Co-formulation and therapeutic evaluation of bioactive plant compounds in Pheroid®

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Dissertation submitted in fulfilment of the requirements for the degree Masters of Science in Pharmaceutical Sciences at the North-West University

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PREFACE AND DECLARATION

This dissertation is presented in article format and consists of two manuscripts peer reviewed for publication. The dissertation was prepared as per the North-West University (NWU) guidelines for postgraduate studies and the requirements of the publishing journals.

All *in vitro* and *in vivo* biological studies were carried out by myself after successful completion of short courses on basic cell culturing techniques and animal handling and the principles of research on animals. Dr Wihan Pheiffer assisted with the experimental design, data analysis and interpretation. Mitochondrial health assessment was carried out in the mitochondrial lab, Department of Biochemistry, NWU, with the assistance of Belinda Fouché and Michelle Mereis. Interpretation of the results was done by myself and Dr Wihan Pheiffer.

The animal study was carried out in accordance with the NWU code of conduct for researchers with ethics number NWU-00167-18-A5 in the AAALAC accredited animal facility (Department of Science and Technology/North-West University Preclinical Drug Development Platform Vivarium; PCDDP). Inoculation of animals with cancer cells was performed by Dr Ambrose Okem. Intraperitoneal administration of cisplatin to animals, oral gavage and animal euthanasia were carried out by the vivarium laboratory animal technologists; Cor Bester, Jacob Mabena and Kobus Venter. I was also involved in the oral gavage, tumour and body mass measurements, monitoring of animal well-being and harvesting of tumour samples at the end of the study.

Histopathological examination of tumour samples for future studies was performed by PathCare. Dr. John Takyi-Williams provided expertise in the instrumentation, method development and optimization, analysis and interpretation of results for the quantification of the actives using LC–MS/MS. The compatibility study was performed at the Center of Excellence for Pharmaceutical Sciences (Pharmacen™), NWU. The experiment was run by Prof. Wilna Liebenberg and data analysis and interpretation was done using the TAM Assistant v 2.0.156 software package by Dr. Marique Aucamp, School of Pharmacy, University of Western Cape.

Captured *in vitro* and *in vivo* data were sent to Prof. Faans Steyn for statistical analysis at the Statistical Consultation Services, Potchefstroom Campus, NWU. Part of the study was presented at the Drug Safety Africa meeting held in Potchefstroom, South Africa (November, 2018); Safety Pharmacology Society annual meeting held in Barcelona, Spain (September, 2019); and Academy of Pharmaceutical Sciences South Africa held in Pretoria, South Africa (October, 2019).

I hereby declare that the dissertation "**CO-FORMULATION AND THERAPEUTIC EVALUATION OF PLANT BIOACTIVE COMPOUNDS IN PHEROID®**" is my own work and that all resources used were acknowledged and referenced in accordance with the NWU referencing guideline. This research has not been submitted before for any degree or examination at any university.

BISRAT SISSAY BEKELE **DATE**

25/11/2019

DEDICATION

This study is dedicated in loving memory to my mother Tsigereda G/Michael. You are the best mother one can ever ask for and you will forever be in my heart.

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I would like to extend my most revered regards to the following who have contributed directly or indirectly towards the successful completion of the study:

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ABSTRACT

Cancer is a global health burden; of which lung cancer is the most frequently diagnosed type and has the highest mortality. The survival profile of lung cancer patients at all stages is dismal despite the availability of various treatment options. This underscores the dire need for alternative treatment options that have better treatment modalities. In this regard, phytochemicals have been used as an emerging treatment strategy to combat all cancer types. Curcumin and ginger extract (GE) phenolics — particularly [6]-shogaol (6SG) — have been shown to have promising chemopreventive activity, the latter being the most potent one. A combination of phytochemicals often produces a more profound anticancer activity than single agent treatment. However, the clinical utility of phytochemicals is restricted owing to their poor physicochemical properties, which can be enhanced by using drug delivery systems such as the Pheroid® technology.

In this study, the therapeutic activity of combined Meriva®; a curcumin phytosome, and GE Pheroid® formulations were investigated against the human lung cancer adenocarcinoma A549 cell line both *in vitro* and *in vivo*. The contents of curcumin in Meriva® and 6SG in GE were quantified. In the *in vitro* study, cellular uptake, cell viability, apoptosis, oxidative stress markers and mitochondrial health were assessed. Furthermore, the formulation administered to the animals was characterised in terms of particle size and distribution, zeta potential and compatibility assays. An *in vivo* study was conducted using an established athymic nude mice xenograft model. Accordingly, male and female athymic nude mice were inoculated with viable A549 cancer cells. Once the tumour volume reached a palpable size, mice were allocated into four groups and received a daily oral gavage of saline, Pheroid® only and phytochemical combination in Pheroid® for 14 days. Cisplatin was injected intraperitonially once a week.

The amounts of principal actives — curcumin and 6SG — in Meriva® and GE were found to be 400 mg/g and 11 mg/g, respectively. Zeta potential and compatibility studies indicated that the phytochemicals were stable in Pheroid® and that no drug-excipient interactions were observed. Confocal microscopy revealed co-localisation of phytochemicals within the Pheroid® vesicles. *In vitro* results indicated that Pheroid® significantly enhanced cellular uptake, anti-proliferative and apoptotic effects of phytochemical combination compared to individual actives and the free active DMSO formulations. From the mitochondrial health assessment, it was noted that Meriva® but not GE was responsible for the mitochondrial dysfunction, and the effect was more pronounced in the Pheroid® formulation than in the free forms. In addition, the anticancer activity observed with combined phytochemicals in Pheroid® was without induction of oxidative stress, indicating the potential safe use of the formulation. The *in vivo* study demonstrated that daily oral gavage with the Pheroid® formulated phytochemical combination non-significantly reduced the tumour growth and burden in mice compared to the Pheroid® only treatment. However, cisplatin significantly

reduced tumour growth compared to the saline negative control. The sub-therapeutic effect observed with the phytochemical combination treatment can be attributed to the suboptimal dose of curcumin and 6SG principal actives administered in the formulation. In addition, treatment with cisplatin was accompanied with a reduction in body mass. Research has indicated that the side effects of chemotherapeutic drugs such as cisplatin can be overcome by co-administration of phytochemicals. The present *in vitro* and *in vivo* study conclusively show the potential anticancer activity of combined Meriva® and ginger extract phenolic compounds. Pheroid® significantly improved the biological activity of the phytochemicals. In addition, the study opens an opportunity to further investigate the anticancer activity of cisplatin co-administered with therapeutic doses of curcumin and 6SG phytochemicals against lung cancer.

Keywords: *Cisplatin; Curcumin; Cellular uptake; Drug delivery system; Ginger extract; in vitro and in vivo models; Meriva®; Mitochondria; Pheroid®; Tumour volume; xenograft; [6]-shogaol*

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LIST OF SYMBOLS

%	Percentage
$d_{0.1}$, $d_{0.5}$ and $d_{0.9}$	Particle size at the 10^{th}, 50^{th} and 90^{th} percentile
g	gram
g	Relative gravitational force
g/kg	Gram per kilogram
M	Molar
m/z	Mass-to-charge ratio
mg	Milligram
mg/g	Milligram per gram
mg/mL	Milligram per millilitre
min	Minute
mL	Millilitre
mL/min	Millilitre per minute
mM	Millimolar
mm^3	Cubic millimetre
msec	Millisecond
mV	Millivolt
N	Normal
ng/mL	Nanogram per millilitre
ngSOD/mg	Nanogram superoxide dismutase per milligram
nM	Nanomolar
nm	Nanometre
psi	Pounds per Square Inch
v/v	Volume per volume
Δ	Delta or change
µg/mL	Microgram per millilitre
µL	Microliter
µM	Micromolar
µmol H_2O_2/min/mg	Micro mole hydrogen peroxide per minute per milligram
µW/g	Microwatt per gram
Ψm	Mitochondrial membrane potential

LIST OF EQUATIONS

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATIONS

A

A549	Human non-small cell lung adenocarcinoma
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AB	Apoptotic body
ADC	Adenocarcinoma
AFRO	WHO African Region
ANOVA	Analysis of variance
AO	Acridine orange
ATP	Adenosine triphosphate

B

Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
bDMC	bisdemethoxycurcumin

C

C	Concentration
CAO	Central airway obstruction
CAT	Catalase
CC	Chromatin condensation
CLSM	Confocal laser scanning microscopy
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CTCF	Corrected total cell fluorescence

D

DMC	Demethoxycurcumin
DMEM	Dulbecco`s Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriamine pentaacetate

E

EFAs	Essential fatty acids
EMRO	WHO’s East Mediterranean region
ER	Endoplasmic reticulum
ESI	Electrospray Ionisation
EtBr	Ethidium Bromide

ETC	Electron transport chain
EURO	WHO's Europe region

F

F	Formulated
FCCP	Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone

G

GE	Ginger extract
GPX	Glutathione peroxidase

H

H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
H_2SO4	Sulphuric acid
HPLC	High performance liquid chromatography

I

IC_{50}	Concentrations reducing cell viability by 50%, relative to an untreated control
ICH	International Conference on Harmonisation
IP	Intraperitonial
ISO	International Organization for Standardization
IVC	Individual ventilated cage

K

$KMnO_4$	Potassium permanganate

L

L	Tumour measurement at longest point
LA	Late apoptotic
LC	Liquid chromatography
LCC	Large cell carcinoma

M

M	Mass of extract in gram
MB	Membrane blebbing
MMP	Mitochondrial membrane potential
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
mtDNA	Mitochondrial DNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

N

N	Necrotic
N_2O	Nitrous oxide
NF	Nuclear fragmentation
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NR	Neutral red
NSCLC	Non-small cell lung cancer

O

OCR	Oxygen consumption rate
$OH^{\bullet}$	Hydroxide radical
OXPHOS	Oxidative phosphorylation
O_2^-	Superoxide anion

P

PBS	Phosphate buffered saline
PSA	Penicillin-streptomycin and amphotericin B mixture
PTP	Permeability transition pore

R

RFU	Relative fluorescence unit
ROS	Reactive oxygen species
rpm	Rotation per minute
RTV	Relative tumour volume

S

SCLC	Small cell lung cancer
SD	Standard deviation
SEM	Standard error of the mean
SLN	Solid lipid nanoparticle
SOD	Superoxide dismutase
SQC	Squamous cell carcinoma
SRC	Spare respiratory capacity
STAT3	Signal transducer and activator of transcription 3

T

T	Content in mg/g
T/C	Treatment over control tumour growth ratio
TAM	Thermal activity monitor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand

U

UF	Unformulated
UGT	Uridine glucuronosyl transferases

V

V	Volume of solution
V_0	Tumour volume at day 0
V_n	Tumour volume at corresponding day

W

W^2	Tumour measurement at widest point
WHO	World Health Organisation

Miscellaneous

µC	Mean slope of the growth rates for the control group
µT	Mean slope of the growth rates for the treatment group
6SG	[6]-shogaol

CHAPTER 1 RESEARCH SCOPE

1.1 Background

Cancer is a multi-gene, multi-step disease emanating from an aberrant proliferation of mutant cells. Successive mutations and selective expansion of tumour cells lead to the formation of tumour mass and growth, which in time break from the surrounding basal membrane resulting in metastasis (Hejmadi, 2009). As a non-communicable disease, cancer is the foremost cause of death in the world (Bray *et al.*, 2018). Family history, lifestyle, infections such as cervical, stomach and liver infections, and environmental pollutants are common determinants for the prominent rise of the disease in the world.

Lung cancer is the most frequently diagnosed cancer type among other cancers and it is by far the main cause of cancer related deaths, worldwide (Bray *et al.*, 2018). Over the years, trends in lung cancer incidence and mortality show a decline in developed countries and a rise in newly industrialised and developing countries such as China and India (Kanavos, 2006). In Africa, epidemiological information on lung cancer is scarce. However, existing data show a lower mortality rate in Western and Middle Africa but a higher rate in Northern and Southern Africa (Parkin *et al.*, 2014).

Based on the histological morphology of the cells, lung cancer is classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). In general, NSCLC accounts for the majority of the incidence and mortality of all lung cancer cases (Zappa and Mousa, 2016). According to literature, lung cancer has the lowest survival profile, where the survival rate of patients at all stages after one year and five years are 44% and 17%, respectively (Townsend *et al.*, 2017; Wong *et al.*, 2017). Tobacco smoking is the primary risk factor accounting for over 80% of all lung cancer cases in the world (WHO, 2018). Currently, surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy are employed as the mainstay treatment strategies of lung cancer (Hirsch *et al.*, 2017).

The use of combinatorial targeted therapy for treating lung cancer often fails to provide satisfactory treatment outcomes due to chemo-resistance, unfavourable drug toxicity, high treatment costs and associated poor quality of life (Bharti *et al.*, 2018; Townsend *et al.*, 2017). This underscores the need to explore novel treatment approaches that have better treatment modalities: a favourable efficacy-to-toxicity profile, for improving lung cancer treatment. In this regard, complementary herbal medicines such as phytochemicals, provide an alternative treatment approach to modern allopathic medicine for treating and/or preventing diseases including cancer.

Nutraceuticals – considered as food or part of food – have a multitude of physiological benefits and provide protection against chronic diseases (Shahidi, 2012). The bioactive ingredients found in nutraceuticals are called phytochemicals and their combination is often more potent than single agent treatment (Shukla and George, 2011; Sung *et al.*, 2012). The herbaceous perennial plants, turmeric *(Curcuma longa)* and ginger (*Zingiber officinale*), possess a wide variety of pharmacological and physiologic functions including anti-inflammatory, antioxidant, anti-microbial, chemo-preventive and chemo-therapeutic activities (Hatcher *et al.*, 2008; Mashhadi *et al.*, 2013). The principal and biologically active compounds found in the rhizomes of ginger include [6]-gingerol, [6]-shogaol and zingerone and, in turmeric, curcumin polyphenols (Surh *et al.*, 1998).

Despite the aforementioned therapeutic benefits, *in vivo* efficacies of phytochemicals are often limited due to their poor physicochemical characteristics such as low solubility following oral administration. This in turn, causes a deficiency in the plasma concentration of the bioactive compounds to elicit sustained therapeutic functionalities at the target site of action. To counter-act these challenges, several lipid-based drug delivery systems such as liposomes have been developed over the years. Among these, Pheroid® — a novel drug delivery system — has been widely applied in various pharmaceutical applications such as oral delivery of anti-malaria drugs (Grobler *et al.*, 2014a; Grobler *et al.*, 2014b; Steyn *et al.*, 2011), topical delivery of cytokines and anticancer drugs (Campbell, 2010; Chinembiri *et al.*, 2015), transdermal delivery of anti-tuberculosis drugs and local anaesthetics (Botes, 2007; Nell, 2012) to mention but a few. The Pheroid® delivery system is based on a colloidal emulsion system and is comprised of plants and ethyl esters of essential fatty acids as the dispersed phase and nitrous oxide saturated water as the continuous phase (Grobler, 2009).

1.2 Research problem

Current lung cancer treatment strategies do not offer a satisfactory treatment outcome owing to chemo-resistance, unfavourable toxicity of drugs, high treatment cost and poor quality of life (Bharti *et al.*, 2018; Townsend *et al.*, 2017). This spurs the need to explore alternative novel treatment approaches that have better treatment modalities, enhanced therapeutic efficacy and lower toxicity. Turmeric and ginger nutraceuticals have been extensively studied in the treatment of numerous illnesses including cancer. The principal phytochemicals found in these plants that are responsible for the biological activities include curcumin in turmeric and [6]-gingerol, [6]-shogaol and zingerone in ginger (Magalhães *et al.*, 2009; Surh *et al.*, 1998). These phytochemicals have low intrinsic toxicity and independently possess anticarcinogenic, anti-inflammatory, pro-oxidant, and antioxidant properties (Magalhães *et al.*, 2009; Surh *et al.*, 1998). However, extensive research findings show that a combination of phytochemicals often has a more pronounced anticancer action than single agent treatment (Klein and Fischer, 2002; Shukla

and George, 2011; Zhou *et al.*, 2003). Furthermore, the physicochemical properties of these phytochemicals impede their clinical applicability and therefore will require the use of a drug delivery system to potentiate the clinical outcome.

1.3 Aim and objectives

1.3.1 Aim

- To evaluate the *in vitro* and *in vivo* chemopreventive activity of Pheroid® formulated Meriva® and ginger extract combinations against the human adenocarcinoma A549 cancer cell line.

1.3.2 Objectives

The following objectives were considered necessary to achieve the aim:

- To formulate and characterize the phytochemical combinations in Pheroid®.
- To conduct cytotoxicity, cellular uptake, apoptosis, oxidative stress markers and mitochondrial health assays using relevant *in vitro* methods.
- To measure *in vivo* chemopreventive activity using xenograft mouse model of lung cancer.

1.4 Study design

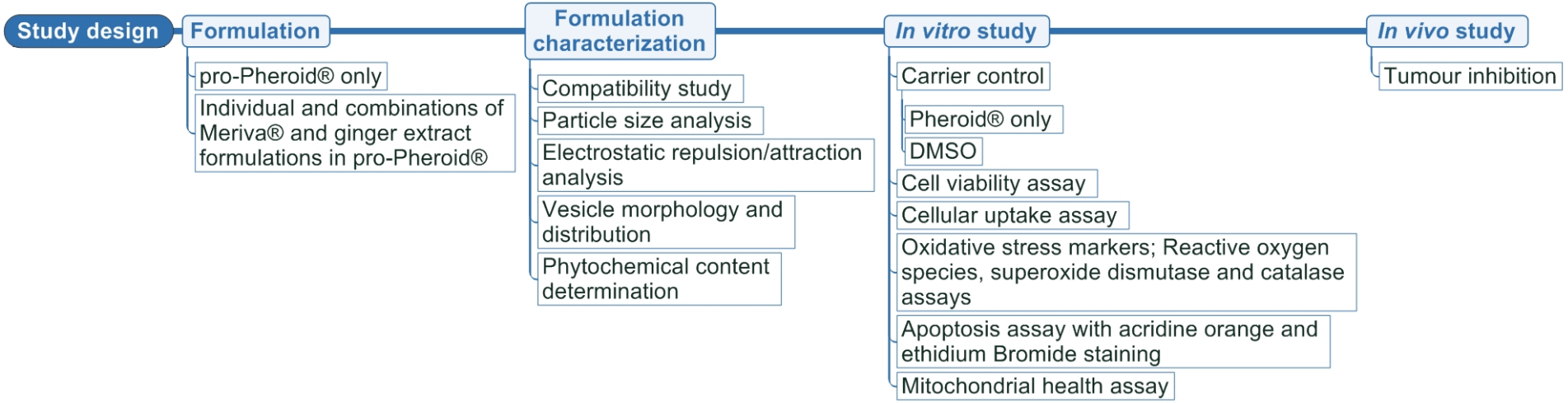

Figure 1.4.1. Graphical abstract representing the outline of the study project

The study consists of four phases namely formulation, characterization, *in vitro* and *in vivo* studies (Figure 1.4.1). Pro-Pheroid® only and phytochemical formulations in pro-Pheroid® were prepared. Then, particle size, zeta potential, vesicle morphology and distribution, and compatibility assays were conducted to characterize the formulation. In addition, the contents of curcumin and [6]-shogaol — plant bioactive compounds — in Meriva® and ginger extract (GE) were determined. The anticancer activity of different phytochemical combinations in Pheroid® and in free form — dissolved in DMSO — were assessed *in vitro* using the A549 lung cancer cell line. Firstly, the concentration of Pheroid® and DMSO carriers that has no effect on cell growth were predetermined for further studies. Secondly, the effect of phytochemicals on the proliferation and viability, oxidative stress markers, apoptosis and mitochondrial health were investigated. Moreover, the uptake of phytochemicals by the cells in presence and absence of Pheroid® was assessed. *In vivo* chemopreventive activity of the phytochemical combination in Pheroid® was conducted in an established athymic mice xenograft model. The study was approved by the North-West university AnimCare ethics committee, Appendix A (NWU-00167-18-

A5).Accordingly, male and female mice were inoculated with viable A549 cells in the right hind leg. Once the tumour reached a palpable size, the animals were categorised into four groups each consisting of four male and female mice. The first, second and third groups received a daily oral gavage of saline, Pheroid® only and Meriva® (70 mg/kg) and GE (100 mg/kg) in Pheroid®, respectively. The fourth group received cisplatin (4.5 mg/kg) once a week via the intraperitoneal route. The total duration of the study was 14 days (Figure 1.4.2).

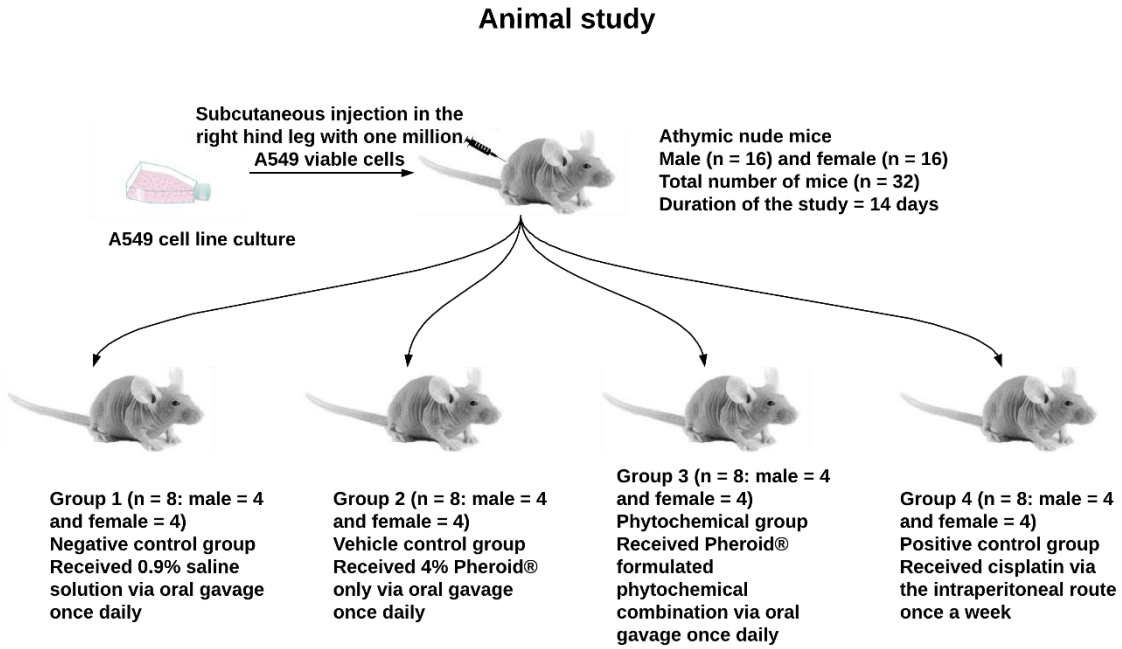

Figure 1.4.2. Experimental design for evaluating the *in vivo* anticancer activity of phytochemical combination in Pheroid® delivery system using an established A549 xenograft model. Figure generated using Lucidchart (https://www.lucidchart.com)

It was hypothesized that phytochemical combination in Pheroid® would produce more effective chemopreventative activity than the free form counterparts and individual treatments in the *in vitro* study. Furthermore, it was hypothesized that Pheroid® would improve the bioavailability and anticancer activity of phytochemical combinations in the *in vivo* study through enhancing their poor physicochemical properties.

1.5 Dissertation outline

Chapter 1 (current chapter) provides a brief research background, problem statement, aims and objectives, framework of the study, and this dissertation outline.

Chapter 2 provides a comprehensive review on lung cancer and its molecular pathogenesis, with particular emphasis made on the function of the mitochondria in lung cancer and role of reactive oxygen species. Furthermore, in this chapter, the plant bioactive compounds of interest and their therapeutic efficacy against different cancer types were highlighted.

Chapter 3 is presented in standard manuscript format for publication in *Pharmaceutics: an open access Journal from Multidisciplinary Digital Publishing Institute (MDPI)*. The manuscript was written according to the instruction for authors' guideline. In this chapter, the *in vitro* anticancer efficacy of unformulated, and Pheroid® formulated Meriva® and ginger extract were investigated against the human lung cancer adenocarcinoma A549 cell line.

Chapter 4 is presented as a manuscript for publication according to the instruction for authors' guideline of *Pharmaceuticals: an open access Journal from MDPI*. In this chapter, the chemopreventative activity of Pheroid® formulated Meriva® and ginger extract combination was investigated in an established lung cancer murine xenograft model. Furthermore, phytochemical content, compatibility study and formulation characteristics were determined and presented.

Chapter 5 is the concluding chapter of the dissertation and contains a summary of the main results written in line with the aim and objectives of the study. In addition, future remarks and recommendations were provided.

References are presented at the end of each chapter. All resources and materials used in this study are referenced in accordance with the NWU referencing guideline and the journals referencing style.

The **Appendices** — attached at the end of the dissertation — contains the AnimCare ethics approval letter, ethics training and animal handling course certificates, instruction for authors' guideline, certificate of analysis and conference presentations.

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CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

Cancer is a global health concern. According to the 2018 GLOBOCAN report there were about 18.1 million new cancer cases and 9.6 million cancer deaths in the world (Bray *et al.*, 2018; WHO, 2018a). In this report, the mortality of cancer in Asia and Africa was higher than the incidence when compared to other regions of the world (Bray *et al.*, 2018). Factors such as population growth and ageing, as well as socioeconomic development play a significant role towards the increasing cancer burden. The top three cancer types based on incidence rate are; lung cancer, female breast cancer, and colorectal cancer and together are responsible for one third of the cancer incidences and mortality burdens in the world (Bray *et al.*, 2018). However, in terms of mortality, lung cancer is ranked first among the top five cancer types with breast cancer as the fifth and colorectal as the second.

2.2 Lung cancer

Lung cancer, a highly aggressive and malignant neoplasm, is the primary cause of cancer related deaths in the world (WHO, 2018a). Patients with lung cancer are often diagnosed late; when the disease is well advanced and treatment options are scarce (Youlden *et al.*, 2008). According to Yoder (2006), more than 90% of adults are symptomatic upon diagnosis. A small number of lung cancer patients present direct signs and symptoms caused by the primary tumour, while the majority present either nonspecific systemic symptoms or metastatic symptoms (Figure 2.1) (Collins *et al.*, 2007; Yoder, 2006).

Many lung cancers occur in the central airways; leading to central airway obstruction (CAO). It has been reported that patients with CAO present with stridor, atelectasis, pneumonia, dyspnea, respiratory failure and hemoptysis (Verma *et al.*, 2018). CAO has a very poor prognosis and the median survival of patients with malignant CAO is approximately 8 months where patients receive palliative support (Chhajed *et al.*, 2006; Verma *et al.*, 2018). Dyspnea develops early in 60% of lung cancer patients, while hemoptysis is present in an estimated 6–35% of patients (Yoder, 2006). A small number of patients have also been reported to present with paraneoplastic syndromes such as hypercalcemia, Cushing's syndrome, neurologic syndromes and pulmonary hypertrophic osteoarthropathy (Collins *et al.*, 2007; Varricchio, 2004).

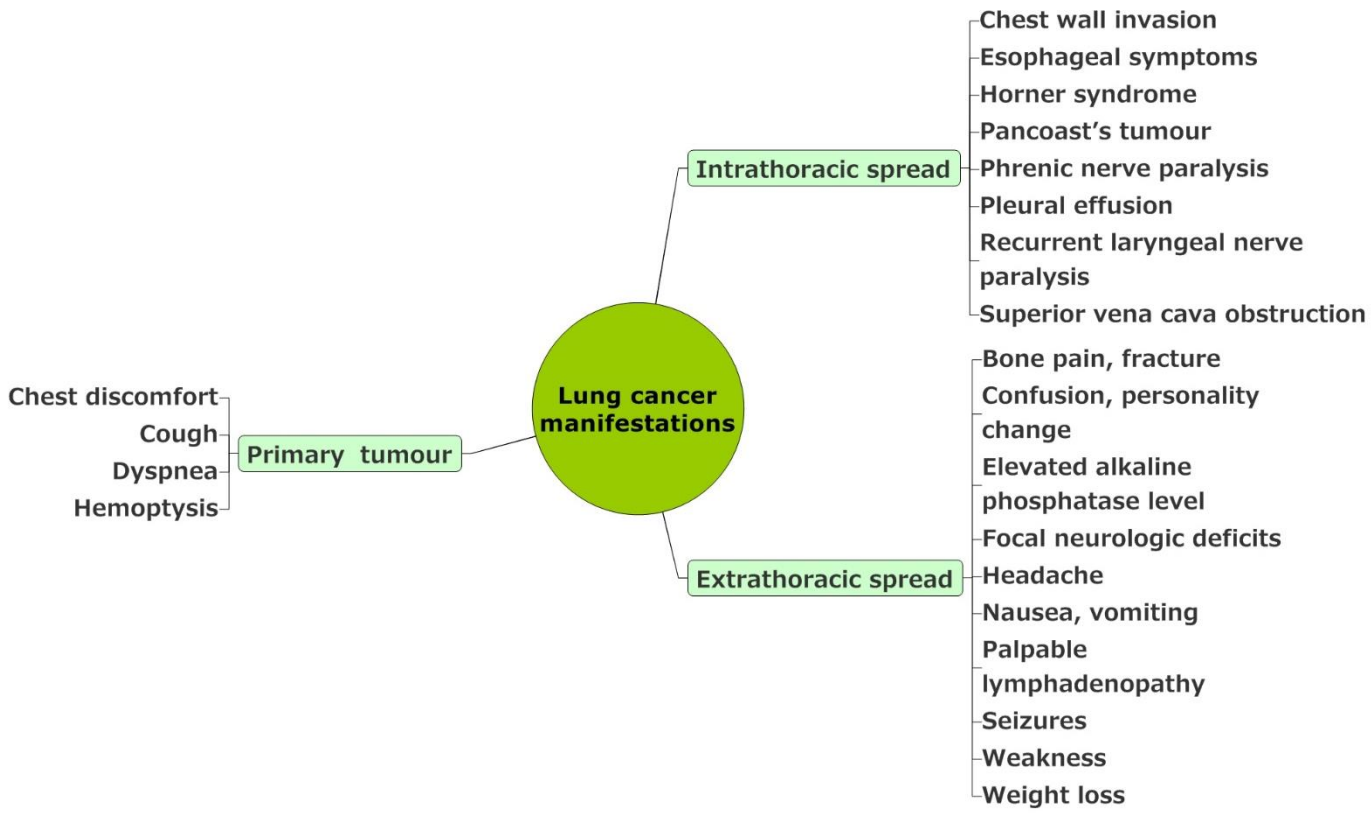

Figure 2.1: Signs and symptoms of lung cancer. Adapted from Beckles *et al.* (2003)

2.3 Epidemiology

According to the World Health Organization WHO (2018a), lung cancer accounts for 1.7 million deaths every year, more than the mortality of colorectal and breast cancers combined. Research indicates that lung cancer patients have a poor survival profile, where the one year and five year survival rate of lung cancer patients at all stages is only 44% and 17%, respectively (Townsend *et al.*, 2017; Wong *et al.*, 2017).

