver) cell lines. A study has shown that cardamom furthermore disrupts cancer cell growth by inducing apoptosis (Vutakuri and Somara, 2018).

Sanghuangporus vaninii has been used in China as a natural remedy for almost 2000 years (Zhu *et al.*, 2019). The polysaccharide found within *S. vaninii* has shown to contain anticancer properties, especially against the MCF-7 cell line (Wan *et al.*, 2020). The mechanisms of action include inhibiting cell proliferation, which causes cell cycle arrest within the G2 phase and induction of apoptosis. Furthermore, *S. vaninii* promotes its anticancer properties by influencing the p53 signalling pathway, as well as repressing the synthesis of MMPs (Wan *et al.*, 2020).

The popularisation of herbal remedies in African countries continues to grow (Huang *et al.*, 2015). Interest in these herbal remedies has not only developed under scientists, but under the public as well (Fouche *et al.*, 2008). These resources are usually provided by traditional healers to treat a wide range of female reproductive and genital complications including breast abnormalities (Steenkamp, 2003). The stem bark of *Tabernaemontana stapfiana* is currently being used to treat breast cancer in Kenya (Ochwang'i *et al.*, 2014). This extract is prepared

by drying and pounding it into a powder after which it is then mixed with alcohol. The mixture is then topically administered once a day for a month (Ochwang'i *et al.*, 2014). Other natural ingredients that have a promising effect on breast cancer cells include the stems of *Acokanthera oppositifolia* and *Xanthium strumarium*. These extracts inhibit MCF-7 cell proliferation (Lukong, Ogunbolude and Kamdem, 2017).

2.6 Natural adjuvant treatments for breast cancer

The prolonged outcome of using a combination of natural and synthetic drugs is to increase the efficacy of the treatment but simultaneously reduce side effects (Wanchai and Armer, 2020), as well as resistance in current chemotherapy (Lin *et al.*, 2020).

Caffeine — a psychoactive compound most commonly found in popular beverages such as tea and coffee (van Dam, Hu and Willett, 2020) — have shown anticancer properties against the MCF-7 breast cancer cell line (Niknafs, 2011). It is proven that caffeine induces oxidative stress and apoptosis in both MCF-7 and MDA-MB-231 breast cancer cell lines. Although, DNA lesions have been caused by the induction of oxidative stress in the MCF-7 cell line, the same observation was not made in the MDA-MB-231 cell line (Machado *et al.*, 2020). In addition, a study by Funakoshi-Tago *et al.* (2020) has shown that caffeine reduces ER α gene expression and greatly enhances the efficacy of tamoxifen against breast cancer, concluding that caffeine could be used as adjuvant therapy.

Lauryl gallate (LG) — derived from a plant triphenol — consists of tumour growth inhibition properties without influencing normal human tissue, as well as the ability to induce apoptosis in tumour cells (Cheng *et al.*, 2016). By using LG as adjuvant therapy with chemotherapy drugs such as cisplatin, synergistic effects could be observed (Dhima *et al.*, 2018). A study has proven that LG increased tamoxifen's ability to express the SIRT-1 gene, but the synergistic co-operation of these drugs induces apoptosis to a greater extent via the BCL-2 (B-cell lymphoma-2) gene (Samani *et al.*, 2020).

Piperine, an alkaloid present Piperaceae seeds, has shown to have predominant activity against metastasis *in vitro* and *in vivo* (Lai *et al.*, 2012). Furthermore, piperine suppresses drug metabolism by inhibiting a P-glycoprotein, which is responsible for drug reflux. This leads to an increase in the bioavailability of many drugs (Makhov *et al.*, 2012). Finally, piperine not only reduces side effects of co-administered drugs, but it has also anti-proliferative effects in cancer cells and contributes to reverse drug resistance (Pachauri *et al.*, 2015).

2.6.1 Turmeric extract

Turmeric (*Curcumuma longa*) is cultivated and used for its culinary properties in traditional Asian dishes. Turmeric contains curcuminoids (2–7% of its composition), which attributes the distinctive yellow colour of turmeric (de Carvalho *et al.*, 2020). Furthermore, turmeric is predominantly used in self-remedies to treat an extensive range of diseases (Pachauri, Gupta and Ghosh, 2015).

A recent study has confirmed the contents of an ethanolic turmeric extract, which includes sterols, tannins, alkaloids, flavonoids, phenols, and glycosides (Maithilikarpagaselvi, Sridhar and Sripradha, 2020). Mono- and sesquiterpenoids, the compounds in volatile oil and curcuminoids are the two active components in turmeric (Sharifi-Rad *et al.*, 2020). The curcuminoids consists of diferuloylmethane (curcumin I), which will be used as the analytical marker, and its two counter parts desmetoxycurcumin (curcumin I & II) and bisdemetoxycurcumin (curcumin III). These phenolic compounds are responsible for the antibacterial, anti-inflammatory, and anticancer activities of the plant (Amalraj *et al.*, 2017, Singh, Maurya and Rai, 2019).

Some ethnomedical and pharmacological uses of *Curcuma longa* includes hepatoprotective, anti-diabetic, anticancer, antibacterial, anti-inflammatory, antioxidant, antidepressant effects, as well as promising results against gastrointestinal illnesses such as dyspepsia, gastric ulcers and irritable bowel syndrome (Yallapu *et al.*, 2015, Sanghvi *et al.*, 2020).

Curcumin has numerous strategies to promote anticancer effects. In the MCF-7 cell line, curcumin arrests cells in the G₁ phase reducing cell proliferation (Dasiram *et al.*, 2017). Curcumin also initiates apoptosis and contributes to anti-proliferative effects via p53-dependent and independent pathways of breast cancer cell lines (Fan *et al.*, 2016).

Another strategy includes curcumin's ability to induce cell cycle arrest in G₂/M cells via the modulation of Wnt/ β -catenin signalling, sequentially causing the loss of cyclin D1 resulting in anticancer effects (Song *et al.*, 2019). In addition, curcumin down regulates the NF- κ B signalling pathway, which may partially inhibit the maturation and expropriation of breast cancer tumours (Stanić, 2017).

2.6.2 Ginger extract

Ginger (*Zingiber officinale*) is an herbaceous and edible plant, originated from India. Besides for its culinary uses, ginger has also been used as traditional medicine for ailments such as

colds, headaches nausea, rheumatism (Mishra, Kumar and Kumar, 2012), diabetes, constipation, gingivitis, asthma, toothache, and stroke (Tanaka *et al.*, 2015).

Ginger contains bioactive compounds that consist of [6]–gingerol, [8]–gingerol, and [10]–gingerol (Sang *et al.*, 2009, Zhang *et al.*, 2017). In a study where these bioactive compounds were used to treat cancerous cells, [10]–gingerol reduced triple-negative breast cancer (TNBC) cells through S-phase cell cycle arrest, as well as apoptosis causing tumour cell death (Ryu and Chung, 2015). Further studies showed that [10]–gingerol also suppressed the growth of MCF-7 and T47D oestrogen receptor-bearing breast cancer cells (Bernard, McConnery and Hoskin, 2017). Ginger's anticancerous activity is due to its capability to alter cell growth regulatory proteins, which includes transcription factors (NF- κ B, STAT3), cellular inhibitor of apoptosis proteins (cIAP-1, XIAP), enzymes (PI3K, ERK1/2, MAPK, MMP-9, COX-2, cdk, caspases), cytokines (TNF- α), signalling pathways (Akt), proto-oncogens (cyclin D1) and other signalling molecules (Rashmi, Tripathi, and Rawal, 2018).

According to Ko *et al.* (2019) ginger consists of biological active ingredients such as shogaol, gingerol, paradol and zingerone. Another study has shown that 6–shogaol, is the predominant anticancer compound of all the other analogues of shogaols and gingerols (Kemkar *et al.*, 2018). It promotes apoptosis and growth inhibition of breast and colon cancer cells by activating PPAR γ and causes the suppression of NF- κ B activity in both MCF-7, as well as HT29 cells (Tan *et al.*, 2013). The principal mode of cell death induced by 6–shogaol in breast cancer cells is autophagy (Ray, Vasudevan and Sengupta, 2015).

From literature, it is evident that adjuvant therapy is beneficial. Adjuvant therapies do not only have anticancer effects, but some enhance the bioavailability of chemotherapeutic drugs, as well as help to lower drug resistance. In this study, turmeric- and ginger extract will be the focus as natural and adjuvant therapy.

2.7 The effect of natural turmeric extract and ginger on oestrogen in the body

Oestrogenic actions can be divided into two subgroups namely selective oestrogen receptor modulators (SERM's) and silent oestrogens. SERM's are substances, which may have selective agonistic or antagonistic oestrogenic activity, while silent oestrogen compounds change oestrogen signalling by utilising oestrogen genes but depriving cell proliferation (Kiyama and Zhu, 2014)

In a recent study by Moreira-Pinto *et al* (2020) to determine varying effects of curcumin, it was determined that in low doses curcumin does not have a significant effect on hormonal levels, but in higher doses, an increase in oestradiol levels was observed (Moreira-Pinto *et al.*, 2020).

Ginger rhizomes have also shown to have increased the oestradiol levels (Tchoffo *et al.*, 2017). Although ginger consists of compounds that may have oestrogenic and anti-oestrogenic activity, much of these compounds are too small to interact with ERs (Kiyama, 2017). Some ingredients found in ginger such as limonene derivatives have shown to have ER α / β selectivity, but was thus proposed to be SERM's (Kiyama, 2020).

2.8 Drug delivery systems

Despite both curcumin and ginger's potential, there are factors that limit the use of these ingredients for cancer therapy. Curcumin for instance has low bioavailability and solubility, weak tissue absorption, accelerated degradation at physiological pH, as well as systemic elimination following oral administration (Elbially *et al.*, 2020). These barriers could be overcome by drug delivery systems, as they improve drug bioavailability, pharmacokinetics and decrease biological degradation. Physical and chemical properties of drugs are also more protected in these systems (Ma and Williams, 2018).

Self-micro emulsifying drug delivery Systems (SMEDDS) are comprised of oils, surfactants and co-surfactants mixed to be homogenous and isotropic (Kim *et al.*, 2019). SMEDDS can greatly increase the oral bioavailability of drugs having poor water solubility. This drug delivery system also increases drug absorption by increasing the solubilisation of drugs, which enhances the penetrability of drugs through the gastrointestinal membrane and expanding lymphatic medication uptake (Li *et al.*, 2017). The mechanism behind this system can be summarised as follows: The SMEDDS formulation will start to self-emulsify when the formulation gets in contact with the gastrointestinal fluids producing nano-sized emulsions (Feeney *et al.*, 2016). Finally, effective drug release via rapid lipid digestion is possible due to the increased surface area of the droplet (Sunazuka *et al.*, 2018). SMEDDS also have a few limitations including unpredictable *in vitro* results, drug precipitation *in vivo*, lipid oxidation and polymorphism-related issues, as well as storage problems (Rajpoot *et al.*, 2020).

Liposomes are also a drug delivery system that are being used to assist in delivering low soluble substances to target areas in the human body, while increasing bioavailability and reducing side effects of the drug (Lee and Thompson, 2017). These lipid-based bilayer vesicles can modify the pharmacokinetics of the desired substance to ensure effective and sufficient drug delivery (Dimov *et al.*, 2017). Liposomes have a few limitations such as time

dependent stability, preparation and mass production problems, ineffective encapsulation (mainly of hydrophilic antioxidants) and low skin permeability (Van Tran *et al.*, 2019).

Phytosomes are compounds consisting of herbal active ingredients bonded to phospholipids to increase the absorption, stability, and the bioavailability of natural substance (Singh, Upadhyay and Upadhyay, 2018). Unlike liposomes, where the constituents are encapsulated by various phosphatidyl choline units, in phytosomes the ratio between the phytoconstituents and phospholipids are 1:1 or 1:2. (Pawar and Bhangale, 2015). An example of a phytosome is Meriva[®], which is a curcumin phytosome. According to Smagurauskaite (2019), the use of phytosomal curcumin is beneficial as curcumin is not only protected from rapid conjugation, but its affinity to biological membranes, as well as its transport through plasma membranes are increased.

The Pheroid[®] drug delivery system is a dynamic yet highly stable lipid bilayer colloidal system. It contains a plant and essential fatty acid dispersed phase, and nitrous oxide saturated water continuous phase (Slabbert *et al.*, 2011). The Pheroid[®] system consists of a hydrophilic core in which hydrophilic ingredients that have weak permeability characteristics/properties are encapsulated. This system is also used to enhance the absorption of oral, dermatological, and biological medicines (Kilian *et al.*, 2015). In addition, pro-Pheroid[®] has many advantages such as polyphilic and efficient drug entrapment, as well as increased drug bioavailability and targeting (Erasmus, 2019). In a previous study conducted by Bekele *et al.* (2019), this system was successfully used to incorporate plant bio-actives for anticancer treatments resulting in promising effects.

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CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Ginger extract (GE) (GR20180505) containing more than 10% gingerols was obtained from Xi'an B-Thriving I/E Co., Ltd. (Shaanxi, China). The powdered turmeric (01/16) was sourced from Warren Chem Specialities. The dl-tocopherol (UT19060054) and the Kolliphor® EL (4017202440) were acquired from BASF (Berlin, Germany). The vitamin F ethyl ester (3194426) was obtained from IMCD (Rotterdam, The Netherlands). For cell culturing and assay purposes DMEM (41965–039) and RPMI (11875101) were purchased from Gibco (Dun Laoghaire, Ireland); FBS (16000036) from Thermo Fisher Scientific (Waltham, MA, USA), penicillin and streptomycin (17-745E), non-essential amino acid solution (NEAA) (BE 13-114E), D-and 2mM l-glutamine (BE17-605E) were acquired from Whitehead Scientific. Trypsin (Sigma, 59427C) and phosphate-buffered saline (PBS) (P4417) were purchased from sigma-Aldrich (Missouri, USA). Furthermore, tricine (T5816-25G), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (23,039-1), dithiothreitol (DTT) (D11000-5.0), beetle luciferin (E160C), coenzyme A (MEL-C70800-0.1), adenosine triphosphate (ATP) (B3003), EDTA-disodium salt (318884-500ML) and $\text{Mg}(\text{CO}_3)$, $\text{Mg}(\text{OH})_2$ solution (M5671-500G), 17β -oestradiol (E_2) (Sigma, E8875), Fulvestrant (ICI 182 780) and lysis buffer (Promega, ADE3971) were also obtained from Gibco (Dun Laoghaire, Ireland), and Sigma-Aldrich (Missouri, USA). In addition, all reagents used in this study were of analytical grade and the raw materials for formulations were of pharmaceutical grade.

3.2 Research methods

The compounds used in the formulations tested against a cancer cell line were turmeric and ginger extracts. Turmeric's active phytochemical is curcumin (curcuminoids). The turmeric extract obtained, resulted in a more concentrated product. Curcumin extracts were prepared using Soxhlet extractions. The ginger used in this study was in extract form with known phytochemical content. Various unformulated concentrations of turmeric and ginger extracts were gauged against normal green monkey kidney cells, the Vero cell line (normal, non-cancerous cells), to determine the concentration where these compounds were not toxic to normal cells. These concentrations were formulated into pro-Pheroid® and characterised. The formulations were then tested *in vitro* for anti-proliferative effects in oestrogen active breast cancer cells (MCF-7) and Vero cells. Using these results, the cancer selectivity index was determined. The anti-proliferative properties were confirmed with an apoptosis assay. To ascertain if the mechanism of action in cell death was due to oestrogen inhibition, an oestrogen receptor inhibition assay was completed on genetically modified oestrogen active breast

cancer cells (T47D-KBluc). Based on the combined *in vitro* results, a final formulation was chosen and administered to an MCF-7 tumour bearing mouse model. The potential of this formulation as an adjuvant treatment was evaluated by co-administration with cisplatin, a known antineoplastic drug.

3.2.1 Soxhlet extraction of curcumin

From literature (Degot *et al.*, 2020), the highest curcumin percentage yield, was obtained via the Soxhlet extraction. The method was modified from Degot *et al.*, 2020, Gururaj *et al.*, 2002 and Patil *et al.*, 2019. Briefly, pure turmeric was accurately weighed off (320 g) and divided equally into the extraction thimbles. Each round bottom flask contained 250 mL of 99% ethanol (Gururaj *et al.*, 2002) and was heated and maintained at 60°C. The extraction process was continued for 8 hours. The principle of this extraction method is that the solvent evaporates from the solvent flask and is collected in the extraction chamber using the condenser. Once the solvent reaches the overflow of the Soxhlet extraction chamber, a siphon forms and the extract travels through the siphon

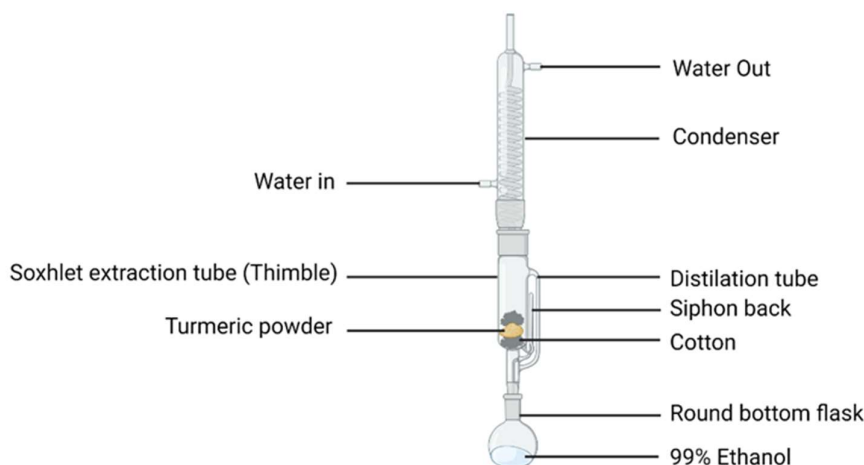


Figure 3.1: Soxhlet extraction setup for the extraction of curcumin from turmeric. This image was generated using Biorender.com

back and into the reservoir (Figure 3.1). The solvent returned to the round bottom flask is evaporated and the cycle is repeated (Shirsath *et al.*, 2017), the extracted compounds are concentrated in the reservoir. The percentage yield was calculated according to the following formula:

$$\text{Percentage yield} = \frac{\text{Total mass of extract powder}}{\text{Total mass of raw material}} \times 100 \quad (1)$$

3.2.2 Liquid Chromatography tandem mass spectrometry (LC–MS/MS): The quantification of curcumin and [6]-shogaol

The ginger and turmeric extracts were prepared by weighing out 6 mg of the turmeric and ginger extract on the Sartorius Praxium balance after which each compound was dissolved in 6 mL and 15 mL of methanol, respectively. A rotary-mixer was then used to mix the solutions for 10 minutes after which it was centrifuged in a benchtop centrifuge (Hermle Centrifuge) for 10 minutes at 2000 *g*. The aliquots were diluted with deionised water to obtain a final concentration of 1 µg/mL turmeric extract and 10 µg/mL ginger extract. For the final analysis, 20 µL of each of the final solutions were injected into the LC–MS/MS (CTC PAL Autosampler-1290 Agilent UPLC-AB Sciex QTRAP 4000). This method was validated using the European Medicines Agency guideline on bioanalytical method validation (EMA, 2011).

Calibration standards (62.5, 125, 250, 500, 1000 ng/ml) of both curcumin and [6]-shogaol were prepared from 1 mg/ml stock solutions of each compound. The content of curcumin in turmeric extract and [6]-shogaol in ginger extract were calculated by the following equation:

$$T = C \left(\frac{V}{M} \right) \quad (2)$$

where, T represents the content of the desired compound in each extract (mg/g), C is the concentration obtained from the calibration curve, V is the volume of the solution and M represents the mass of extract measured in gram.

Calibration standards and extracts were separated using an Agilent 1290 Infinity UPLC system containing a Phenomenex Kinetex™ C18 column (30 mm × 2.1 mm, 1.7 µm) fitted with a Phenomenex guard-column (UHPLC C18, 2.1 mm ID). A CTC PAL HTx-xt autosampler with a 20 µL sample loop was used for the injection of the sample extracts into the UPLC (ultra-performance liquid chromatography) system. The separation was performed by using a gradient elution method with water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid. The flow rate and mobile phase composition changes were determined by a precursor study (Bekele *et al.*, 2019). The flow rate was set at 0.4 mL/min. The gradient elution conditions were as follows: 0–1.5 min 30 to 90% B; 1.5–1.9 min: 90% B; 1.90–1.91 min: 90 to 100% B; 1.91–2.30 min: 100% B; 2.30–2.31 min: 100 to 30% B; 2.3–3.0 min: 30% B.

The tandem mass spectrometry (MS/MS) was performed by using the AB SCIEX 4000 QTRAP mass analyzer equipped with a Turbo Ion Spray source (SCIEX, Toronto, Canada)

operating in electrospray ionization (ESI) positive mode. The instrument control was carried out via analyst software (version 1.6), data acquisition and the quantitative analyses were performed by MultiQuant™ 2.1 Software. The multiple reaction monitoring (MRM) mode was used to detect and quantitate the analyte. The instrumental settings were set to its optimum: temperature, 450 °C, ionization mode, positive; curtain gas, 275.8 kPa; CAD gas, medium; nebuliser gas (GS1), 310.264 kPa, heater gas (GS2), 50 psi and the ion spray voltage at 5.5 kV.

A gas generation system (Peak Scientific Nitrogen Generator, model AB-3G, PA, USA) was used to produce the nitrogen flow. The transition has occurred in a dwell time of 150 msec. MS/MS parameters have occur in the positive ESI mode and optimised by direct infusion of curcumin and 6-shogol reference standard solutions at a flow rate of 10 µL/min. MRM transitions was as follows: curcumin (369.1 → 177.2) and ginger (m/z 277.1 → 137.1). The MS/MS settings was set to collision energy at 29/17 volts, collision exit cell potential at 8/22 volts, declustering potential at 336/116, and entrance potential at 10 volts.

3.2.3 Formulation of plant bio-actives in Pheroid® delivery systems

Initially, the extracts were screened for the presence of any biological active compounds. Subsequently, the quality and characterisation control of these compounds were performed. Finally, these biological active compounds in each extract were quantified.

3.2.3.1 Extract concentration screening

The concentrations used in the formulations were predetermined using the unformulated extracts to disqualify any cytotoxic concentrations. Concentrations ranging between 5–75 µg/mL were used to determine inhibitory concentrations (IC₂₅, IC₅₀ and the IC₇₅) using cell viability assays (cf. Section 3.7 Cell viability) in non-cancer cells (Vero cells). The inhibitory concentrations were combined into nine formulations (Table 3.1).

Table 3.1: Pheroid® formulation concentrations based on the combination of the inhibitory concentration values of the respective extracts. Formulations represent as F_{A-I}

Ginger extract	Curcumin			
		IC ₂₅	IC ₅₀	IC ₇₅
	IC ₇₅	F _A = 75:25	F _D = 75:50	F _G = 75:75
	IC ₅₀	F _B = 50:25	F _E = 50:50	F _H = 50:75
	IC ₂₅	F _C = 75:25	F _F = 25:50	F _I = 25:75

3.2.3.2 Pheroid® formulation

The combination concentrations indicated in Table 3.1 were formulated in pro-Pheroid®. Firstly, pro-Pheroid® was formulated following the method used by Bekele *et al.* (2019). Briefly, vitamin F ethyl ester (70% w/w), Kolliphor® EL (29% w/w) and dl- α -tocopherol (1% w/w) were mixed and gassed under pressure with nitrous oxide (N₂O). Secondly, the Pro-Pheroid® mixture and the active ingredients were mixed until homogeneity was achieved. Afterwards, the formulated product was gassed with N₂O under pressure. The pro-Pheroid® and combination formulations were then diluted 8 000 times in supplemented DMEM leading to a 0.0125% Pheroid® concentration. The 0.0125% Pheroid® concentration was used as final carrier concentration for *in vitro* studies based on viability assays. Formulations for the *in vivo* study was diluted in N₂O gassed water to 4% Pheroid®.

3.3 Particle size distribution

The particle size analysis was done by following the method of Knijnenburg *et al.*, (2018) with slight modifications. Each formulation was analyzed using a Malvern Mastersizer 2000 fitted with a Hydro 2000 SM dispersion unit. Refractive index input data for the analyses was set for Vitamin F (Refractive index 1.473) and the dispersant was distilled water. A 4% v/v Pheroid® concentration of each formulation was prepared from the pro-Pheroid® formulation in a 0.1 N hydrochloric acid. The acid medium was used to simulate gastric pH conditions. The measurements were taken in triplicate after each of the samples were injected into the distilled water dispersion media, which was stirred at 2 000 RPM. Sample was added until an obscuration value was obtained, between 0–10%. The uniformity of the particle sizes in the system was measured as a function of the span value using the equation below. A small span value indicates a narrower distribution.

$$\text{Span} = \frac{(d_{0.9} - d_{0.1})}{d_{0.5}} \quad (3)$$

where, $d_{0.9}$ and $d_{0.1}$ are where 90% and 10% of the volume of distribution are below these values respectively. Furthermore, $d_{0.5}$ indicates the volume median diameter.

3.4 Electro-kinetic potential analysis

The zeta potential is a crucial characteristic controlling emulsion stability that indicates the amount of electrostatic repulsion/attraction between particles (Bahadur *et al.*, 2020). The electro-kinetic characteristics of the formulations were determined by measuring the zeta (ζ)-potential using the Malvern Nano ZSP Series Zetasizer Zen5600 (Malvern Instruments Ltd,

Malvern, Worcestershire, UK). The samples were diluted in deionised water (90% v/v) after which the zeta (ζ)- potential of each of the formulations were obtained in triplicate. Dispersions with a zeta potential of ± 30 mV is considered physically stable. Dispersions with a zeta potential of lower than 20 mV are considered to have limited stability (Suganthi and Rajan, 2012).

3.5 Vesicle morphology

Vesicle morphology is used to qualitatively confirm that Pheroid[®] vesicles were formed. This was done by optical and confocal laser scanning microscopy (Knijnenburg *et al.*, 2018) using a Nikon D-Eclipse C1 Confocal inverted microscope TE2000-Efited with a diode (409 nm), an Argon Ion (488 nm), and Helium-Neon (543 nm) polarized lasers. Nile red was used as the fluorescence marker (excitation: 515–560 nm, emission: >590 nm), 50 μ L 4% formulation samples were labelled with 1 μ L Nile Red and mixed vigorously. After 15 minutes, 20 μ L was mounted on a glass slide and imaged. Images were taken with a medium pinhole (30 mm), and a 3us/scan speed under a 60x ApoPlanar oil immersion objective. All confocal images were acquired using identical settings on Nikon EZ 200 software.

3.6 Cell culturing and assay preparation

Both the human breast cancer cell line, MCF-7 (ATCC HTB-22), and the African green monkey kidney, Vero cells (ATCC CCL-81-VHG), were cultured in DMEM, supplemented with 10% FBS, 1% antibiotics (100 U/mL penicillin & 100 μ g/mL streptomycin), 1% antimycotic (250 μ g/mL amphotericin B), 1% NEAA, D-and 2mM L-glutamine.

Cells were cultured in T-75cm² cell culture flasks. Once cells reached a confluency of 70–80%, they were used in the subsequent experiments. When 70–80% cell confluency was reached, the old media was aseptically removed and washed with 15 mL PBS. After the PBS was discarded, 5 mL trypsin was pipetted onto the cells and incubated for 4 minutes at 37°C and 5% CO₂. The trypsin was deactivated by adding 10 mL of pre-warmed fresh media. The cells were then transferred to a 15 mL tube and centrifuged for 4 minutes at 2 000 rpm. The supernatant was discarded, and the cells were resuspended in 1 mL media. Thereafter, 50 μ L of the cell suspension was seeded into a T-75cm² with 20 mL of fresh pre-warmed media.

The genetically modified human breast cancer cell line, T47D-KBluc (CVCL_K229), were used to perform the oestrogen inhibition assay. Roswell Park Memorial Institute (RPMI-1640) (R8755, Sigma-Aldrich) medium supplemented with 10% FBS was used to culture these cells. In addition, for the purposes of the oestrogen inhibition assay, the cells were grown in hormone-stripped RPMI media, which was prepared by using HyClone's charcoal/dextran-

treated (CDT) media with a 10% FBS supplement. This was done to avoid the influence from other inherent hormones and to witness the compounds effects to bind to the oestrogen receptor. The 10% stripped media was diluted to 5%, which was used as an assay media to perform the bioassays.

When the cell viability assay was performed, cells were seeded into a transparent 96-well plate at seeding densities of 1×10^4 cells/well and 8×10^3 cells/well (200 μ L) for the MCF-7 and Vero cells, respectively. The optimal seeding densities were predetermined for each cell line (Appendix 1). Apoptosis assays were performed by seeding 1×10^6 cells per well (1 mL) in a 12 well plate. The TK47-KBluc cells were seeded into a white walled 96-well microplate with a flat transparent bottom at a seeding density of 1×10^3 cells per well (100 μ L). All microtiter plates used in this study were tissue culture treated.

3.7 Cell viability

Cell viability studies are done to determine if the test substances are non-cytotoxic. The neutral red assay is used to estimate the quantity of viable cells and is based on the principle of a dye binding to the lysosomes in live cells. If a cell dies, the pH gradient changes and the dye is no longer able to bind. Thus, the viable cells can be quantified to the number of cells that contain the red dye relative to an untreated control (Repetto *et al.*, 2008). The cell viability was measured to disqualify cytotoxic concentrations of the raw extracts (cf. Section 4.2.2 Extract concentration screening). This was determined using the Vero cells to ensure that the treatments are safe for normal cells. Subsequently, the MCF-7 (cancer line) and the Vero (normal line) were treated with the formulated extract combinations, and the cell viability for each was determined.

3.7.1 Neutral red cellular uptake assay

Neutral red assay was used to determine the cell viability and was performed by following the method modified by Repetto *et al.* (2008). After seeding, the cells were allowed to attach for 24 hrs, the growth media (supplemented DMEM) was removed, and the test compounds (200 μ L) made up in plain media DMEM was added. The negative control received only media. The blank control consisted of no cells and each well received only PBS. After a 24 hour exposure, the cells were inspected for any traces of toxicity by using a microscope. The formulations were aspirated, and the wells were washed three times with 200 μ L of PBS. After washing was completed, a 0.33% v/v neutral red solution was added to the cells and incubated for three hours at 5% CO₂ at a relative humidity of 90%. After incubation, the cells were inspected for the formation of the neutral red dye incorporating into the cells. The neutral red

solution was aspirated and discarded. The wells were washed three times with 200 μ L of PBS. The Neutral red solubilisation solution (200 μ L) (50% ethanol, 49% distilled water and 1% acetic acid) was added into all the wells to perforate the cells, releasing the Neutral red solution. To ensure optimal mixing of the Neutral red solubilisation solution, the plate was shaken on a plate shaker for 15–30 min at room temperature. The SpectraMax® Paradigm® Multi-Mode Detection Platform spectrophotometer was used to measure the absorbance at 560 nm and 630 nm. Each formulation was tested in triplicate. The measurements were normalised by subtracting the background reading. Afterwards the cell viability was calculated using the following equation:

$$\% \text{Cell viability} = \frac{A_{\text{Experiment}} - A_{\text{Blank}}}{A_{\text{Control}} - A_{\text{Blank}}} \times 100 \quad (4)$$

Where $A_{\text{Experiment}}$ represents the absorbance of the treated cells, A_{Blank} is the absorbance of the blank (PBS) and A_{Control} is the absorbance of the untreated cells.