Since 1985 the number of lung cancer cases in the world had increased by 51% where women (76%) had a larger increase compared to men (44%) (Cruz *et al.*, 2011). Among men, lung cancer has the highest incidence where it accounts for a yearly 28% death, while in women it is the third most common cancer accounting for 26% of all cancer deaths (Cruz *et al.*, 2011; Sadeghi-Gandomani *et al.*, 2017; Stewart and Wild, 2014).

Geographically, more developed countries show a decline in the incidence and mortality rate of lung cancer, while a rapid increase in lung cancer cases was observed in developing nations due to the endemic use of tobacco (Youlden *et al.*, 2008). In 2012, about 58% of lung cancer cases occurred in less developed regions (Ferlay *et al.*, 2015), as compared to the 69% that occurred in developed countries in 1980 (Cruz *et al.*, 2011). Until 2035, it is predicted that the number of lung cancer mortalities will rise globally by 86% (Didkowska *et al.*, 2016). During this time, WHO's

East Mediterranean region (EMRO) and WHO's Africa region (AFRO) will have the highest increase; 123% and 108%, respectively, while the lowest increase is predicted for WHO's Europe region (EURO), 37% (Didkowska *et al.*, 2016).

2.4 Etiology

According to the WHO (2018b), tobacco smoke is the primary risk factor for more than 80% of all lung cancer cases. Tobacco smokers, when compared with non-smokers, are 30-fold more likely to develop cancer (Walser *et al.*, 2008). On the other hand, a dose-dependent exposure to secondhand smoke or environmental tobacco smoke is another risk factor for developing lung cancer among the non-smoker population (Cruz *et al.*, 2011). Host genetic milieu is another risk factor that plays an essential role in the pathophysiology of lung cancer. A study conducted by Matakidou *et al.* (2005) indicated that family history was significantly associated with the development of lung cancer.

On another note, inflammatory diseases of the airways such as chronic obstructive pulmonary disease (COPD) could potentially contribute to the pathogenesis of lung cancer (Cruz *et al.*, 2011). In addition, occupational hazards such as exposure to certain noxious chemicals including arsenic, asbestos, cadmium, chromium, nickel, silica and diesel exhaust waste, could have been identified as potential carcinogens (Cruz *et al.*, 2011; Field and Withers, 2012; Loomis *et al.*, 2018). Air pollution — in the form of either indoor or outdoor combustion — has the propensity to increase the risk of lung cancer in humans (Cruz *et al.*, 2011).

2.5 Types of lung cancer

Lung cancer is categorized into two major types; non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), of which NSCLC accounts for the majority (85%) of all lung cancer cases (Pore *et al.*, 2013; Yong *et al.*, 2015; Zappa and Mousa, 2016). Furthermore, NSCLC is subdivided into adenocarcinoma (ADC), squamous cell carcinoma (SQC) and large cell carcinoma (LCC) types. Among these subtypes, ADC is the most frequently diagnosed (40%) NSCLC in both sexes of smokers and non-smokers (Zappa and Mousa, 2016), and it originates in the periphery of the lung from small airway epithelial cells (Lemjabbar-Alaoui *et al.*, 2015; Yokota and Kohno, 2004; Zappa and Mousa, 2016). LCC is a poorly differentiated tumour, lacking glandular or squamous maturation, and often arise in the center of the lungs and sometimes from nearby lymph nodes, chest walls and distant organs (Rossi *et al.*, 2014; Zappa and Mousa, 2016). On the other hand, SQC arise from bronchial epithelial cells, in the center of the lungs, through squamous dysplasia (Yokota and Kohno, 2004). Both SQC and LCC are strongly associated with smoking (Zappa and Mousa, 2016).

SCLC originates from epithelial cells with neuroendocrine features and is the most differentiated cancer type (Yokota and Kohno, 2004). SCLC tends to be a central mediastinal tumor and is extremely aggressive; rapidly disseminating into sub-mucosal lymphatic vessels and regional lymph nodes (Lemjabbar-Alaoui *et al.*, 2015), through which metastasis to other organs of the body is achieved. Similar to the aforementioned cancer types, SCLC is also implicated by cigarette smoking.

2.6 The molecular pathogenesis of lung cancer and mitochondria as the novel target for treating lung cancer

The pathogenesis of lung cancer in humans involves a multitude of interlinked steps comprised of aberrant cellular growth, angiogenesis, and metastasis (Aggarwal *et al.*, 2008; Cai *et al.*, 2011). Nuclear Factor-Kappa B (NF-κB), a redox-sensitive transcription factor, plays a major role in the development and progression of cancer including lung cancer (Cai *et al.*, 2011; Simone *et al.*, 2011). Its activation results in the expression of several target genes such as cell cycle regulatory genes (cyclin D1), apoptosis suppressor proteins (Bcl-2 and Bcl-xL) and matrix metalloproteinases (MMPs), that are crucial in the development of aggressive cancer types (Aggarwal *et al.*, 2008; Alvira, 2014; Cooper *et al.*, 2013; Panov, 2005; Rivas-Fuentes *et al.*, 2015; Shtivelman *et al.*, 2014; Sung *et al.*, 2012).

Cells require energy to carry out normal homeostasis functions such as division and proliferation. This energy is produced mainly by glycolysis and oxidative phosphorylation pathways. Oxidative phosphorylation generates more adenosine triphosphate (ATP) molecules per substrate than the glycolysis pathway. Most cancer types utilize the glycolytic pathway, even in the presence of oxygen, to generate energy and other metabolic products important for tumour progression (Yu *et al*., 2017). The electron transport chain (ETC) found in the inner membrane of the mitochondria is responsible for facilitating reactions associated with oxidative phosphorylation. Unlike other cancer types, lung cancer cells heavily rely on mitochondrial respiration for generating energy that is vital for rapid cellular growth and metastasis (FitzGerald *et al.*, 2017). Therefore, inhibiting the mitochondrial function consequently starves the cancer cells of energy. On the contrary, normal lung cells have drastically lower levels of oxidative phosphorylation and energy requirements, and are not as strongly impacted as lung cancer cells (FitzGerald *et al.*, 2017).

The mitochondrion hosts multiple redox-active complexes and metabolic enzymes. It is the major source for generating endogenous reactive oxygen species (ROS) such as superoxide anion (O_2^-) as an end product of aerobic metabolism via the ETC (Sullivan and Chandel, 2014). Since lung cancer cells heavily rely on the mitochondrial oxidative phosphorylation (OXPHOS) to generate energy, the increased metabolic activity resultantly increases the ROS level. These reactive oxygen species act as secondary messengers and at low to modest levels are involved

in the regulation of biological and physiological processes such as cell cycle progression and proliferation, immune signalling, apoptosis, metabolism, aging and hypoxic signalling (Boonstra and Post, 2004; Wojtovich and Foster, 2014; Zhang *et al.*, 2016). However, higher relative ROS levels, facilitates carcinogenesis and cancer progression.

To balance the elevated ROS level, cancer cells often increase expression of antioxidant proteins (Figure 2.2) (Sullivan and Chandel, 2014). When cancer cells fail to maintain the elevated intracellular redox homeostasis, an irreversible oxidative damage to proteins, lipids, nucleic acids, membranes and organelles such as mitochondria will occur, which induces cancer specific cell death via activation of apoptosis, necrosis or autophagy pathways (Redza-Dutordoir and Averill-Bates, 2016; Zhang *et al.*, 2016). This is why cancer cells are extremely susceptible to slight changes in ROS and antioxidant levels, where either suppression of ROS production or antioxidant treatment — or vice versa — can lead to cancer specific cytostasis or oxidative cell death (Liou and Storz, 2010; Sullivan and Chandel, 2014).

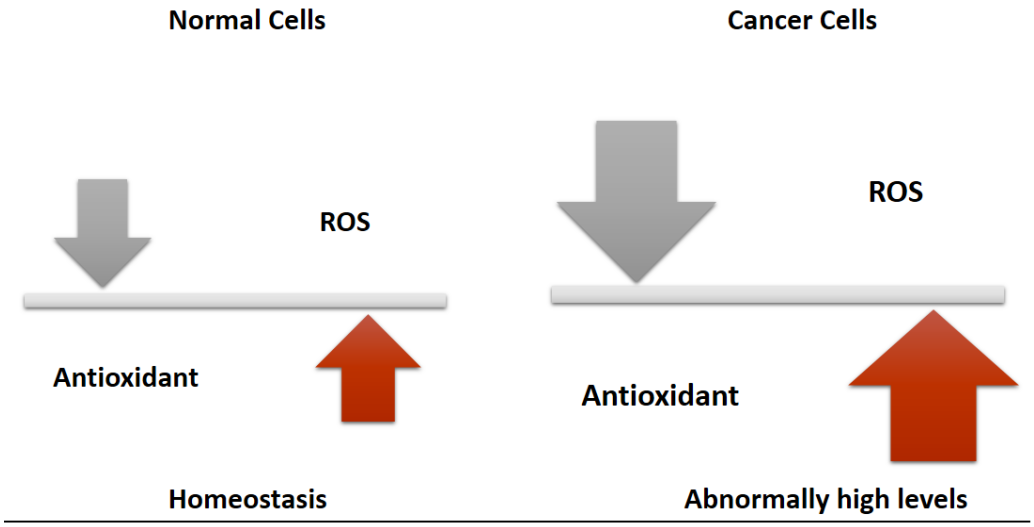

Figure 2.2: ROS and antioxidant production between normal and cancer cells. Adapted from Sullivan and Chandel (2014)

Apoptosis, necrosis and autophagy are known as classical forms of cell death pathways and operate either distinctly or cross-talk through interconnecting signalling pathways to regulate different types of cell death. Apoptosis is referred to as a controlled cascade of self-destruction and subsequent removal of cellular debris by neighbouring cells (Redza-Dutordoir and Averill-Bates, 2016; Renehan *et al.*, 2001). It is a tightly regulated and highly conserved process that is essential for maintaining normal cellular homeostasis. Apoptosis is usually activated to discard potentially harmful cells that either acquired mutation or infected by pathogens (Redza-Dutordoir and Averill-Bates, 2016). Apoptotic cell death takes place via three main pathways; death receptor (extrinsic), mitochondrial (intrinsic) and endoplasmic reticulum (ER) pathways. Studies show that

ROS trigger apoptosis via the three main apoptotic pathways (Circu and Aw, 2010; Redza-Dutordoir and Averill-Bates, 2016). The characteristic morphological features of apoptotic cells include cell shrinking, chromatin condensation, membrane blebbing and nuclear fragmentation (Ly *et al.*, 2003; Redza-Dutordoir and Averill-Bates, 2016; Reed, 2000; Vermes *et al.*, 2000) (Figure 2.3). In contrast to apoptosis, necrotic cell death is regarded as accidental and results from nonspecific stress inducers. The characteristic features of necrotic cell death are enlargement of cellular organelles and rupture accompanied by an inflammatory response (Chen *et al*., 2018). On the other hand, autophagy is a degradation process that involves eventually leads to cell death.

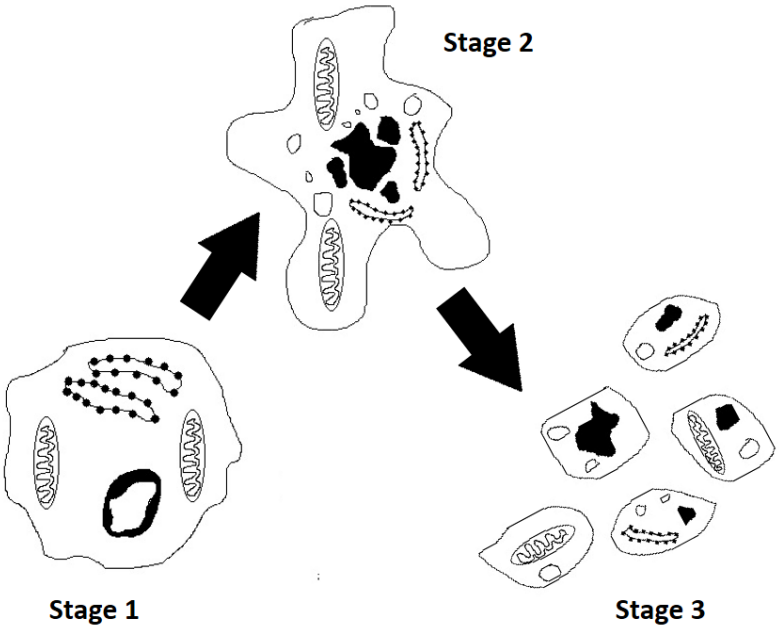

Figure 2.3: Schematic representation of cells undergoing apoptotic morphological change. Adapted from Häcker (2000). In the early apoptotic morphological change, the chromosomes of the nucleus condense and an outgrowth of rounded shapes known as blebs appear on the surface of the membrane (***stage 1***). Then, the cell shrinks in size, the nucleus condenses completely and fragments into small pieces, and cytoplasmic vacuoles form (***stage 2***). Finally, the cell disintegrates into apoptotic bodies containing cellular components which are rapidly eliminated by neighbouring cells through phagocytosis (***stage 3***)

Mitochondrial membrane potential (ΔΨm) plays an essential role in the survival of the cell because it drives ATP synthesis, calcium ion (Ca^{2+}) uptake and storage, and generation and detoxification of ROS (Nicholls, 2004). Under conditions of oxidative stress, components of the mitochondrial permeability transition pore (PTP) undergo oxidative modifications, resultantly stimulating opening of the PTP and significantly impacting mitochondrial anion fluxes (Circu and Aw, 2010). This event causes a brief increase in the mitochondrial membrane hyperpolarization which initiates the collapse of the ΔΨm and translocation of certain mitochondrial apoptogenic factors such as cytochrome c into the cytosol of the cell (Circu and Aw, 2010). In addition,

significant loss of cytochrome c from the inner mitochondrial membrane consequently increase ROS production due to a disturbance in the ETC thereby exacerbating the intracellular oxidative stress and loss of ΔΨm (Circu and Aw, 2010).

2.7 Current lung cancer treatment strategies

Although the mainstay treatment for both SCLC and NSCLC is chemotherapy, advanced lung tumors are resistant to chemotherapy (Kim, 2016). Generally, the recommended treatment option for patients with NSCLC is surgery, if diagnosed at an early stage (Hirsch *et al.*, 2017; Yokota and Kohno, 2004). However, the outcome of surgical treatment remains unsatisfactory due to post-operative complications (Bendixen *et al.*, 2016; Noguchi *et al.*, 1995; Pawlak *et al.*, 2018). In contrast to NSCLC patients, the cornerstone treatment options for SCLC patients remains a platinum based etoposide chemotherapy and radiotherapy, respectively (Alvarado-Luna and Morales-Espinosa, 2016; Gridelli *et al.*, 2005).

In addition to the aforementioned treatment options; immunotherapy and targeted therapy are applied in both lung cancer types to culminate the disease progression (Hirsch *et al.*, 2017). Immunotherapy targets to boost host's anti-tumour immune response in one of two ways. The first approach is to increase the body's immune system, while the second approach is administering proteins and antibodies, which are part of the immune system, to aid the immune system's fight against the cancer. In most instances, cancer immunotherapy drugs are expensive and are effective to only some patients and cancer types (Ventola, 2017). Targeted therapy is where molecular pathways are blocked that are essential for tumour development (Vanneman and Dranoff, 2012). However, Yan and Liu (2013) emphasises that molecular identification, drug resistance, and finding reliable biomarkers are some of the challenges faced by targeted therapies. It is axiomatic that the current lung cancer treatment strategies have their success. However, because of the urgency for effective treatment against cancer, an alternative novel treatment approach is needed to complement the existing treatment regimen in order to increase treatment efficacy and maximize patient quality of life.

2.8 Nutraceuticals and phytochemicals

Nutraceutical — as defined by Dr. Stephen DeFelice — are foods that have medicinal or health benefits (Kalra, 2003). For this reason, they have been extensively exploited in the treatment of numerous illnesses including cancer (Allegri *et al.*, 2018; Pandey *et al.*, 2017). In addition, nutraceuticals are relatively inexpensive and are well tolerated by the human body (Ranzato *et al.*, 2014). Plant bioactive compounds, also referred to as phytochemicals, are responsible for the many pharmacological actions that nutraceuticals possess (Prakash and Sharma, 2014).

In vitro and *in vivo* studies have shown that combination of phytochemicals possess a profoundly stronger cytotoxic activity against cancer cells than single-agent treatment (Klein and Fischer, 2002; Shukla and George, 2011; Zhou *et al.*, 2003). This enhanced anticancer activity of combined phytochemicals stem from their complementary and overlapping mechanisms of action in the modulation of multiple targets involved in tumorigenesis (Sung *et al.*, 2012). An example of these combinations is a turmeric, ginger and garlic aqueous extract mixture that, in comparison to the reference drug Tamoxifen significantly increased apoptosis in MCF-7 and ZR-75 breast cancer cell lines (Vemuri *et al.*, 2017).

Similarly — in mice bearing androgen-sensitive human prostate tumour — there was a significant inhibition in prostate tumorigenicity, final tumour mass and metastases after treatment with a combination of soy phytochemical concentrate and black tea (Zhou *et al.*, 2003). In addition, phytochemicals have been shown to be effective against refractory tumours that are not responding to initial chemotherapeutic agents. For example, a combination of *Curcumae longae* rhizome (Turmeric) or *Coptidis sp.* rhizome (CR) aqueous extracts with tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) showed increased cytotoxicity against human alveolar adenocarcinoma (A549) TRAIL-resistant NSCLC cell lines (Chiang *et al.*, 2018). Researches showed that curcumin from turmeric and shogaol from ginger block multiple pathways involved in tumorigenesis (Aggarwal *et al.*, 2008). Furthermore, these phytochemicals were shown to strongly inhibit the mitochondrial function and in turn induce apoptosis in several neoplastic cell lines including the A549 cancer cell line (Annamalai *et al.*, 2016; Chen *et al.*, 2010).

2.8.1 Turmeric extract

Turmeric is a nutraceutical that is regularly used in many households as a spice in culinary dishes. Curcumin, the principal polyphenolic curcuminoid extracted from the rhizomes of turmeric, is one of the most powerful and promising chemopreventive and anticancer phytochemicals (Ye *et al.*, 2012). Curcumin and its counterpart's, demethoxycurcumin (DMC) and bisdemethoxycurcumin (bDMC) are called curcuminoids (Figure 2.4) and make up 1-6% of turmeric by weight. Of these phytoconstituents 60-70% is curcumin, while 20-27% and 10-15% is DMC and bDMC, respectively (Nelson *et al.*, 2017).

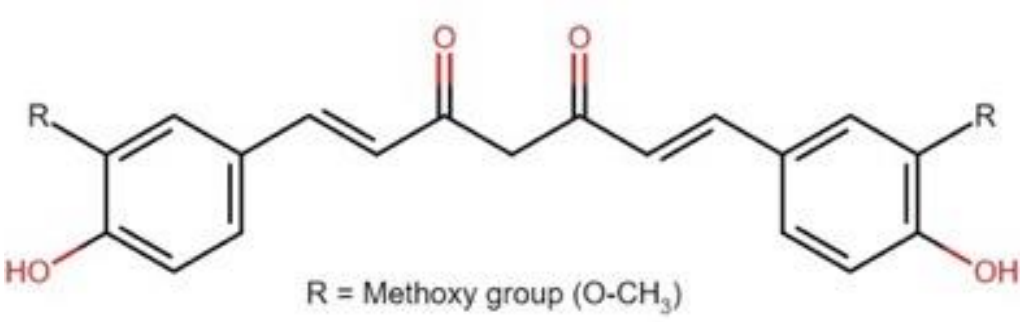

Figure 2.4: Chemical structure of curcuminoids; Curcumin (contains both methoxy functional groups), DMC (only one methoxy group), and bDMC (methoxy group is absent). Figure generated using Chemspider (http://www.chemspider.com/StructureSearch.aspx)

In addition to its anticancer activity, curcumin also has potent anti-inflammatory, antioxidant and pro-oxidant activity (Ahsan *et al.*, 1999). Studies have shown that curcumin exerts its anticancer activity through modulation of transcription factors, growth factors, protein kinases, proteasomes, epigenetic changes and pro-inflammatory mediators involved in tumour initiation, promotion, angiogenesis, and metastasis (Aggarwal *et al.*, 2008; Kunnumakkara *et al.*, 2017). The compound is also pharmacologically safe; human phase II clinical trials against pancreatic cancer indicated no toxicity when administered at doses up to 10 g/day (Dhillon *et al.*, 2008).

Studies have shown that curcumin mediates its apoptotic effect in A549 cancer cell lines through an increase in ROS levels and a resultant fall in the MMP ($\Delta\Psi m$) (Chen *et al.*, 2010; Cheng *et al.*, 2017). Wu *et al.* (2010) also showed that curcumin induced apoptosis in NCI-H460 NSCLC cell lines via intrinsic, extrinsic and endoplasmic reticulum pathways (Figure 2.5). In addition, Yang *et al.* (2012) reported that curcumin induced apoptosis in NCI-H446 SCLC cells by causing a rapid decrease in MMP and an increase in intracellular ROS levels. These *in vitro* results jointly reveal the therapeutic versatility of curcumin in both NSCLC and SCLC cell types. Despite the potential therapeutic activity and low intrinsic toxicity, the clinical utility of curcumin is hampered by its poor solubility at low pH, instability at neutral and high pH, multidrug efflux pump effects and low bioavailability as a result of extensive metabolism and rapid elimination (Patil *et al.*, 2015).

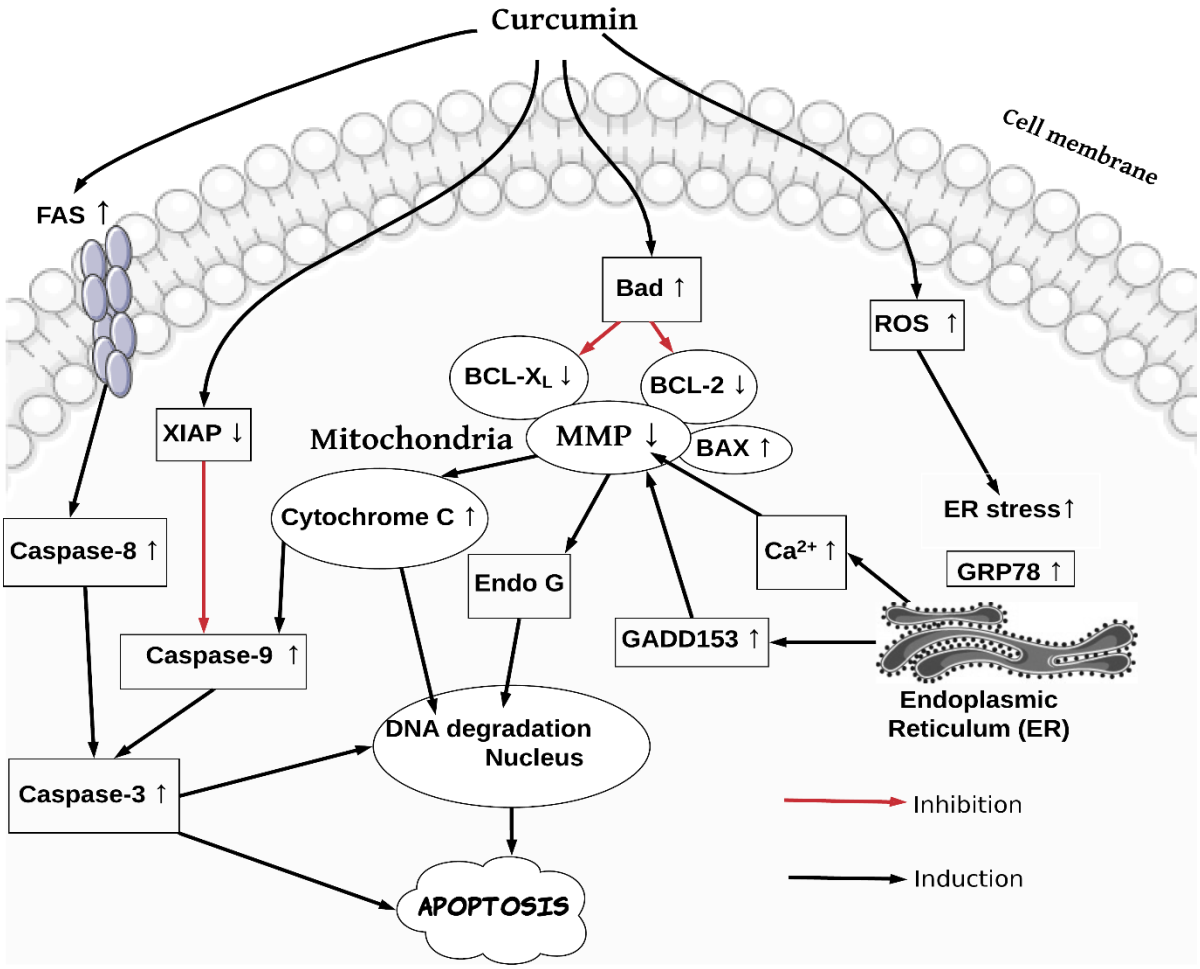

Figure 2.5: Mechanism of curcumin–induced apoptotic cell death via intrinsic, extrinsic and endoplasmic reticulum (ER) pathways. Adapted from (Wu *et al*., 2010). MMP = mitochondrial membrane potential, FAS = first apoptosis signal receptor, ROS = reactive oxygen species, Bcl-2 = B-cell lymphoma 2, BCL-X_L = B-cell lymphoma-extra-long, , BAD = Bcl-2 associated agonist of cell death, BAX = Bcl-2 associated X protein, DNA = deoxyribonucleic acid, XIAP = X-linked inhibitor of apoptosis, Endo G = endonuclease G, GADD153 = growth arrest- and DNA damage-inducible gene 153, GRP78 = 78-kDa glucose regulated protein. Figure generated using Lucidchart (https://www.lucidchart.com)

2.8.2 Ginger

Ginger (*Zingiber officinale*), has been used in traditional medicine dating as far back as 2 500 years (Shukla and Singh, 2007). The principal phytochemicals found in the rhizomes of ginger include [6]-gingerol, [6]-shogaol and zingerone polyphenols, respectively (Surh *et al.*, 1998). Previous experimental studies showed that [6]-shogaol (6SG) —constituents found in dried ginger — has a more potent antioxidant, and anti-inflammatory activity than gingerols (Dugasani *et al.*, 2010; Guo *et al.*, 2014). The authors corroborated that the presence of a α- and β-unsaturated ketone moiety in the molecular structure of the compound (Figure 2.6) bestowed its enhanced therapeutic activity. In the A549 cancer cell line and mice-bearing A549 cells, the pro-oxidant role

of 6SG increased intracellular oxidative stress, which activated the release of mitochondria-associated apoptotic molecules such as cytochrome c, caspase-3 and -9 (Kou *et al.*, 2018) (Figure 2.7). In another study, 6SG-induced apoptosis was accompanied by enhanced ROS levels, altered MMP, and increased DNA damage in laryngeal cancer cells (HEp-2) (Annamalai *et al.*, 2016).

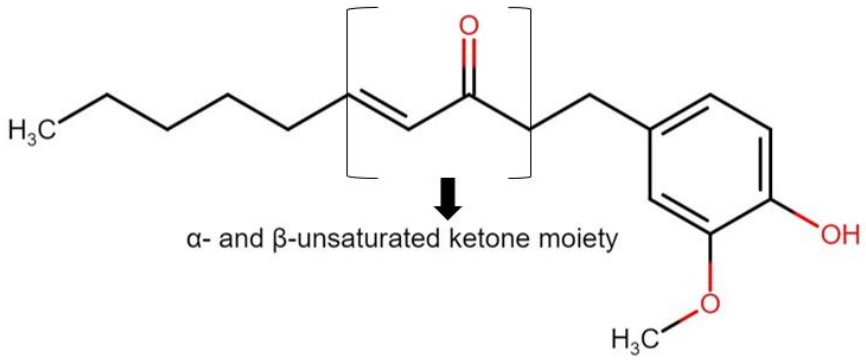

Figure 2.6: Chemical structure of 6SG and its pharmacophore (α- and β-unsaturated ketone moiety). Figure generated using Chemspider (http://www.chemspider.com/StructureSearch.aspx)

Like its counterpart curcumin, 6SG also has anti-proliferative, anti-metastatic, and pro-apoptotic activities. Kim *et al.* (2015) observed that 6SG is a potent inhibitor of STAT3 activation in cancerous cells and when administered alone, it significantly suppressed tumour growth. Nedungadi *et al.* (2018) showed that 6SG induced extensive cytoplasmic vacuolation and cell death in MDA-MB-231 (breast) and A549 cancer cell lines. In the same study, 6SG halted the cell cycle at the G1 phase in MDA-MB-231 cells, and significantly decreased the cell viability in both the A549 and MDA-MB-231 cancer cells. Also, Nedungadi *et al.* (2018) showed that 6SG selectively induced cytotoxicity to tumour cells without having an effect on normal human embryonic lung epithelial cells and human blood cells. In an *in vivo* human clinical trial, Zick *et al.* (2008) reported that doses up to 2 g per day of ginger constituents 6-gingerol, 8-gingerol, 10-gingerol, and 6SG showed low toxicity.