3.8 Calculation of inhibition concentration

The inhibition concentration was calculated for each unformulated extract using the percentage cell viability results. The cell viabilities were plotted on a log scale and the straight-line equation ($y=mx+c$) determined, as well as the coefficient of determination (R^2). The IC_{50} is represented as the antilog of x , which was mathematically determined when $y=50$. An inhibition of 25% means that the total cell viability must be 75%. Thus, the remaining IC - values were calculated with $y=25$ for IC_{75} , and $y=75$ for IC_{25} , respectively.

3.9 Flow cytometric analysis for apoptotic phase detection

The nuclear morphology and apoptosis assay was done according to Liegler *et al.* (2005) with some adjustments. Acridine orange (AO) and ethidium bromide (EtBr) was used for the dual staining differential assay, which was used to evaluate apoptosis. The MCF-7 cells were exposed to the different formulations for 24 hours. After the treatment, the supernatant was gently removed, and the cells were washed with 200 μ L of PBS. Finally, a 100 μ L AO/EtBr staining solution (100 μ g/mL of each dye in PBS) was added to each sample. Apoptosis quantification was done with flow cytometry in triplicate, counting a minimum number of 1×10^4 cells for each treated and untreated group. The emission of AO and EtBr was detected with a 525/20 nm filter (FL1) and a 635/20 nm filter (FL3), respectively, after excitation at 488 nm. Cell counting was done by using FACSVerse™ with FACSuite™ software (BD Biosciences, San Jose, CA, USA) using the gating strategy described by Liegler *et al.* (2005).

3.10 Cancer selectivity index (CSI)

The cancer selectivity index (CSI) was calculated by following the method described in Rashidi *et al.* (2017). This index indicates the cytotoxic selectivity of the test compounds towards cancer cells rather than normal cells (Nguyen and Ho-Huynh, 2016). However, since our formulations are based on the inhibitory concentrations, the equation for the cancer selectivity index was adjusted to calculate the ratio using percentage cell viability.

The following formula was used to calculate the CSI:

$$\text{Cancer selectivity index} = \frac{\% \text{Cell viability}_n (\text{Normal cells})}{\% \text{Cell viability}_n (\text{Cancer cells})} \quad (5)$$

Where, n represents the formulation used as the treatment. If the CSI is ≥ 1 , the formulation shows anticancer properties (selective towards cancer). If the CSI is < 1 , then the formulation is not selective.

3.11 Oestrogen inhibition assay

The oestrogen receptor inhibition assay was performed by following the method described in Van Zijl *et al.* (2017). The T47D-KBluc cells are genetically modified human breast cancer cells to emit light — in the presence of the appropriate substrate — when oestrogen binds to the oestrogen receptor (ER). This emitted light is measured via luminescence. Before the assay was initiated, the cells were grown in RPMI media supplemented with 10% dextran-charcoal treated (hormone-free) FBS two weeks prior to the assay. The cells were seeded at 1×10^5 cells/mL in dosing media that was modified by reducing the dextran charcoal treated FBS to 5%. After a 24 h attachment period, the media was then substituted with 100 μL /well of the test formulations prepared in dosing media and incubated at 5% CO_2 in a humidified atmosphere for 24 hours. Luciferase assay reagent (LAR) was prepared following the method described in Villeneuve *et al.* (2000). This was done by adding 20 mM tricine, 2.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 33.3 mM DTT, 470 μM beetle luciferin, 270 μM coenzyme A, 530 μM ATP, 0.1 mM EDTA-disodium salt and 1.07 mM $\text{Mg}(\text{CO}_3)_4/\text{Mg}(\text{OH})_2$ solution to deionized water (18.2 M Ω .cm), which was then removed from the -80°C freezer and allowed to thaw.

Stock solutions of selected formulations were prepared earlier (selected based on the CSI). Two standards, an ER agonist E_2 and an ER antagonist were also prepared at 1×10^3 $\mu\text{g/mL}$. To determine the anti-oestrogenic ability of the test samples, they were co-incubated with an ER agonist, oestradiol (E_2) (20 pM that creates 100% induction of the luciferase activity). The cells were exposed to a series of dilutions of an ER antagonist, ICI 182 780 (1047)

(Fulvestrant) 0.13 nM, 0.41 nM, 1.23 nM, 3.7 nM, 11.1 nM and 33.3 nM. The test samples (0.0125% v/v) were serially diluted six times, at a 1:1 ratio. The cells were exposed to the samples and standards at 37°C and a relative humidity of 5% for 24 hours.

At the end of the assay, the test samples and standards were aspirated, and the cells were washed thrice with DPBS supplemented with Mg^{2+} and Ca^{2+} . Subsequently, 25 μ L reporter lysis buffer was added to the wells and placed in the -80°C freezer for at least 10 minutes. Afterwards, the plates were removed from the freezer and was allowed to thaw thoroughly by placing the plates on a plate shaker for 20 minutes. The rest of the assay commenced in the dark.

A Berthold multimode microplate reader (model LB941) was used to measure luminescence, which was primed with LAR. After the plates were thawed the plate reader injected 100 μ L luciferase assay reagent (LAR). The luciferase activity (luminescence) was quantified as relative light units (RLU). The amount of light elicited is directly proportional to the number of agonists present in the test compound (Denison *et al.*, 2004). Finally, the injector was washed with deionized water (18.2 M Ω .cm) as instructed on the microplate.

3.12 Final formulation selection

The final formulation was chosen on the results of the formulation's cell viability assay, cancer selectivity index, and apoptosis assay results. A formulation with a higher cancer selectivity index indicated that it was more cytotoxic towards cancerous cells. To confirm if the formulation was suitable to use *in vivo*, it had to indicate relatively high apoptotic effects.

3.13 Animal study

3.13.1 Experimental animals and husbandry

The animal study was conducted at the DSI/NWU Preclinical Drug Development Platform Vivarium. A total of 25 animals were used to conduct this study. Five female mice were randomly housed per individual ventilated cage (IVC). Corn cob was used for bedding and laboratory tissue was used for making a nest. Animals had access to water and rodent food *ad libitum*. Polyvinyl chloride (PVC) tubing and sufficient nesting material were included for environmental enrichment. The IVC was regularly cleaned. The light of the room changed in a 12 hr cycle between light and dark. The temperature and the humidity in the rodent's rooms were maintained at 22 $\pm$ 2°C/55 $\pm$ 10%, according to the SOP_Viv_Anim 14: Routine husbandry of rodent.

3.13.2 Sample Size

A total of five groups consisting of five mice each were used in the study. The sample sizes were determined using an evidence-based estimation. Previous studies of similar design and treatments have shown that five animals per study group are sufficient for tumour-based studies (Kang *et al.*, 2010, Lv *et al.*, 2016, Lu *et al.*, 2017). Additionally, for the precursor study—NWU-00167-18-A5—the power analyses done by the NWU Statistical Consultation Services showed that sample size between five and nine animals within different groups was sufficient to successfully perform the animal study (Bekele, 2019). The final sample size used in this study is also supported by Mead's resource equation (Mead, 1988).

3.13.3 Cell culture and inoculation in athymic nude mice

MCF-7 cells were cultured and harvested as previously discussed (cf. Section 3.6 Cell culturing and assay preparation). The inoculation was conducted according to SOP_Viv_Anim 30: Establishment of Rodent Xenograft Model. Briefly, animals were acclimatised for five days and then received an oestrogen supplement (3 mg/kg IP between shoulder blades) weekly. Once ready, two and a half million (2.5×10^6) viable MCF-7 cells in 100 μ L plain DMEM and Matrigel (1:1) cell suspension was injected subcutaneously in the right hind leg. The health of all the animals was monitored daily according to the SOP_Viv_Anim 27: Determining pain and distress in laboratory rodents. Tumour growth was measured every second day and noted on monitoring sheet 1(A): key for assignment of health check scores and monitoring sheet 1(B): log sheet for assignment of health check scores.

3.13.4 Experimental design

To avoid any possible biases to occur, after inoculation, the mice were randomly and equally divided into five groups, so that each group contained five female mice. Only after the tumours reached a size of 100 mm³ (enrolment size) the animals were enrolled into the study and that specific day was assigned as day zero. The study happened in a staggered approach since not all the tumours reached the enrolment size at the same time. The treatments for the study included the selected Pheroid® formulated plant bio-active combination and a cisplatin combination treatment therapy. Group 1 served as a negative control group and Group 2 served as a vehicle control group, receiving 0.9% saline and a blank Pheroid® formulation, respectively. The selected Pheroid® formulated plant bio-active was administered orally to Group 3. Group 4 served as the positive control group and received cisplatin (4.5 mg/kg), which was administered intraperitoneally (IP). Group 5 received a combination treatment therapy, which included a full dose of cisplatin IP and full dose of the phytochemical

combination therapy (ginger and curcumin), administered orally. Oral administration was done daily, and cisplatin was administered once a week. The tumour volume was measured with a linear digital calliper and recorded every second day. The tumour volume was calculated using the equation below:

$$\text{Tumour volume} = W^2 \times \frac{L}{2} \quad (6)$$

where, W represents tumour width, measured from its widest point and L represents tumour length measured from its longest point recorded on tumour monitoring sheet given (Addendum 2: tumour monitoring data collection sheet) (cf. Appendix 2).

The study was performed over 15 days. After euthanasia (see below), the final tumour volume (excised) was weighed and measured. Both relative tumour volume (RTV) and tumour inhibition rate (TIR) was determined using the method described by Yin *et al.* (2012) and calculated using the formula below:

$$\text{RTV} = \frac{V_n}{V_0} \quad (7)$$

Where, V_n represents the tumour volume measured at the same day and V_0 , the tumour volume measured at day 0.

The TIR was calculated using the formula:

$$\text{TIR} = \frac{T_{\text{RTV}}}{C_{\text{RTV}}} \quad (8)$$

where, T_{RTV} represents the RTV of the experimental group and C_{RTV} represents the control group's RTV.

3.13.5 Monitoring, humane endpoints, euthanasia and sample collection

At the end of the study, all animals were euthanised according to SOP_Viv_Anim 1: Euthanasia of Rodents. Euthanasia was done through cervical dislocation by the veterinarian or laboratory animal technologist (LAT). Health checks were performed prior to and routinely during the study according to SOP_Viv_Anim 27: Determining pain and distress in laboratory rodents and recorded in addendum 1(A and B) (Monitoring sheet) (cf. Appendix 2). daily. The observations considered appearance, food and water intake, respiration and cardiac activity,

and natural and provoked behaviour. The scoring in addendum 1(A) and 1(B) gives the clinical indication for humane euthanasia. Humane end point was noted when either the length or the width of the tumour reaches 20 mm or volume exceeding 400 mm³. Some clinical signs include hindered movement as a result of the size of the tumour and weight loss. In addition, more clinical signs are described in addendum 1(A), which includes an increase or decrease in respiratory rate. Any provoked behaviour such as depression, exaggeration of response, signs of violence or comatose was monitored. If a score of two in addendum 1(A) was obtained, the frequency of observations was doubled. If a score of three for an animal is achieved, euthanasia was considered, or a veterinarian performed the final assessment. Any abnormal conditions were reported to the veterinarian and animals were humanely euthanised as per the AnimCare Research Ethics Committee approved SOP_Viv_Anim 1: Euthanasia of rodents (Approval no: NWU-0076-15-A5) if deemed necessary and an ethics incident report prepared.

The experienced LATs involved in the study were responsible for observing and recording the signs of pain and discomfort as outlined above. The project head (study leader) was responsible for ensuring that the monitoring was conducted as per the SOP and that the monitoring was accurately documented in the monitoring sheets. Only trained and experienced personnel performed humane euthanasia.

3.14 Statistical analysis

The data obtained during this study were expressed as mean $\pm$ the standard deviation (SD). The experimental groups were compared to the relevant control groups by using the Mann-Whitney U- test and the Bonferroni's *post hoc* test determine statistical differences. For time dependent datasets repeated measure ANOVAs was used. Significance was tested at a 95% confidence level. Statistical analysis and graph generation were done on GraphPad Prism version 5 (GraphPad, San Diego, CA, USA).

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CHAPTER 4: RESULTS AND DISCUSSION

This chapter will discuss the evaluation of bio-active phytochemicals against oestrogen active breast cancer. The aim of this study included, proving that these compounds possessed anticancer properties, as well as proving them to be non-cytotoxic to normal tissue (represented by non-cancer cell culture). The results of possible mechanisms of action for the formulations' anticancer properties *in vitro*, as well as the final *in vivo* results will be addressed.

4.1 The extraction of curcumin

From literature, various methods for extracting curcuminoids are available, such as Soxhlet extraction, accelerated solvent extraction, ultrasonic- and microwave extraction (Shuya *et al.*, 2020, Tsaltaki *et al.*, 2019). From a previous study, the Soxhlet extraction method has proven to be the most effective to isolate curcumin (Degot *et al.*, 2020). In the current study, the Soxhlet extraction process resulted in a 4.021% yield (12.868 g extract). Fernández-Marín *et al.* (2021), obtained an 8.44% yield by using the similar extraction method for 6 hours with 150 mL ethanol. Priyadarshi *et al.* (2018), obtained a similar yield as our study (5.95%) by following the same method but rather using a non-polar solvent (hexane) for 24 hours. With this in mind, it was expected that the percentage yield obtained from our extraction would be similar compared to Fernández-Marín *et al.* (2021). A possible reason for the low percentage yield is that some of the mixture in the Soxhlet extraction process could have boiled out of the apparatus. It is also a possible that after the evaporation process, not all the residue extract was collected.

4.2 Liquid chromatography tandem mass spectrometry (LC-MS/MS): The quantification of curcumin and [6]-shogaol

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to quantify curcumin and [6]-shogaol using multiple reaction monitoring. Multiple reaction monitoring increased the overall sensitivity and selectivity of the analysis by monitoring the molecular ions of curcumin and [6]-shogaol, and their specific fragment ions (Zheng, Mandal, and Wishart, 2018). This was achieved by a triple quadrupole mass filter within the mass analyser, made up of Q1, Q2 and Q3 regions that specifically filter out any unwanted mass ions (Kutscher *et al.*, 2017). The curcumin (m/z 369.1) and [6]-shogaol (m/z 277.1) molecular ions move from Q1 towards Q2, which acts as a collision cell that fragments the molecular ions into intense fragments of m/z 177.2 and m/z 137.1 for curcumin and [6]-shogaol respectively. The Q3 region of the triple quadrupole specifically relays these fragments of interest towards the detector for quantification (Grebe and Singh, 2011).

The elution times of curcumin and [6]-shogaol was 1.13 and 1.32 min, respectively (Figure 4.1 A & B).