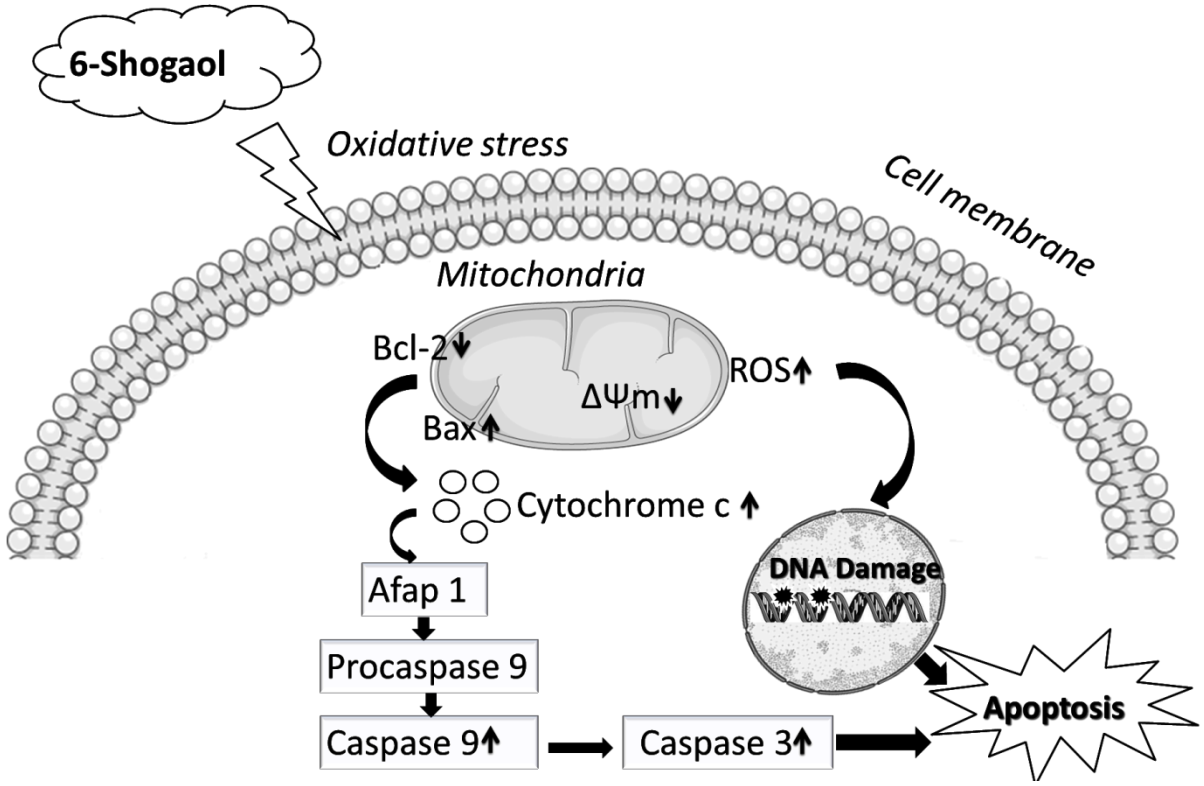

Figure 2.7: [6]-shogaol induces apoptosis by increasing intracellular oxidative stress, lowering mitochondrial membrane potential and activating the caspase cascade. Adapted from (Annamalai et al., 2016). Afap1 = Actin filament-associated protein 1). Δ Ψm = mitochondrial membrane potential, ROS = reactive oxygen species, Bcl-2 = B-cell lymphoma 2, BAX = Bcl-2 associated X protein, DNA = deoxyribonucleic acid. Figure generated using Servier Medical Art (http://smart.servier.com)

2.9 The use of drug delivery systems to improve the biological efficacy of nutraceuticals

The oral route of administration is a favoured route for chronic diseases including cancer, since it is convenient, non-invasive and cost-effective (Ting *et al.*, 2014). However, like many other phytochemicals the lipophilic nature of curcumin, and ginger extract phenolics impedes their dissolution rate in the physiological fluid. This resultantly leads to poor absorption and high excretion profile. For instance, a study conducted by Shoba *et al.* (1998) showed that an oral dose of curcumin (2 g/kg) in rats and human volunteers (weighing 50–75 kg), produced a peak plasma concentration of 1.35±0.23 µg/mL at time 0.83 hours in rats and an extremely low serum curcumin concentration (0.006±0.005 µg/mL) after one hour in humans.

Over the years, multifarious nano and micro drug delivery systems such as solid lipid nanoparticles (SLN) and liposomes have been designed to improve the biological uptake of hydrophobic pharmaceutical agents. One of them is the novel Pheroid® technology, a colloidal emulsion system comprised of plant and ethyl esters of essential fatty acids (EFAs) as the

dispersed phase and nitrous oxide saturated water as the continuous phase (Slabbert *et al.*, 2011; Steyn *et al.*, 2011). Unlike the other delivery systems such as polymeric delivery systems, Pheroid® is made from inexpensive raw materials that are safe and do not promote toxic accumulation during chronic consumption. Moreover, the delivery system has the capacity to entrap both hydrophobic and hydrophilic drug molecules, protect against instability in physiological environments and rapid metabolic transformations, target specific treatment areas, facilitate lymphatic uptake thus promoting higher bioavailability of parental compounds and decrease drug resistance (Grobler, 2009). In addition, the Pheroid® delivery system have been used to encapsulate active ingredients derived from plant compounds for application in the cosmetics, nutritional supplements and agricultural purposes. The clinical applicability of the Pheroid® formulation containing plant derived active compounds was investigated in the current study.

2.10 Summary

Globally, cancer is a major public health concern, with the number of new cancer cases and deaths rising rapidly (Bray *et al.*, 2018; WHO, 2018a). Lung cancer, is the most frequently diagnosed cancer type and a primary cause of cancer related death in the world (Townsend *et al.*, 2017; WHO, 2018a; Wong *et al.*, 2017). Current lung cancer therapy involves chemotherapy, surgery, immunotherapy, radiation therapy and targeted therapy. The modern paradigm of cancer therapy is shifting towards precision medicine in order to effectively treat cancer based on an understanding of the patient's and the disease's genetic milieu (Ginsburg and Phillips, 2018). However, high treatment costs and undesired toxicity of chemotherapeutic drugs often put patients in palliative care (Bharti *et al.*, 2018; Townsend *et al.*, 2017).

Traditional medicine has long been used as the cornerstone for treating many illnesses including cancer. Curcumin from turmeric and 6SG from ginger blocks multiple pathways involved in tumorigenesis (Magalhães *et al.*, 2009; Surh *et al.*, 1998). A profound pharmacologic action is achieved by combining two or more phytochemicals (Klein and Fischer, 2002; Shukla and George, 2011; Zhou *et al.*, 2003). The enhanced therapeutic action achieved by combining phytochemicals stem from their overlapping and complementary mechanism of actions (Sung *et al.*, 2012). Apart from their efficacy, several studies also demonstrated that these phytochemicals are safe (Dhillon *et al.*, 2008; Yong *et al.*, 2015; Zick *et al.*, 2008). However, the clinical application of these compounds is greatly diminished owing to their limited physicochemical activities such as solubility following oral administration. The oral route is a favoured route of administration because it is non-invasive, inexpensive and has the highest patient compliance. Multifarious lipid based drug delivery systems such as the novel Pheroid® technology have been developed over the years to overcome such challenges. To the best of our knowledge, the current study is the

first to co-formulate Meriva® (a patented curcumin formulation) and ginger extract using a novel Pheroid® delivery system and assess the clinical significance of the combined phytochemical formulation against A549 lung cancer cells both *in vitro* and in *vivo*.

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CHAPTER 3

Chapter 3 consists of a manuscript to be submitted for publication to *Pharmaceutics*; an open access journal from MDPI. The manuscript is written in US English and according to the Instruction for Authors guideline (Appendix B). This chapter is presented in the template provided by the journal. For ease of reading, the tables and figures are inserted at their relevant positions.

Type of the Paper (Article)

Evaluating the chemopreventive activity of Meriva® and ginger extract combination in Pheroid® against lung cancer: an *in vitro* study

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Abstract: Cancer is a global health concern and the complexity of the disease limits its effective treatment. Therefore, there is a continuous search for supplementary medicines that can complement current cancer therapy. Phytochemicals have been shown to have remarkable chemopreventive activities and their combination often produces robust anticancer effects. However, the clinical application of phytochemicals is restricted as a result of their poor physicochemical properties. In the present study, the chemopreventive activity of a combined Meriva® and ginger extract in the Pheroid® drug delivery system was investigated against the human adenocarcinoma A549 lung cancer cell line through cell viability, mitochondrial metabolic function and respiration, oxidative stress and apoptosis assays. Combinations of phytochemicals in Pheroid® produced a more robust anticancer effect than individual treatments. The apoptotic effect observed was accompanied by a marked decrease in mitochondrial metabolic function and respiration, as well as cell proliferation. In addition, Pheroid® reduced the pro-oxidant effects of individual and combination of phytochemicals and as a result decreased intracellular oxidative stress. The anticancer effect of the Meriva® and ginger extract combination in Pheroid® was comparable with the effects observed with cisplatin treatment and further animal studies are therefore required to assess its effectiveness in the biological system.

Keywords: A549 cells; Anticancer effects / chemopreventive; Curcumin; Drug delivery system; Ginger extract; Lung cancer; Meriva®; Mitochondria; Pheroid®; Phytochemicals; [6]-shogaol

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in men and women worldwide accounting for 1.7 million deaths every year [1]. In contrast to other cancer types, lung cancer has the lowest survival profile; with the one year and five year survival rates of lung cancer patients at all stages at 44% and 17%, respectively [2, 3]. Based on the histological morphology of the cells, lung cancer is classified into two major types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), of which NSCLC accounts for the majority of the incidence and mortality cases.

Lung cancer cells rely heavily on mitochondrial respiration to generate energy that is vital for rapid cellular growth and metastasis [4]. The mitochondrion contains multiple redox-active complexes and metabolic enzymes. It is the major source for the generation of endogenous reactive oxygen species (ROS) such as the singlet oxygen radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•) as by-products of aerobic metabolism via the electron transport chain [5]. Compared to normal cells, cancer cells display higher levels of ROS. To balance the elevated ROS level, cancer cells often exhibit an increase in the

expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) [5]. These enzymes are regarded as indispensable first line of defense antioxidants.

When cancer cells fail to maintain the elevated intracellular redox homeostasis, irreversible oxidative damage to proteins, lipids, nucleic acids, membranes, and organelles such as mitochondria will occur, which in turn induces cell death via activation of apoptosis, necrosis or autophagy/mitophagy [6, 7]. Apoptotic cell death can take place via three main mechanisms, namely death receptor (extrinsic), mitochondrial (intrinsic) and endoplasmic reticulum (ER) pathways. Studies have shown that ROS triggers apoptosis via all three of these main apoptotic pathways [6, 8].

Chemotherapy is the mainstay standard treatment for lung cancer disease [9]. However, in advanced lung tumors chemoresistance may develop and alternative treatment approaches such as radiotherapy, immunotherapy, targeted therapy and surgery are often employed to combat the cancer. In most cases, the surgical approach remains unsatisfactory due to post-operative complications [10–12] while cancer immunotherapy drugs are expensive and only effective in some patients and cancer types [13]. On the other hand, molecular identification, drug resistance, and the discovery of reliable biomarkers are some of the challenges faced in targeted therapies [14]. Therefore, because of the urgency of the need for effective treatment against lung cancer, there is a quest for assessing supplementary medicines that have better treatment modalities such as a favorable efficacy-to-toxicity profile.

In this regard, complementary herbal medicines have proven to be a promising treatment approach in modern allopathic medicine for the treatment and/or prevention of diseases, including cancer. The herbaceous perennial plants, turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*), which are commonly used as spices in culinary dishes, possess a wide variety of pharmacological and physiologic functions including anti-inflammatory, antioxidant, anti-microbial, chemo-preventive and chemo-therapeutic activities [15–17]. The principal and biologically active compounds, known as phytochemicals, are found in the rhizomes of turmeric and ginger. Specifically curcumin is found in turmeric, and [6]-gingerol, [6]-shogaol and zingerone polyphenols are found in ginger [18].

Over the years, multifarious nano- and micro drug delivery systems have been designed to improve the biological uptake of hydrophobic pharmaceutical agents. Among them is the Pheroid® drug delivery system. Pheroid® is a colloidal emulsion system comprised of plant and ethyl esters of essential fatty acids as the dispersed phase and nitrous oxide saturated water as the continuous phase [19, 20]. It has the capacity to entrap both hydrophobic and hydrophilic drug molecules [21], enhance the cellular uptake and bioavailability of drug compounds [22–24], and decrease drug resistance and toxicity [25].

The aim of this study was to investigate the comparative anticancer potential of Pheroid® formulated Meriva® (curcumin phytosome) and ginger extract (GE) combinations against the human adenocarcinoma A549 lung cancer cell line. This was achieved by measuring the cytotoxicity, apoptosis, oxidative stress markers, and functional mitochondrial bioenergetics parameters.

2. Materials and Methods

2.1. Materials

Meriva® was a kind gift from Formul8 Pharma (Pty) Ltd (Johannesburg, South Africa). Ginger extract (GE) with >10% gingerols was purchased from Xi'an B-Thriving I/E Co., Ltd. (Shaanxi, China). Vitamin F ethyl ester was purchased from IMCD (Rotterdam, The Netherlands), dl-α-tocopherol and Kolliphor® EL were purchased from BASF (Berlin, Germany). The human non-small cell lung cancer adenocarcinoma A549 cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The reagents and consumables for cell culturing and assays were obtained from Thermo Fisher Scientific (Waltham, MA, USA), Gibco (Dun Laoghaire, Ireland), and Sigma-Aldrich (Missouri, USA). The raw materials of the Pheroid® are graded raw materials for the manufacturing of human products. All other chemicals used were of analytical reagent grade. The absorbance and fluorescence measurements were done using a SpectraMax® Paradigm® multimode detection platform (SpectraMax, Molecular devices).

2.2. Formulation of Meriva® and ginger extract in pro-Pheroid®

Pro-Pheroid®, a precursor of Pheroid® which is comprised of an oil mixture, was prepared following the method described by Grobler [24]. Briefly, Vitamin F ethyl ester, Kolliphor® EL and dl-α-tocopherol (70%, 29% and 1% *w/w*) were mixed thoroughly and gassed with nitrous oxide (N_2O) under pressure. The concentrations of Meriva® and GE were selected from literature on the basis of a preclinical study: Meriva® (70 mg/kg) was used as a putative chemopreventative agent for lung cancer and GE (100 mg/kg) was used in the management of prostate cancer [26, 27]. Using these dosages as guidelines, a panel of four Meriva® and GE combinations, and individual actives in pro-Pheroid® were formulated. The final products (pre-gassed pro-Pheroid® and phyto-actives) were mixed until homogeneous and gassed with N_2O under pressure. These pro-Pheroid® formulations were diluted 5 000 times in supplemented DMEM to give a 0.02% Pheroid® concentration and final Meriva® and GE concentrations are presented in Table 1. Pheroid® at 0.02% and DMSO at 0.125% were used as optimum final carrier concentrations based on the cell viability assay performed on the A549 cells (Figure S1).

Table 1. Treatment concentrations of individual and combinations of Meriva® and ginger extract (DMSO dissolved and Pheroid® formulated), Pheroid® only and cisplatin

Formulation	Concentration (μg/mL)						Pheroid® only	Cisplatin
	Meriva®	GE	Meriva®/GE combination					
	52.5	75	52.5/75	75/52.5	52.5/52.5	75/75	0.02%	30 μM
DMSO	UF1	UF2	UF3	UF4	UF5	UF6		
Pro-Pheroid®	F1	F2	F3	F4	F5	F6		

UF = Actives dissolved in 0.125% DMSO and F = Formulated actives in 0.02% Pheroid®.

2.3. Cell culture

The human lung adenocarcinoma cell line, A549 (ATCC® CCL-185™) was maintained in supplemented growth medium: Dulbecco's Modified Eagle's Medium (DMEM) with 10% *v/v* fetal bovine serum, 2 mM L-glutamine, 1% *v/v* penicillin 100 units/mL, streptomycin 100 μg/mL, and amphotericin B (0.25 μg/mL mixture) in a humidified incubator (5% CO_2, at 37°C) [28]. Cells at 85% confluence were used in subsequent experiments. For the neutral red uptake and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays, cells were seeded in clear 96 well tissue culture treated plates at a seeding density of 1 x 10^4 cells per well (200 μL). For the ROS assay, cells were seeded at the same density but in a black 96 well tissue culture treated plate with a clear bottom. For the SOD and CAT assays, cells were cultured in a 24 well tissue culture treated plate at a seeding density of 5 x 10^4 cells per well (1 mL). Apoptosis and nuclear morphology assays were performed on cells seeded in a 12 well tissue culture treated plate seeded at 1 x 10^5 cells per well (1 mL).

2.4. Cell viability and proliferation assay

The cell viability was determined using the neutral red assay according to the method described by Repetto *et al.* [29]. After cell attachment, the supplemented growth media was removed and cells were exposed to the respective Meriva® and GE concentrations (free form and formulated), cisplatin (positive control) at an IC_{50} of 30 μM [30] and Pheroid® only (Table 1) for 24 hours. Filtered neutral red (NR) dye (0.033%) of 200 μL was added to the cells and cells were incubated for 3 hours. Cells were rinsed thrice with PBS and 200 μL of neutral red solubilisation solution (50% ethanol, 49% distilled water and 1% acetic acid) was added to each well. The plate was then incubated at room temperature for 20 minutes on a plate shaker and the absorbance was measured at a wavelength of 540 nm. The background absorbance was measured at 690 nm for each sample. The absorbance measurements were taken in triplicates for each treatment concentration as well as for the untreated cells (n = 4). The experiment was done independently

in duplicate. Measurements were performed by subtracting the background, and the cell viability was determined by the following equation:

$$\%\text{Cell viability} = \frac{A_{Experiment}-A_{Blank}}{A_{Control}-A_{Blank}} \text{ x } 100 \text{ to get } \% \quad (1)$$

Where; $A_{Experiment}$ refers to the absorbance of the treated cells, A_{Blank} is the absorbance of the blank (PBS) and $A_{Control}$ is absorbance of the untreated cells.

The effect of the formulated and DMSO dissolved phytochemical combination on the mitochondrial metabolic activity of A549 cells was quantified colorimetrically using the MTT assay according to the method described by Mosmann [31]. After a 24 hour exposure to treatment (Table 1), the spent media was removed and the cells were washed three times with PBS. Next, 100 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (0.5 mg/mL) was added per well and the plate was incubated for 3 hours at 37°C. After incubation, the supernatant was removed and the formazan crystals were dissolved in 200 µL of DMSO and left on the plate shaker for 20 minutes. The absorbance was measured at wavelengths of 560 nm and 630 nm (background). Measurements were made in triplicate, blanked, and percentage MTT reduction was calculated according to equation 1 above.

2.5. Oxidative stress markers

2.5.1. Reactive oxygen species (ROS)

The generation of ROS after 24 hour exposure to different treatment conditions (Table 1) was evaluated according to the method described by Yao [32]. One hour prior to the assay, the untreated cells (negative control) were stimulated with 0.03% of hydrogen peroxide (H_2O_2) [33]. The treatment media was removed and cells were washed three times with PBS and stained with 10 µM 2'-7'dichlorofluorescin diacetate (H_2DCFDA), and incubated for 30 minute at 37°C in the dark. Thereafter, the H_2DCFDA was removed and cells were washed gently once with PBS, after which 100 µL of PBS was added to each well and fluorescence was measured with excitation and emission wavelengths of 480 nm and 535 nm, respectively. The relative fluorescence unit (RFU) of the intracellular ROS was calculated as the difference between experimental and blank measurements. The fluorescence measurements were done in triplicate for each treatment concentration and for the controls.

2.5.2. Catalase activity

The catalase (CAT) activity was determined according to Cohen [34] with adaptations. After a 24 hour exposure period, media was removed and cells were washed twice with PBS, trypsinized and centrifuged at 1 000 *g* for four minutes. The cell pellet was resuspended in ice cold phosphate buffer [0.09 M K_2HPO_4 adjusted to pH 7.4 with 0.09 M KH_2PO_4] (270 µL). The cells were lysed by sonication using a 702 model ultrasonic cleaning bath (United scientific, South Africa) at a medium intensity for 20 seconds, followed by the addition of 25 µL of 5% Triton X, and centrifugation at 10 000 *g* for 10 min at 4°C. A 10 µL sample of the supernatant was taken for CAT analysis; the remaining sample was stored for protein determination. The samples were added to 93 µL H_2O_2 (6 mM) each in a 96 well plate and incubated for three minutes, after which the reaction was stopped by the addition of 19 µL of 6N H_2SO_4. Finally, 130 µL $KMnO_4$ (1.9 mM) was added to each well and the absorbance was measured within 30 to 60 seconds at 490 nm. The protein concentration of each sample was determined using Bradford's method [35]. All tests were done in triplicate and results were reported as a mean of three readings and expressed as µmol H_2O_2/min/mg protein [34].

2.5.3. Superoxide dismutase (SOD) activity

The superoxide dismutase (SOD) activity was determined according to the method of Del Maestro [36] with modification. Cells were seeded and exposed as for the CAT determination. After exposure, cells

were lysed with 0.2% Triton X-100 and centrifuged at 10 000 *g* for 10 min at 4°C. Tris buffer (50 mM, pH 7.5) was mixed with 1 nM diethylene triamine penta-acetic acid (DTPA) in a 49:1 (*v*/*v*) ratio. The mixture was aerated with air for 20 minutes and the pH was adjusted to 8.2. A 4 µL cell lysate sample and Tris buffer blank were added in triplicate to the wells in a 96 well tissue culture plate in triplicate and 245 µL of DTPA/Tris buffer mixture was added. The reaction was initiated by adding 4 µL pyrogallol (24 nM in 10 mM HCl) to each well and the optical density was measured at 560 nm every 30 seconds for 5 minutes. The SOD activity was expressed as ngSOD/mg protein [36].

2.6. Nuclear morphology and apoptosis assay

The nuclear morphology anomalies and apoptosis were evaluated using the acridine orange (AO) and ethidium bromide (EtBr) differential staining assay according to the method described by Ribble [37] with modifications. The AO/EtBr dual staining assay is an economic and effective method for detecting apoptosis compared with flow cytometry [38]. The A549 cells were exposed to the different treatment parameters (Table 1) for 24 hours. After treatment, the supernatant was gently removed and the cells were washed twice with PBS. A 100 µL AO/EtBr staining solution (100 µg/mL of each dye in PBS) was added and cells were incubated at room temperature for 5 min and washed once with PBS. Images were captured immediately using a fluorescence microscope (Olympus Co., Japan) at 100x magnification with excitation and emission wavelengths of 488 nm and 550 nm, respectively. The experiment was performed once and apoptosis quantification was done in triplicate, counting a minimum number of 300 cells for each treated and untreated group. Cell Counting was done using ImageJ v1.52p software [39] according to the method described by Helmy Iman and Azim Adel [40]: The images were first converted to 8-bit, the threshold was set, and watershed was applied to split closely touching cells. A bright green fluorescence with organized chromatin structure indicated healthy cells, green or orange-red fluorescence with condensed or fragmented chromatin indicated apoptotic cells and red fluorescence indicated necrotic cells [41]. The percentage of apoptotic cells were calculated using the following formula:

$$\text{Percentage of apoptosis} = \frac{\text{Total number of apoptotic cells}}{\text{total number of cells counted}} \times 100 \quad (2)$$

2.7. Seahorse XF^e Mito stress test

The overall mitochondrial health of the A549 cells was evaluated by measuring the oxygen consumption rate (OCR) according to the method described by Decleer [42] with adaptations, using the Seahorse™ XF^{e}96 Extracellular Flux Analyser (Agilent Seahorse Bioscience, MA, USA). Prior to running the assay, the Seahorse XF^e sensor cartridge was hydrated by adding 200 µL Seahorse XF^e calibrant in the XF^e utility plate. Cells were seeded at an optimized seeding density of 2.5 x 10^4 per well (160 µL) onto a 96 well Seahorse microplate. Cells were exposed to Pheroid® formulated and unformulated individual and combinations of phytochemicals, Pheroid® only and cisplatin (Table 1) one hour after seeding and incubated for 24 hours in humidified air at 37°C in 5% CO_2. After treatment, the growth media was removed and the cells were washed twice with 200 µL Seahorse assay media (un-buffered base medium) using the XF^e Prep station. To each well of the plate, 180 µL of assay media was added and the plate incubated for 1 hour at 37°C in a CO_2 free incubator. Following calibration, the baseline OCR was measured and the cells were treated in succession with oligomycin (1 µM), carbonyl-cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.75 µM) and rotenone/antimycin A (0.5 µM each), while measuring the ATP production, maximal respiration and spare respiratory capacity (SRC) bioenergetic parameters in between the additions. Following the assay, the media was removed and the cells were frozen at -80°C for normalization by the CyQUANT® GR assay. Hereafter, the cells were thawed and 100 µL of CyQUANT® GR dye/cell-lysis buffer was added per well, and the plates incubated in the dark for five minutes at room temperature. Then, 95 µL of this cell suspension was transferred into a black spectrophotometer microplate and fluorescence was measured using Synergy™ HT Multi-detection microtiter plate reader (BioTek® Instruments, VT, USA) at 485/20 nm excitation and 528/20 nm emission wavelengths.

Basal respiration is strongly controlled by the ATP turnover and it changes in response to ATP demand [43]. Therefore, a reduction in basal respiration indicates lower oxygen consumption and ATP production, which indicates impairment of normal mitochondrial respiration. FCCP-stimulated oxygen consumption rate measures maximal respiration and a decrease in this response is strongly tied with mitochondrial dysfunction [43]. FCCP is an uncoupling agent that disrupts mitochondrial membrane potential (MMP), thus enabling complex V to consume maximum oxygen. The spare respiratory capacity (difference between basal and maximum respiration) measures the cells ability to meet with increased energy demand when under stress and a lower SRC is suggestive of mitochondrial dysfunction [43].

2.8. Statistical analysis

The data obtained was expressed as the mean ± standard error of the mean (SEM). Levene's test was used to determine the homogeneity of the variance and significant differences between groups were calculated using either the Kruskall-Wallis ANOVA or the Unequal N HSD test (modified Tukey HSD test), using Dunnett's test as a *post hoc* test. The results were considered statistically significant if $p < 0.05$. Statistical analysis was done on Statistica (StatSoft Inc., Germany) and graphs were produced using GraphPad Prism6 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Anti-proliferative effects of Meriva® and GE on A549 cells

Assessing the viability of cancer cells is an imperative *in vitro* parameter for screening novel anticancer agents [44]. One way of achieving this is via the neutral red uptake assay, a highly sensitive indicator of cell viability as a function of cytostatic or cytotoxic effects [44]. At high concentrations, Pheroid® interferes with the cell's viability by forming an oily layer on the surface of the culture media and thereby preventing gaseous exchange [24, 45]. In contrast, DMSO at a concentration of 0.1%–0.5% is regarded as non-toxic to cells [46]. To avoid false positive results, the Pheroid® only and DMSO working concentrations were optimised. Pheroid® at a maximum concentration of 0.02% and DMSO at 0.125% showed no effect on the viability of A549 cells when compared to untreated cells (Figure S1). Therefore, these concentrations were selected as optimum working concentrations for loading the phytochemicals. According to the International Standard Organisation (ISO), cell viability above 80 percent is non-cytotoxic; between 80 percent to 60 percent is considered weakly cytotoxic; 60% to 40% moderate and below 40% strongly cytotoxic [47]. Combinations of Meriva® and GE in Pheroid® (F3 [52.5 μg/mL Meriva® and 75 μg/mL GE], F4 [75 μg/mL Meriva® and 52.5 μg/mL GE] and F5 [52.5 μg/mL Meriva® and 52.5 μg/mL GE]) resulted in significant ($p < 0.005$) growth inhibition of A549 cells when compared to untreated cells and their DMSO dissolved counterparts (Figure 1A). These combinations gave moderate to weak cytotoxicity: F3 (59%), F4 (60%), F5 (58%), respectively. By contrast, treatment with UF6 (75 μg/mL Meriva® and 75 μg/mL GE) and F6 (75 μg/mL Meriva® and 75 μg/mL GE) showed a significant ($p < 0.05$) reduction in cell viability when compared to the untreated cells, however, no significant difference was observed between UF6 and F6. When compared to its free form (UF1 [52.5 μg/mL]), Pheroid® formulated Meriva® (F1 [52.5 μg/mL]) elicited a significant ($p < 0.05$) decrease in A549 cell viability (Figure 1A). Cisplatin demonstrated strong ($p < 0.00001$) cytotoxicity against A549 cells when compared to the untreated cells with cell viability decreasing to 30%.

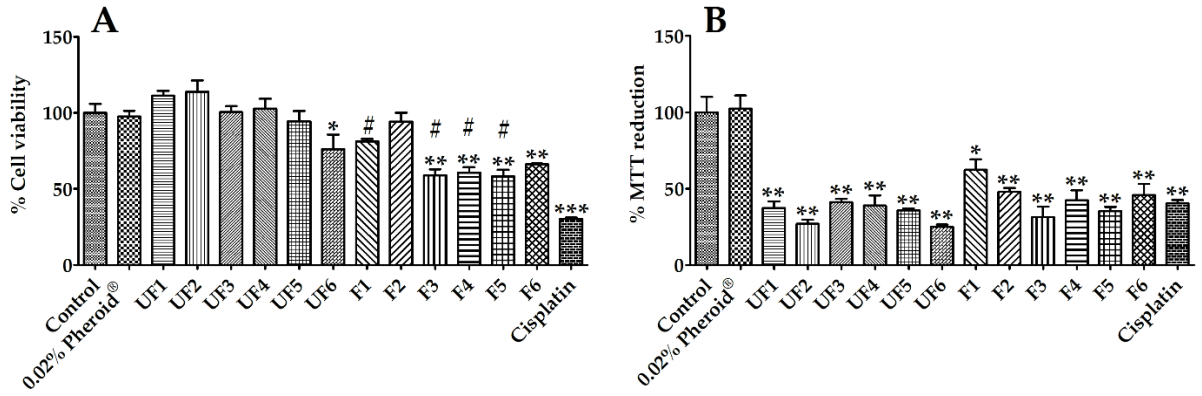

Figure 1. Cell viability, as indicated by Neutral Red (A) and MTT (B) assays, of A549 cells treated for 24 hours with individual and combinations of Meriva® and ginger extract. UF = DMSO dissolved; F = Pheroid® formulated; (n = 4) ± SEM; Asterisk (*) indicates statistical significance relative to the control where * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.00001$); hash (#) indicates statistical significance relative to the DMSO dissolved counterpart ($p < 0.05$)

3.2. Mitochondrial metabolic activity

Mitochondria are responsible for the majority of cellular ATP production and play a critical role in the proliferation and survival of cells. The effect of Pheroid® formulated and DMSO dissolved phytochemicals, either alone or in combination, on the mitochondrial metabolic function of A549 cells was assessed using the MTT assay. Cells treated with 0.02% Pheroid® only displayed no observable effect ($p > 0.5$) when compared to the untreated control. This indicated that Pheroid® alone does not interfere with the cells' mitochondrial metabolic activity. Therefore, any effect observed can be regarded as the result of the response to phytochemical treatment. All treatment groups significantly ($p < 0.05$) reduced the mitochondrial metabolic activity of A549 cells when compared to the untreated cells (Figure 1B).

3.3. Assessing the effect of Meriva® and GE on intracellular ROS, SOD and CAT levels

Antioxidant defense enzymes such as SOD and CAT preserve the balance between ROS generation and elimination. When this delicate redox homeostasis is disturbed either by an increase in ROS, or a decrease in antioxidant levels, it leads to oxidative stress (OS). To evaluate the OS markers, cells were exposed to treatment (Table 1) for 24 hours. The intracellular ROS was measured using a cell permeant H_2DCFDA fluorogenic dye. Once inside the cell, H_2DCFDA is converted to a nonfluorescent compound, which later is oxidised by ROS to fluorescent 2'-7'dichlorofluorescein (DCF). The fluorescence intensity can then be directly correlated to intracellular ROS activity. Individual and combinations of DMSO dissolved Meriva® and GE treatment significantly ($p < 0.005$) increased ROS when compared to the untreated control (Figure 2A); with UF1 (52.5 µg/mL Meriva®) demonstrating the highest ($p < 0.00001$) increase. Cisplatin treated cells also exhibited a significant ($p < 0.00001$) increase in ROS levels when compared to the untreated cells. In contrast, cells treated with 0.02% Pheroid® only significantly ($p < 0.005$) reduced ROS (Figure 2B). In general, a reduction in ROS was observed in Pheroid® formulated phytochemical treatment (Figure 2). Nonetheless, treatment with individual actives in Pheroid® displayed a significant ($p < 0.005$) increase in ROS compared to the untreated control, while their combinations in Pheroid® showed no observable change ($p > 0.1$) (Figure 2B). Hydrogen peroxide (H_2O_2) was used as a positive control and exposure of cells to this chemical resulted in a significant ($p < 0.00001$) increase in intracellular ROS compared to the untreated control (Figure 2).

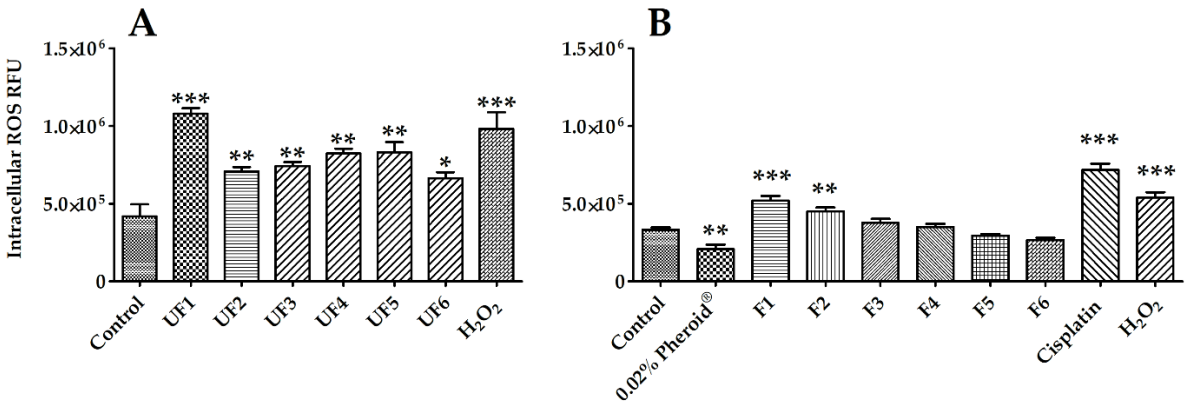

Figure 2. Intracellular ROS levels measurement in A549 cells after 24 hour exposure to free forms of phytochemicals (A); Pheroid® only, phytochemicals in Pheroid® and cisplatin (B); (n = 3) ± SEM; UF = DMSO dissolved and F = Pheroid® formulated; Asterisks (*) indicate statistical significance relative to the control * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.00001$)

The SOD activity was quantified in a kinetic reaction using pyrogallol auto-oxidation [48]. When compared to the untreated control, SOD levels were significantly ($p < 0.05$) decreased in UF2 (75 µg/mL GE) treated cells (Figure 3A). Using H_2O_2 as the substrate for enzymatic reaction and $KMnO_4$ as a titrant for the reaction with excess peroxide, CAT activity was quantified photometrically [34]. No significant ($p > 0.1$) decrease or increase in CAT levels were observed for any of the treatment groups when compared to the untreated cells (Figure 3B).

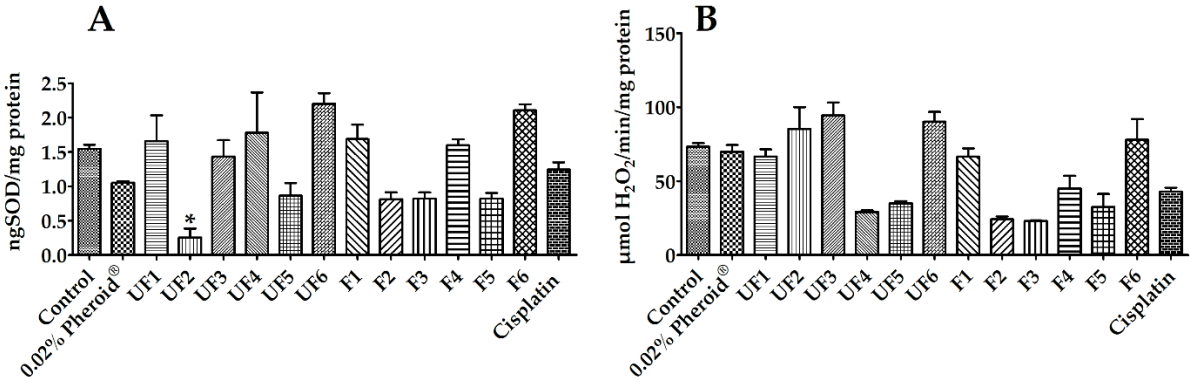

Figure 3. The measurement of superoxide dismutase (A) and catalase (B) levels in A549 cells after treatment with individual and combinations of phytochemicals in Pheroid® (F) and in DMSO (UF). A549 cells were exposed to treatment for 24 hours; (n = 3) ± SEM; Asterisk (*) indicate statistical significance relative to the control ($p < 0.05$)

3.4. Nuclear morphology and apoptosis assay

The apoptotic effect of Meriva® and GE combinations (Table 1) on A549 cells was investigated using nucleic acid specific AO/EtBr double stain fluorochromes. Acridine orange (AO) easily traverses the membrane and stains the cytoplasm and nuclear DNA of live cells green, while EtBr only passes through membranes with poor integrity and stains the nucleus red [37]. In addition to staining nuclear DNA, AO also concentrates in acidic lysosomes and intercalates with nuclear RNA, thereby producing bright red fluorescence [41, 49]. Morphological changes such as membrane blebbing, the formation of apoptotic bodies, the condensation of chromatin and the fragmentation of nuclear DNA are characteristic features of cells undergoing apoptosis [37, 49] (Figure 4).

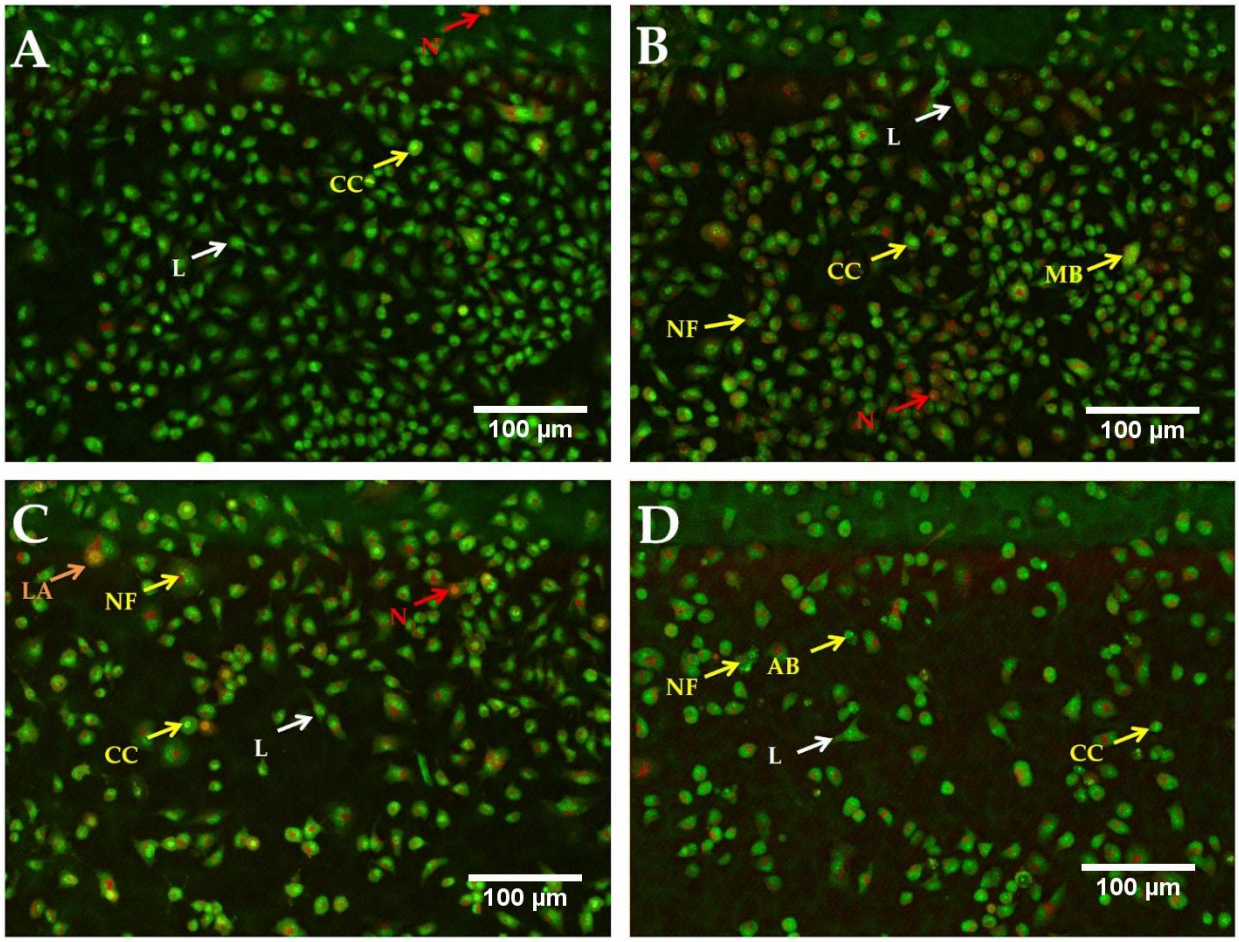

Figure 4. Fluorescence microscopic detection of apoptosis in A549 cells using Acridine Orange/Ethidium Bromide double staining fluorochromes. Cells were either untreated (A), or exposed to DMSO dissolved phytochemical combination UF3 (B), Pheroid® formulated phytochemical combination F3 (C), and cisplatin (D) for 24 hours. Images were captured at 100x magnification. The characteristic apoptotic morphological changes observed include chromatin condensation (CC), nuclear fragmentation (NF), membrane blebbing (MB), and apoptotic bodies (AB). Orange and red cells respectively indicate late apoptotic (LA) and necrotic (N) cells, while green cells indicate live (L) cells with intact nuclear structure. Scale bar = 100 µm

Bright green fluorescence with nuclear fragmentation and chromatin condensation is a sign of early apoptosis. In contrast, late apoptotic events can be identified by fragmented orange chromatin, while necrotic cells appear red with a normal nuclear structure (Figure 4). These characteristic apoptotic morphological features were observed in the presence of UF3, F3 and cisplatin treatments. Cisplatin displayed a significant apoptotic effect in A549 cells ($p < 0.00001$) when compared to the untreated cells. In general, Pheroid® enhanced the apoptotic effects of combined phytochemicals (F3, F4, F5 and F6) ($p < 0.05$) in comparison to their DMSO dissolved counterparts, with F3 having a robust ($p < 0.00001$) apoptotic profile when compared to untreated cells (Table 2). The enhanced apoptotic effect exhibited by F3 formulation could be as a result of the suitable combination ratios between Meriva® and ginger extract that potentiates either synergistic or additive mechanism of action between the phytochemicals. In the DMSO dissolved treatment group, UF3 demonstrated a significant ($p < 0.0005$) apoptotic effect when compared to the untreated control.

Table 2. The apoptotic effects of Meriva® and ginger extract on A549 cells after 24 hour of treatment measured using AO/EtBr double staining

Response (%)	Treatment group							
	UF1	**UF2**	**UF3**	**UF4**	**UF5**	**UF6**	**Pheroid® only**	**Untreated**
Live	80*	83	75**	81	79*	85	93	91
Dead	20*	17	25**	19	21*	15	7	9
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**Cisplatin**	
Live	79*	85	60***	74**	80*	77*	54***	
Dead	21*	15	40***	26**	20*	23*	46***	
	52.5 μg/mL Meriva®	**75 μg/mL GE**	**52.5/75 μg/mL Meriva®/GE**	**75/52.5 μg/mL Meriva®/GE**	**52.5/52.5 μg/mL Meriva®/GE**	**75/75 μg/mL Meriva®/GE**		

UF = Actives dissolved in 0.125% DMSO and F = Formulated actives in 0.02% Pheroid®; Asterisk (*) indicates statistical significance relative to the control * ($p < 0.05$), ** ($p < 0.0005$) and *** ($p < 0.00001$)

3.5. Mitochondrial health

The mitochondrial health of A549 cells following 24 hour treatment with Meriva® and GE was measured using the Seahorse Mito stress test. The basal cellular respiration, along with other bioenergetic modulators such as ATP production, maximum respiration, and SRC were evaluated as key parameters of mitochondrial dysfunction. The DMSO dissolved Meriva® and GE combination (UF6) significantly ($p < 0.001$) reduced ATP production while UF1 and UF2 significantly ($p < 0.01$) increased maximal respiration and SRC (Table 3).

Table 3. The effect of Meriva® and ginger extract on mitochondrial bioenergetics parameters of A549 cells after 24 hour treatment exposure

Treatment groups	Bioenergetics Parameters			
	Basal Respiration	**ATP Production**	**Maximal Respiration**	**Spare Respiratory Capacity**
Untreated	68±3	57±4	139±17	72±16
0.02% Pheroid®	63±3	50±2	149±9	86±7*
UF1	67±8	55±7	166±17**	99±10***
UF2	74±4	62±4	174±8**	100±7***
UF3	70±3	59±3	153±13	83±13
UF4	70±5	59±4	147±16	77±15
UF5	74±8	63±5	155±7	80±7
UF6	57±15	42±14**	118±29	60±15
F1	34±6***	23±5***	76±14***	42±10**
F2	66±5	55±3	169±13**	103±9***
F3	43±5***	31±4***	102±10**	59±5
F4	45±4***	35±4***	113±9	68±6
F5	43±6***	34±5***	110±9*	67±4
F6	41±3***	31±3***	104±11**	63±11
Cisplatin	58±3	48±2	139±6	82±4

(n = 5–6) ± SEM; Asterisk (*) indicate statistical significance relative to the untreated group where * ($p < 0.05$), ** ($p < 0.005$), *** ($p < 0.00005$); UF = DMSO dissolved and F = Pheroid® formulated

The Pheroid® formulated Meriva® (F1) treatment led to a significant ($p < 0.005$) reduction in all of the bioenergetics parameters (Table 3 and Figure 5). In addition, the phytochemical combinations — F3, F5, and F6 — in Pheroid® also displayed a significant ($p < 0.01$) reduction in the ATP production, basal and maximal respiration, while F4 treatment caused a significant ($p < 0.0001$) reduction in the basal respiration and ATP production. Pheroid® only treatment at 0.02% concentration displayed a marked increase ($p < 0.01$) in the SRC when compared to untreated cells. This indicates that the cells are able to produce more ATP and circumvent oxidative stress. Cisplatin treatment produced no significant changes in the mitochondrial respiration of A549 cells.

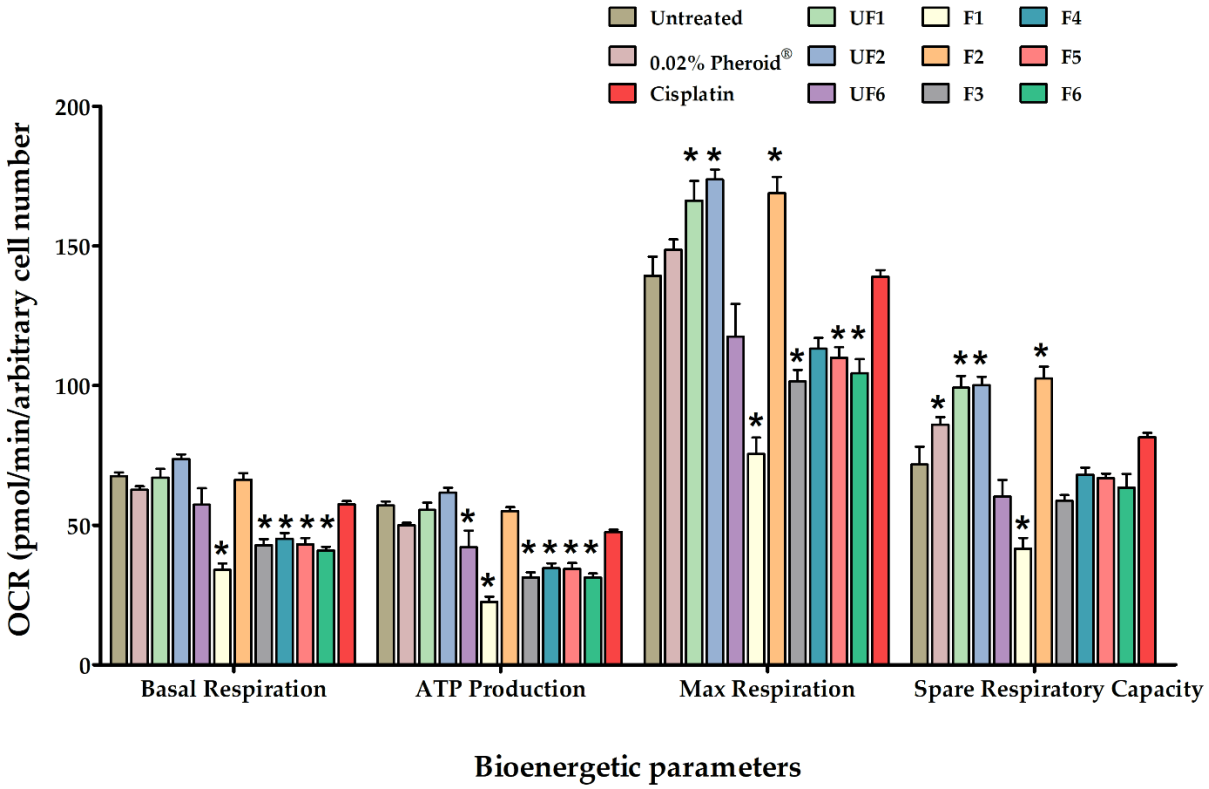

Figure 5. The effect of Meriva® and ginger extract on the mitochondrial bioenergetics parameters of A549 cells after 24 hour treatment exposure; (n = 3) ± SEM; Asterisks (*) represents significant ($p < 0.05$) changes when compared to the untreated cells; UF = DMSO dissolved and F = Pheroid® formulated; Graph only represent treatments with significant change in bioenergetic parameters

4. Discussion

Curcumin and ginger extract (GE) phenolics are promising chemopreventive and anticancer agents [50, 51]. In addition to being safe and inexpensive, these agents have the ability to selectively target and induce apoptosis in rapidly proliferating cells, either independently or in combination [52]. A combination of phytochemicals, however, usually exhibits a stronger cytotoxic effect against cancer cells compared to single-agent treatment [53, 54]. This is often due to the overlapping and synergistic mechanism of action of individual actives on multiple cell death pathways [53].

In this study, the effect of Meriva® and GE on the mitochondrial metabolic rate of A549 cells was investigated using the MTT assay. In this assay, metabolically viable cells (i.e. cells that have intact membranes and normal mitochondrial function) are estimated based on their ability to reduce MTT to insoluble formazan [55]. The results indicated that all treatments (Table 1) significantly reduced mitochondrial metabolic activity in the A549 cells (Figure 1B). To further investigate the anti-proliferative effects of the phytochemicals, the neutral red (NR) uptake assay was also performed. This assay provides a quantitative estimation of viable cells based on their ability to incorporate the supravital NR dye into the

lysosome depending on both the pH gradient and ATP production [44]. During cell death or in the case of a reduced pH gradient, NR cannot be retained and consequently diffuses from the lysosome. In this assay, Pheroid® significantly enhanced the growth inhibitory effects of combined Meriva® and GE phytochemicals — F3, F4, F5, and F6 — when compared to the untreated cells and their DMSO dissolved counterparts (Figure 1A). The mitochondrial cell viability (MTT) and growth inhibition (NR) assays therefore demonstrated contrasting results in cell viability. A study conducted by Lim *et al*. [55] reported that — in the absence of cells — vitamin E isomers markedly reduced MTT into purple formazan and therefore may not be a suitable assay to determine cell viability. However, in the present study, the final treatment concentration of dl-α-tocopherol in the Pheroid® formulations was lower than where Lim *et al*. [55] observed reversed cell viability profiles. Another study conducted by Triglia *et al.* [56], reported that inconsistency between NR and MTT results emanates from the different mechanisms of action of the drugs investigated. In that study, the antibiotic monensin showed cytotoxicity in a three dimensional culture of dermal fibroblast when measured by the NR assay, but was non-cytotoxic when assessed by the MTT assay. In addition, some herbal extracts have been found to potentially interfere with the MTT assay [57, 58].

On another note, agents that interfere with cell cycle phase redistribution, for example by inhibiting G2/M transition, significantly influence the mitochondrial number and/or metabolic function as a consequence of the amount of formazan produced [59]. Previous studies have demonstrated that curcumin and GE constituents — in particular [6]-shogaol — individually mediate cell death via oxidative stress, G2/M arrest, DNA damage, endoplasmic reticulum stress, and a decline in mitochondrial function in A549 cells [60–64]. It is therefore possible that the results observed in the MTT assay may be due to the inhibitory effect of treatments on the G2/M cell cycle, which alters the number and/or function of mitochondria involved in the conversion of MTT to formazan crystals, rather than interference from dl-α-tocopherol or the phytochemicals with the redox reaction on which the assay is based. This could mean that the cells are cytostatic and viable, as shown in NR assay (Figure 1A), but that they are experiencing metabolic stress which could potentially lead to cell death via apoptosis [65]. This is supported by the AO/EtBr double staining apoptosis assay (Figure 3), which showed typical apoptotic morphological features such as membrane blebbing, cell shrinking, chromatin condensation and nuclear fragmentation [6, 66–68] (Figure 3). In this assay, UF3, F3, F4 and F6 produced a robust apoptotic effect when compared to untreated cells. The apoptotic effect observed as a result of the F3 treatment was also comparable to that of cisplatin (Table 2). This affirms that the Meriva® and GE combination in Pheroid® displays potential chemopreventive activity against the A549 cancer cell line.

At low to moderate levels, ROS is involved in a number of normal physiological processes, including multiple cellular signalling systems, mitogenesis and protection of the cells against noxious agents [69]. However, when ROS levels supersede those of the enzymatic and non-enzymatic antioxidant detoxification mechanisms, it results in oxidative stress. Such stress may cause potential damage to lipids, proteins and cellular DNA, which are all factors implicated in a number of diseases, including cancer. Cancer cells exhibit higher levels of ROS and antioxidant enzymes than normal cells [70]. Therefore, a slight disturbance in this delicate equilibrium will induce cell death. The use of antioxidant agents in cancer therapy has, however, led to much controversy over the years [71]. This is mainly because ROS forms an integral part of the body's innate defence system, and suppressing its level with chronic antioxidant use has detrimental consequences on normal physiological and redox responses [72]. Despite this, ROS-mediated cytotoxicity is a common first line anticancer therapy [73, 74]. Cisplatin, a standard anti-neoplastic agent, is frequently used in the treatment of many solid tumours, including lung cancer [75, 76]. The mechanism used by cisplatin to induce cell death is complex and, as of yet, not fully understood. However, studies show that cisplatin damages nuclear DNA by intercalating with the nuclear material and forming DNA adducts [77]. In addition to this mechanism, Marullo *et al.* [78] demonstrated that cisplatin has a direct effect on the mitochondrial DNA of A549 cells by inhibiting its transcription and reducing the expression of electron transport chain proteins, which consequently increases intracellular and mitochondrial ROS. This finding agreed with the present study in which a significant increase in intracellular ROS was observed in A549 cells after a 24 hour exposure to cisplatin (Figure 2B).

SOD and CAT are regarded as first line defence antioxidant enzymes and play an essential role in the detoxification of ROS. SOD functions by converting the superoxide anion radical to H_2O_2, which is then further decomposed to harmless oxygen and water molecules by the H_2O_2 scavenger enzyme, CAT. In this study, cisplatin-mediated ROS generation in A549 cells was accompanied by a non-significant decrease in the expression of SOD and CAT (Figure 4). The imbalance observed between the increased ROS generation and the reduced antioxidant enzymes levels implies that oxidative damage to the cellular components and ROS-mediated activation of the intrinsic and extrinsic apoptotic pathways occur [79]. As cisplatin lacks the ability to distinguish between normal and cancerous cells, it may induce severe side effects, including ototoxicity, neurotoxicity and nephrotoxicity, of which the latter is the dose limiting adverse effect [75, 80]. Previous *in vivo* studies have shown that combining cisplatin with curcumin [81] and GE [82] reduces the cisplatin-induced toxicity. Accordingly, this provides the platform for the Meriva® and GE combination to be used in conjunction with cisplatin in the treatment of lung cancer.

Curcumin and GE, in particular [6]-shogaol, have further been shown to be powerful ROS scavenging phytochemicals, although both may exhibit pro-oxidant activity depending on the concentration, exposure time and environmental conditions applied [83–86]. In the present study, the ROS and antioxidant enzyme assays conclusively demonstrated the pro-oxidant nature of unformulated phytochemicals in A549 cells. Pro-oxidant effects occur when a biological or exogenous substance induces oxidative stress through the induction of ROS or the inhibition of antioxidant enzymes [87]. Treatment of the A549 cell line with unformulated Meriva® and GE, either individually, or in combination, resulted in a significant increase in intracellular ROS (Figure 2A). This increase was accompanied by a non-significant decrease in the SOD levels following combined Meriva® and GE (UF5), and GE (UF2) treatments, while a non-significant decrease in CAT levels was observed following combined UF4 and UF5 phytochemical treatments. These findings were in accordance with previous studies in which curcumin was shown to significantly increase ROS in A549 cells in a dose dependant manner following a 24 hour exposure time [60, 88], and in which [6]-shogaol induced a marked increase of ROS in Caki cells [89], Mahlavu cells [90] and COLO 205 cells [91]. In addition, studies report that the ginger constituent, [6]-gingerol, displays a significant increase in ROS in AGS cells following 24 hour of treatment [92]. Curcumin — in its antioxidant capacity — mimics the SOD enzyme and enhances the dismutation of superoxide radicals [93]. Yusof and Abdul-Aziz [94] also showed that GE displays antioxidant activity by significantly decreasing SOD and CAT activities in the HepG2 cancer cell line.

By contrast, Meriva® and GE phytochemicals in Pheroid® displayed a substantial decrease in intracellular ROS generation (Figure 2B). This phenomenon could be due to the antioxidant properties of Pheroid® itself, in which a significant decrease in ROS and a non-significant decrease in SOD levels were observed in comparison to the untreated cells (Figure 4A). Such a decrease in ROS, SOD and CAT levels is characteristically associated with reduced oxidative stress [95]. The antioxidant properties of Pheroid® can be attributed to the presence of dl-α-tocopherol, which functions as one of the excipients in the formulation. According to literature, the dl-α-tocopherol isoform has an antioxidant activity [96] and possesses anticancer properties [97]. These findings demonstrate that Pheroid® alleviated the potential oxidative stress effects of combined phytochemicals on the A549 cells, while maintaining the anticancer efficacy of the phytochemical combination (Figure 1 and Figure 2). This is essential in cancer treatment, as it potentiates the safe and effective use of anticancer agents without displaying adverse toxic effects in normal cells.

Unlike other cancer types that rely on the glycolytic pathway, lung cancer cells are more heavily dependent on mitochondrial respiration to generate the energy required for rapid cellular growth and metastasis [4]. Apart from ATP production, mitochondria play a role in other physiological processes, including calcium and redox homeostasis, apoptotic cell death and inflammation [98]. The Seahorse XF^e Mitochondrial stress test revealed that Meriva® in Pheroid® (F1) and its combinations with GE in Pheroid® — F3, F4, F5 and F6 — significantly inhibited basal respiration, ATP production, maximal respiration and SRC, with F1 displaying the most pronounced effect across all the parameters (Figure 5). The ginger extract in Pheroid® and without Pheroid® did not have an effect on the mitochondrial respiration of A549 cells (Table 3). This finding is in accordance with a study by Deng *et al.* [99], which reported that

GE increased the electron transport chain complex activity and ATP production in HepG2 cells, while simultaneously reducing cell viability . This indicates that GE phenolics induce cell death in cancer cells, via pathways other than the inhibition of mitochondrial ATP, including ROS-mediated autotic cell death [100] and the p53 pathway [101]. This is in contrast to curcumin, which suppresses mitochondrial oxidative respiration and causes a metabolic shift towards glycolysis in the MCF-7 breast cancer cell line [102]. Cisplatin treatment did not induce any significant change in the mitochondrial bioenergetics of A549 cells.