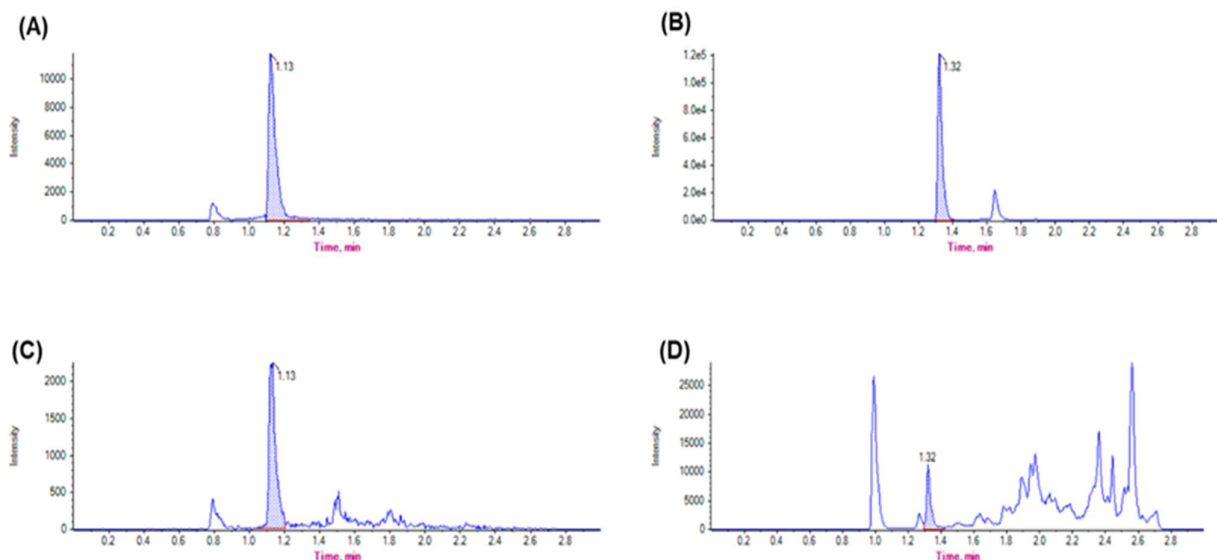


Figure 4.1: Mass chromatograms reporting elution times (min) for curcumin and [6]-shogaol in the curcumin standard solution (A), [6]-shogaol standard solution (B), turmeric extract (C), and ginger extract (D)

Both *cis* and *trans* [6]-Shogaol have the same mass transition (m/z 277→137.1), however the geometric isomerism of *trans* [6]-Shogaol made it slightly more hydrophobic than its *cis*-isomer and therefore eluted at a retention of 1.65 min (Figure 4.1 B). *Cis* [6]-shogaol was used for quantification since it was in a higher abundance than its *trans*-isomer. A standard concentration range of 62.5–1000 ng/ml was used to construct the calibration curve for the determination of both curcumin and [6]-shogaol in their respective extracts with regression values of 0.996 and 0.995, respectively (Table 4.1; Appendix 3). The calculated concentrations were within accordance with the guidelines set aside by the FDA (Food and Drug Administration, 2018) and EMA (Agency, 2011) for the acceptable accuracy criteria of a calibration curve to be used for quantification of an analyte. The guideline stipulates that the accuracy of the lowest calibrator (62.5 ng/ml) must be within 20% of its nominal value, and 15% for the other calibrators. The content of curcumin in the turmeric extract was found to be 231.17 mg/g, and the content of [6]-shogaol in the ginger extract was found to be 34.7 mg/g.

Table 4.1: Calculated concentration and accuracy of curcumin and [6]-Shogaol. Standard calibrators used for quantification

Sample	Analytic endpoint	Calibration range				
Standards	Nominal concentration (ng/mL)	62.5	125.0	250	500	1000
Curcumin	Calculated concentration	73.9	110.3	232.8	484.2	1036.4
	Accuracy	118.2	88.2	93.1	96.8	103.6
	R ²	0.996				
[6]-Shogaol	Calculated concentration	75.1	108.0	217.9	479.1	1041.4
	Accuracy	120.1	86.4	87.1	95.8	104.1
	R ²	0.995				

Normally, the content of curcuminoids in turmeric may vary between 2–9%, depending on different environmental conditions, such as the weather and climate conditions (Priyadarsini *et al.*, 2014). Previously, Gantait *et al.* (2011) determined the curcumin content in five different brands of turmeric powder. The different brands were extracted five times with 20 mL methanol. Subsequently, the content of curcumin was determined using the high-performance thin layer chromatography (HPTLC) method and obtained a very high curcumin content ranging from 21–34 % in their individual extract. In our study, a comparable percentage content was achieved. A total of 23% curcumin was found in the turmeric extract.

In addition, a 3.47% 6-shogaol content was quantified in the ginger extract powder. Wang *et al.* (2009) previously quantified various active ingredients in CO₂ supercritical fluid extracted ginger oleoresin and obtained a 6.4% 6-shogaol via the HPLC-VWD (high performance liquid chromatograph with a variable wavelength detector) method. Although the quantified amount of [6]-shogaol is remarkable lower in this study, super critical extraction will deliver a more accurate result, but it is a much more advanced and expensive extraction method.

4.3 Antiproliferation effects and potencies of turmeric and ginger extracts on non-cancer Vero cell line

The evaluation of cell viability is a crucial parameter to determine test samples' cytotoxic effects. The neutral red uptake assay is one of the most frequently used cytotoxicity assays, with a diverse range of biomedical and environmental uses (Repetto *et al.*, 2008). The neutral red uptake assay has proven itself time and again as reliable (Abd El-Wahab *et al.*, 2013, Gomez-Gutierrez *et al.*, 2021) and was used to perform all cell viability assays in this study. The measurements of cell viability are best described by the South African National Standards, which are based on the International Standard Organisation standards) (SANS, 2010). To interpret the potential

cytotoxicity of a sample, cell viability reduced to below 70% is considered to be cytotoxic (SANS, 2010). Additionally, we set a threshold of below 50% cell proliferation as severe cytotoxic.

4.3.1 Solvent and carrier control

Because Pheroid® has high lipid content; it cannot be administered at a too high concentration in *in vitro* studies. High lipid samples create an impenetrable layer on the culture media, which impedes gaseous exchange, resulting in the suffocation of cells (Chelopo *et al.*, 2017). To prevent a false positive result, different concentrations of Pheroid® were evaluated. Pheroid® from 0.1% to 0.025% proved to be too high in concentration and resulted in cell viabilities lower than 50% (Figure 4.2). This severely cytotoxic reaction was significantly different to the control ($p < 0.05$). However, 0.0125% and 0.00625% concentrations gave 129.05% and 148.88% cell viabilities, respectively, proving not to be cytotoxic. Therefore, the final Pheroid® concentration chosen was 0.0125%. This is because, at this concentration, we aimed to have a high enough carrier concentration, while preventing direct influence of the dilution media effect on the study's results. DMSO is a common solvent used to help dissolve test samples into cell culture media. Machana *et al.* (2011) dissolved various herbal plants, which was evaluated against the hepatocarcinoma (HepG2) cell line. At a final concentration of 1% v/v DMSO, cytotoxicity of less than 10% was observed in both HepG2 and Vero cells. In the current study, a DMSO concentration of 0.6% delivered a cell viability above 70% (83%) (Figure 4.2), which was used to completely dissolve turmeric and ginger. At this concentration, DMSO had no cytotoxic effects (Figure 4.2).

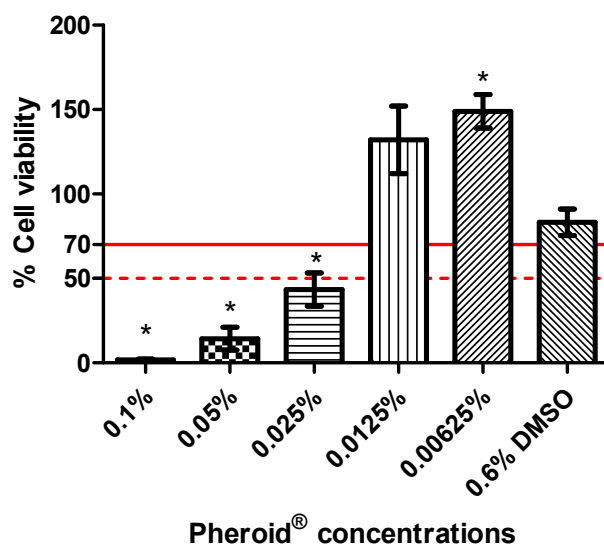


Figure 4.2: Percentage cell viability of Vero cells after a 24h treatment of the carrier (Pheroid®) at serial dilution concentration range (0.00625 – 0.1% v/v), and solvent (0.6% v/v DMSO) using the neutral red cellular uptake assay. All results are given in terms of the control. Asterisk (*) indicates significant differences ($p < 0.05$) compared to the control. The solid red line indicates 70% cell viability (threshold for cytotoxicity), and the dashed line indicates 50% cell viability (threshold for severe cytotoxicity)

Bekele *et al.* (2019) determined a working Pheroid® concentration of 0.02% also using the neutral red uptake assay. This was also tested against the non-cancer cell line, Vero cells. This corroborates the results obtained in Figure 4.2, as a similar working concentration of 0.0125% was obtained.

4.3.2 Extract concentration determination for formulation

The cell viability of unformulated turmeric (TE) and ginger (GE) extract dissolved in 0.6% DMSO were tested against Vero cells (Figure 4.3). For both extracts a dose dependent response was observed — sequential decrease in cell viability with increase in concentration. At 5 and 7 $\mu\text{g/mL}$, TE proved to be non-cytotoxic ($>70\%$) (Figure 4.3 A). At a concentration of 12.5 $\mu\text{g/mL}$, TE initially displayed cytotoxic (67%) and higher concentration (15–75 $\mu\text{g/mL}$) of TE resulted in severe cytotoxic effects ($<50\%$) (Figure 4.3 A). In Figure 4.3 B, GE proved to have non-cytotoxic effects in concentrations ranging from 5–12.5 $\mu\text{g/mL}$. At 15 $\mu\text{g/mL}$, GE started to deliver cytotoxic effects (64%). Severe cytotoxicity was seen when GE was administered at concentrations higher than 15 $\mu\text{g/mL}$ ($<50\%$) (Figure 4.3 B). All TE concentrations except for 5 $\mu\text{g/mL}$ and 7.5 $\mu\text{g/mL}$ concentrations displayed a statistically significant cytotoxicity compared to the control ($p<0.05$). On the other hand, only 50 $\mu\text{g/mL}$ concentration of GE gave a statistically significant cytotoxicity compared to the control ($p<0.05$).

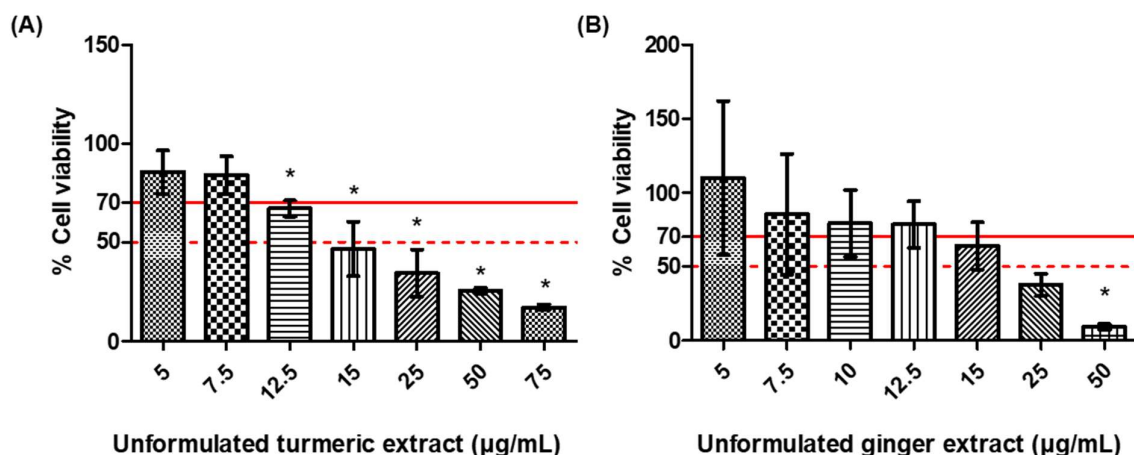


Figure 4.3: Percentage cell viability of Vero cells after a 24h treatment of unformulated concentrations of turmeric (A) and ginger (B) extract on Vero cells via the neutral red cellular uptake assay. All results are given in terms of the control. Asterisk (*) indicates significant differences ($p<0.05$) compared to the control. The solid red line indicates 70% cell viability (threshold for cytotoxicity) and the dashed line indicates 50% cell viability (threshold for severe cytotoxicity)

TE displayed a sharper decline in percentage cell viabilities when compared to GE. The TE had cytotoxic effects at a concentration of 12.5 $\mu\text{g/mL}$ and severe cytotoxicity was initially seen at a concentration of 15 $\mu\text{g/mL}$ (Figure 4.3 A). The GE started to display cytotoxic effects at 15 $\mu\text{g/mL}$.

Only at a much higher concentration (25 µg/mL) GE displayed severe cytotoxic effects. From these results it is evident that the turmeric extract is potentially more potent than the ginger extracts. This will be confirmed in the following section.

4.3.3 Inhibitory concentration (IC) determination

The cell viability as a function of log concentration of the TE and GE with their linear equation is calculated in Figure 4.4. The resulting coefficient of determination (R^2) of TE and GE was 0.9230 and 0.9803, respectively. This indicates a strong correlation between the results obtained and the graph model (Senthilnathan, 2019).

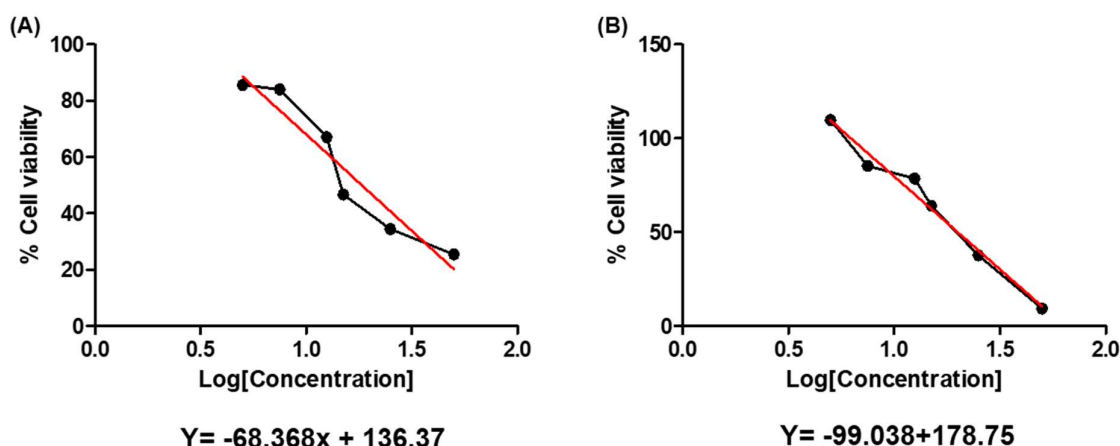


Figure 4.4: Vero cell viability log graphs for turmeric extract (4.3 A) and ginger extract (4.3 B) with their linear equations, respectively, which was used to determine the different inhibitory concentrations (IC_{25} , IC_{50} and IC_{75}). The cell viability assays were done via the neutral red cellular uptake assay after 24h exposure to different treatments

The linear equation of each graph (Figure 4.4 A & B) was calculated and used to determine the IC_{25} , IC_{50} and IC_{75} values of each extract (Figure 4.4). The IC_{25} of TE and GE were calculated to be 7.90 and 11.16 µg/ml, respectively. Inhibition at 50% was achieved at 18.34 µg/ml for turmeric extract and 19.95 µg/ml for ginger extract. The final increment, 75% cell proliferation inhibition, was reached at 42.56 µg/ml TE and 35.68 µg/ml ginger extract. These concentration values were then applied and formulated into pro-Pheroid® in different combinations (Table 4.3).