The results obtained from the Mito stress test were further supported by that of the NR assay. Since lysosomal NR dye retention is ATP dependent, the reduction in ATP production (Figure 5) observed in Pheroid® formulated phytochemical combinations F3, F4, F5 and F6 may explain the significant decline in NR retention (Figure 1A). This observation was also true for the UF6 treatment group, which displayed a significant decrease in ATP production and a reduced cell viability in comparison to the rest of the unformulated phytochemical treatments (Figure 1A and Table 3). The improved chemopreventive activity observed for combined Meriva® and GE in the formulated treatment group was likely due to the enhanced cellular uptake imparted by the Pheroid® drug delivery system. This was confirmed by confocal microscopy (Figure 6). Since the F3 phytochemical combination in Pheroid® displayed the most promising anticancer effect, cellular uptake was compared against its free form counterpart, UF3 (concentrations reported in Table 1). Cells were cultured onto coverslips in 6 well tissue culture–treated plates at a seeding density of 5 x 10^5 cells per well (1 mL) [103] and harvested after 1, 4 and 6 hour treatment exposure intervals with UF3 and F3 phytochemical combinations. The background fluorescence setting was configured using untreated control cells to avoid interference and to standardise the settings for all measurements using the green 530±30 nm wavelength at which curcumin autofluoresces following excitation at 488 nm [104]. Cellular uptake was found to be greater for the formulated phytochemicals than their unformulated counterparts as early as 1 hour following exposure (Figure 6). The overall trend indicated that F3 accumulates in the cells at a higher rate, to reach a point of saturation after 4 hour, whereas UF3 displayed a similar uptake to F3 (at 4 hour) following 6 hour of treatment exposure (Figure 6)

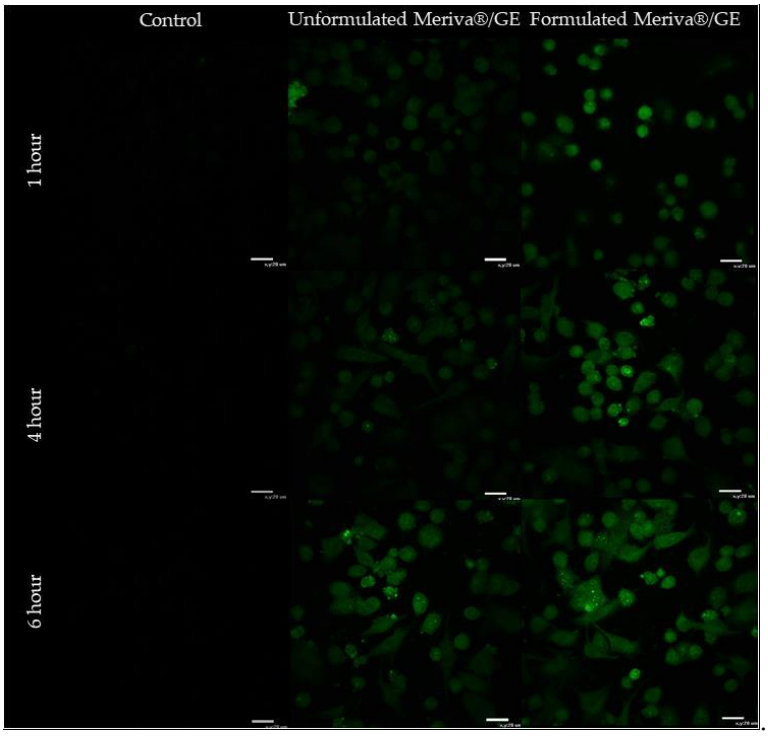

Figure 6. Confocal microscopy images of cellular uptake following 1, 4, and 6 hours of treatment exposure with free 52.5 μg/mL Meriva® and 75 μg/mL ginger extract (UF3), Pheroid® formulated 52.5

μg/mL Meriva® and 75 μg/mL ginger extract (F3). The image was captured at 600x magnification (60x/1.40 Plan Apo VC oil objective). Scale bar = 20 μm

Thereafter, the fluorescence of each cell was quantified using ImageJ [39]. In brief, each cell was selected and the area integrated intensity was determined. A non-fluorescent area was used as background and the corrected total cell fluorescence (CTCF) was calculated [105]. The saturation observed in Figure 6 was confirmed in Figures 7A and B. Pheroid® significantly enhanced the uptake of the phytochemicals by A549 cells over all time-points in comparison to the control (Figure 7). Interestingly, for each time interval assessed, a significant uptake of the unformulated combination was observed when compared to the control, which can be attributed to the phytosome formulation. However, the combinations in Pheroid® displayed a significantly greater cellular uptake relative to their unformulated counterparts and to the control at each interval (Figure 7). The mechanism responsible for the enhanced cellular uptake includes the binding of Pheroid®, a fatty acid, to fatty acid membrane binding proteins (FABPs) where Pheroid® specifically interacts with the glycolipoprotein lipid microdomains [24]. Once inside the cell, intracellular Pheroid® vesicles are then metabolised either by the mitochondria or peroxisomes, depending on the vesicle morphology, which releases the entrapped active compounds. The more rapid delivery observed with the Pheroid® formulation will therefore improve the onset of action of the Meriva® and GE phytochemicals.

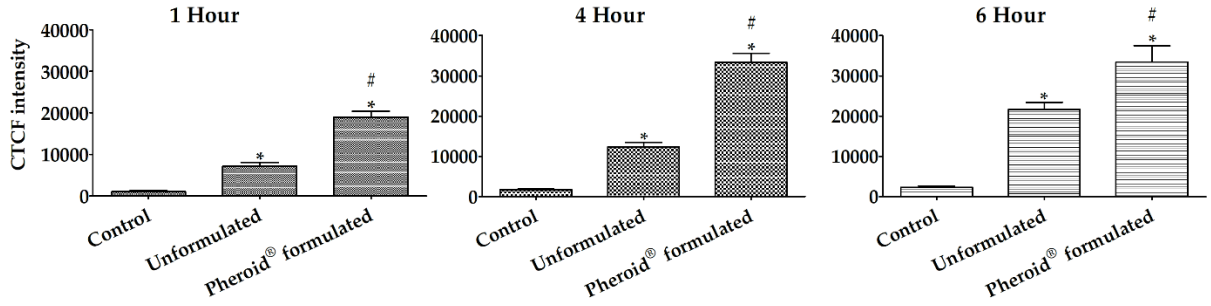

Figure 7. Corrected total cell fluorescence intensities (CTCF) of cellular uptake following 1, 4, and 6 hours of treatment exposure with free 52.5 μg/mL Meriva® and 75 μg/mL ginger extract (UF3), Pheroid® formulated 52.5 μg/mL Meriva® and 75 μg/mL ginger extract (F3) and an untreated control. Result are expressed as means and error bars indicate SEM. Asterisk (*) indicates statistical significance relative to the control ($p < 0.05$); hash (#) indicates statistical significance relative to the DMSO dissolved counterpart ($p < 0.05$)

In summary, Pheroid® significantly enhanced the cellular uptake, anti-proliferative and apoptotic effects of combined Meriva® and GE phytochemicals in A549 cells. In addition, these anticancer effects did not result in oxidative stress. Meriva® and GE, either individually, or in combination, further significantly reduced the mitochondrial metabolic activity of A549 cells, however, a robust anti-proliferative effect was observed when combination treatment in Pheroid® was applied. This enhanced effect could be explained by a reduction in the mitochondrial respiratory capacity. Since lung cancer cells rely heavily on mitochondrial oxidative phosphorylation for energy production, dysfunction in the mitochondria has a detrimental consequence on cell proliferation. In this study, Meriva® was found to be responsible for the mitochondrial dysfunction observed, while GE exhibited no effect on mitochondrial respiration. GE, however, may have enhanced the activity of Meriva® via other mechanisms involved in cell death.

5. Conclusions

The results obtained in the present study conclusively show that the combination of Meriva® and GE in Pheroid® yields a better chemopreventive activity than individual treatments or the unformulated combinations. The enhanced anticancer activity is likely the result of the additive or synergistic

mechanisms of action of the individual phytochemicals on multiple pathways involved in the pathogenesis of lung cancer. While cisplatin is a standard of care in lung cancer treatment, it causes severe side effects due to its non-selective induction of oxidative stress to both cancerous and normal cells. The combined phytochemical treatment in Pheroid® displayed anti-proliferative and apoptotic effects, comparable to those observed for cisplatin treatment in A549 cells, without causing oxidative stress. These findings provides an opportunity to further investigate the safe and effective use of Pheroid® formulated Meriva® and GE combination in lung cancer xenograft animal models.

Supplementary Material: Figure S1 indicates A549 cell viability under different Pheroid® and DMSO concentrations over a 24 hour period using the Neutral Red assay.

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Supplementary Material

Aim: To determine working Pheroid® and DMSO concentrations that has no apparent effect on the growth and proliferation of A549 cells.

Method: Pro-Pheroid® was prepared following the method described by Grobler [1]. Briefly, Vitamin F ethyl ester, Kolliphor® EL and dl-α-tocopherol (70%, 29% and 1% w/w) were mixed at 70°C and gassed with nitrous oxide gas. A panel of 15 Pheroid® concentrations was then prepared by diluting pro-Pheroid® in Dulbecco`s Modified Eagle media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-amphotericin B mixture. Three DMSO concentrations at 0.375%, 0.25% and 0.125% were also prepared in the growth media. A549 cells were seeded onto a 96 well tissue culture treated plate at a seeding density of 1 x 10^4 cells per well (50 000 cells/mL) and incubated overnight in humidified air with 5% CO_2 at 37°C. The following day, cells were treated with different Pheroid® and DMSO concentrations. After 24 hours of incubation, old media was removed and cells were washed thrice with phosphate buffer saline (PBS). Filtered neutral red dye of 200 µL was added to the cells, which were then incubated for three hours. Cells were rinsed thrice with PBS and 200 µL of neutral red solubilisation solution was added into each well. The plate was then placed on a plate shaker at room temperature for 20 minutes [2]. Absorbance was measured at 540 nm and 690 nm wavelengths. A mean of three independent experiments was made to determine cell viability.

Result: In general, low DMSO concentrations in the range of 0.1% to 0.5% are non-toxic to cells while higher concentrations have deleterious effects on cell viability [3]. In the present study, all tested DMSO concentrations indicated cell viability greater than 90%. Pheroid® up to 0.06% exhibited cell viability above 80%, with the highest (112.8%) and lowest (80.1%) viability noted at 0.018% and 0.06% concentrations, respectively. Whereas, Pheroid® concentrations at 0.1% ($p < 0.001$) and 0.2% ($p < 0.0001$) significantly reduced cell viability compared to the untreated control, respectively.

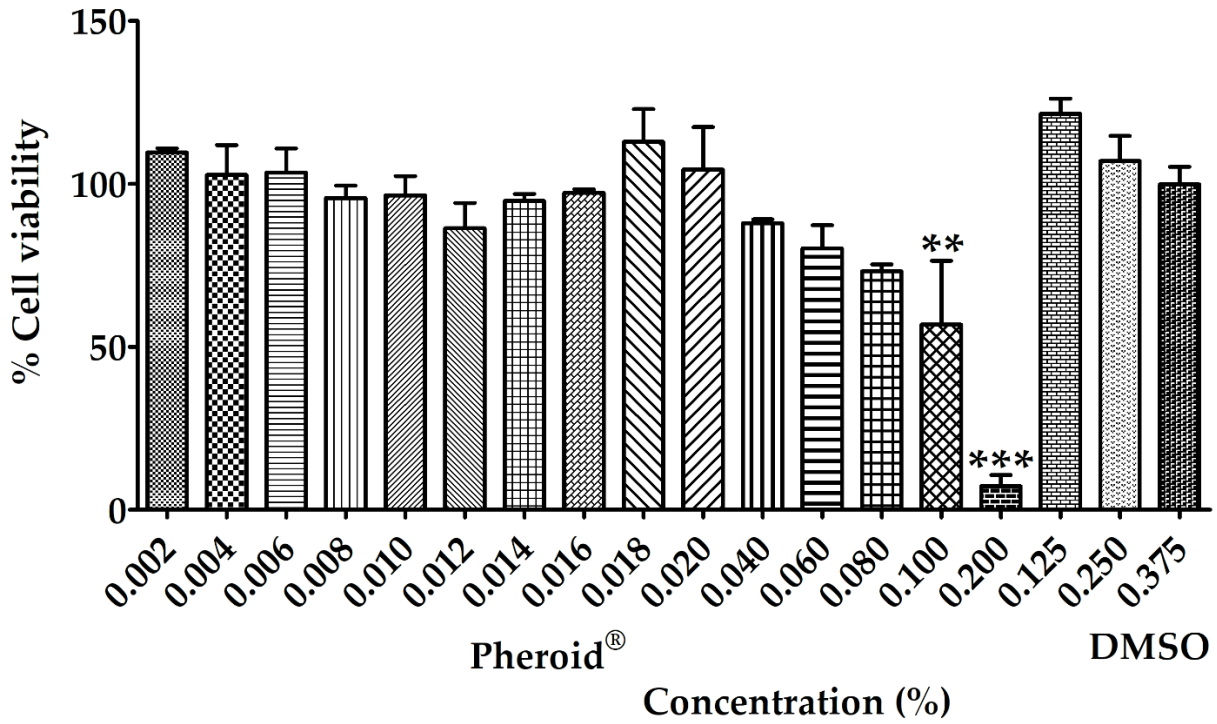

Figure S1: A549 cell viability under different Pheroid® and DMSO concentrations over a 24 hour period using NR assay; (n = 3) ± SEM; Asterisk (*) indicates statistical significance compared to the control ** ($p < 0.001$) and *** ($p < 0.0001$)

Conclusion: Pheroid® and DMSO concentrations at 0.02% and 0.125% were selected for further *in vitro* experiments as suitable final carrier concentrations. Pheroid® at 0.02% concentration provides the maximum amount for constituting Meriva® and GE in the delivery system while in itself it does not induce antiproliferative effects. Although all DMSO concentrations had no effect on the growth of A549 cells, the lower concentration was selected as a precautionary measure.

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CHAPTER 4

Chapter 4 is written according to the instruction for Authors guideline of *Pharmaceuticals*; an open access journal from MDPI. The manuscript is written in US English and guidelines are provided in Appendix B. This chapter is presented in the template provided by the journal. For ease of reading, the tables and figures are inserted at their relevant positions. This manuscript will only be submitted for publication once additional study groups have been completed.

Research Article

Evaluating the chemotherapeutic effect of Pheroid® formulated Meriva® and ginger extract combination in lung cancer murine xenograft model

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Abstract: Cancer therapy using phytochemicals is an emerging treatment strategy owing to their chemopreventive activity and safety profile. Curcumin and [6]-shogaol (6SG) have potent anti-tumorigenic activity, the latter being more potent. However, the clinical application of phytochemicals is greatly reduced by their poor physicochemical properties, which can be overcome using lipid based drug delivery systems such as the Pheroid® technology. The aim of the present study was to formulate and characterize Meriva® and ginger extract (GE) combination in Pheroid® and evaluate its anticancer effect in non-small cell lung cancer (NSCLC) bearing athymic nude mice. The phytochemical combination had a mean particle size of 26±0.13 μm and zeta potential of -28±3.26 mV. No drug – excipient interactions were noted, indicating compatibility. The mice treated daily with Pheroid® formulated Meriva® (70 mg/kg [28 mg/kg curcumin]) and GE (100 mg/kg [1.1 mg/kg 6SG]) combination for 14 days, demonstrated a non-significant reduction in tumor growth and burden compared to Pheroid® only treatment. This was attributable to the suboptimal doses of curcumin in Meriva® and 6SG in GE. Thus, the use of therapeutic dose combinations of curcumin and 6SG phytochemicals in Pheroid® could enhance the treatment outcomes in future studies in xenografted NSCLC mice.

Keywords: A549; Ginger extract; Lipid based drug delivery system; Meriva®; Non–small cell lung cancer; ; Pheroid®; Xenograft

1. Introduction

Lung cancer accounts for the majority of cancer related deaths worldwide [1]. Current treatments of lung cancer include surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapy [2]. However, the survival profile of lung cancer patients at all stages is poor compared to other cancer types [3, 4]. In addition, the use of combinatorial therapy for treating lung cancer often fails to provide satisfactory treatment outcomes due to chemo-resistance, unfavourable anticancer drug toxicity, high treatment costs and associated poor quality of life [3, 5]. This underscores the need for supplementary novel treatment approaches to improve lung cancer treatment.

Naturally obtained phytochemicals such as curcumin and ginger extract (GE) phenolics have anti-inflammatory, antioxidant and anticancer properties [6, 7]. Research has shown that curcumin and GE independently demonstrated significant anti-tumor activity in several *in vivo* cancer models. For instance, oral administration of curcumin at 100 mg/kg in athymic nude mice inoculated with the human adenocarcinoma NCI-H460 lung cancer cell line showed a significant reduction in tumour size and weight [8]. In another study, oral feeding with GE at 100 mg/kg inhibited tumour growth and progression of prostate cancer PC-3 in xenografted athymic nude mice [9]. Curcumin mediates its anticancer activity through modulation of transcription factors, growth factors, protein kinases, proteasomes, epigenetic

changes and pro-inflammatory mediators involved in tumour initiation, promotion, angiogenesis, and metastasis [10, 11]. The compound is also pharmacologically safe; human phase II clinical trials against pancreatic cancer indicated no toxicity when administered at doses up to 10 g/day [12].

[6]-shogaol (6SG) is one of the predominant constituents in dried ginger and has more potent antioxidant, and anti-inflammatory activity than gingerols and curcumin [13–15]. Like curcumin, 6SG exhibits anti-proliferative, anti-metastatic, and pro-apoptotic activities by regulating multiple targets such as transcription factors, including NF-kB and STAT3; growth factor receptors, kinases, and inflammatory mediators [16]. Kim *et al* [17] observed that 6SG is a potent inhibitor of STAT3 activation and when administered alone, significantly suppressed tumor growth. In a human clinical trial, Zick *et al.* [18] reported that doses up to 2 g per day of ginger constituents 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol showed very low toxicity and high tolerability with mild gastrointestinal complaints.

Despite the extraordinary therapeutic benefits of curcumin and GE, their translation into a potential clinical application is limited because of poor aqueous solubility at acidic pH, P-glycoprotein efflux and, extensive *in vivo* metabolism following oral administration [19, 20]. In addition, curcumin undergoes rapid deprotonation under neutral and alkaline pH showing instability [21]. The oral route of administration is a favoured route for chronic diseases, like cancer, since it is convenient, non-invasive and cost-effective [22]. However, the lipophilic nature of the phytochemicals impedes their dissolution in the physiological fluid leading to incomplete biological uptake, poor absorption and high excretion profile. Overall, this causes a deficiency in the plasma bioactive concentration to elicit sustained therapeutic functionalities at the target site of action. To improve the oral bioavailability of compounds such as curcumin and GE phytochemicals, various drug delivery systems have been developed over the years. Some of these are phospholipid and emulsion based delivery systems, chemical modifications (conjugation of a chemical substance to the phytochemical as a means of carrier system) and other delivery means such as chitosan based delivery system and nano-dispersion [22].

Pheroid® is a colloidal drug delivery system that is comprised of plant derived essential fatty acids as the dispersed phase and nitrous oxide saturated water as the continuous phase [23, 24]. Pheroid® is made from safe pharmaceutical grade raw materials and its formulation is proved to be non-toxic during acute and chronic administration to animals at doses up to 2 000 mg/kg via intravenous and 50 mg/kg oral administrations [25]. Moreover, the delivery system has the capacity to entrap both hydrophobic and hydrophilic drug molecules, protect against instability in physiological environments and against extensive metabolic transformations [26]. The presence of nitrous oxide in Pheroid® provides an added advantage in the stability of the formulation at higher and lower pH, miscibility of the vesicles in the dispersal medium and assists in the self-assembly of the vesicles [26]. An *in vitro* study demonstrated that Pheroid® significantly enhanced the anticancer activity of Meriva® (a curcumin phytosome) and GE combination against the A549 lung cancer cell line [27]. The present study aims to further investigate the *in vivo* anticancer potential of Meriva® and GE combination in Pheroid® against lung cancer in an athymic nude mouse xenograft model and compares the effect with a standard chemotherapeutic agent, cisplatin. Formulation characteristics, such as morphology, particle size, electro kinetic potential, and pH were assessed. Furthermore, a compatibility study and the quantification of curcumin in Meriva® and 6SG in GE in Pheroid® were done.

2. Results

2.1. pH measurement

The pH of Pheroid® formulated Meriva® and GE was measured to assess the chemical stability of curcumin in the final Pheroid® formulation. Curcumin is stable in acidic environment and undergoes rapid degradation under neutral and alkaline pH [21]. Ginger phenolic compounds however are stable over a wide pH range [20]. The results show that the phytochemical formulation in Pheroid® has a weakly acidic pH (Table 1), assuring the stability of curcumin and ginger phenolic compounds.

Table 1. The pH of Pheroid® only and Pheroid® formulated Meriva® and ginger extract

Formulation	pH			Mean±SD
	Trial			
	1	2	3	
Pheroid® only	6.65	6.60	6.69	6.65±0.05
Meriva® and GE in Pheroid®	5.90	5.90	5.87	5.89±0.02

2.2. Particle size, zeta potential, and vesicle morphology and distribution analysis

Particle size, distribution and zeta (ζ) potential analyses were performed to characterize the phytochemical formulation. In the particle analysis, Pheroid® only showed two populations in the size distribution curve with peak volumes of 8% and 3% at 0.2 μm and 4 μm sizes, respectively (Figure 1a). The mean particle size of these populations is 2±0.39 μm (Table 2). In contrast, phytochemical loaded Pheroid® vesicles showed a single population with peak volume of 6% at 17 μm and a mean particle size of 26±0.13 μm (Figure 1b). The span value, as an index of polydispersity, shows a broader population distribution in Pheroid® only and Pheroid® formulated ginger extract as opposed to Pheroid® formulated Meriva® and phytochemical combination formulations (Table 2). The span value indicates presence of various nano and micro particles in Pheroid® only and ginger extract in Pheroid® formulations.

Table 2. Measurement of population width (span) for Pheroid® only, individual and combination of Meriva® and ginger extract in Pheroid® formulations

Pheroid® formulation	$d_{0.1}$(μm)	$d_{0.5}$ (μm)	$d_{0.9}$ (μm)	Mean(μm)	Span
Pheroid® only	0.11±0.001	0.25±0.018	7.02±1.165	2.00±0.390	27.82±3.315
Ginger extract	0.10±0.004	0.26±0.007	8.12±0.209	3.00±0.074	31.62±1.070
Meriva®	3.89±0.561	19.78±0.484	71.98±0.446	30.15±10.000	3.44±0.051
Combination	5.19±0.844	18.87±1.336	56.20±3.059	26.00±0.130	2.72±0.407

Zeta potential measures the electrostatic attraction and repulsion at the border plane between the particles and the solvent. This analysis gives an indication of the colloidal stability of the formulation. A formulation with a high surface charge difference (>±10 mV) has greater interparticle repulsion and shows resistance to aggregation [28]. The results of the electrokinetic potential analysis revealed that both Pheroid® only and phytochemical entrapped Pheroid® vesicles had a mean ζ-potential of -18±1.04 mV and -28±3.26 mV, respectively (Figure 1 c and d).

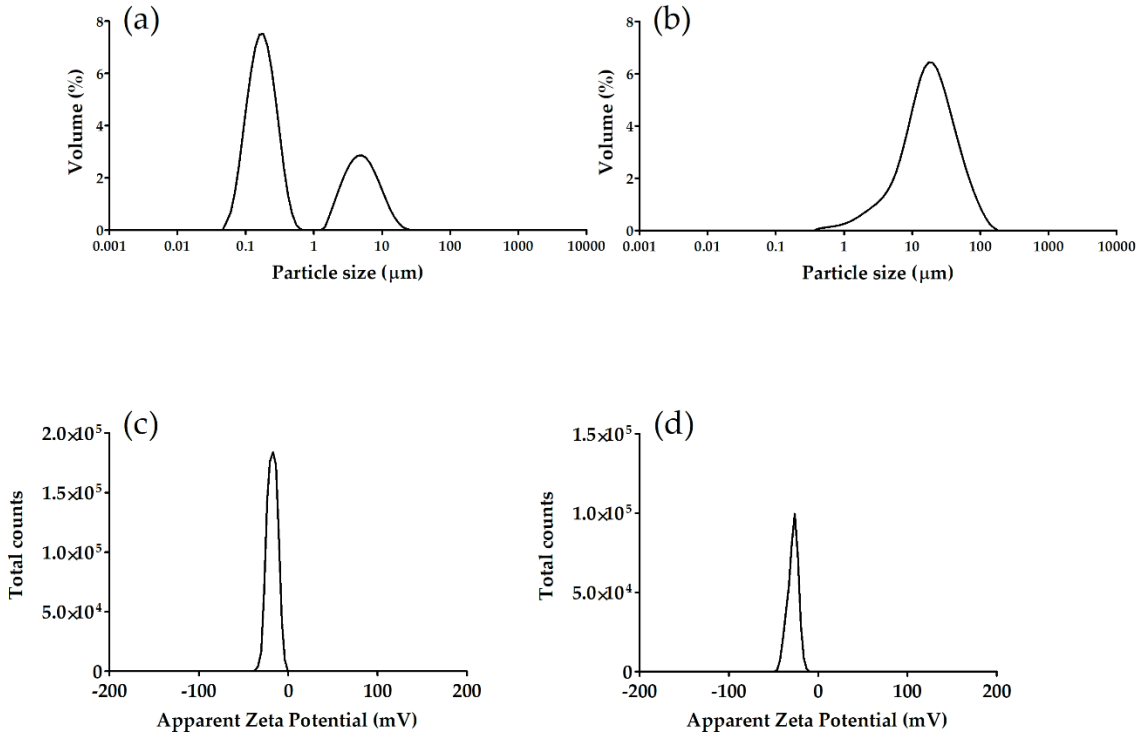

Figure 1. Mean particle size and ζ-potential analysis of Pheroid® only (a and c, respectively) and phytochemical loaded Pheroid® vesicles (b and d, respectively)

Entrapment of phytochemicals by Pheroid® vesicles was confirmed visually using confocal laser scanning microscopy (CLSM). The Pheroid® vesicles were labeled with the lipophilic Nile Red dye (excitation: 515–560 nm, emission: >590 nm), which emits a strong red fluorescence, while curcumin entrapped in the vesicles autofluoresces green at excitation and emission wavelengths of 488 nm and 530±30 nm, respectively (Figure 2d). Morphologically, phytochemical combination entrapped vesicles looked larger in size and irregularly shaped than Pheroid® only vesicles (Figure 2c). This attests the internalization of phytochemicals by the vesicles. In addition, there appears to be no green fluorescence detected outside of the vesicles, thus confirming the co-localization of phytochemicals within the vesicles without any apparent leakage.

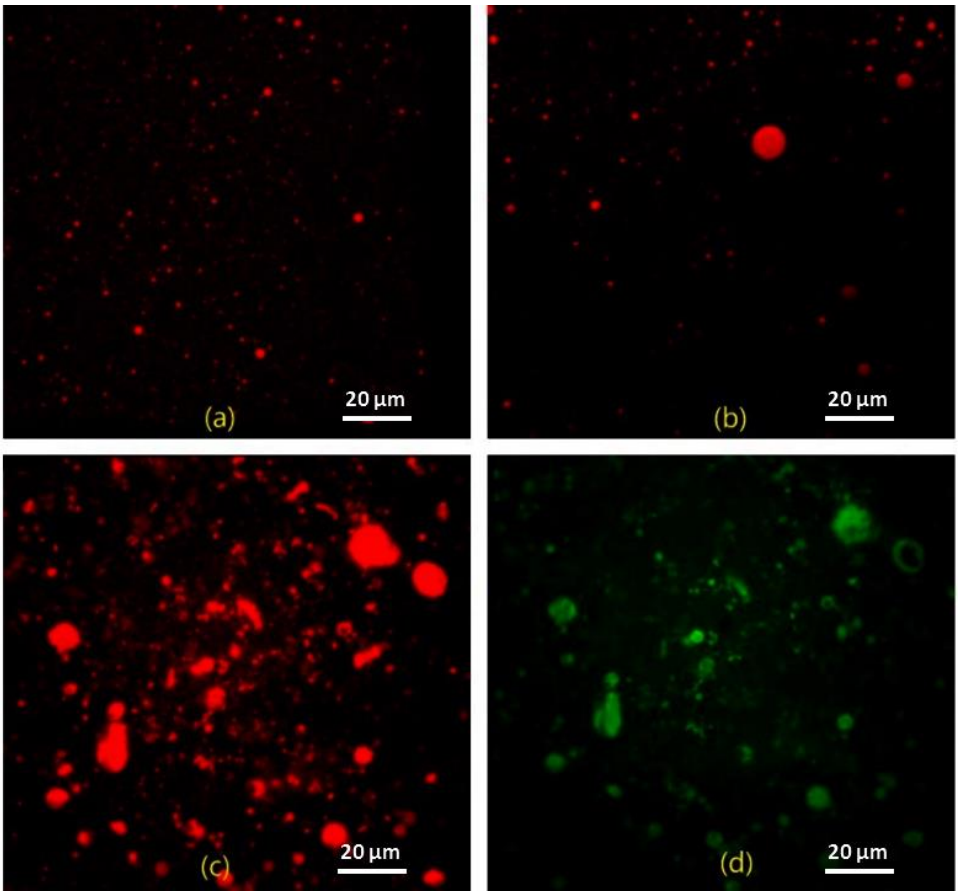

Figure 2. Confocal images of Pheroid® only (a) and entrapped phytochemicals in Pheroid® vesicles (b-d). Samples were stained with Nile Red for red fluorescence (excitation maximum: 549 nm; emission maximum: 628 nm), curcumin autoflouresces green at 530±30 nm. The image was captured at 600x magnification (60x/1.40 Plan Apo VC oil objective)

However, it is evident that ginger extract constituents do not show autofluorescence and thus cannot be visually confirmed if present within the vesicles. By nature, ginger extract is lipophilic and its formulation in Pheroid® creates a lipid microenvironment within which it concentrates when in contact with an aqueous medium. To confirm this hypothesis, the mean particle size, ζ-potential and morphological analysis were performed on pro-Pheroid® formulated ginger extract. The results revealed similar trends with Pheroid® only formulation where two populations with distinct particle size were observed with peak volumes of 6% and 3% at 0.2 µm and 5 µm, respectively (Figure 3a), and a mean particle size of 3±0.074 µm. The formulation also had a broader population distribution with a mean span value of 31.62±1.070 (Table 2). Surface charge analysis showed good stability with a mean ζ-potential of -28±2.34 mV (Figure 3b). Confocal microscopy showed presence of various spherically shaped vesicles that are similar to the Pheroid® only vesicles (Figure 2b). This indicates that, incorporation of the ginger extract within the vesicles did not affect the morphology of the vesicles.

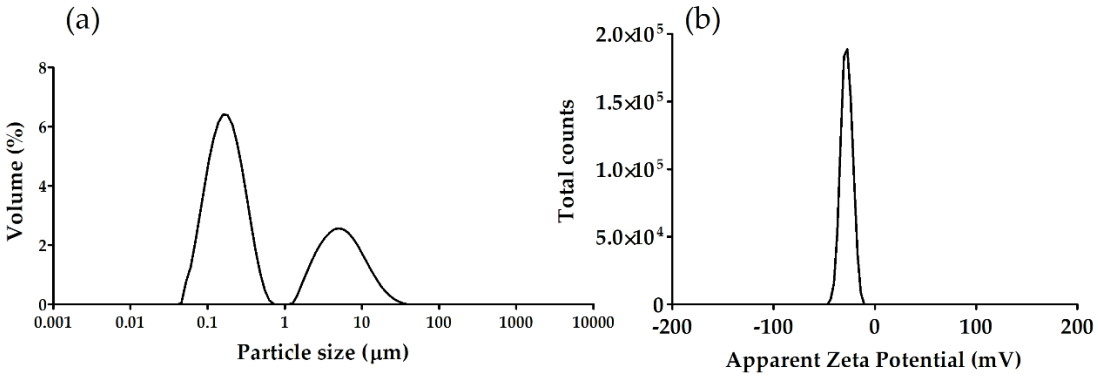

Figure 3. Mean particle size (a) and ζ-potential (b) analysis of Pheroid® formulated ginger extract

2.3. Compatibility study of Meriva® and ginger extract combination in pro-Pheroid®

Compatibility between Meriva® and ginger extract and with Pheroid® excipients are reported as heat flow diagrams (Figure 4a and b). An interaction average heat flow value of -17.6 μW/g with an interaction error of 22.9 μW/g was measured for the first Meriva® and ginger extract sample (Figure 4a). Thereby resulting in a heat flow difference of 5.3 μW/g between the measured heat flow and the theoretically calculated heat flow. This low interaction value is indicative of compatibility between the two compounds.

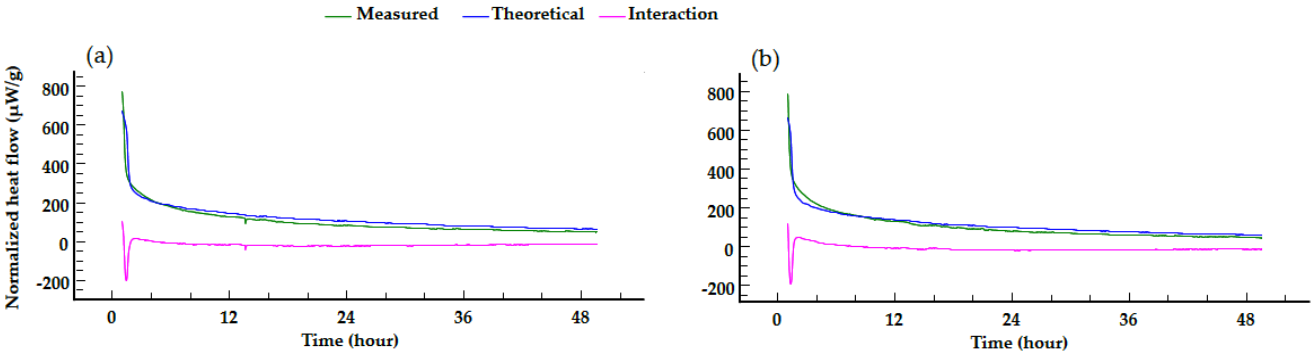

Figure 4. The heat flow graph obtained with the combination of Meriva® and ginger extract combined in a pro-Pheroid® formulation for two independent tests (a and b)

An interaction average heat flow value of -10.6 μW/g with an interaction error of 20.9 μW/g was measured for the second test (Figure 4b). Thereby resulting in a heat flow difference of 10.3 μW/g between the measured heat flow and the theoretically calculated heat flow. This interaction heat flow is double that obtained with the first test (Figure 4a). Although it is higher no incompatibility can be identified due to the similarity in the heat flow curves of the measured heat flow versus the theoretically calculated heat flow. The negative peak at the start of the compatibility run could be of concern and therefore a components graph was constructed (Figure 5). The components graph depicts the heat flow curve measured for each individual component. All four curves follow a similar downwards slope with no sudden increase or decrease in the heat flow. Therefore, the negative peak seen on the interaction graph could be ascribed to the experiment being started prior to complete baseline capturing or it could be ascribed to an attribute of the pro-Pheroid® base, causing a slight uptake of heat during the initial exposure to a higher than ambient temperature. From the heat flow data, it can be concluded that no incompatibility exists between Meriva® and ginger extract when mixed together in a pro-Pheroid® formulation.

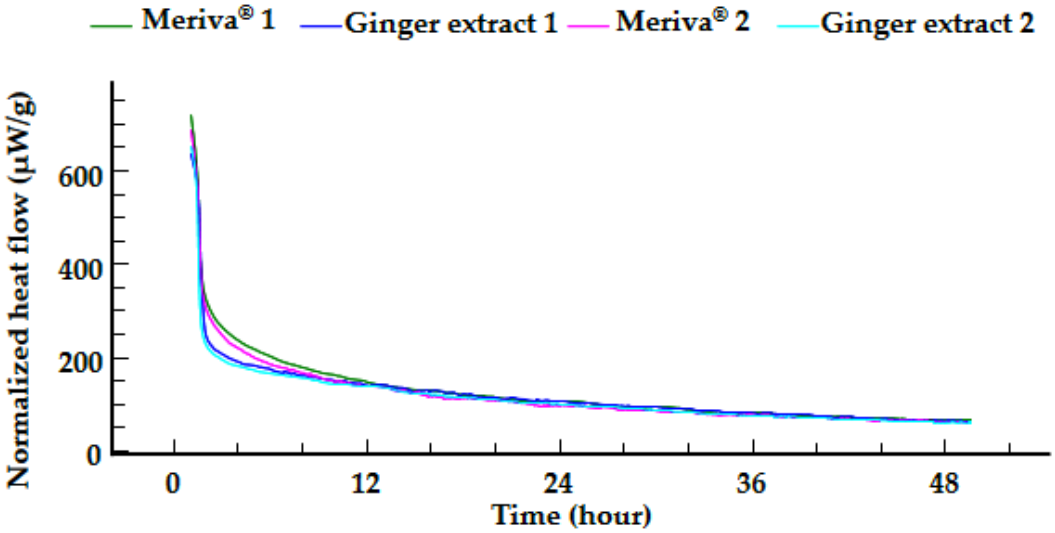

Figure 5. Components graph depicting the heat flow curves obtained with each individual compound

2.4. Quantification of curcumin and [6]-shogaol using LC–MS/MS method

The contents of curcumin and [6]-Shogaol in Meriva® and GE powders were determined using LC–MS/MS method. The LC-MS/MS method development initiated with the selection of MRM transition for both curcumin and [6]-Shogaol by infusion method. Moreover, compound dependent parameters were optimized. For the chromatographic separation, 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) were selected after optimization. A linear gradient of 30% to 90% solvent B in 1.5 min on a Phenomenex Kinetex™ C18 column gave the best peak shapes for both compounds. The elution times of curcumin and [6]-Shogaol were 1.21 and 1.40 min, respectively (Figure 6a and b). Furthermore, as observed from Figure 6b and also shown in literature [29], [6]-Shogaol has two isomers, namely cis-[6]-Shogaol (predominant peak) and trans-[6]-Shogaol (small peak) which eluted at 1.40 and 1.70 min, respectively. Hence, cis-[6]-Shogaol with the predominant peak was selected and utilized for quantification. The mass chromatograms of curcumin, [6]-Shogaol, Meriva® powder and GE powder are shown in Figure 6. The curcumin content of Meriva® powder was determined to be 400 mg/g extract, whilst the [6]-Shogaol content of GE powder was determined to be 11 mg/g extract.

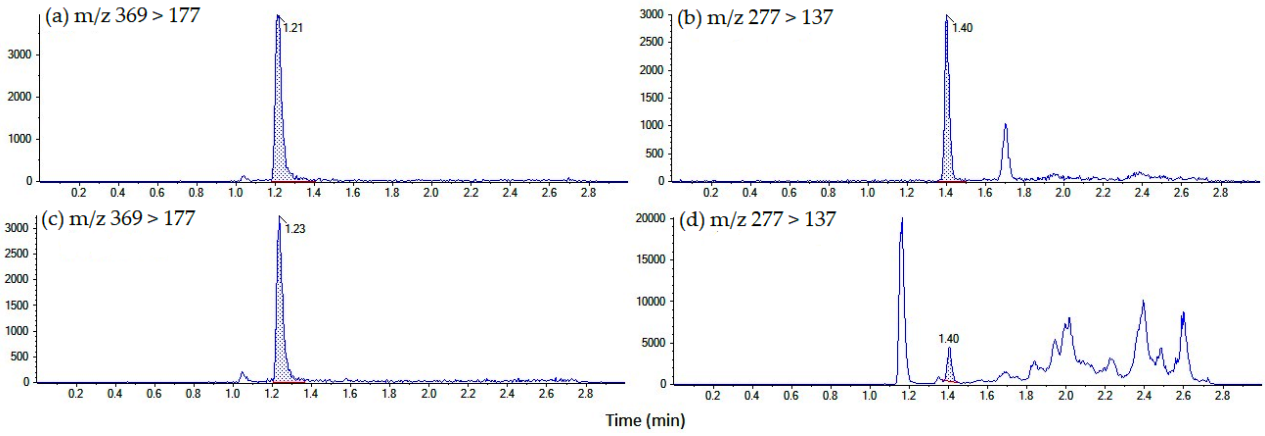

Figure 6. Mass chromatograms of (a) curcumin standard solution; (b) [6]-Shogaol standard solution; (c) the methanol extract of Meriva® powder; (d) the methanol extract of ginger extract powder

2.5. In vivo animal study

To investigate the *in vivo* chemotherapeutic effect of Pheroid® formulated Meriva® and ginger extract combination, male and female BALB/c athymic nude mice were subcutaneously injected with 1 x 10^6 A549 cells in Matrigel® in the right flank. After inoculation, mice were allocated into saline (n=5; three males and two females), Pheroid® only (n=7; four male and three female), cisplatin (n=8; four male and four female) and Pheroid® formulated phytochemical combination (n=8; four male and four female) treatment groups. Treatment commenced once the tumor volume was palpable and continued for 14 days. Accordingly, the first and second groups received daily oral gavage with 100 µL 0.9% normal saline and 4% Pheroid® only per body mass. The third group received cisplatin treatment via intraperitoneal (IP) route of administration at 4.5 mg/kg once a week and group four received 4% Pheroid® formulated Meriva® (70 mg/kg) and GE (100 mg/kg) combination daily via the oral route. Doses for Meriva® and GE were selected from literature on the bases of a preclinical study of Meriva® used as a putative chemo-preventative agent for lung cancer and GE used in the management of prostate cancer [30, 31].

In this study, gender had no influence on changes in tumor volume and body mass for all treatment groups. However, a relationship between treatment and treatment period was noted. The tumor volume was normalized relative to the first measured tumor in the respective groups using equation 1 (Figure 7a). The tumor growth curve shows that the saline negative control group has a lower tumor volume and slower growth rate over time compared to the vehicle control (Pheroid® only) and phytochemical combination in Pheroid® treatment groups. Although the tumor development and growth in this group was not satisfactory, the tumor did not regress over the course of the treatment period.

$$RTV = V_n/V_0, \quad (1)$$

Tumor volume in the vehicle control (Pheroid® only) group grew exponentially over the 14 day treatment period. Daily oral gavage with phytochemical combination in Pheroid® and saline displayed a significant ($p < 0.001$) tumor reduction compared to the vehicle control group from day 11 until the end of the treatment period (Figure 7a). On the other hand, intraperitonealcisplatin treatment significantly reduced the tumor growth after the first week of administration ($p < 0.001$) compared to the vehicle control treatment group. However, this potent anticancer effect was accompanied with a decline in animal body mass. Cisplatin causes anorexia and cachexia as a side effect [32], which affects the body mass. In this group, the food intake and feeding efficiency was considerably affected by cisplatin administration, especially during the second week of treatment with the lowest mean body mass recorded at the end of the treatment period ($p < 0.001$) compared to the vehicle control group (Figure 7b). In the saline, Pheroid® only and Pheroid® formulated phytochemical treatment groups, the murine body mass remained unchanged (Figure 7b), indicating that these treatments did not cause any adverse effects in the animals that could have affected their normal feeding behavior.

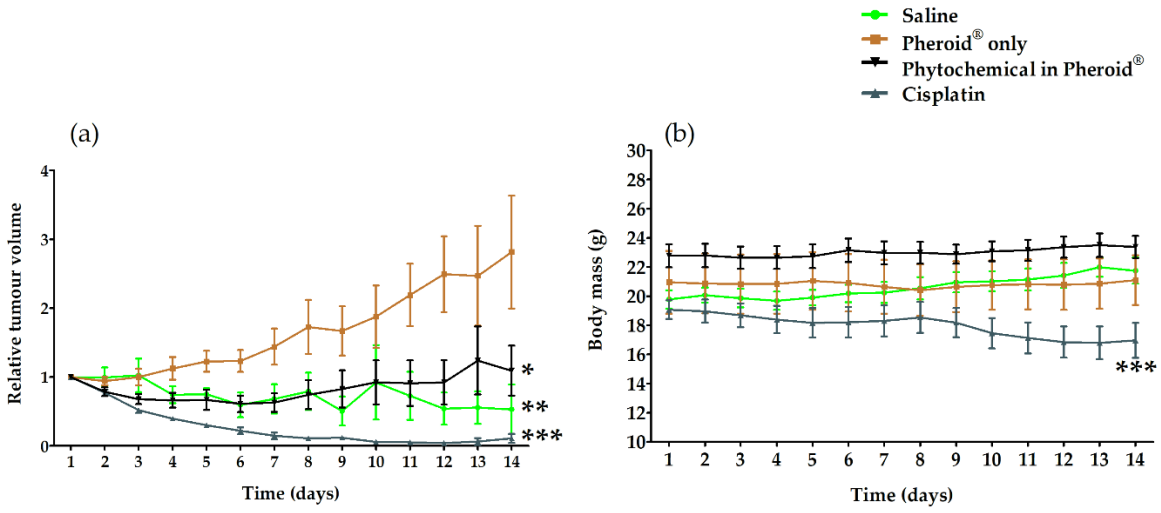

Figure 7. Tumor volume (a) and body mass (b) of individual treatment groups. Values represent mean ± SEM; (Saline, n = 5; Pheroid® only, n = 7; Phytochemical in Pheroid®, n = 8; cisplatin, n = 8)

The tumor was excised the day after the last treatment. It indicated that cisplatin significantly ($p < 0.05$) reduced tumor growth compared to the vehicle control (Pheroid® only) and phytochemical combination in Pheroid® treatment groups (Figure 8).

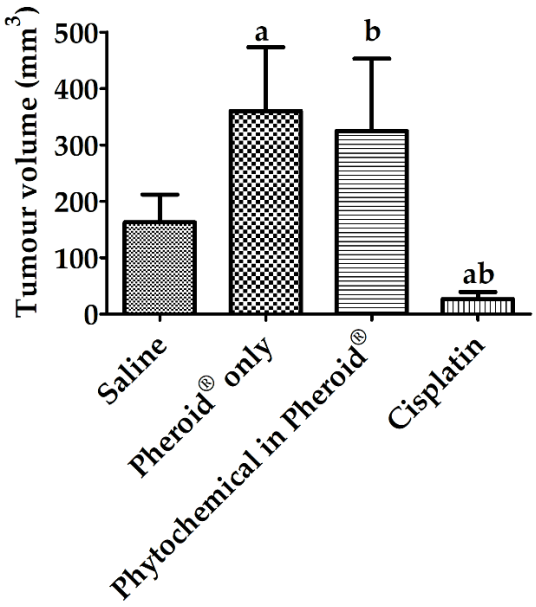

Figure 8. Excised tumor volume of individual treatment groups. Values represent mean ± SEM. Common symbols indicate significant differences between treatment groups ($p < 0.05$)

The biological anticancer activity was analyzed using the rate-based T/C method as described by Hather *et al* [33]. T/C was computed using equation 2 based on the ratio of the fitted tumor growth rates of treated versus control groups at a specified time, normalized to the 14 day study period. To avoid extreme data, low tumor volumes were truncated to 50 mm^3. The tumor volumes were then log transformed to fit the data into the model. From the log transformed values, the slope of the tumor volume with time was calculated and its mean was used to calculate the rate-based T/C.

$$\text{Rate-based T/C} = 10^{(\mu T - \mu C) \times 14 \text{ days}} \quad (2)$$

Where μT is the mean slope of the growth rates for the treatment group, and μC is the mean slope of the growth rates for the control group.

It was assumed that the tumor volume in each group followed an exponential growth. Agents that result in a mean tumor volume T/C of ≤ 0.15 are highly active; T/C > 0.15 but ≤ 0.45 have intermediate activity; and those with T/C > 0.45 are considered to have low levels of activity [34]. In this analysis, cisplatin exhibited an intermediate activity when compared to the negative control saline group; T/C of 0.25 ($p = 0.004$), (Table 3). Pheroid® only and phytochemical combination in Pheroid® had lower tumor inhibition activity compared to the negative control saline group with Pheroid® only showing a higher T/C value (Table 3). When compared to the vehicle control group, cisplatin showed a higher activity with a mean T/C value of 0.12 ($p < 0.0001$), whereas Pheroid® formulated phytochemical combination and saline treatment groups showed lower activities T/C of 0.52 ($p > 0.05$) and 0.47 ($p > 0.05$), respectively. Comparison between cisplatin and phytochemical treatment showed that cisplatin significantly ($p < 0.001$) reduced tumor growth with T/C value of 0.22

Table 3. Rate-based T/C compared to the saline negative control

Group	Dose (mg/kg)	Rate-based T/C	*p* value
Cisplatin	4.500	0.250	0.004
Pheroid® only	-	2.140	0.071
Combination	70 Meriva® and 100 ginger extract	1.110	0.779

3. Discussion

Traditional herbal medicine is the mainstay of primary healthcare in about 80% of the worlds' population — mainly in developing countries — because it is readily accessible and thought to function better in the human physiological system without inducing any major side effects [35, 36]. There has also been growing consumption of herbal medicines in developed countries for their health benefits and for use as an alternative natural drug source [37, 38]. Turmeric — known as the golden spice — and ginger have been used as spices in culinary dishes and their principal active compounds curcumin and ginger extract (GE) phenolics have been used in traditional folk medicine for their anti-inflammatory, antioxidant and anticancer properties [39, 40].

Meriva® is Indena's patented phytosome technology containing a mixture of curcumin and soy lecithin at 1:2 mixing ratio with two parts of microcrystalline cellulose to impart flowability. Literature indicated that Meriva® increased the bioavailability of native curcumin by five folds in rodents [41]. Here we report for the first time the combined *in vivo* anticancer potential of Meriva® (70 mg/kg) and GE (100 mg/kg) in the Pheroid® drug delivery system against lung cancer. The content of curcumin in Meriva® was quantified and found to be 400 mg/g. [6]-shogaol (6SG) — among other GE constituents and curcumin — has a strong anti-tumorigenic effect [15, 42] and its content in GE was quantified as 11 mg/g. Therefore, the concentration of individual actives in the formulation administered to the animals was 28 mg/kg curcumin and 1.1 mg/kg 6SG. Although the goal of combination therapy is to reduce the dose of individual drugs, the amount of actives administered to the animals in the present study were suboptimal according to the literature concentrations of curcumin and 6SG. Oral treatment with curcumin at a dose of 100 mg/kg for 28 days significantly reduced tumour growth and weight in NCI-H460 bearing athymic nude mice [8]. In another study, oral gavage with 6SG at a dose of 30 mg/kg five times a week for seven weeks significantly reduced tumour growth and burden compared to the vehicle control in A549 bearing athymic nude mice [43]. Therefore, in future studies, oral doses of curcumin and 6SG individually as determined by *in vivo* literature studies should be formulated in Pheroid® in order to compare their combined therapeutic effect against lung cancer.

Electrokinetic potential analysis and compatibility study revealed that Meriva® and GE are stable in Pheroid® and that no drug–excipient interactions were observed. This is of paramount importance since it allows for direct measurement of the pharmacological effects of the phytochemicals without interference from the delivery system. Among other formulation parameters, measurement of particle size and distribution are important indices for evaluating the quality and suitability of a formulation. Moreover, this bivariate distribution influences the stability of the formulation, entrapment efficiency, drug release profile, biodistribution, mucoadhesion and cellular uptake [44, 45]. *In vitro* cellular uptake study is one important prerequisite physicochemical criterion to be taken into account before *in vivo* study [46]. In the previous *in vitro* study, it was observed that Pheroid® significantly enhanced the cellular uptake of Meriva® and GE combination [27]. Therefore, based on this preliminary *in vitro* biological uptake result, the *in vivo* study was conducted without incorporation of the free (unformulated) Meriva® and GE combination treatment group.

In the particle size analysis, Meriva® and GE combination in Pheroid® were bigger in size compared to Pheroid® only formulation. This is thought to be as a result of the excipients found in Meriva®. To confirm this assumption, the particle size of Meriva® in Pheroid® was measured. The analysis showed a single population with a mean particle size of 30.15±10 μm and a mean span value of 3.44±0.051. Since Pheroid® formulated GE showed similar particle size with Pheroid® only, it can be concluded that the particle size of combined phytochemical formulation is largely influenced by Meriva®. To determine the successful incorporation of Meriva® and GE combination by the Pheroid® vesicles, quantifying the

entrapment efficiency is of paramount importance. In this study, although sufficient, entrapment of phytochemicals by Pheroid® vesicles was confirmed using confocal laser scanning microscopy. Therefore, additional analysis focussing on the concentrations of the actives entrapped is suggested for future studies.

The pH of a formulation is another critical factor that can potentially influence the pharmacokinetic profile of active ingredients and the safe use of the formulation in the biological system. When designing an oral formulation for preclinical study in laboratory animals, the pH should lie within the recommended range, 2–9. A formulation that has a pH outside of this range — either too acidic or alkaline — can result in tissue injury and vascular thrombosis [47]. In the present study, the pH of both Pheroid® only (6.65 ± 0.05) and Pheroid® formulated phytochemical combination (5.89 ± 0.02) were within this range. Curcumin is more structurally stable in an acidic environment, however, at neutral and alkaline pH, it undergoes rapid degradation [21]. On the other hand GE phenolics are stable at various pH conditions and in simulated gastric and intestinal fluids, indicating their suitability for oral administration [20]. The study further highlighted that *in vivo* sub-therapeutic concentrations of GE phenolics can be overcome by using drug delivery systems and selective uridine glucuronosyl transferases (UGTs). As such, incorporation of Meriva® and GE in Pheroid® is a plausible strategy to improve their chemical stability and systemic exposure.

Of note, the vehicle control group had a statistically non-significant ($p > 0.05$) increase in tumor growth compared to the negative control group (saline) (Figure 7a and Figure 8). Drug carrier systems are not inert in nature and have some degree of physiological effect [48]. Additionally, the ingredients used in some drug carrier systems have been proven to promote cancer cell growth based on nutritional abilities [49]. Therefore, the effect seen with Pheroid® only treatment can further be investigated to delineate the mechanism with which it is associated in the promotion of cell proliferation. Pheroid® formulated phytochemical combination displayed similar effect with saline treatment where a reduced tumor growth and burden was observed compared to the vehicle control group from the 11th day of treatment period (Figure 7a and Figure 8). This sub-therapeutic anticancer effect could be mainly due to the insufficient amounts of curcumin (28 mg/kg) and 6SG (1.1 mg/kg) actives administered to the animals. Unlike normal tissue, the tumour microenvironment is perfused with highly dense vasculature that is permeable and leaky, heterogeneous in distribution, hypoxic, and has low pH value and overexpressed proteins [45, 50]. These characteristic features enable functional carriers to sense the tumour microenvironment and alter their conformation. An example of such conformational change by carriers is pH responsive drug release, whereby the low pH of the tumour microenvironment triggers morphological change in the lipid bilayer of the carrier [51]. Therefore, this could be one possible mechanism to use in order to enhance the combined phytochemical anticancer effect in the Pheroid® delivery system.

Cisplatin is an effective antineoplastic drug. In the present study, cisplatin significantly ($p < 0.05$) reduced the tumour growth compared to the negative control group (Figure 7a). The excised tumour also revealed that cisplatin significantly ($p < 0.05$) reduced the tumour volume compared to Pheroid® only and phytochemical in Pheroid® treatment groups (Figure 8). However, cachexia and nephrotoxicity are the main dose limiting side effects of cisplatin [32, 52]. In this study, mice treated with cisplatin showed a decline in body mass during the treatment period (Figure 7b). The side effects of standard chemotherapeutic drugs like cisplatin can be reduced with the addition of phytochemicals in the treatment regime. This combination treatment also has the potential to improve the anticancer activity of the synthetic drugs through either synergistic or additive mechanism of action. Kumar *et al*. [53] showed that curcumin ameliorates cisplatin induced nephrotoxicity and increased its anticancer effect. In addition, concomitant use of cisplatin with ginger in the form of an extract and as an essential oil showed a protective effect against cardiotoxicity, hepatotoxicity and nephrotoxicity caused by cisplatin [54, 55]. The current *in vivo* pilot study shows that combination of Meriva® and GE in Pheroid® demonstrated some degree of anticancer activity, which can be potentiated with combination of therapeutic doses of individual actives. This provides an opportunity to further investigate the anticancer effect of cisplatin co-administered with Pheroid® formulated curcumin and 6SG combination against lung cancer.

4. Materials and Methods

4.1. Materials

Human non-small cell lung cancer adenocarcinoma A549 cell line (ATCC® CCL-185™) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cell culture media, reagents and consumables were obtained from Thermo Fisher Scientific (Waltham, MA, USA), Gibco (Dun Laoghaire, Ireland), and Sigma-Aldrich (Missouri, USA). Curcumin (98%) and [6]-shogaol (96%) standards were purchased from Laboratory of the Government Chemist (LGC) standard (UK). Meriva® was kindly donated by Formul8 Pharma (Pty) Ltd (Johannesburg, South Africa). Ginger extract with >10% gingerols was purchased from Xi'an B-Thriving I/E Co., Ltd. (Shaanxi, China). Vitamin F ethyl ester was purchased from IMCD (Rotterdam, The Netherlands). Kolliphor® EL and dl-α-tocopherol were purchased from BASF (Berlin, Germany). All chemicals used were of analytical reagent grade.

4.2. Formulation and characterization of Meriva® and GE combination in Pheroid®

Meriva® and GE were formulated in Pheroid® according to the method described in the previous *in vitro* study [27]. Briefly, 13.15 g of Meriva® and 18.81 g of GE were mixed with 45.61 g of pro-Pheroid® and homogenized at 1 000 rpm at 50°C until in solution. The mixture was then gassed under pressure with nitrous oxide for four days.

4.2.1. pH measurement

The pH of Pheroid® only and Pheroid® formulated GE and Meriva® combination were determined using a bench top Mettler Toledo pH meter (Ohio, USA). A 4% Pheroid® concentration was prepared by diluting the oil concentration 25 times in nitrous oxide gassed water. Before each measurement, the pH was calibrated using pH 4, 7 and 10 standard buffers. Results were presented as the mean of three measurements.

4.2.2. Particle size and ζ-potential analysis

The particle size and ζ (electrokinetic) potential measurements were made using Malvern hydro 2000 SM Mastersizer and Malvern Nano ZSP Series Zetasizer ZEN5600 (Malvern Instruments Ltd, Malvern, Worcestershire, UK), respectively. Before analysis, a 4% Pheroid® concentration was made from the pro-Pheroid® formulation in 0.1 N hydrochloric acid aqueous medium (1:25 v/v). Particle size measurements were made in triplicates after injecting the samples in distilled water dispersion media at 10–20% obscuration value. Equation 3 was used to calculate the span value, which measures the width of the population distribution. The smaller the span value, the narrower the distribution will be. The ζ potential measurements were made in triplicates by diluting the samples in deionized water (1:5000 v/v).

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

Where; $d_{0.9}$,$d_{0.5}$ and $d_{0.1}$ are particle sizes at the 90th, 50th and 10th percentile, respectively.

4.2.3. Vesicle morphology and size distribution

The morphology and vesicle size distribution of Pheroid® only vesicles and phytochemical entrapped Pheroid® vesicles were determined by Nikon D–Eclipse C1 Confocal inverted microscope TE2000-E equipped with a diode, an Argon Ion and Helium–Neon polarized lasers with an excitation wavelengths of 409 nm, 488 and 543 nm, respectively [56]. Using Nile red as the fluorescence probe, Pheroid® vesicle images were taken with a 30 μ m pinhole and a 60 x 1.40 ApoPlanar oil immersion objective. A 50 μL 4% Pheroid® concentration sample was labeled with a 5 μL Nile red fluorescent marker and vortexed briefly. After storing the sample in the dark for 15 minutes, 20 μL of the mixture was mounted onto the glass slide with a coverslip and imaged at 60x magnification.