Table 4.2: Calculated inhibitory concentration (IC₂₅, IC₅₀ and IC₇₅) using predetermined x-and y values. The final concentration (µg/mL) of the active ingredient in turmeric extract (curcumin) and ginger extract ([6]-shogaol) are reported

Turmeric extract	Y-values	X-value	Concentrations (µg/ml) (Antilog X)	Concentration curcumin (µg/ml)
IC ₂₅	75	0.898	7.90	1.83
IC ₅₀	50	1.227	18.34	4.24
IC ₇₅	25	1.592	42.56	9.84
Ginger Extract				Concentration [6]- shogaol (µg/ml)
IC ₂₅	75	1.05	11.16	1.55
IC ₅₀	50	1.30	19.95	2.77
IC ₇₅	25	1.55	35.68	4.95

Wiji Prasetyaningrum *et al.* (2018) synthesized and evaluated the effects of curcumin and its derivatives against various cell lines including the Vero and MCF-7 cell lines. In that study, they proved that curcumin was more cytotoxic towards the MCF-7 cancer cell line than the Vero cells, obtaining IC₅₀ values of 3.80 µg/mL and 11.41 µg/mL, respectively. Although the IC₅₀ value against the Vero cell line obtained by Wiji Prasetyaningrum *et al.* (2018) was lower when compared to the current study, it is known that turmeric extracts consist of many active constituents but in small quantities (Edwards *et al.*, 2020). Tyagi *et al.* (2015) identified β-sesquiphellandrene, a compound found in turmeric (*Curcuma longa*), which displayed anti-proliferative effects against various cancer cell lines (human multiple myelomas: U266 and MM.1), human colon cancer (HCT116), human non-small cell lung cancer cell line (A549), and human chronic myeloid leukaemia (KBM-5) cells), thus confirming that curcumin is not the only anticancer agent found in turmeric. The ginger extract proved to be quite potent (IC₅₀=19.95 µg/ml) against the Vero cell line in this study. A comparable value of 9.68 µg/mL was obtained when Osman *et al.* (2020) evaluated an ethanolic ginger extract's anticancer activity against the MCF-7 breast cancer cell line. Ahmed, Gonda and Hunyadi, (2021) proved that semisynthetic [6]-shogaol was non-cytotoxic against normal human fibroblast cells (TIG-1 cells) compared to cancer cell lines. In addition, Sakpakdeejaroen *et al.* (2009) showed that [6]-shogaol had an IC₅₀ value of 9.8 ± 2.37 µg/mL against the MCF-7 cancer cell line. In another study by Mahomoodally *et al.* (2021), a new derivative of [6]-shogaol, (Z)-6-oxo-[6]-shogaol delivered a very low IC₅₀ value against the MCF-7 breast cancer cell line (1.73 µg/mL). The increased potency of ginger extracts compared to the isolated active [6]-shogaol, can be attributed to the complex mixture of bio-actives present in the extract. The literature above compared with our results, supports that ginger, and its actives have more anti-proliferative effects in cancer cells rather than in normal cells. Finally, when

comparing the IC₅₀ values of the current study and literature, it may be concluded that the turmeric extract (TE) is the more potent phytochemical overall, when compared to the ginger extract (GE). The different inhibition concentrations calculated for the turmeric and ginger extracts were used in various combinations and formulated in Pheroid® (Table 4.3) and was characterized.

Table 4.3: Determining Formulations with various combined concentrations of ginger (GE) and turmeric (TE) extracts

Formulation	IC combination formulation	Weighed off ingredients			
		Ginger extract (g)	6-shogaol (mg)	Turmeric extract (g)	Curcumin (mg)
A	GE(IC ₇₅):TE(IC ₂₅)	1.427	49.52	0.316	73.05
B	GE(IC ₅₀):TE(IC ₂₅)	0.798	27.69	0.316	73.05
C	GE(IC ₂₅):TE(IC ₂₅)	0.446	15.48	0.316	73.05
D	GE(IC ₇₅):TE(IC ₅₀)	1.427	49.52	0.734	169.69
E	GE(IC ₅₀):TE(IC ₅₀)	0.798	27.69	0.734	169.69
F	GE(IC ₂₅):TE(IC ₅₀)	0.446	15.48	0.734	169.69
G	GE(IC ₇₅):TE(IC ₇₅)	1.427	49.52	1.702	393.47
H	GE(IC ₅₀):TE(IC ₇₅)	0.798	27.69	1.702	393.47
I	GE(IC ₂₅):TE(IC ₇₅)	0.446	15.48	1.702	393.47

4.4 Formulation characterisation

The particle size was measured to evaluate the size distribution of particles in each formulation. Additionally, the zeta potential of each formulation was determined, which acts as a measurement for formulation stability. The particle size distribution and zeta potential of Formulation A–I is reported in Table 4.4. The vesicle morphology is reported in Figure 4.6.

4.4.1 Particle size distribution and zeta potential

All formulations displayed bimodal distributions (Figure 4.5 A–I). The d_{0.1} for all the formulations displayed similar results and measured below 80 nm. The d_{0.5} value of all the formulations measured between 1.30 (Formulation E) and 1.89 µm (Formulation B).

Table 4.4: Particle size distribution, span and zeta potential of Pheroid® Combination formulations

Combination formulation	d _{0.1} (µm)	d _{0.5} (µm)	d _{0.9} (µm)	Span	Vol. Weighted Mean D [4,3]:	Zeta potential mV ± SD
A	0.073	0.174	1.893	10.446	0.807	-27.1 ± 11.2
B	0.074	0.189	1.762	8.37	0.703	-27.1 ± 23.3
C	0.070	0.149	1.666	10.720	0.842	-19.2 ± 6.92
D	0.071	0.139	1.450	9.921	0.438	-25.9 ± 6.5
E	0.071	0.130	1.367	9.530	0.4	-25.7 ± 5.71
F	0.066	0.138	1.263	8.650	0.386	-25.2 ± 13
G	0.072	0.133	1.313	9.321	0.373	-32.4 ± 4.26
H	0.076	0.134	1.279	8.960	0.363	-27.5 ± 4.81
I	0.064	0.126	0.965	7.126	0.279	-32.3 ± 5.84

For the 90th percentile (d_{0.9}) of the particle size distribution, the formulations delivered results ranging from 0.965 µm (formulation I) to 1.893 µm (formulation A). All the formulations displayed a span value higher than seven. The wide span at all samples is due to the bimodal distributions (Figure 4.5).

The zeta potential of the formulations ranged between -19 mV and -33 mV (Table 4.4). From literature, there are different parameters to indicate zeta potential stability. According to Suganthi and Rajan, (2012), nano-dispersions obtaining a zeta potential of ±30 mV are stable. In addition, Jokerst *et al.* (2011) reported that if formulations display a zeta potential greater than ±10 mV, they can be considered to be stable — since there are more inter-particle repulsion and agglomeration was unlikely. Bogdan *et al.* (2011) indicated that colloidal dispersions having a zeta potential of ±25 mV were also considered to be stable. Thus, there is no clear standardised threshold of zeta potential to indicate stability. We used a range derived from Bogdan *et al.* (2011) and Suganthi and Rajan, (2012) of ±25 to ±30 mV as stable dispersions.

The mean zeta potentials of all the formulations except for Formulation C (-19.2±6.92) was within the stable range (Table 4.4). It is also to be noted that this formulation had the widest span in particle size (10.720). Formulation D–F indicated zeta potentials of -25.9, -25.7, -25.2 mV, indicating less stable comparing to formulations A, B, G, H, I. However, according to Jokerst *et al.* (2011) and Bogdan *et al.* (2011), these formulations can still be considered stable in terms of their thresholds.

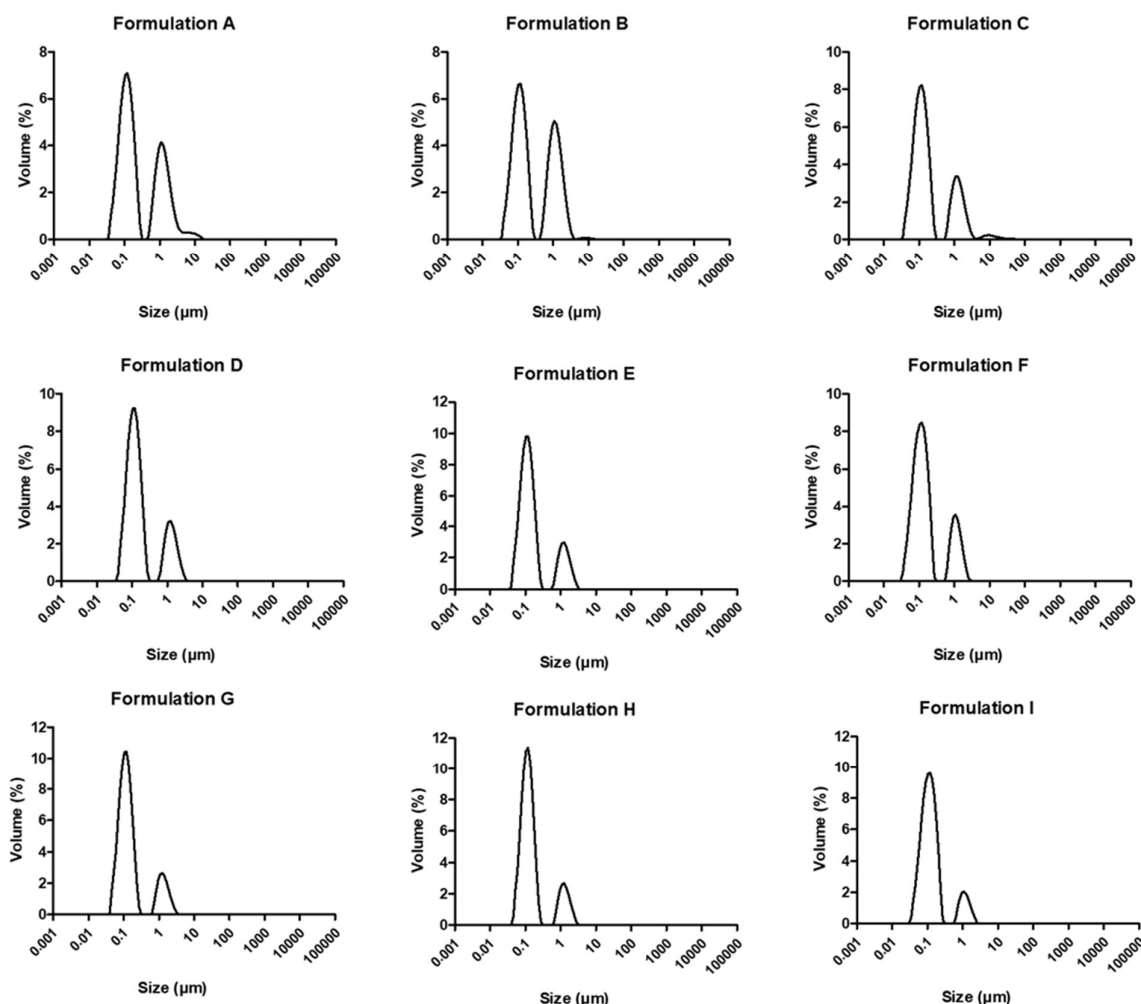


Figure 4.5: Particle size distribution for Pheroid® formulations A-I using the Malvern 2000 Mastersizer. Measurements were calculated as an average of triplicate readings for each formulation

Other studies that used the Pheroid® drug delivery system for oral use obtained comparable zeta potentials. Kleynhans *et al.* (2019), investigated the oral toxicity of Pheroid® in rodents and reported an electrokinetic potential of -23.3 ± 0.8 mV. Erasmus, (2020), used the same drug delivery system to improve the delivery of a peroral cannabinoid, and reported a zeta potential of 32.90 ± 1.13 mV. The results in these previous studies corresponds with the resulting range obtained in this study (-19 – -32 mV). Other Pheroid® related research (Du Plessis *et al.*, 2010, Steyn *et al.*, 2010, Krause, Grobler and Goldring, 2015) did not report particle size distributions nor zeta potential analysis.

4.4.2 Vesicle morphology

Confocal images were obtained as quality control in characterization. Figure 4.6 shows both a confocal micrograph (A) and a light micrograph (B) of formulation I (all formulations exhibited similar morphology and therefore one formulation's (I) were reported as representative sample). Formation of vesicles was confirmed as indicated in Figure 4.6 A. The incorporation of the phytochemicals was qualitatively confirmed (Figure 4.6 B). In addition, different vesicle sizes were seen (Figure 4.6 A & B), which confirms the results reported in Table 4.3 and Figure 4.5. From all samples, no coagulation between vesicles was seen (Appendix 4), which supports the formulations' stability results in Table 4.4.

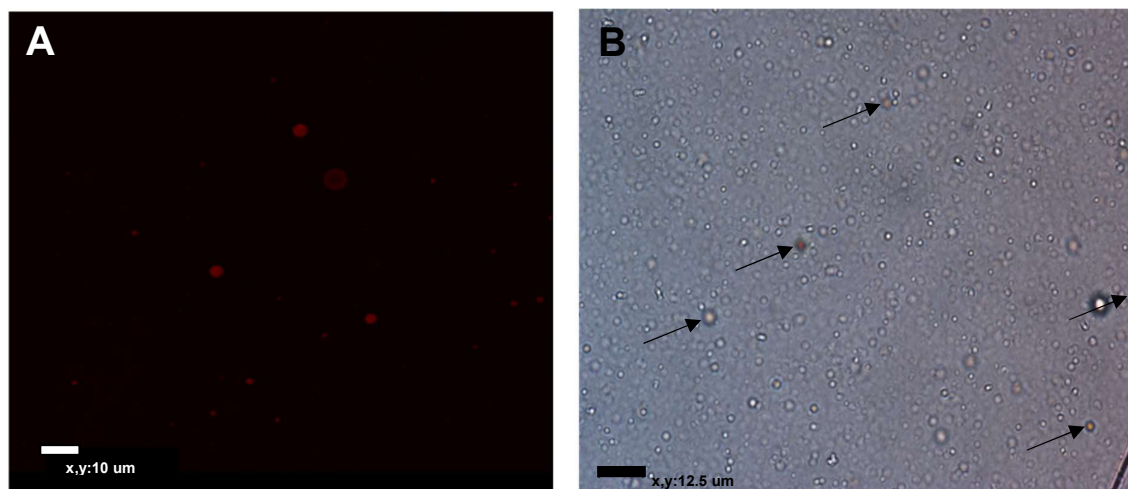


Figure 4.6: Confocal (A) and light micrograph (B) of Pheroid® formulations (representing image used of Formulation I) obtained by using Nikon D-Eclipse C1 Confocal inverted microscope TE 2000-fitted with a Helium–Neon (543 nm) polarized laser. Nile red was used as the fluorescence marker (excitation: 515–560 nm, emission: >590 nm). The Scale bar represents 10 μm of micrograph A, and 12.5 μm of micrograph B. All arrows in light micrograph B indicates the entrapment of phytochemicals in Pheroid®

4.5 Anticancer effects of turmeric and ginger combination formulations against MCF-7 breast cancer cells, relative to non-cancer Vero cells

The cell viability of different combination Pheroid® formulations were assessed against the MCF-7 and Vero cell lines to determine the formulations' anticancer properties against the cancer line, and the non-cytotoxicity against the non-cancer cells. Formulation A showed weak cytotoxic effects against the Vero cell line (63% viability) (Figure 4.7 A). Formulations B–F were proven to be non-cytotoxic (>70% viability). Severe cytotoxicity was seen after treatments with Formulations G–I (22%, 18%, and 15.47% viability, respectively) (Figure 4.7 A). When these same formulations were tested against the MCF-7 cancer cell line (Figure 4.7 B), formulations A–C proved to be non-cytotoxic (77%, 105%. and 79.53% viability, respectively). Formulation D–I resulted to be severely cytotoxic (<50%). No clear dose response was seen for the Vero cells after the treatments

(Figure 4.7 A), but when the treatments were administered to the cancer line a steep decline in percentage cell viability was observed (Figure 4.7 B).

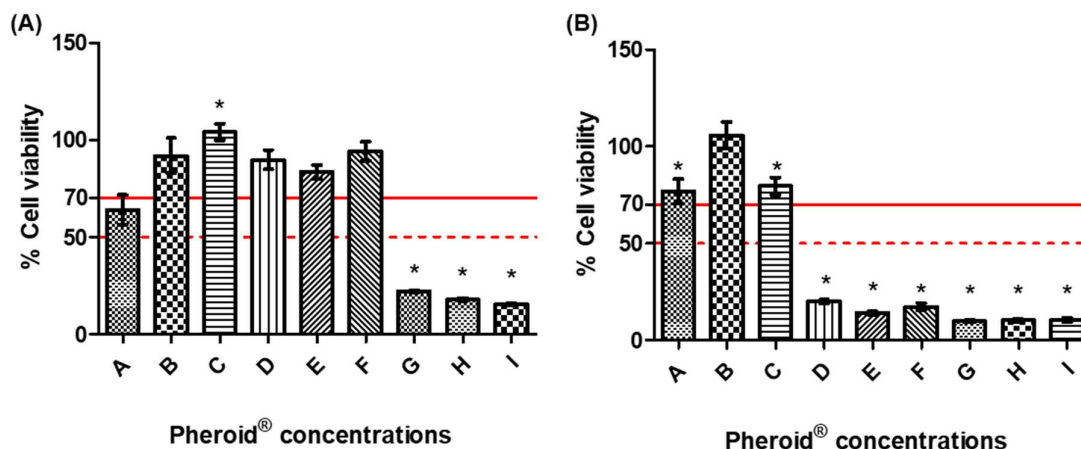


Figure 4.7: Percentage cell viability of Pheroid® formulations tested against the non-cancer cell line, Vero cells (A) and the breast cancer cell line, MCF-7 (B) using the neutral red cellular uptake assay after cells were treated for 24h. All results are given in terms of the control. Asterisk (*) indicates significant differences ($p<0.05$) compared to the control. The solid red line indicates 70% cell viability (threshold for cytotoxicity), and the dashed line indicates 50% cell viability (threshold for severe cytotoxicity). Formulation A-I were formulated based on their GE and TE potencies. All formulations were formulated in their respective inhibitory concentration combinations (GE:TE). Formulation A (75:25), B (50:25), C (25:25), D (75:50), E (50:50), F (25:50), G (75:75), H (50:75), I (25:75)

Formulation B and C did not have an effective cell proliferation inhibition in any cell line. Although Formulation A was cytotoxic against the Vero cell line (Figure 4.7 A), the same result was not achieved against the cancer cell line (Figure B). Formulation G–I had the same severe cytotoxic result in both cell lines (Figure 4.7 A & B). These severe cytotoxic effects may be contributed to the high turmeric extract content (Table 4.3) containing the more potent constituent, curcumin. Tabatabaei Mirakabad *et al.* (2016) evaluated the cytotoxic effects of pure curcumin and curcumin loaded in the PLGA-PEG-PLGA (poly dl-lactide-co-glycolide-b-ethylene glycol-b-dl-lactide-co-glycolide) drug delivery system against the MCF-7 breast cancer cell line. In that study, it was confirmed that by using the drug delivery systems, a more cytotoxic effect was achieved. This corresponds with the results obtained in the current study that the majority of the Pheroid® formulations also displayed high levels of cytotoxicity against the MCF-7 breast cancer cell line (Figure 4.7 B).

Interestingly, Formulation D–F displayed opposite effects in the MCF-7 cells compared to the Vero cells (Figure 4.7 A & B). This indicates that the phytochemicals formulated at these concentrations have the ability to be severely cytotoxic towards the cancer cell line but not towards the normal cells. To interpret the results obtained in Figure 4.7 the cancer selectivity index was calculated.

4.6 Cancer Selectivity Index

The Cancer Selectivity Index (CSI) is an indication whether a treatment is more toxic to cancer cells than to normal cells. The CSI is based on the principle of the ratio of IC_{50} 's of the normal cells against the cancer cells (Peña-Morán *et al.*, 2016). In our study, we modified the approach to determine the CSI. Since the inhibitory concentrations (IC_{25} – IC_{75}) of both the turmeric and ginger extracts are already incorporated into the formulations, the percentage cell viabilities per formulation — from Figure 4.5 — served as the endpoint used to calculate the ratio. The resulting ratios are reported in Table 4.5.

Table 4.5: The cancer selectivity index of the combination formulations (Formulations A–I) of turmeric extract and ginger extract in Pheroid® calculated from the inhibitory effect in MCF-7 breast cancer cells and Vero cells (non-cancer cells). CSI values greater than 1 given in bold

Formulation	CSI
A	0.83
B	0.87
C	1.31
D	4.48
E	5.97
F	5.43
G	2.20
H	1.72
I	1.46

4.7 Apoptotic effects of turmeric and ginger combination formulations on MCF-7 breast cancer cells

The apoptosis assay was done to deduce whether the decrease in cell viability reported in Figure 4.7 B was due to apoptosis or necrosis (i.e. mechanism of cell death). The apoptosis assay was performed via the double staining method using ethidium bromide and acridine orange. Figure 4.8–4.10 displays the quantitative results obtained from the flow cytometer.

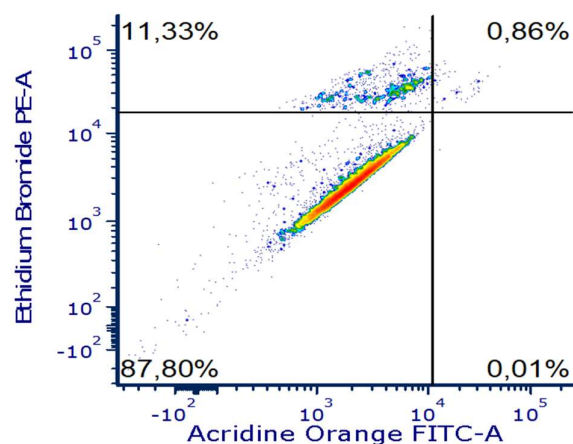


Figure 4.8: Representative scatter graph for the negative control group, displaying the percentage of MCF-7 cell population in various apoptotic phases after 24h culturing in plain DMEM. The flow cytometry analysis was measured with acridine orange (AO) and ethidium bromide (EtBr). The emission of AO and EtBr was detected with a 525/20-nm filter (FL1) and a 635/20-nm filter (FL3), respectively, after excitation at 488 nm. The lower left quadrant (Q1), represents the percentage viable cells, the upper left quadrant (Q2) represents the percentage early apoptosis, the upper right quadrant (Q3) represents the percentage apoptotic cells and the lower right quadrant (Q4), the percentage late apoptotic/necrotic cells

In Figure 4.8 the predominant number of cells are viable (87.8%). Early apoptosis was detected to a lesser extent (11.33%), compared to the viable cells in the control group. Apoptosis and late apoptosis/necrosis were detected in less than 1% percent of cells (Figure 4.8). In a study by Sun, Lin and Chang, (2016), the apoptotic effects of colchicine in MCF-7 cells were evaluated. In that study, the negative control displayed 6.6% and 7.3% apoptosis after 24 hours and 48 hours, respectively. Thus, it is expected that apoptosis may be seen in a lesser extent in the negative control group, as it is a normal cell process.

According to Akkoç *et al.* (2015), curcumin induces apoptosis weakly alone, thus the total apoptotic effects obtained was the result of both turmeric and ginger extract, which was not expected to be very rapid. Apoptosis in MCF-7 cells was the greatest after exposure to Formulation A–C, where Formulation B displayed the highest rate of apoptosis (Figure 4.9 & 4.10). Formulation D and E induced early apoptosis the most effective, with Formulation E leading in this result (Figure 4.9 & 4.10).

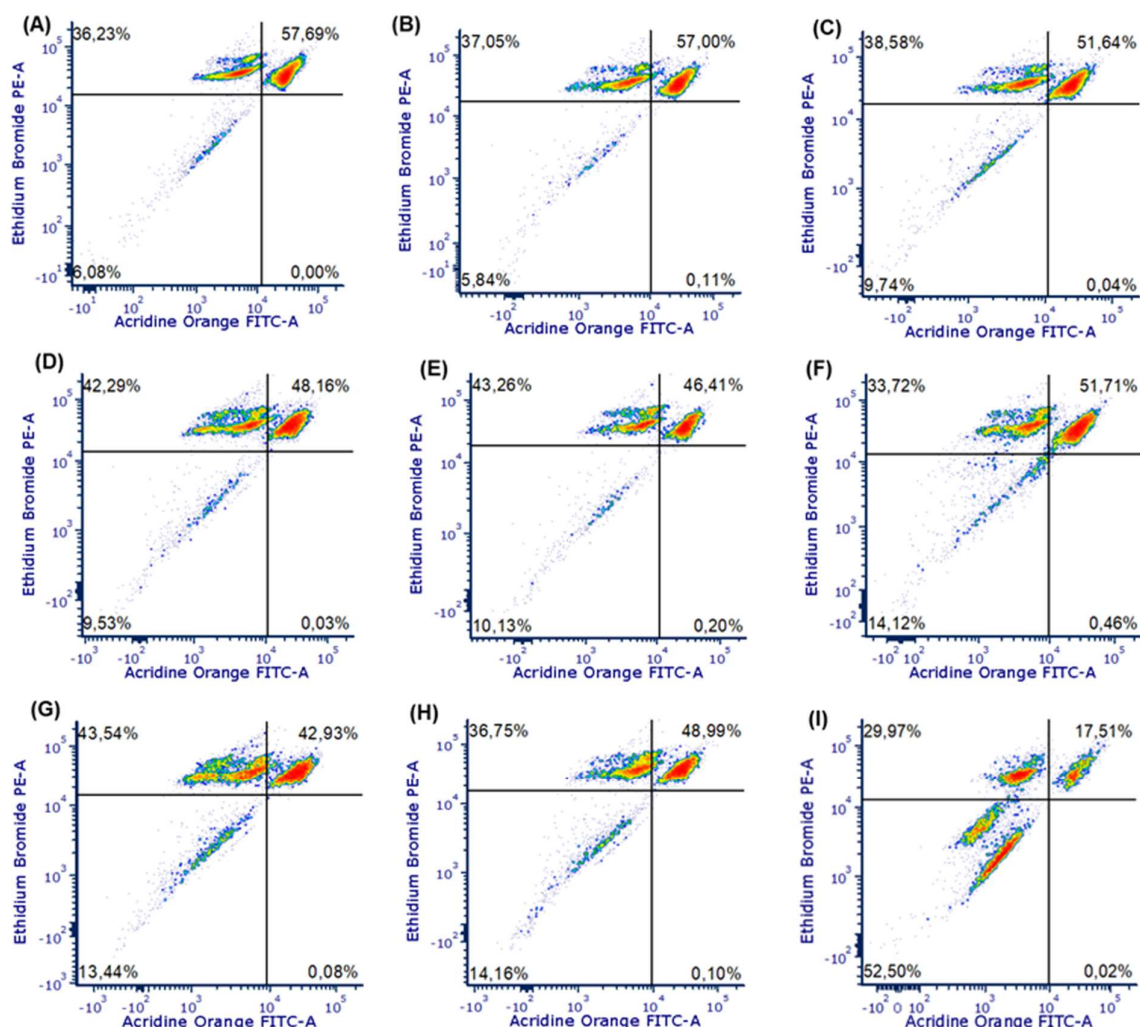


Figure 4.9: Representative scatter graphs displaying the percentage of the MCF-7 cell population in various apoptotic phases after a 24h exposure to formulation A–I. The flow cytometry analysis was done with acridine orange (AO) and ethidium bromide (EtBr). The emission of AO and EtBr was detected with a 525/20-nm filter (FL1) and a 635/20-nm filter (FL3), respectively, after excitation at 488 nm. The lower left quadrant (Q1), represents the percentage viable cells, the upper left quadrant (Q2) represents the percentage early apoptosis, the upper right quadrant (Q3) represents the percentage apoptotic cells and the lower right quadrant (Q4), the percentage late apoptotic/necrotic cells

Late apoptosis/necrosis (Q4) was not induced by any of the formulations (Figure 4.9 & 4.10). Although most of the cell bulk analysed were measured in the early apoptotic (Q2) and apoptotic (Q3) phases, a large percentage of cells in the early apoptotic stage is very close to the border of the apoptotic phase (Figure 4.9). This indicates that the cells in the early apoptotic cells are transitioning into the apoptotic state. The total combined percentage of cells in Q2 and Q3 ranged from 47% (Figure 4.9 I) to 94% (Figure 4.9 B). Thus, all formulations, except Formulation I indicated high levels of apoptosis (Figure 4.9).

When cells are in their early phase of apoptosis, cells start to decrease in size and pyknosis initialise (Elmore, 2007) indicating the cell proliferation to decrease. Zhang *et al.* (2018) confirmed

that flavonoids, like curcumin (Meng *et al.*, 2014), inhibit cell proliferation and induce apoptosis through various signalling pathways. This corresponds with the results obtained in Figure 4.7–4.10 that Pheroid® formulations of turmeric and ginger extract do inhibit cell proliferation and induce apoptosis. In a study conducted by Lv *et al.* (2014), curcumin at the highest concentration (100 µM) displayed lower than 50% apoptosis after a 48h treatment period against the MCF-7 and the MDA-MB-231 breast cancer cell line. Although in apoptosis in the current study was evaluated after a 24h treatment period, similar apoptotic effects (Q3) were observed. Furthermore Ray, Vasudevan, and Sengupta, (2015) showed that [6]-shogaol induced apoptosis more effectively over a period of 48 hours than 24 hours. An in-house study by Bekele *et al.* (2019) evaluated the apoptotic effects of Pheroid® formulated and unformulated combination of Meriva® and ginger extract. It was proved that the combination of Meriva and ginger extract (52.5/75 µg/mL) formulated in Pheroid® induced apoptosis much more effectively, when compared to the same concentration of the components when not formulated. It can thus be concluded that the combined effects of Pheroid® formulated turmeric and ginger extract induce apoptosis much quicker when apoptosis was evaluated for each unformulated component separately.