4.2.4. TAM III compatibility assay

Thermal Activity Monitor (TAM) is a sensitive and accurate instrument for detecting incompatibilities and instabilities between active pharmaceuticals (APIs) and/or excipients. Compatibility between the phytochemicals and Pheroid® excipients was determined with a 2277 Thermal Activity Monitor (TAM III; TA Instruments, United States of America), equipped with an oil bath with a stability of ±100 μK over 24 h. The calorimeter's temperature was maintained at 40°C. During the compatibility study, the heat flow was measured for the single components, as well as for the mixtures. The assay was performed in duplicate for each phytochemical and their combination in pro-Pheroid® as shown in Table 4 below. Data was analyzed using TAM Assistant v 2.0.156 software package.

Table 4. Amounts of actives and pro-Pheroid® used in compatibility study

Trial	Individual (mg)		Combination (mg)	Volume of pro-Pheroid® (mL)
	Meriva®	GE	Meriva®/GE	
1	101.25	101.20	41.28/58.64	3
2	101.33	101.40	41.83/58.90	

4.3. Determination of phytochemical content

The contents of curcumin in Meriva® and [6]-Shogaol in GE were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS) according to previously described methods [57–59] with some modifications.

4.3.1. Preparation of standards and samples

Curcumin and [6]-shogaol standards were accurately weighed and dissolved in methanol to obtain a stock solution of 1 mg/mL and 0.4 mg/mL, respectively. The stock solutions were diluted serially with deionized water to give 62.5, 125, 250, 500 and 1000 ng/mL concentrations to constructing the calibration curve.

For the analysis of curcumin and [6]-shogaol in Meriva® and GE, respectively, 5 mg of Meriva® and GE powder were weighed on an analytical balance (Axis model AGN220C, Gdansk, Poland) and dissolved in 5 mL of methanol to give a 1 mg/mL concentration. The mixtures were rotated on the rotor-mixer for 10 min and then centrifuged for 10 min at 2 000 × g using a benchtop centrifuge (Allegra X-30R Beckman Coulter, USA). Aliquots of each supernatant were further diluted with deionized water to give final concentrations of 1 μg/ml Meriva® and 10 μg/ mL GE, and 20 μL of each injected for LC–MS/MS analysis.

4.3.2. Liquid chromatography tandem mass spectrometry (LC–MS/MS) conditions

The separation of the extracted sample was performed on Agilent 1290 Infinity HPLC system and CTC PAL HTx-xt autosampler with a 20 μL sample loop. The analytes were separated on a Phenomenex Kinetex™ C18 column (30 mm × 2.1 mm, 1.7 μm) with a pre-column (UHPLC C18, 2.1 mm ID) at room temperature using gradient elution with water (A) and acetonitrile (B), both with 0.1% formic acid. The flow rate was 0.4 mL/min and the gradient was as follows: 0.0–1.5 min: linear from 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.

Tandem mass spectrometry (MS/MS) was performed using SCIEX API 4000 QTRAP mass analyzer equipped with a Turbo Ion Spray source (SCIEX, Toronto, Canada) operating in electrospray ionization (ESI) positive mode. Analyst software (version 1.6) was used for instrument control, data acquisition; and quantitative analyses was performed with MultiQuant™ 2.1 Software. Detection and quantitation of analyte was achieved using multiple reaction monitoring (MRM) mode. Optimized instrument settings were as follows: ionization mode, positive; curtain gas, 40 psi; CAD gas, medium; nebulizer gas (GS1), 45 psi, heater gas (GS2), 50 psi; ion spray voltage, 5.5 kV; temperature, 450 °C. The nitrogen flow was produced by a gas generation system (Peak Scientific Nitrogen Generator, model AB-3G, PA, USA). A dwell time of

150 msec was used for the transition. MS/MS parameters were optimized by direct infusion of curcumin and [6]-shogaol reference standard solutions at a flow rate of 10 μL/min, in the positive electrospray ionization (ESI) mode. MRM transitions selected for curcumin and [6]-shogaol quantification were m/z 369.1 → 177.2 and m/z 277.1 → 137.1, respectively. The MS/MS settings were as follows: collision energy at 29/17 volts; collision exit cell potential at 8/22 volts; declustering potential and entrance potential were 336/116 and 10 volts, respectively.

4.3.3. Analysis of sample extracts

An LC-MS/MS method was developed and validated as per ICH guidelines [60] for curcumin and [6]-shogaol content analysis. Five calibration solutions were injected in duplicate and their analyses were performed. Calibration curves were constructed using linear regression analysis of the peak area (Y) corresponding to curcumin and [6]-shogaol versus their concentration (X) in ng/mL. The curcumin and [6]-shogaol contents of the samples were determined using the corresponding calibration curves. The content of curcumin in Meriva and [6]-shogaol in ginger extract were calculated according to the equation 4.

$$T = CxV/M, \quad (4)$$

Where; T is content in mg/g, C is concentration established from the calibration curve, V is volume of solution prepared and M is the mass of extract in gram

4.4. Animal study

The A549 cell line was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum and 1% penicillin-streptomycin-amphotericin B mixture at 37 °C with 5% CO_2 and 90% relative humidity. Cells were sub-cultured at least three times before inoculation. Male and female BALB/c athymic nude mice approximately six to eight weeks old were obtained from an AAALAC accredited vivarium animal facility (DST/NWU PCDDP, Potchefstroom, South Africa). The animal experiment was approved by the AnimCare Research Ethics Committee of North-West University (NWU-00167-18-A5). Animals were housed in Individual Ventilated Cages (IVC). The internal temperature and humidity of the IVC was maintained at 22 °C and 44% relative humidity, respectively. The IVC was regularly cleaned and animals had *ad libitum* access to water and commercial rodent chow in a 12 h light and dark cycle controlled room. After three days of acclimatization, mice were subcutaneously injected in the right hind leg with 0.1 mL of cell suspension in Matrigel® containing 1 x 10^6 viable A549 human adenocarcinoma lung cancer cells. Once the tumor volume reached 100 mm^3, the mice were allocated into four groups each containing four males- and four females (n = 8 per group) and that day was designated as "Day 0". Accordingly, group one and two received 100 μL of 0.9% saline solution and 4% Pheroid® only per body mass *per os* per day, respectively, while group three received Pheroid® formulated Meriva® (70 mg/kg) and GE (100 mg/kg) combination daily via the same route. The fourth group received cisplatin at a dosage of 4.5 mg/kg via intraperitoneal injection once a week. Tumor volume was measured every day with a linear digital calliper and calculated using the equation given below:

$$\text{Tumor volume} = W^2 \times L/2, \quad (5)$$

Where W is the tumor measurement at the widest point and L is the tumor measurement at the longest point. At the end of the 14 day treatment period, mice were euthanized via cervical dislocation and the tumors were excised and measured.

4.5. Statistical analysis

Data obtained were expressed as mean ± standard error mean (SEM). The raw data in the animal study were log transformed and the intercept and slope calculated. The homogeneity of the variance was determined using Levene's test. Significant differences between treatment groups were determined by using either the Analysis of Covariance (ANCOVA) or the Unequal N Honestly Significant Difference

(HSD) test —modified Tukey HSD test) —with Dunnett's test as post hoc test. Excised tumor were tested for normality using Kolmogorov-Smirnov test. Significance between groups was tested using Kruskal–Wallis (KW), with Dunnett's as post hoc test. The results were considered statistically significant if $p < 0.05$. Statistical analysis was done on Statistica (StatSoft Inc., Germany) and graph were produced using GraphPad Prism6 (GraphPad, San Diego, CA, USA).

5. Conclusions

Curcumin and ginger extract (GE) phenolics have been shown to have potent chemopreventive activities despite their poor physicochemical properties. [6]-shogaol (SG) — among GE phenolics and curcumin — has potent anti-tumorigenic activity. Confocal microscopy showed entrapment of the phytochemicals within the Pheroid® vesicles. In addition, the phytochemicals were found to be stable and compatible with Pheroid® excipients. Daily oral treatment of mice with Pheroid® formulated Meriva® and GE combination exhibited a non-statistically significant reduction in tumor growth and burden compared to the vehicle control treatment group. However, the amount of active phytochemicals — curcumin and 6SG — administered to the animals were suboptimal compared to literature values. Cisplatin is a standard chemotherapeutic drug and demonstrated a statistically significant reduction in tumor growth compared to the negative control saline group. However, this anticancer effect was accompanied with a decline in body mass. Literature indicates that the side effects of cisplatin can be overcome by co-administration with phytochemicals such as curcumin and GE phenolic compounds. The present study thus provides an opportunity to further investigate the anticancer effect of cisplatin co-administered with therapeutic dose combination of curcumin and 6SG in Pheroid® against lung cancer.

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CHAPTER 5 STUDY CONCLUSION AND FUTURE RECOMMNEDATIONS

5.1 Study conclusion

The present study aimed to evaluate the chemopreventive activity of Meriva®; a curcumin phytosome, and ginger extract (GE) combinations in Pheroid® against lung cancer in both *in vitro* and *in vivo* models. This was done by quantifying the contents of principal phytochemicals; formulating and characterising combined phytochemicals in Pheroid®; conducting cytotoxicity, cellular uptake, apoptosis, oxidative stress markers, and mitochondrial health assays using relevant *in vitro* methods. The *in vivo* anticancer activity was determined using athymic nude mice xenograft lung cancer model.

Meriva® and GE combinations were formulated in pro-Pheroid® — a precursor of Pheroid® — and this formulation was characterised. Electrokinetic potential analysis and compatibility study indicated that the phytochemicals were stable in Pheroid® and that no drug–excipient interactions were observed. This result entails that the therapeutic effect measured from formulated phytochemical combination treatment is without interference from the delivery system. Confocal microscopy showed co-localization of the phytochemicals within the Pheroid® vesicles. In addition, the particle size of phytochemical entrapped Pheroid® vesicles were larger in size compared to Pheroid® only vesicles. This was due to the presence of microcrystalline cellulose and soy lecithin excipients in Meriva®. The contents of curcumin in Meriva® and [6]-shogaol (6SG) in GE were quantified as 400 mg/g and 10.63 mg/g, respectively. Based on this, the actual amounts of curcumin and 6SG in Pheroid® formulated Meriva® and GE combinations administered to the animals were 28 mg/kg and 1.1 mg/kg, respectively. Compared to literature values, the dose of these actives is insufficient. A limiting factor in the formulation process was a rapid increase in viscosity with an increase of added phytochemicals. This limited the therapeutic concentration of Meriva® and GE in the Pheroid® given to the animals, as it needed to be in a liquid form for oral gavage.

The *in vitro* study demonstrated that the phytochemical combination in Pheroid® showed a more robust chemopreventive activity than the individual actives and free forms. In this study, 52.5 µg/mL Meriva® and 75 µg/mL GE showed strong anti-proliferative and apoptotic effects. This enhanced effect was due to a reduction in the mitochondrial respiration capacity. Unlike other cancers, lung cancer cells heavily rely on mitochondrial oxidative phosphorylation for energy production and a dysfunction in the mitochondria has a detrimental consequence on cell proliferation. A mitochondrial health assay revealed that Meriva® was responsible for the mitochondrial dysfunction, while GE exhibited no effect on mitochondrial respiration. However,

incorporation of GE may have amplified the activity of Meriva® through other mechanisms involved in cell death either by synergistic or additive effects.

Cellular uptake is an important physicochemical parameter to be considered before *in vivo* study. By means of confocal microscopy, it was confirmed that Pheroid® significantly enhanced the internalisation of phytochemicals by the cells compared to the free forms. This is indicative of improved biological function of the phytochemicals imparted by formulation in the Pheroid® drug delivery system. Despite the presence of higher content of phytochemicals within the cells, Pheroid® reduced the intracellular oxidative species in phytochemicals combination treatment. The antioxidant property of Pheroid® could be attributed to the presence of dl-α-tocopherol, as one of the excipients in its formulation. Therefore, it can be concluded from the *in vitro* study that Pheroid® improves the chemopreventive activity of phytochemical combination while reducing oxidative stress. This is essential in cancer therapy as it potentiates the safe and effective use of anticancer agents without having the adverse toxic effects to normal cells.

A single preclinical modality usually does not provide complete information of a given treatment substance. To investigate the *in vivo* anticancer activity of Meriva® and GE phytochemicals in Pheroid®, male and female athymic nude mice were inoculated with viable A549 cells. The concentrations of Meriva® (70 mg/kg) and GE (100 mg/kg) were selected from literature on the bases of a preclinical study of Meriva® used as a putative chemo-preventative agent for lung cancer and GE used in the management of prostate cancer. Overall, gender had no influence on the treatment outcome for all the groups. Treatment of mice with a daily oral gavage of phytochemical combination in Pheroid® demonstrated a non-statistically significant reduction in tumour growth and burden compared to the vehicle control (Pheroid® only). This sub-therapeutic effect is due to the suboptimal amounts of curcumin and 6SG actives present in their respective samples. Although daily oral feeding with suboptimal concentrations of phytochemical formulation in Pheroid® resulted in a sub-therapeutic effect, it displayed a good promise that if the combination of therapeutic dose of phytochemicals were used, there would be a decrease in tumour volume that could be comparable to the results of cisplatin therapy. Of note, a non-statistical increase in tumour growth was seen in the vehicle control group compared to the negative control saline group. Shah et al. (2017) reported a similar finding in which treatment with empty liposomes — vehicle control — had no effect on tumour growth compared to saline therapy.

Cisplatin is a standard platinum based anticancer drug frequently used in the treatment of many solid tumours including lung cancer. However, it causes severe side effects such as anorexia, ototoxicity, peripheral neuropathy, and nephrotoxicity due to oxidative stress and lack of selectivity between cancerous and normal cells. Intraperitoneal injection of cisplatin (4.5 mg/kg) once a week significantly reduced the tumour growth compared to the saline group. However, in

this group, cisplatin induced anorexia and cachexia were observed as manifested by a decline in body mass. These adverse effects can be addressed by co-administering phytochemicals, which also has the potential to improve cisplatin anticancer activity.

The study's aims and objectives were achieved through the aforementioned *in vitro* and *in vivo* results. The *in vitro* results indicate the chemopreventive potential of Meriva® in the Pheroid® delivery system against lung cancer in combination with ginger extract. In addition, incorporation of phytochemicals in Pheroid® improved their biological uptake and anticancer activity compared to the free forms. This indicates the potential applicability of oral Pheroid® formulations since the oral route is a favoured route of administration. In conclusion, this study has demonstrated the development of enhanced formulation of Meriva® and GE in Pheroid® as a potential adjuvant therapy for lung cancer.

5.2 Future recommendations

- The amounts of plant bioactive — curcumin and [6]-shogaol (6SG) — compounds in the Pheroid® formulation administered to the animals as Meriva® and ginger extract (GE) were suboptimal when compared to literature values. Therefore, for future studies increasing Meriva® and GE concentrations or using therapeutic doses of curcumin and 6SG is suggested.
- To determine the successful incorporation of Meriva® and GE combination by the Pheroid® vesicles, quantifying the entrapment efficiency is important. In this study, entrapment of phytochemicals by Pheroid® vesicles was confirmed through confocal laser scanning microscopy and size analysis. Although sufficient, additional analysis focussing on the concentrations of the actives entrapped is suggested for future studies.
- The pharmacokinetic profile of phytochemical combination formulation in Pheroid® should be investigated to determine the plasma concentration of the actives and to further elucidate drug-drug interactions.
- Co-administration of cisplatin with curcumin and ginger phenolic phytochemicals have the potential to increase the anticancer activity of cisplatin and improve its toxicity profile (Damião et al., 2013; Ismail and Attyah, 2012; Kumar et al., 2017). Literature has shown that 6SG has a more potent anti-tumerigenic activity than other GE phenolics and curcumin (Chen et al., 2012; Wu et al., 2010). This opens an opportunity to further investigate the anticancer effect of cisplatin co-administered with Pheroid® formulated curcumin and 6SG combination against lung cancer.
- In cancer therapy, employing passive and active targeting nanotherapy is an amicable route to achieve maximum therapeutic index with minimum toxicity. However, this mechanism is largely dependent on the particle size and distribution of a formulation.

Particles that are above 200 nm are prone to rapid hepatic clearance and opsonisation. Therefore, combination of phytochemicals in delivery system should be optimised to have a particle size that is in the range of 10–200 nm for better treatment in future studies.

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Appendix A

Ethics approval and certificates of ethics training

NWU®
NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT
YUNIBESITI YA BOKONE-BOPHIRIMA

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

Health Sciences Ethics Office for Research, Training and Support

Animal Care, Health and Safety in Research Ethics Committee (AnimCare)
Tel: 018 299 2234
Email: Tiaan.Brink@nwu.ac.za

Dr W Pheiffer
Pharmaceutical Sciences
DST/NWU Preclinical Drug Development Platform (PCDDP)

26 October 2018

Dear Dr Pheiffer

APPROVAL OF YOUR APPLICATION BY THE ANIMCARE COMMITTEE OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00167-18-S5

Kindly use the ethics reference number provided above in all future correspondence or documents submitted to the administrative assistant of the Animal Care, Health and Safety in Research Ethics Committee (AnimCare).

Study title: Co-formulation and therapeutic evaluation of three bioactive plant compounds in Pheroid®

Study leader: Dr W Pheiffer

Student: BS Bekele-31045308

Application type: Single study

Project Category *(impact on animal wellbeing)*	**NA**	**0**	**1**	**2**	**3**	**4**	**5**
						X	

Expiry date: 31 October 2019 (monitoring report is due at the end of October annually until completion)

You are kindly informed that after review by the AnimCare committee, Faculty of Health Sciences, North-West University, your ethics approval application has been successful and was determined to fulfil all requirements for approval. Your study is approved for a year and may commence from 26/10/2018. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation. A monitoring report should be submitted two months prior to the reporting dates as indicated i.e. annually for Category 0-4 studies, six-monthly for category 5 studies, to ensure timely renewal of the study. A final report must be provided at completion of the study or the AnimCare committee, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimMonitoring@nwu.ac.za. Annually, a number of studies may be randomly selected for an internal audit.

The AnimCare committee, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the AnimCare committee, Faculty of Health Sciences prior to implementing these changes. These requests should be submitted to Ethics-AnimCare@nwu.ac.za with a cover letter with a specific subject title indicating "Amendment request: NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted. The amendments made should be indicated in yellow highlight in the amended documents (or in the fillable MSWord format application forms where a yellow highlighter may not be visible, change the text colour to red). The *e-mail*, to which you attach the documents that you send, should have a *specific subject*

line indicating that it is an amendment request e.g. “Amendment request: NWU-XXXXX-XX-XX”. This e-mail should indicate the nature of the amendment. This submission will be handled via the expedited process.

Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form to Ethics-AnimCareIncident-SAE@nwu.ac.za. The *e-mail*, to which you attach the documents that you send, should have a specific subject line indicating that it is a notification of a serious adverse event or incident in a specific project e.g. “SAE/Incident notification: NWU-XXXXX-XX-XX”.

Please note that the AnimCare committee, Faculty of Health Sciences has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research. The AnimCare committee, Faculty of Health Sciences reserves the right to visit sites where approved studies will be conducted and any animal housing facility under the authority of NWU as often as it deems necessary, either announced or unannounced.

The AnimCare committee, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the South African National Standard (SANS) document 10386:2008 entitled, “The care and use of animals for scientific purposes”, the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimCare@nwu.ac.za.

Yours sincerely

Prof Christiaan B Brink
Chair: AnimCare

Prof Minrie Greeff
Head: Ethics Office

Current details: (13009230) G:\My Drive\9.1.5.1.1 Ethics\NWU-00167-18-S5\\9.1.5.4.1_Approval Letter_ AnimCare.docm
26 October 2018

File reference: 9.1.5.3.3

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South Africa 2520

Tel: +2718 299-1111/2222
Web: http://www.nwu.ac.za

Health Sciences Ethics Office for Research, Training and Support
Tel: +2718 299 2089
Email: minrie.greeff@nwu.ac.za

7 March 2018

Dear Mr Bisrat Bekele

PROOF OF ATTENDANCE AND ASSESSMENT

This letter certifies that you have attended the 2 day ethics training and successfully completed the associated assessment.

The Basics of Health Research Ethics

(Accreditation number: PSB002/037/01/2018 from University of Free State CPD accreditation department accredited by the HPCSA)

Presenter: Prof Minrie Greeff (Head of the Health Sciences Ethics Office for Research, Training and Support) on **22 and 23 January 2018.**

This letter of attendance, as proof of ethics training and assessment, is valid for 3 years and expires on 31 Janaury 2021 (Where applicable, Ethics CEUs awarded: 14 CEUs).

Yours sincerely,

Prof Minrie Greeff
Head of Health Sciences Ethics Office
for Research, Training and Support

Prof Awie Kotzé
Executive Dean: Faculty of Health Sciences

Original details: (10187308) C:\Users\NWUUSER\Google Drive\9. Research and Postgrad Education\9.1.5.7 Training\Templates\9.1.5.7.5_LOA_BCHRE_Jan2018.docm
7 March 2018

File reference: 9.1.5.7.5

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South Africa 2520

Tel: +2718 299-1111/2222
Web: http://www.nwu.ac.za

Health Sciences Ethics Office for Research, Training and Support
Tel: +2718 299 2089
Email: minrie.greeff@nwu.ac.za

7 March 2018

Dear Mr Bisrat Bekele

PROOF OF ATTENTANCE

This letter certifies that you have attended the half-day ethics training, entitled:

The SANS document: As regulation for research with animals

presented by Prof Christiaan B Brink (Chairperson of AnimCare) on 24 January 2018.

This proof of attendance, as recognised by AnimCare and the Ethics Office, Faculty of Health Sciences, NWU, is valid for 3 years and expires on 24 January 2021.

Yours sincerely,

Prof Minrie Greeff
Head of Health Sciences Ethics Office
for Research, Training and Support

Prof Awie Kotzé
Executive Dean: Faculty of Health Sciences

Original details: (10187308) C:\Users\NWUUSER\Google Drive\9. Research and Postgrad Education\9.1.5.7 Training\Templates\9.1.5.7.5_LOA_SANSDOC_Jan2018.docm
7 March 2018

File reference: 9.1.5.7.5

NWU®
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YUNIBESITI YA BOKONE-BOPHIRIMA

Faculty of Health Sciences

Vivarium

This is to certify that

BISRAT BEKELE
Passport nr EP4619552

has attended the Introductory Short Course on

Animal Handling and the Principles of Research on Animals

11 – 13 April 2018

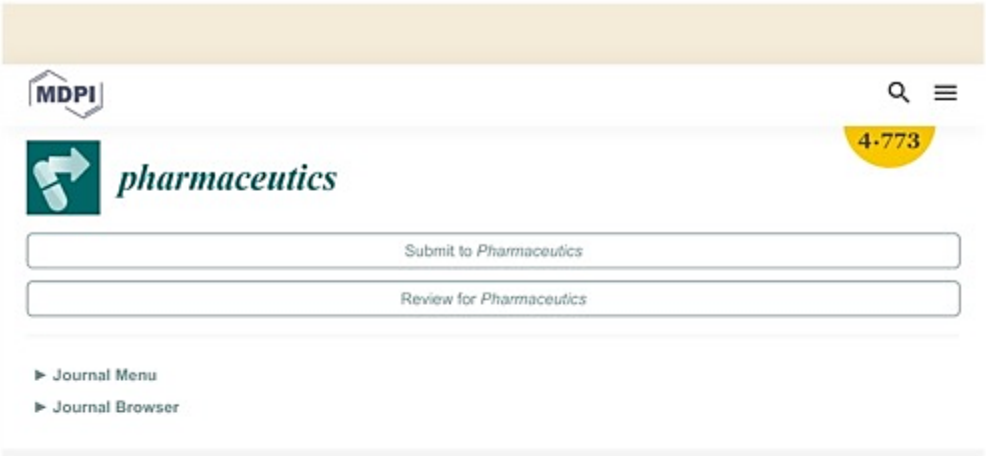
Dean:
Faculty of Health Sciences

Head:
Vivarium (PCDDP)

Appendix B

Pharmaceutics; an open access journal from MDPI

https://www.mdpi.com/journal/pharmaceutics/instructions



Instructions for Authors

Shortcuts

Submission Checklist

Please.

1. read the Aims & Scope to gain an overview and assess if your manuscript is suitable for this journal;
2. use the Microsoft Word template or LaTeX template to prepare your manuscript;
3. make sure that issues about publication ethics, research ethics, copyright, authorship, figure formats, data and references format have been appropriately considered;
4. ensure that all authors have approved the content of the submitted manuscript.

Manuscript Submission Overview

Types of Publications

Pharmaceutics has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Pharmaceutics* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on Supplementary Materials and references to unpublished data).

Manuscripts submitted to *Pharmaceutics* should neither been published before nor be under consideration for publication in another journal. The main article types are as follows:

- *Articles:* Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. Authors should not unnecessarily divide their work into several related manuscripts, although Short *Communications* of preliminary, but significant, results will be considered. Quality and impact of the study will be considered during peer review.
- *Reviews:* These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA guidelines.

Submission Process

Manuscripts for *Pharmaceutics* should be submitted online at susy.mdpi.com The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the

submission website. Once you have registered, click here to go to the submission form for *Pharmaceutics*. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors must use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the editorial office pharmaceutics@mdpi.com. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Pharmaceutics* Microsoft Word template file must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the *Pharmaceutics* LaTeX template files. You can now also use the online application writeLaTeX to submit articles directly to *Pharmaceutics*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery.
- *Supplementary files:* May be any format, but it is recommended that you use common, non-proprietary formats where possible (see below for further details).

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

Note for Authors Funded by the National Institutes of Health (NIH)

This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/).

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Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
  - Front matter: Title, Author list, Affiliations, Abstract, Keywords
  - Research manuscript sections: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
  - Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.
- **Review manuscripts** should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.
- **Graphical abstract:** Authors are encouraged to provide a graphical abstract as a self-explanatory image to appear alongside with the text abstract in the Table of Contents. Figures should be a high quality image in any common image format. Note that images displayed online will be up to 11 by 9 cm on screen and the figure should be clear at this size.
- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text, and in figure or table captions and used consistently thereafter.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible

- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and of Expression Data.
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
- **Research Data and supplementary materials:** Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about Supplementary Materials and Data Deposit for additional guidelines.
- **Preregistration:** Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.
- **Guidelines and standards:** MDPI follows standards and guidelines for certain types of research. See https://www.mdpi.com/editorial_process for further information.

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Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- **Conclusions:** This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

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Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.
- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.
  For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the CRediT taxonomy for the term explanation. For more background on CRediT, see here. **"Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully".**
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *Pharmaceutics* does not publish studies funded by the tobacco industry. Any projects funded by pharmaceutical or food industries must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study".
- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, ReferenceManager or Zotero to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for Endnote and Zotero are available.

References should be described as follows, depending on the type of work:

- Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range.

- Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

- Unpublished work, submitted work, personal communication:

4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript in preparation).
5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).
6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.

- Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

- Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

- Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year).

Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:

10. Title of Site. URL (archived on Day Month Year).

See the Reference List and Citations Guide for more detailed information.

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Preparing Figures, Schemes and Tables

- File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Pharmaceutics* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

[Return to top]

Supplementary Materials, Data Deposit and Software Source Code

Data Availability

In order to maintain the integrity, transparency and reproducibility of research records, authors must make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository or uploading as supplementary information to the publication. The name and version of all software used should be clearly indicated.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable, however we recommend that common, non-proprietary formats are used where possible.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, ...) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- *New microarray data* must be deposited either in the GEO or the ArrayExpress databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).

All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

[Return to top]

Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/), revised in 2013. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. At a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Editors reserve the right to reject any submission that does not meet these requirements.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

A written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A template permission form is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. Alternatively, you may provide a detailed justification of why informed consent is not necessary. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under

open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Pharmaceutics endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist.

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf.

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oriza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

Publication Ethics Statement

Pharmaceutics is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Pharmaceutics* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Pharmaceutics* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding publication of publishing addenda and corrections.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the Rights and Permissions page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Pharmaceutics* submissions.
      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

      If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, we may publish a correction or retract the paper.
    - **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.
      Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

      If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

  Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

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Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

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English Corrections

To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. Advice on some specific language points can be found here.

If you are not a native English speaker, we recommend that you have your manuscript professionally edited before submission or read by a native English-speaking colleague. This can be carried out by MDPI's English editing service. Professional editing will enable reviewers and future readers to more easily read and assess the content of submitted manuscripts. All accepted manuscripts undergo language editing, however **an additional fee will be charged** to authors if very extensive English corrections must be made by the Editorial Office: pricing is according to the service here.

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Preprints and Conference Papers

Pharmaceutics accepts articles that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates *Preprints*, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* instructions for authors for further information.

Expanded and high quality conference papers can be considered as articles if they fulfil the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Pharmaceutics* does not publish pilot studies or studies with inadequate statistical power.

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Authorship

MDPI follows the International Committee of Medical Journal Editors (ICMJE) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or revising it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements. More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE).

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

Reviewers Recommendation

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflict of interests before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

Editors and Journal Staff as Authors

Editorial independence is extremely important and MDPI does not interfere with editorial decisions.

Editorial staff or editors shall not be involved in the processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other editorial board members who do not have conflict of interests with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

Conflict of Interests

According to The International Committee of Medical Journal Editors, "Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors' access to all of the study's data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose."

Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. If there is no role, please state "The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".

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Editorial Procedures and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether they are properly prepared and whether they follow the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals' *Editor-in-Chief* or *Associate Editors* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the *Editor-in-Chief*.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, however once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this

opportunity as proof of the rigorous process employed in publishing their research. To guarantee an impartial refereeing the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. Usually, only one round of major revisions is allowed. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments.
- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the Editor-in-Chief, Associate Editor, or Editorial Board member. The academic Editor being consulted will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage is final and cannot be reversed.