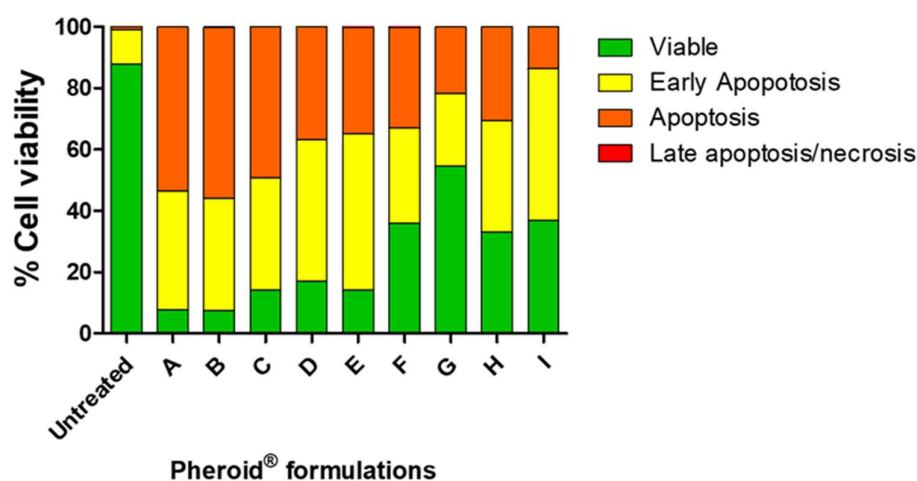


Figure 4.10: Percentage composition of different induced apoptotic phases in MCF-7 cells after a 24h exposure to Formulation A–I. The flow cytometry analysis was done with acridine orange and ethidium bromide

From the results obtained and reported in Figure 4.7–4.10 and Table 4.5, Formulations D–F showed the most promising effects. These formulations have shown to be non-cytotoxic to Vero cells and severely cytotoxic to MCF-7 cells (Figure 4.7). These three formulations have also indicated the highest calculated CSI value (4.48, 5.97 and 5.43, respectively). Additionally, these formulations showed promising effects in the apoptosis assay indicating sufficient early apoptotic

and apoptotic effects. Thus, formulations D, E, and F were tested selected to as candidates for the in vivo study. These formulations where then used to test for oestrogen receptor inhibition.

4.8 Potential oestrogen receptor inhibition by turmeric and ginger combination formulations

Oestrogen has proliferative effects on breast cancer cells mediated by the ER α (oestrogen receptor alpha) in ER α -positive breast cancer cell lines such as MCF-7 (Liao *et al.*, 2014) and plays an integral role in its cell functions. Thus, the oestrogen receptor inhibition assay was completed to determine whether the apoptosis resulted from the inhibition of the ER α . The inhibition of the ER is reported in Figure 4.11. None of the treatments displayed effective ER inhibition.

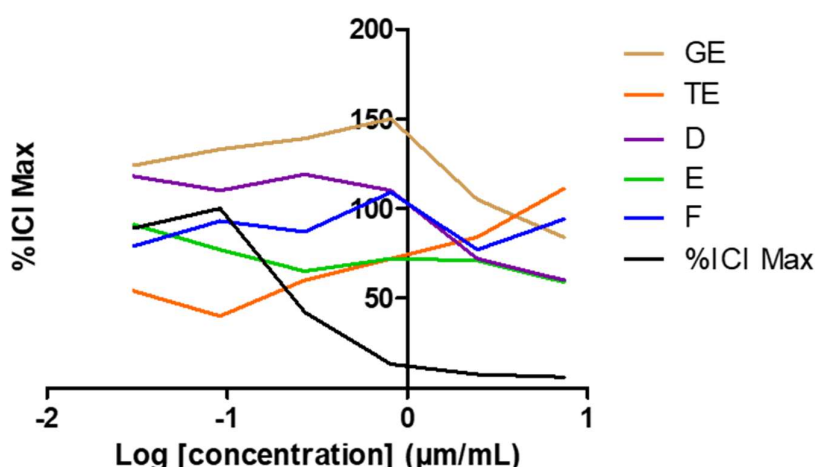


Figure 4.11: Oestrogen receptor inhibition in TK47-KBluc cells after 24h exposure to candidate formulations, where GE represents ginger extract, TE represents turmeric extract. Formulations were formulated in their respective inhibitory concentration combinations (GE:TE). Formulation D (75:50), E (50:50), F (25:50)

Hallman *et al.* (2017) evaluated the effects of curcumin on the ER (oestrogen receptor) and discovered that curcumin down regulates ER α proteins significantly. This result was not obtained in our study (Figure 4.11) since the formulations tested contained turmeric extracts and not pure curcumin as in the case of Hallman *et al.* (2017). In another study, Sharif *et al.* (2018) simulated and used computer analysis to test the ability of [6]-gingerol and [6]-shogaol to bind and inhibit the ER α . Both components displayed binding and inhibition of the receptor with 6-gingerol leading in the effect. It was also concluded that these components did not inhibit the receptor sufficiently enough to replace tamoxifen as treatment for breast cancer induced by hormonal replacement therapy. Thus, it can be concluded from our results and literature that the turmeric and ginger extracts' actives have effects on the ER α , but it will not be sufficient interaction to induce apoptosis, seen in section 4.5. This interaction will rather assist with inhibition of cell proliferation.

4.9 Determination of the final formulation to be administered during *in vivo* study

The final formulation that was chosen for the *in vivo* study was based on the results obtained in the *in vitro* study. This formulation needed to provide sufficient evidence to be non-cytotoxic to normal cells, while effectively inhibiting cell proliferation in the cancer cell line. This formulation should also have a relative high cancer selective index, confirming to be more cytotoxic to cancer cells rather than normal cells. Furthermore, this formulation had to provide evidence of inducing apoptosis. In addition, if this formulation had promising effects in inhibiting the oestrogen receptor, the result of that assay would support the efficacy of the specific formulation.

Formulation E met the above-mentioned criteria the best. This formulation proved to be non-cytotoxic to Vero cells (Figure 4.7 A) and severely cytotoxic to MCF-7 cells (Figure 4.7 B). This formulation has also obtained the highest cancer selective index (5.97) (Table 4.5), proving to be severely cytotoxic to cancer cells rather than normal cells. Although this formulation did not induce the highest overall apoptotic effects, it still induced sufficient evidence of early apoptosis (43%) and apoptotic (46%) effects (Figure 4.7 & 4.8). This indicates that the predominant number of cells are in the apoptotic phases pertaining anticancer effects. Finally, no formulations displayed effective oestrogen receptor inhibition at the tested concentrations. This confirms that Formulation E is a suitable choice for the *in vivo* study.

4.9.1 Formulation and characterisation of turmeric-ginger final formulation for animal study

Formulation E was freshly formulated and characterised for the purpose of the animal study and will be referred to as Turmeric-Ginger final formulation (TGFF).

4.9.1.1 Particle size distribution and zeta potential

The stability of the formulation was measured by assessing the particle size distribution and zeta potential. The results were reported in Table 4.6. The formulation displayed a single peak distribution (monomodal). The particle size distribution at $d_{0.1}$, $d_{0.5}$, $d_{0.9}$ were measured at 1.390, 3.405 μm and 7.292 μm , respectively.

Table 4.6: Particle size distribution, span and zeta potential of TGFF

Formulation	$d_{0.1}$ (μm)	$d_{0.5}$ (μm)	$d_{0.9}$ (μm)	Span	Vol. Weighted Mean D [4,3]:	Zeta potential		
						mV	$\pm$	SD
Formulation TGFF	1.390	3.405	7.292	1.734	3.950	-36.5	$\pm$	5.68

The formulation indicated uniformity with a low span value of 1.734 (Table 4.6). According to Jokerst *et al.* (2011), Bogdan *et al.* (2011) and Suganthi and Rajan, (2012), this formulation

proved to be very stable. There are a few differences between the characterisation results for Formulation E (Table 4.4 & Figure 4.5) and TGFF (Table 4.6 & Figure 4.12).

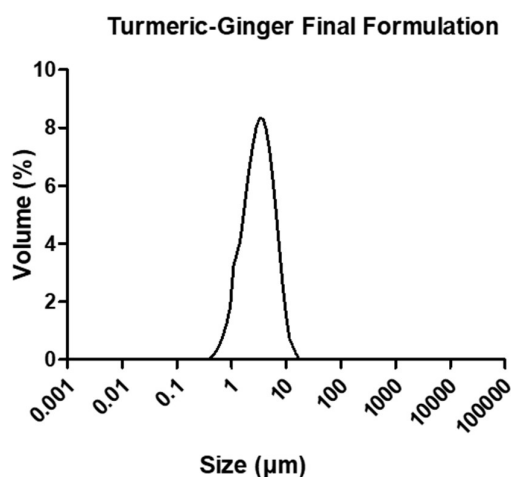


Figure 4.12: Average particle size distribution for TGFF using the Malvern 2000 Mastersizer. Measurements were calculated as an average of triplicate readings for each formulation

Firstly, there is a bimodal distribution in Figure 4.5 and a monomodal distribution Figure 4.12, which is corroborated in Table 4.4 with a much wider particle size span (9.530) than in Table 4.6 (1.734). Although, a bimodal distribution (Figure 4.5) was initially obtained, a similar effect was seen in the previous study (Bekele *et al.*, 2019). In this study a bimodal distribution and a monomodal distribution was obtained when Pheroid® and phytochemical loaded Pheroid® was evaluated, respectively. This indicates some intra study variations.

4.9.1.2 Vesicle morphology

As previously stated, confocal images were obtained for the purpose of quality control in characterisation. Figure 4.13 shows both a confocal micrograph (A) and a light micrograph (B) of the Final Formulation. No coagulation between vesicles was witnessed, which indicates the formulation's stability and corroborated the results given in Table 4.6.

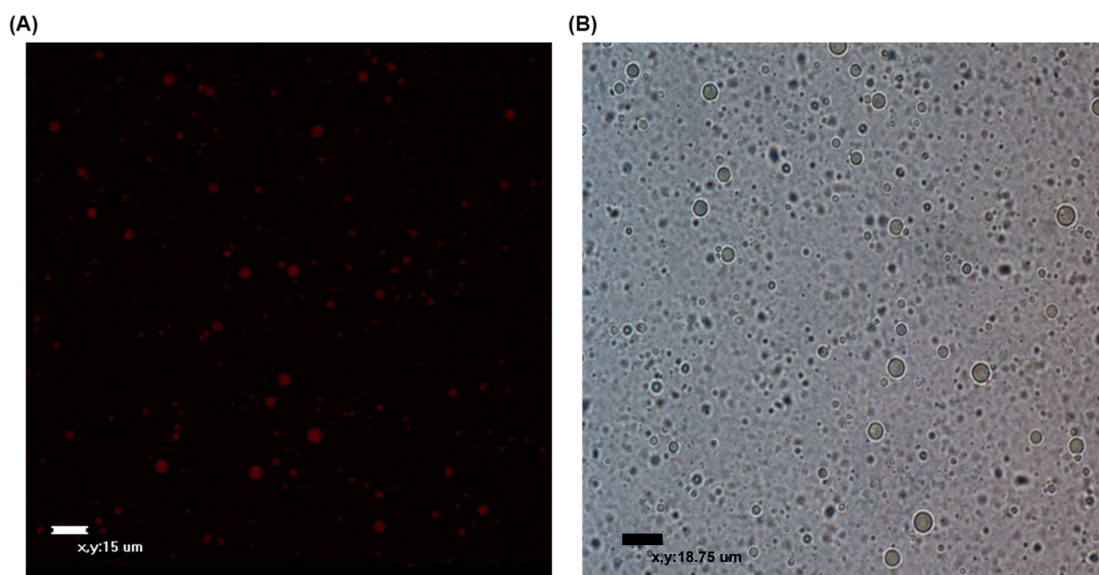


Figure 4.13: Confocal (A) and light micrograph (B) of TGFF obtained by using Nikon D-Eclipse C1 Confocal inverted microscope TE2000-fitted with Helium–Neon (543 nm) polarized laser. Nile red was used as the fluorescence marker (excitation: 515–560 nm, emission: >590 nm). Scale bar represents 15 μm of micrograph A, and 18.75 μm of micrograph B

The final formulation indicated to be highly stable with sufficient entrapment of active ingredients in Pheroid® according to our criteria, which indicates that this formulation is suitable for the *in vivo* study.

4.10 Animal study

Twenty-two female nude mice were obtained from the AAALAC accredited DSI/NWU Preclinical Drug Development Platform Vivarium. These animals were between six and eight weeks old. The animal study was approved by the NWU AnimCare REC (NWU-00533-20-A5; Appendix 5). The animals were maintained as described in the materials and methods section (cf. 3.13.1 Experimental animals and husbandry). The inoculation of the animals was according to SOP_Viv_Anim 30. In short, a total of 22 female athymic nude mice were inoculated with 2.5×10^6 cells/animal into the right flank. As per the study design, tumour growth in all animals were checked every second day. However, after 21 days post-inoculation, no tumours developed, and no animals were enrolled into the study. Thus, this part of the project could not be completed. All relevant monitoring sheets in terms of supplementation and inoculation are given in Appendix 2.

The MCF-7 xenograft mouse model is a well-established model used for breast cancer studies (Du *et al.*, 2017, Li *et al.*, 2017 and Wang *et al.*, 2020). Factors that may affect the onset of tumours include cell type, cell densities, animal age, supplementation needed. Oestrogen supplementation is needed for MCF-7 xenograft formation (Salem *et al.*, 2018). Oestrogen supplementation can be administered through various methods.

This includes injections (Salem *et al.*, 2018), oestrogen supplemented drinking water (Bihani *et al.*, 2017) and pellets (Zhao *et al.*, 2017), as well as MedRod implants, which delivers constant daily oestrogen serum levels (Konings *et al.*, 2018). Cell densities are often the major factor for tumour onset. The cell densities used in this study, were based on in-house model establishment (Establishment of tumour-bearing rodent models, NWU-00254-17-A5). The cell densities used was supported by literature (Dall *et al.*, 2015 and Moon, *et al.*, 2008). However, other studies suggest that a much higher cell density should be inoculated (Table 4.7).

Table 4.7: Various cell densities of MCF-7 breast cancer cells used for xenograft studies. The onset days and palpable tumour size are also reported

MCF-7 cell density inoculated/animal	Days of onset before palpable tumour sizes were reached	Study specific palpable tumour size	Reference
5x10 ⁶	±14	50 mm ³	Haggag <i>et al.</i> (2020)
5x10 ⁶	21	200 mm ³	VanWeelden <i>et al.</i> (1998)
1x10 ⁷	42	~70 mm ³	Swami <i>et al.</i> (2011)
1x10 ⁷	28	831–3,080 mg	Johnson <i>et al.</i> (2013)
1x10 ⁷	12	75–80 mm ³	Jiang <i>et al.</i> (2019)
4x10 ⁷	21	72–128 mm ³	Peng <i>et al.</i> (2015)

Table 4.7 shows that successful tumour growth can be achieved with higher cell densities of MCF-7 cells were inoculated, compared to our study. The use of higher inoculation densities however is not always guaranteed. Swami *et al.* (2011), inoculated ten million cells and only achieved tumours roughly 70 mm³ (Table 4.7). These tumours took up to 42 days to form, whereas Haggag *et al.* (2021) had palpable (50 mm³) in 14 days (Table 4.7). The MCF-7 breast cancer cell line is known to be a slow growing cell line and to be unpredictable in xenograft studies (Table 4.7). In our study, even with an oestrogen supplementation, no palpable tumours developed after inoculation. From Table 4.7, it is evident that a cell density of around 1x10⁷ cells per animal should be considered.

4.11 The promise of turmeric and ginger extract as adjuvant breast cancer therapy

As reflected in the results of this study, TE and GE in a drug delivery formulation are complimentary antineoplastic agents. Turmeric and ginger extract contains numerous active ingredients of which curcumin and [6]-shogaol, respectively are the predominant phytochemicals. Many studies have confirmed that these phytochemicals possess anti-tumour abilities (Liju, Jeena and Kuttan, 2014, Marrelli *et al.*, 2015, Gupta *et al.*, 2016, Srinivasan, 2019).