In the case of a special issue, the *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the *Editor-in-Chief* who will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

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Clinical Trials Registration

Registration

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register or and to cite a reference to the registration in the Methods section. Suitable databases include clinicaltrials.gov, the EU Clinical Trials Register and those listed by the World Health Organisation International Clinical Trials Registry Platform.

CONSORT Statement

Pharmaceutics requires a completed CONSORT 2010 checklist and flow diagram as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (http://www.consort-statement.org) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist. Meeting these basic reporting requirements will greatly improve the value of your trial report and may enhance its chances for eventual publication.

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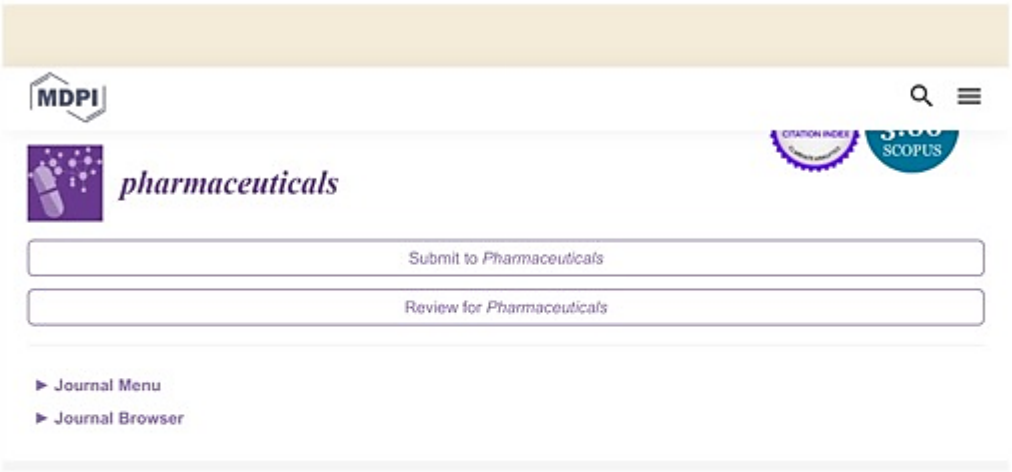
Appendix C

Pharmaceuticals; an open access journal from MDPI

https://www.mdpi.com/journal/pharmaceuticals/instructions



Instructions for Authors

Shortcuts

Submission Checklist

Please.

1. read the Aims & Scope to gain an overview and assess if your manuscript is suitable for this journal;
2. use the Microsoft Word template or LaTeX template to prepare your manuscript;
3. make sure that issues about publication ethics, research ethics, copyright, authorship, figure formats, data and references format have been appropriately considered;
4. ensure that all authors have approved the content of the submitted manuscript.

Manuscript Submission Overview

Types of Publications

Pharmaceuticals has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Pharmaceuticals* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on Supplementary Materials and references to unpublished data).

Manuscripts submitted to *Pharmaceuticals* should neither been published before nor be under consideration for publication in another journal. The main article types are as follows:

- *Articles:* Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. Authors should not unnecessarily divide their work into several related manuscripts, although Short *Communications* of preliminary, but significant, results will be considered. Quality and impact of the study will be considered during peer review.
- *Reviews:* These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA guidelines.

Submission Process

Manuscripts for *Pharmaceuticals* should be submitted online at susy.mdpi.com The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the criteria to qualify for authorship) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the

submission website. Once you have registered, click here to go to the submission form for *Pharmaceuticals*. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

Accepted File Formats

Authors must use the Microsoft Word template or LaTeX template to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the editorial office pharmaceuticals@mdpi.com. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the *Pharmaceuticals* Microsoft Word template file must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.
- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the *Pharmaceuticals* LaTeX template files. You can now also use the online application writeLaTeX to submit articles directly to *Pharmaceuticals*. The MDPI LaTeX template file should be selected from the writeLaTeX template gallery.
- *Supplementary files:* May be any format, but it is recommended that you use common, non-proprietary formats where possible (see below for further details).

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

Note for Authors Funded by the National Institutes of Health (NIH)

This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, http://nihms.nih.gov/).

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Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
  - Front matter: Title, Author list, Affiliations, Abstract, Keywords
  - Research manuscript sections: Introduction, Results, Discussion, Materials and Methods, Conclusions (optional).
  - Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.
- **Review manuscripts** should comprise the front matter, literature review sections and the back matter. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.
- **Case reports** should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
- **Graphical abstract:** Authors are encouraged to provide a graphical abstract as a self-explanatory image to appear alongside with the text abstract in the Table of Contents. Figures should be a high quality image in any common image format. Note that images displayed online will be up to 11 by 9 cm on screen and the figure should be clear at this size.

- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text, and in figure or table captions and used consistently thereafter.
- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible
- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on Deposition of Sequences and of Expression Data.
- **Equations:** If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.
- **Research Data and supplementary materials:** Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about Supplementary Materials and Data Deposit for additional guidelines.
- **Preregistration:** Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript.
- **Guidelines and standards:** MDPI follows standards and guidelines for certain types of research. See https://www.mdpi.com/editorial_process for further information.

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Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the criteria to qualify for authorship.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and

appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any pre-registration codes.

- **Conclusions:** This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.
- **Patents:** This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

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Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to FundRef if the manuscript is finally published.
- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.
  For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.; Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.; Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.; Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the CRediT taxonomy for the term explanation. For more background on CRediT, see here. **"Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully".**
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *Pharmaceuticals* does not publish studies funded by the tobacco industry. Any projects funded by pharmaceutical or food industries must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study".
- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, ReferenceManager or Zotero to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for Endnote and Zotero are available.

References should be described as follows, depending on the type of work:

- Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range.

- Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

- Unpublished work, submitted work, personal communication:

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5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).
6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.

- Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

- Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

- Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year).

Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:
10. Title of Site. URL (archived on Day Month Year).

See the Reference List and Citations Guide for more detailed information.

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Preparing Figures, Schemes and Tables

- File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Pharmaceuticals* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.
- All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).
- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

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Supplementary Materials, Data Deposit and Software Source Code

Data Availability

In order to maintain the integrity, transparency and reproducibility of research records, authors must make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository or uploading as supplementary information to the publication. The name and version of all software used should be clearly indicated.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable, however we recommend that common, non-proprietary formats are used where possible.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal Data also accepts submissions of data set papers.

Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited in one of the following databases: GenBank, EMBL, or DDBJ. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis, ...) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- *New microarray data* must be deposited either in the GEO or the ArrayExpress databases.The "Minimal Information About a Microarray Experiment" (MIAME) guidelines published by the Microarray Gene Expression Data Society must be followed.
- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).

All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/), revised in 2013. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. At a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Editors reserve the right to reject any submission that does not meet these requirements.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

A written informed consent for publication must be obtained from participating patients who can be identified (including by the patients themselves). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not

mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A template permission form is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. Alternatively, you may provide a detailed justification of why informed consent is not necessary. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Pharmaceuticals endorses the ARRIVE guidelines (www.nc3rs.org.uk/ARRIVE) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at www.nc3rs.org.uk/ARRIVEchecklist.

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Available online: http://www.official-documents.gov.uk/document/hc8889/hc01/0107/0107.pdf.

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The MLH1^{+} cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oriza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

Publication Ethics Statement

Pharmaceuticals is a member of the Committee on Publication Ethics (COPE). We fully adhere to its Code of Conduct and to its Best Practice Guidelines.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Pharmaceuticals* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Pharmaceuticals* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our policy regarding publication of publishing addenda and corrections.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the Rights and Permissions page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Pharmaceuticals* submissions.
      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

      If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, we may publish a correction or retract the paper.
    - **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.
      Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

      If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

  Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are

expected to comply with the best ethical publication practices when publishing with MDPI.

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Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

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English Corrections

To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. Advice on some specific language points can be found here.

If you are not a native English speaker, we recommend that you have your manuscript professionally edited before submission or read by a native English-speaking colleague. This can be carried out by MDPI's English editing service. Professional editing will enable reviewers and future readers to more easily read and assess the content of submitted manuscripts. All accepted manuscripts undergo language editing, however **an additional fee will be charged** to authors if very extensive English corrections must be made by the Editorial Office: pricing is according to the service here.

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Preprints and Conference Papers

Pharmaceuticals accepts articles that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates *Preprints*, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* instructions for authors for further information.

Expanded and high quality conference papers can be considered as articles if they fulfil the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Pharmaceuticals* does not publish pilot studies or studies with inadequate statistical power.

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Authorship

MDPI follows the International Committee of Medical Journal Editors (ICMJE) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or revising it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements. More detailed guidance on authorship is given by the International Council of Medical Journal Editors (ICMJE).

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

Reviewers Recommendation

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflict of interests before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

Editors and Journal Staff as Authors

Editorial independence is extremely important and MDPI does not interfere with editorial decisions.

Editorial staff or editors shall not be involved in the processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other editorial board members who do not have conflict of interests with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

Conflict of Interests

According to The International Committee of Medical Journal Editors, "Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors' access to all of the study's data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose."

Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. If there is no role, please state "The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".

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Editorial Procedures and Peer-Review

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether they are properly prepared and whether they follow the ethical policies of the journal, including those for human and animal experimentation. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals' *Editor-in-Chief* or *Associate Editors* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the *Editor-in-Chief*.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board members* and Guest Editors of the journal. Potential reviewers suggested by the

authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, however once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee an impartial refereeing the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. Usually, only one round of major revisions is allowed. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments.
- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the Editor-in-Chief, Associate Editor, or Editorial Board member. The academic Editor being consulted will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage is final and cannot be reversed.

In the case of a special issue, the *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the *Editor-in-Chief* who will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the www.mdpi.com website.

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Clinical Trials Registration

Registration

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register or and to cite a reference to the registration in the Methods section. Suitable databases include clinicaltrials.gov, the EU Clinical Trials Register and those listed by the World Health Organisation International Clinical Trials Registry Platform.

Appendix D

Certificate of analysis

Certificate of analysis for Ginger extract (10% Gingerols)

XI'AN B-THRIVING

B-Thriving

XI'AN B-THRIVING I/E CO.,LTD

CERTIFICATE OF ANALYSIS

Product Name	Ginger Extract	**Country of Origin**	China
Latin name	*Zingiber officinale*	**Ingredient**	Gingerols
Plant part	Root	**Manufacture Date**	2018/05/05
Batch Number	GR20180505	**Expire Date**	2020/05/04
Quantity	600kgs	**Issue Date**	2020/05/08

ANALYSIS	SPECIFICATION	RESULTS	TEST METHOD
Identification	Positive	Complies	HPLC, IR,TLC
Appearance	Light Yellow Fine Powder	Complies	Visual
Particle Size	98% pass 80 mesh	Complies	80 Mesh Screen
Odor	Characteristic	Complies	Organoleptic
Loss on Drying	≤6%	3.55%	5g/105℃/ 2 hrs
Assay	**>10% Gingerols**	**10.10%**	**HPLC**
Heavy Metal	<10ppm	Complies	Atomic Absorption
Pb	<2ppm	Complies	Atomic Absorption
As	<2ppm	Complies	Atomic Absorption
Residual solvent	Comply with EP8.0	Complies	EP8.0
Microbiology			
Total Plate Count	≤3000cfu/g	Complies	CP2015
Yeast & Mold	≤300cfu/g	Complies	CP2015
E.Coli	Negative	Complies	CP2015
Salmonella	Negative	Complies	CP2015

Conclusion Conform with specification .

Storage Store in cool & dry place. Do not Frozen . Keep away from strong light and heat.
After opening please use it immediately, if not please pack it in vaccum .

Shelf life 2 years when properly stored

MR.YUAN	MR.WANG	MISS.LI
Quality Assurance Officer	**Corrector**	**Analyst**

Certificate of analysis for curcumin standard

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON, M3J2J8, CANADA
Tel: (416) 665-9696, Fax: (416) 665-4439
Email:orders@trc-canada.com Website: www.trc-canada.com

1. Identification

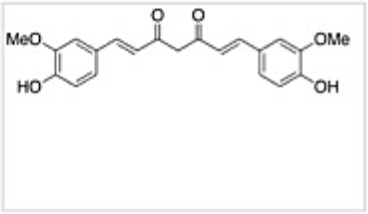
CAS Number: 458-37-7

Cat. Number: C838500

Synonym: (1E,6E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; (E,E)-1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; C Yellow 15; C.I. 75300; C.I. Natural Yellow 3; Curcuma; Curcumin I; Curcumine; Diferuloylmethane; Haidr; Halad; Haldar; Ukon;

Product: Curcumin

Structure:



Molecular Formula: C21H20O6

Molecular weight: 368.38

Source of Product: N/A

2. Analytical Information

Lot Number:	2-JTN-138-1	**Boiling Point:**	N/A
Melting Point:	184.0 - 185.5°C	**Atmosphere:**	Air
Apearance:	Orange Solid	**Solubility:**	DMSO (Slightly), Methanol (Slightly)
Method for Determining Identity:	1H NMR (DMSO-d6) and MS	**Stability:**	Not Determined
Purity:	98%	**Long Term Storage Condition:**	Refrigerator
Additional Info.:	TLC Conditions: SiO2; Dichloromethane : Methanol = 9 : 1; Visualized with UV, KMnO4 and Naked Eye; Single Spot, Rf = 0.75.1H NMR and MS conform to structure.		

Purity is based on the analytical results of the tests performed. NMR and TLC may have an accuracy of +/- 2%. Isotopic purity is based on mass distribution observed.

Philip Chan, Head of Quality Assurance

QC Test Date: 23-Sep-14

Retest Date: 20-Sep-21

Certificate of analysis for [6]-shogaol standard

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON, M3J2J8, CANADA
Tel: (416) 665-9696, Fax: (416) 665-4439
Email:orders@trc-canada.com Website: www.trc-canada.com

1. Identification

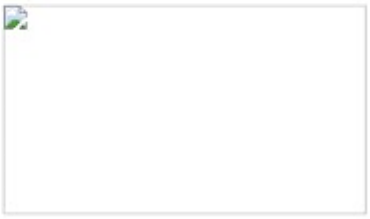
CAS Number: 555-66-8

Cat. Number: S357800

Product: [6]-Shogaol

Synonym: 1-(4-Hydroxy-3-methoxyphenyl)-4-decen-3-one; Shogaol;

Structure:



Molecular Formula: C17H24O3

Molecular weight: 276.37

Source of Product: Synthetic

2. Analytical Information

Lot Number:	8-VKU-17-1	**Boiling Point:**	N/A
Melting Point:	N/A	**Atmosphere:**	Air
Apearance:	Pale Yellow Oil	**Solubility:**	Chloroform (Slightly), Ethyl Acetate (Slightly)
Method for Determining Identity:	1H NMR (CDCl3) and MS	**Stability:**	Not Determined
Purity:	96%	**Long Term Storage Condition:**	Refrigerator
Additional Info.:	TLC Conditions: SiO2; Hexane : Ethyl Acetate = 8 : 2; Visualized with UV and KMnO4; Single Spot, Rf = 0.30.;1H NMR and MS conform to structure.		

Purity is based on the analytical results of the tests performed. NMR and TLC may have an accuracy of +/- 2%. Isotopic purity is based on mass distribution observed.

Philip Chan, Head of Quality Assurance

QC Test Date: 29-Jun-16

Retest Date: 27-Jun-20

Appendix E

Proof of conference participation and abstracts

Published abstract at the first Drug Safety Africa conference in 2018, Potchestroom, South Africa (https://www.sciencedirect.com/science/article/pii/S1056871919303260)

Journal of Pharmacological and Toxicological Methods 98 (2019) 106608

Contents lists available at SciVerse ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Drug Safety Africa Meeting Abstracts

Aqueous extracts of the selected plants will be administered to the animals by oral gavage and the control group will receive solvent blank. The administered doses will exceed the therapeutic range just enough to produce moderate adverse effect. Telemetered rats will be used to assess the effects the selected herbal medicines will have on the core battery such as the cardiovascular, respiratory and CNS systems. All the experiments will follow the ARRIVE guidelines. Behaviours and clinical signals observed in the Irwin test, as well as data from telemetered rats will be used to identify the principle effects of all the test substances. South Africa is at the verge of incorporating traditional medicine into the mainstream healthcare system, robust safety pharmacology studies will ensure the safe of use of herbal medicine.

Yand, J.-Y. & Wang, L.-H. (2010). Chapter 7: Safety pharmacology and toxicity study of herbal medicine. Liu WJH (ed.) Traditional Herbal Medicine Research Methods: Identification, Analysis and Pharmaceutical and Clinical Studies.

Mander, M. et al. (2007). Chapter 13: Economics of the Traditional Medicine Trade in South Africa. Health Systems Trust, Durban, South Africa. [online] URL: http://www.hst.org.za/uploads/files/chap13_07.pdf.

doi:10.1016/j.vascn.2019.07.002

003

Meeting abstracts

Therapeutic use and pharmacological safety of herbal medicines

Bisrat Sissay Bekele, Wihan Pheiffer, Anne F. Grobler
DST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom, South Africa

In general, >80% of the world's population relies on traditional medicine for their primary healthcare [1]. The use of complementary herbal medicine provides an alternative option to modern allopathic treatment for treating or preventing numerous diseases including cancer. According to the literature [2], a combination of two or more active biological compounds obtained from medicinal plants has a more profound therapeutic action than a single agent treatment. However, there is a paucity of information regarding the translation of phytochemicals to a potential phytopharmaceuticals. In addition, poor biological uptake coupled with inadequate safety and efficacy data hamper the clinical utility of most herbal medicines. This underscores the need for a thorough pragmatic study on the pharmacological efficacy and safety of herbal medicines including assessing the cardiovascular, neurological, hematological, respiratory, liver and kidney toxicities. Table 1 shows some examples of biologically active compounds derived from different medicinal plants and their toxicity. In the current study, the effective dose combination of three plant bioactive compounds, will be assessed in an *in vitro* assay against the human alveolar adenocarcinoma (A549) cell line. The biological uptake of the phytochemical combination will be enhanced using a novel Pheroid® drug delivery system. The *in vivo* anticancer activity of combined phytochemical Pheroid® formulation [3], as well as their impact on the peripheral blood parameters, and on liver and kidney functions will be evaluated for possible toxicity. See Table 1 for therapeutic activity and toxicity of herbal medicines [1].

Plant species	Active compound	Therapeutic use	Clinical findings
Aristolochia sp.	Aristolochic Acid (AA)	Arthritis, rheumatism, hepatitis	Nephrotoxicity, upper tract urothelial carcinoma
Gingko biloba L.	Ginkgolides	Alzheimer's dementia	Hepatotoxicity, subcutaneous hematomas and intracranial hemorrhage
Ephedra sinica	Ephedrine alkaloids	Respiratory conditions	Cardiotoxicity, neurotoxicity, hepatotoxicity

It is anticipated that Pheroid® formulated phytochemical combinations will exhibit profound cytotoxic activity against the A549 lung cancer cells with minimal impact on the homeostasis of the body. Although herbal medicines are widely used as promising therapeutic agents, it is imperative to determine their efficacy, and assess potential adverse reactions for safe and rational use.

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004

Meeting abstracts

Elimination of host and dead pathogenic organism DNA for improved diagnostic outcome

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Molecular-based diagnostic techniques used to detect bacterial infections in clinical samples are often unable to discriminate between living and dead bacteria and extracellular DNA. This presents a major problem, leading to reduced specificity and sometimes in a failure to properly monitor the effectiveness of antimicrobial therapy. An additional problem faced by molecular diagnostic assays as well as next generations sequencing (NGS)-based diagnostic approaches is the presence of high quantities of contaminating host DNA, which often obscures the presence of and changes in microbial populations due to the majority of sequencing reads aligning to host DNA. Here we describe the effect of two buffers used to achieve live/dead differentiation and the removal of contaminating host cell DNA. We evaluated these buffers in two different scenarios; one focusing on a previously validated TB diagnostic and one following a NGS-based approach. For the diagnostic method, 101 fresh sputum samples were treated with the live/dead differentiation buffer before lysis and molecular diagnosis by quantitative polymerase chain reaction (q-PCR). For future NGS applications, samples will first be treated with a differential lysis buffer to selectively lyse any host cells present. This will be followed by treatment with a DNA removal buffer and

Certificate of attendance for the annual safety pharmacology society (SPS) conference in 2019, Barcelona, Spain

The Safety Pharmacology Society

Presents

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SPS 19th Annual Meeting

September 23–September 26, 2019

Barcelona, Spain

Michael Engwall, DVM, PhD, DSP

Safety Pharmacology Society, President

Accepted abstract for poster presentation

The Safety and Efficacy of Meriva®and Ginger Extract Combination in Pheroid® Against Lung Cancer

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Introduction

Lung cancer is the leading cause of cancer related deaths globally[1,2]. Cisplatin is frequently used in the treatment of solid tumours, including lung cancer, despite its toxicity[3]. Turmeric and ginger extracts are comprised of a mixture of biologically active compounds—such as curcumin and [6]-shogaol—with established chemopreventive, anti-inflammatory and antioxidant properties[4]. Human clinical safety data shows that turmeric and ginger extracts exhibit low intrinsic toxicity, even at higher doses[4,5]. Pheroid® is a colloidal drug delivery system, comprised of plant based essential fatty acids (EFAs) dispersed phase and a nitrous oxide saturated water continuous phase. The present *in vitro* safety study investigated the chemopreventive activity of combined Meriva® (a curcumin phytosome; M) and ginger extract (G) in Pheroid® against the A549 human lung cancer cell line as potential complementary medicine to be used in combination with conventional therapy.

Materials and Methods

Meriva® and GE were formulated in Pheroid® at a 1:1.43 combination ratio. The concentration of curcumin in Meriva® and [6]-shogaol in ginger extract were quantified using LC-MS/MS. Cell viability was studied using MTT and neutral red uptake assays. Comparative safety of treatment between the Pheroid® formulated phytochemical combination and cisplatin was measured in terms of induction of the following oxidative stress markers: reactive oxygen species (ROS), superoxide dismutase (SOD) and catalase (CAT). Cellular uptake of compounds was determined by using confocal laser scanning microscopy.

Results and Discussion

Individual and combination of phytochemicals in free form (FF) and in Pheroid® formulation (PF) significantly decreased the metabolic activity of A549 cells (Fig.1A). However, only their combination in Pheroid® (PFC) showed a significant decrease in cell viability compared to the control. The decrease, is comparable with cisplatin activity (Fig. 1B). In the oxidative stress assay, only the combination of phytochemicals in Pheroid® had a non-significant change in intracellular reactive oxygen species compared to control (Fig. 2), while overall non-significant changes in antioxidant enzymes for all treatments were noted (Fig. 3). The content of curcumin in Meriva® and [6]-shogaol in ginger extract was 400 mg/g and 10.61 mg/g, respectively.

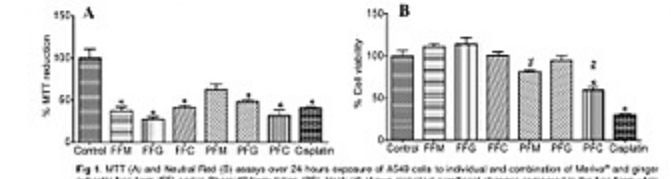

Fig 1. MTT (A) and Neutral Red (B) assays over 24 hours exposure of A549 cells to individual and combination of Meriva® and ginger extractin free form (FF) and in Pheroid® formulation (PF). Hash (#) shows statistical significant changes compared to the free form while asterisks (*) shows statistical significant changes compared to control.

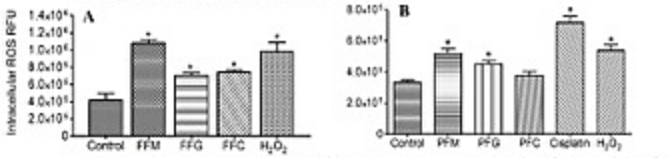

Fig 2. Assessing the reactive oxygen species level in A549 cells after treatment with free form (A) and Pheroid® formulated (B) Meriva® and ginger extract over 24 hours.

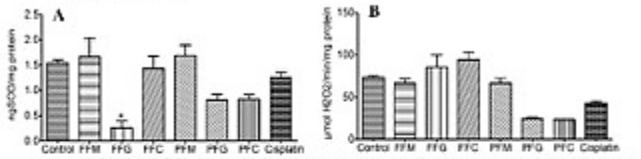

Fig 3. Superoxide dismutase (SOD) (A) and catalase (CAT) (B) levels following treatment with individual and combination of phytochemicals in Pheroid® and without Pheroid®.

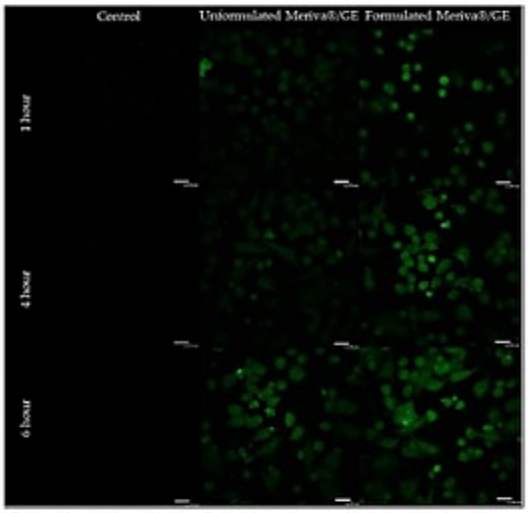

Figure 4. Cellular uptake of free and Pheroid® formulated phytochemical combinations at different time points. Images taken using confocal microscopy at 60x magnification. Curcumin autofluorescence in the green emission spectra at 530±30nm wavelength.

Pheroid® significantly enhanced cellular uptake (Fig. 4) and anticancer efficacy of the Meriva® and GE combination. In the oxidative stress assay, it was evident that the phytochemical combination in Pheroid® did not induce intracellular reactive oxygen species compared to control. In contrast, individual actives in their free forms and in Pheroid®, and cisplatin exhibited pro-oxidant activity. This significant increase in intracellular reactive oxygen species with non-significant changes in antioxidant enzymes will consequently result in oxidative stress. The combination of Meriva® and GE in the Pheroid® formulation proved to be safe (no oxidative stress) despite higher concentration of the actives present in the cells.

Conclusions

Although cisplatin is an effective antineoplastic agent, its side effects stem from non-selective induction of oxidative stress to both cancer and normal cells. The Pheroid® formulated Meriva® and ginger extract combination demonstrated a comparable anticancer activity with cisplatin, without induction of oxidative stress, but still reducing cancer cell viability. It can therefore be used as a safe and effective adjuvant therapy in lung cancer.

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Acknowledgments

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Academy of pharmaceutical society South Africa conference held in Centurion, South Africa, 2019

Author workshop certificate of attendance

Researcher Academy On Campus

Certificate of Attendance

ELSEVIER

This certifies that

Bisrat Bekele

has attended the following

Author Workshop

at Academy of Pharmaceutical Sciences South Africa Conference 2019,
on Wednesday 09 October, 2019

Presented by Anne Marie Pordon, Publisher

Suzanne DeBell
Managing Director, Education
Reference & Continuity Books

Philippe Terheggen
Managing Director, Science,
Technology & Medical Journals

Accepted abstract for oral presentation

NWU® | PCDDP DST/NWU Preclinical Drug Development Platform | science & technology Department: Science and Technology REPUBLIC OF SOUTH AFRICA

The *in vitro* anticancer activity of free and Pheroid® formulated Meriva® and ginger against lung cancer

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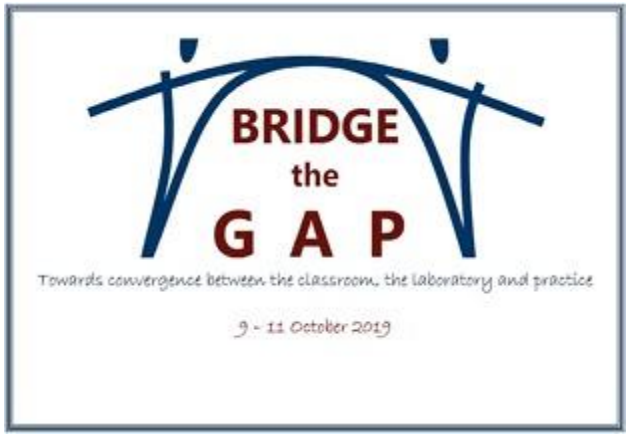

Draft programme – subject to change

Wednesday, 9 October 2019		
09:00 – 10:00	**Registration**	
10:00 – 10:30	**Welcoming address by PSSA President**	
10:30 – 13:00	**Oral session**	**Session chair:** Gareth Kilian
10:30 – 11:15	**David Katerere**	**Plenary lecture:** Substandard and falsified medicines in Africa – what role for pharmaceutical scientists?
11:15 – 11:30	Bisrat Bekele	Comparing the chemopreventive activity of herbal extracts as free-form combinations versus in formulation: an *in vitro* study
11:30 – 11:45	Sarah D'Souza	Design and characterization of biomimetic nanoparticles for the treatment of tuberculosis infected macrophages
11:45 – 12:00	Rovhuya Farisani	Factors which contribute to the wastage of ^{18}fluorine-flourodeoxyglucose and interruption of services at the positron emission tomography/computed tomography centre at Dr George Mukhari Academic Hospital, Gauteng Province
12:00 – 12:15	Tumelo Kgoe	Establishment and characterization of a murine herpes simplex virus type 2 (HSV-2) model for the evaluation of novel microbicides
12:15 – 12:30	Siyabonga Melamane	Formulation optimization of smart thermosetting lamotrigine loaded hydrogels using Box Behken design and artificial neural networks
12:30 – 12:45	Thobile Ngqaneka	The impact of niacin on PCSK9 levels in Vervet monkeys
12:45 – 13:00	Nathaniel Muzamhindo	Cost and safety implications of individual versus bulk ^{18}fluorine-fluorodeoxyglucose doses in the positron emission tomography radiopharmacy at Dr George Mukhari Academic Hospital, Gauteng Province
13:00 – 14:00	Lunch	
14:00 – 17:00		**Session chair:** Alvaro Viljoen
14:00 – 15:30	**Anne-Marie Pordon**	**Workshop 1: Writing a publication paper**
15:30 – 16:00	Coffee break & Poster session	
16:00 – 16:45	**Beverley Summers**	**Plenary lecture: Radiopharmacy**
17:00 – 18:00	**AGM**	
18:00 – 20:00	**Welcome cocktail**	