Curcumin's success as treatment for many ailments including tumour growth may be attributed to its ability to modify molecular targets, such as gene expression, signalling cascades or through physical engagement (Shishodia *et al.*, 2013). Furthermore, curcumin inhibits breast cancer cell proliferation by arresting the cell cycle and inducing apoptosis via p53 gene (Kandoth *et al.*, 2013). Signalling pathways such as Ras, phosphatidylinositol-3-kinase (PI3K), protein kinase B (Akt), mammalian target of rapamycin (mTOR), and Wnt/-catenin are modified (Ettl *et al.*, 2012). In addition, transcription factors such as NF-kB is also inhibited leading to a reduction in cell growth (Murray-Stewart and Casero, 2017). Finally, curcumin inhibits angiogenesis and growth of breast cancer tumours by down regulating VEGF (Vascular endothelial growth factor) isomers (Saber-Karimian *et al.*, 2019).

Like, curcumin, ginger has various methods of inducing anticancer effects. It was discovered that 6-shogaol can activate the peroxisomal proliferator activated receptor (PPAR) leading to inhibition of cell proliferation (Mahomoodally *et al.*, 2021). Another study proved that 6-shogaol caused considerable G2/M cell cycle arrest and apoptosis, associated with decline in G1 phase cells.

It was proven that [6]-shogaol stimulate apoptosis by activating reactive oxygen species, caspases, as well as up regulation of apoptotic genes (Tan *et al.*, 2013). Secondly, apoptosis is induced by activating endoplasmic reticulum (ERM) stress signalling. In addition, the expression of cyclooxygenase-2 (COX-2) and p38 MAPK is down regulated (Hu *et al.*, 2012). This shows that curcumin and 6-shogaol, the main components in turmeric and ginger extract, respectively has the properties to induce anticancer effects.

As surmised from our data and literature, curcumin has many anticancer properties. Furthermore, curcumin may also act as a chemo-preventive agent. Ferguson and Orlando, (2015) used curcumin as adjuvant to 5-fluorouracil to treat breast cancer. Although 5-fluorouracil is an effective treatment, it is regularly countered by its severe toxicity to normal cells (Ramos *et al.*, 2019). Ferguson and Orlando, (2015) proved curcumin to be an effective chemo-preventive agent, as it protected normal cells against severe cytotoxicity in the presence of 5-fluorouracil, thus resulting in higher cell viabilities.

In addition, Woźniak *et al.* (2020), proved that [6]-shogaol enhanced the anticancer effect of 5-fluorouracil, oxaliplatin and irinotecan. Furthermore, El-Ashmawy *et al.* (2018), proved that ginger extract was an efficacious adjuvant to doxorubicin in treating mammary carcinoma. When solid Ehrlich carcinoma bearing mice were treated with a combination of doxorubicin and ginger extract, improved anticancer effects were seen when compared to the mono-treatment of each agent El-Ashmawy *et al.* (2018). The chemo-preventative effects of ginger were reported by Othman *et al.* (2020) as doxorubicin-induced cardiotoxicity in rats was reduced by ginger extract. Finally, Al-Abbasi *et al.* (2016) proved that [6]-gingerol assisted in doxorubicin-induced vascular toxicity.

It may be concluded that enhanced anticancer and chemo-preventive effects were proved by the addition of constituents found in turmeric and ginger extract to known anticancer therapies. These phytochemicals have well-established mechanisms to support their claim of being anticancer agents in *in vitro* and *in vivo* studies. The aim of the *in vivo* study was to show that the combination of ginger and turmeric extracts — formulated in drug delivery systems such as Pheroid® — would increase the antineoplastic effect of known chemotherapy agent, such as cisplatin. From the results obtained in the *in vitro* study, and the available literature on the selected extracts as supplementary treatments, it is highly probable that the final formulation TGFF, could have promising adjuvant therapy potential.

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CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

The results obtained during this study will be summarised and concluded in this chapter. This includes the efficacy of the extraction process and analysis of the extracted products. Furthermore, the success of formulations after characterisation and quality control will also be discussed. In addition, the *in vitro* results, which lead to the selection of the final formulation for the *in vivo* study will be summarised. Finally, future recommendations will also be addressed in this chapter.

5.1 Study outcome and conclusions

The search for natural adjuvant cancer therapies continues to be a prioritised study, with many phytochemicals presenting to be potential candidates. Many of these natural products present poor physico-chemical properties, such as difficulty to dissolve, thus leading to a reduction in bioavailability. However, these problems are surmountable by using drug delivery systems, such as the Pheroid® drug delivery system, to increase bioavailability.

In this study, two extracted natural products were used, where ginger was a commercial extracted product. The second component, turmeric powder was extracted via the ethanol Soxhlet extraction process. The active constituents were quantified using LC/MS-MS, which confirmed that sufficient amounts of curcumin and [6]-shogaol were found in turmeric and ginger extract, respectively.

Quality control on the carrier and solvents was performed. Here we confirmed that DMSO at 0.6% (v/v) dissolved the extracts effectively, while being non-cytotoxic to the Vero cells — the non-cancer cell line. The carrier control assay showed that a Pheroid® concentration of 0.0125% was also non-cytotoxic. Unformulated turmeric and ginger extracts were individually tested for cytotoxicity against the Vero cells to determine serial inhibition concentrations (IC₂₅, IC₅₀, and IC₇₅). The resulting values showed that the turmeric extract was more potent than the ginger extract against Vero cells. These inhibitory concentration values were consolidated into nine different combinations of turmeric and ginger extract into Pheroid®.

These nine formulations were characterised by determining particle size distribution, electrokinetic stability, and vesicle morphology. All formulations displayed a bimodal display in particle size distribution and achieved acceptable zeta potential measurements, indicating stability according to literature.

The nine formulations were evaluated for cytotoxic effects against the MCF-7 oestrogen active cancer cell line and the Vero cells (non-cancer cells). A total of five out of nine formulations was

proved to be non-cytotoxic to the Vero cells, while six out of nine formulations proved to be severely cytotoxic to the cancer cell line. These cytotoxic effects were interpreted as anticancer effects by calculating the cancer selective index. The majority of formulations was classified as anticancer agents, since they were selective cytotoxic to cancer cells rather than to normal cells. Formulation E was the most selective against the cancer cells.

The apoptosis assay reported that all the formulations induced apoptosis. The quantified results were in both the early phase and in the apoptotic phase. The inhibitory effects of the most promising formulations on the oestrogen receptor were evaluated to deduce if the apoptotic effects seen were from ER- α inhibition. Although minimal inhibition of the receptor was displayed, it was not effective enough to make the conclusion that apoptosis was induced by this mechanism. Additionally, it can be debated that the formulations do not utilise this mechanism of action in the inhibition of cell proliferation of oestrogen active breast cancer.

By reviewing the *in vitro* data, Formulation E — formulated according to the IC₅₀ value of both turmeric and ginger extract — was chosen as the overall best performing formulation. This formulation was freshly formulated as the TGFF. This formulation was highly stable according to the zeta potential. A single peak and a low span value in particle size distribution was also obtained, indicating high levels of homogeneity.

Unfortunately, the *in vivo* study did not commence due to insufficient tumour onset. The anticancer effect of these bio-active phytochemicals was proved in the *in vitro* study and comparable to literature. Both turmeric and ginger have previously been used as adjuvant agents. However, the novel combination of these in a drug delivery system increases its potential as adjuvant therapy agents.

To conclude, the aims of this study have been achieved through the *in vitro* data generated — proving anticancer effects of the Pheroid® formulated ginger and turmeric extracts against the oestrogen active MCF-7 breast cancer cell line.

5.2 Recommendations for future studies

- To further investigate the effects on the biochemical level, more mechanisms of action of these phytochemicals can be evaluated and confirmed. These include oxidative stress investigations, changes in mitochondrial membrane potential (MMP), the activation of the caspase cascade, specific gene expressions, and lactate dehydrogenase enzyme (LDH).
- As an additional quality control step, a positive control may be included in the *in vitro* assays.
- Other active constituents in turmeric and ginger extract may be quantified via the LC-MS/MS method to determine the composition of the mixtures.
- To confirm the increased antineoplastic effects of TGFF, the *in vivo* study should be completed. The optimal MCF-7 cell densities for inoculations should be re-evaluated by an in-house study to obtain sufficient tumour growth in animals.
- It is recommended that another method of oestrogen supplementation such as the MedRod implants, which delivers constant levels oestrogen in the animals. This also reduces animal handling and the risk of complications such as emboli from injections leading to the death in animals.
- To determine the bioavailability and pharmacokinetics of the bio-actives, the concentrations of curcumin and [6]-shogaol may also be quantified in the blood plasma.
- To evaluate cancer related biochemical changes in the animal during treatment, specific biomarkers such as the carcinoembryonic antigen (CEA), cancer antigen 15.3 (CA15.3), TRU-QUANT and CA27.29, and CA125, can be measured in the blood plasma.
- To evaluate cellular changes in the tumours after treatment, excised tumours can be analysed via histopathology and histochemistry.

APPENDIX 1: THE OPTIMISATION OF MCF-7 AND VERO CELL SEEDING DENSITIES FOR USE IN BIOASSAYS

Aim:

To determine seeding densities of MCF-7 and Vero cells in a 96-well plate at which sufficient confluency will be achieved after 24 and 48 hrs

Method:

Cells were cultured in T-75cm² cell culture flasks. Once cells reached a confluency of 70–80%, they were used in the subsequent experiments. When 70–80% cell confluency was reached the old media was aseptically removed and washed with 15 mL PBS. After the PBS was discarded, 5 mL trypsin was pipetted onto the cells and incubated for 4 minutes at 37°C and 5% CO₂. The trypsin was deactivated by adding 10 mL of pre-warmed fresh media. The cells were then transferred to a 15 mL tube and centrifuged for 4 minutes at 2 000 RPM. The supernatant was discarded, and the cells were resuspended in 1 mL media. Thereafter, cells were seeded into a transparent 96-well plate at seeding densities ranging between 2×10^3 – 2×10^4 and 2×10^3 – 12×10^3 of 1×10^4 cells/200µL/ for the MCF-7 and Vero cells, respectively in each well.

Results:

After 24 hours, the lowest percentage confluency of the MCF-7 and Vero cells were as expected at a cell density of 2×10^3 cells/well for both cell lines (Figure A1 A & C). At 17.5×10^3 cell/well 100% confluency was reached for the MCF-7 cell line (Figure A1 A). The Vero cells at a seeding density of 12×10^3 cells/well obtained the highest confluency after 24 hours (Figure A1 C). After 48 hours, the lowest percentage confluency of the MCF-7 and Vero cells were at a cell density of 2×10^3 cells/well for both cell lines (Figure A1, B & D). A 100% confluency was reached at cell 1×10^4 cells/well and 8×10^3 for the MCF-7 and Vero cells, respectively

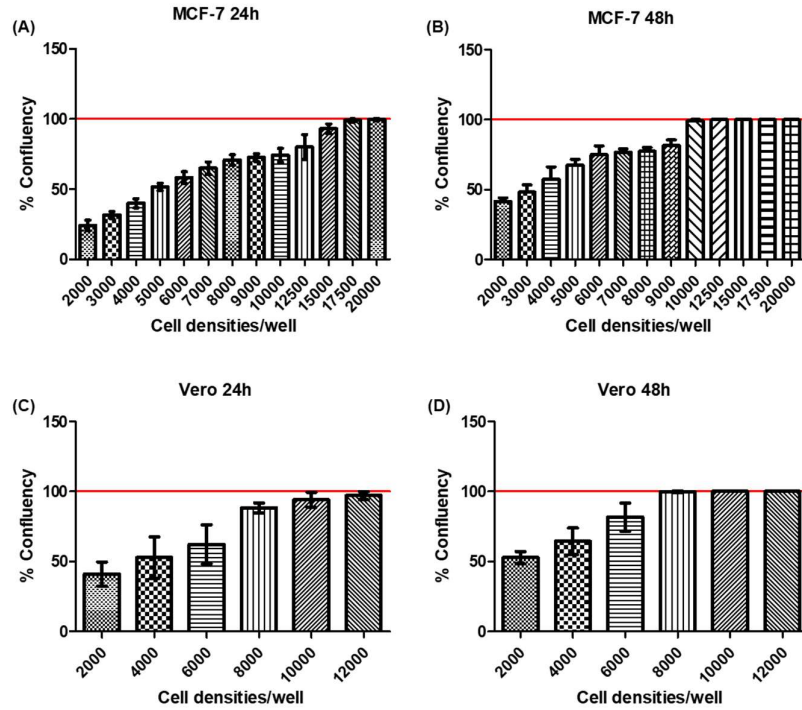


Figure A1: Seeding cell densities of MCF-7 and Vero cells after 24hr (A and C) and 48 hrs (B and D), respectively. The solid red line indicates 100% confluency

The seeding densities of MCF-7 and Vero cells were selected at 10×10^3 and 8×10^3 (Figure A1) as these cell densities obtained 70–80% confluency, which is required when seeding for bioassays.

APPENDIX 2: ANIMAL WELFARE MONITORING SHEETS

This appendix contains all the log sheets relevant for the *in vivo* study. These include the template forms for health monitoring once animals were enrolled into the study. Additionally, all log sheets completed for the oestrogen supplementation as well as the inoculation log sheets have been included in this appendix. Finally, all relevant SOPs of the PCDDP Vivarium referenced in this dissertation are included.

#	HEALTH INDICATOR	SCORE	
		VALUE	EXPLANATION
1.	Loss in Body Mass (BM)	0	Gain/ No Loss in BM
		1	Loss in BM $\leq$ 5%
		2	5% < Loss in BM $\leq$ 20%
		3	Loss in BM > 20%
2.	Appearance	0	Normal
		1	Lack of grooming
		2	Rough coat, nasal/ ocular discharge
		3	Very rough coat, abnormal posture, enlarged pupils
3.	Clinical Signs	0	Normal
		1	Small changes of potential significance
		2	Respiratory Rate (RR) $\uparrow$ or $\downarrow$ 30%
		3	RR $\uparrow$ or $\downarrow$ 50%
4.	Natural Behaviour	0	Normal
		1	Minor changes
		2	Abnormal, reduced mobility, decreased alertness, inactive
		3	Unsolicited vocalisations, self-mutilation, either very restless or immobile
5.	Provoked Behaviour	0	Normal
		1	Minor depression/ exaggeration of response
		2	Moderately abnormal responses
		3	Violent or comatose
Total Possible Score		15	Consider euthanasia whenever a score of 3 is assigned or get
Veterinarian for final assessment. Double frequency of observations for any animal that scored a 2 for any indicator.			

Table A1: Addendum 1(a): key for assignment of health check scores

Table A2: Addendum 1(b): log sheet for assignment of health check scores

Select score for each health indicator by circling appropriate score using the Key for assignment of Health Check Scores as is described in Addendum 1(A).

ID	Ear Map	Body Mass (g)	Loss in Body Mass				Appearance				Clinical Signs				Natural Behaviour				Provoked Behaviour				Total Score	Time	Notes
			0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3			
			0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3			
			0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3			
			0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3			
			0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3			
			0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3			

Notes:		
Animal Technician Name:	Signature:	Date:
Study Director Signature:		Date:

Table A3: Addendum 2: tumour monitoring data collection sheet

Mouse number	Date	Weight (g)	Tumour length (mm)	Tumour width (mm)	Tumour volume (mm ³) $\frac{1}{2}(\text{length} \times \text{width}^2)$

Signatures

Print name

Sign

Study director sign

Date