EVALUATION OF *RICINUS COMMUNIS* SEMI-PURIFIED EXTRACTS' POTENTIAL AS ANTI-METASTATIC AGENTS USING METASTATIC BREAST (MCF-7) CANCER CELLS

by

RIXILE FOREVER MABASA

[redacted]

Research Dissertation

Submitted in fulfilment of the requirements of the degree of

MASTER OF SCIENCE

in

BIOCHEMISTRY

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Molecular and Life Sciences)

at the

UNIVERSITY OF LIMPOPO

SUPERVISOR: Dr VG Mbazima

CO-SUPERVISOR: Dr TM Matsebatlela

2017

DECLARATION

I declare that the study entitled **EVALUATION OF *RICINUS COMMUNIS* SEMI-PURIFIED EXTRACTS' POTENTIAL AS ANTI-METASTATIC AGENTS USING METASTATIC BREAST (MCF-7) CANCER CELLS** is my own work. This report is being submitted for the degree of Master of Science in Biochemistry at the University of Limpopo. This report has not been submitted to any other University and I further declare that all sources quoted are indicated and acknowledged by means of a comprehensive list of references.

Rixile Forever Mabasa (Mr) ....

Full names **Signature**

[redacted] ..... February 2016 .....

Student number **Date**

DEDICATION

I wish to dedicate this work to my spirit man and to the strength that drives my aspirations in accordance to the divine purpose of God for my life.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to everybody who made a significant contribution to the success of this project.

- I begin by thanks giving and praise to the eternal, only wise God, Him who founded the earth and its fullness by His Word.
- I appreciate my supervisor, Dr Vusi Mbazima, for driving this project towards its intended end and for your patience and persistence even in my endless delays. Receive my prayers for many breakthroughs in your career as a well-grounded scientist.
- Prof Phineas Mokgotho, you have always kept me very close to you like a mentor would and I've learned many administrative skills from you because you had the confidence to fraction some of your responsibilities to my hands.
- Dr Moss Matsebatlela and Mr Raymond Makola for committing yourselves to assist me with operations of the fluorescence microscope and of course making the biscuits available when I needed them for coffee.
- Dr Victor Bagla, thank you sir for your assistance with the solvent-solvent fractionation procedure.
- My humble regards to the entire BMBT department, you have created numerous opportunities for me to gather market-related skills and I am now an asset of value to many corporate sets.
- To my family, friends and the mysterious "superwoman", I am very grateful for your faith in me. You always believe in the potential I have to do better even when I didn't.
- The National Research Foundation (NRF), for funding my MSc studies for the period of two years.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABREVIATIONS

ATCC	American Type Culture Collection
BEA	Benzene: ethanol: ammonium hydroxide
BM	Basement membrane
But	*n*-Butanol
CEF	Chloroform: ethyl acetate: formic acid
CO_2	Carbon dioxide
CXCL	Chemokine (C-X-C motif) ligand
DCFH	2,7-Dichlorofluorescin
DCFH-DA	2,7-Dichlorofluorescin diacetate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
EMW	Ethyl acetate: methanol: water
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
H_2O_2	Hydogen peroxide
Hex	*n*-Hexane
MMPs	Matrix metalloproteinases
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NO	Nitric oxide
^oC	Degrees Celsius
PBS	Phosphate-buffered saline
PIGF	Placental growth factor
PSN	Penicillin, streptomycin and neomycin
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
TIMPs	Tissue inhibitor of metalloproteinases
TLC	Thin layer chromatography
TSP	Thrombospondin
uPA	Urokinase-type plasminogen activator
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

ABSTRACT

The malignancy of cancer cells is responsible for the high death rate in patients diagnosed with metastatic cancers. Medicinal plants represent a reservoir of bioactive compounds that can be useful in the management of cancer. In this study, semi-purified extracts of *Ricinus communis* leaves were evaluated for their potential to serve as an anti-metastatic agent by using *in vitro* assays that tested their effects on a number of processes related to metastasis. The exhaustive extraction procedure was employed to generate the crude acetone extracts of *R. communis* leaves. The crude extracts were then subjected to solvent-solvent fractionation to yield six semi-purified extracts (*n*-butanol, Chloroform, Ethyl-acetate, *n*-hexane, Methanol + H_2O and H_2O). Thin layer chromatography (TLC) was done to determine the phytochemical composition of the semi-purified extracts as well as their antioxidant potential. Non-polar fractions showed to have a diverse mixture of phytochemicals with, however, very limited antioxidant activity. On the other hand, polar fractions showed to have phytochemical compounds with strong antioxidant potential. TLC guided the selection of *n*-hexane and *n*-butanol as fractions of great phytochemical diversity and antioxidant activity, respectively. The selected fractions were then assessed for their effect on the viability of normal fibroblasts (BUD-8) and breast (MCF-7) cancer cells using the MTT assay. The *n*-butanol fraction was shown to significantly decrease the viability of BUD-8 at concentrations above 200 µg/ml. The *n*-hexane fraction, however, showed to significantly affect the viability of the cells even at lower concentrations. On the positive side, the reduced viability of BUD-8 cells after exposure to both fractions was followed by an increase in cell proliferation after 24 hours suggesting that the extracts exhibited cytostatic rather than cytotoxic effects. Treatment of MCF-7 cells with different concentrations (100-500 µg/ml) of the fractions showed a dose- and time-dependant decrease in cell viability. Hoechst stain also confirmed the non-toxicity of the fractions to MCF-7 cells at 100 and 200 µg/ml. The fractions also showed to possess free radical scavenging activities by reducing the amount of intracellular ROS as demonstrated by the DCFH-DA fluorescent assay. Fluorescence intensity was strongly reduced in cells treated with the fractions and elevated in H_2O_2-treated and untreated MCF-7 cells. The effect of the fractions on metastasis was assessed by determining their effects on MCF-7 cell migration, attachment and invasiveness using wound healing assay, adhesion assay and Boyden chamber invasion assay, respectively. The wound healing assay showed the fractions to have strong inhibitory activities on the migration of MCF-7 cells. The

ability of the cells to attach to cell culture treated plates was also greatly reduced in cells treated with the fractions. The *n*-butanol fraction was demonstrated to exhibit a time- and dose-dependent inhibition on MCF-7 cell invasion by reducing the cells' capability to penetrate through the matrigel matrix to the bottom of the porous membrane. Gelatin-zymography was done to assess the effect of the *n*-butanol fraction on activity of MMP-2 and MMP-9. The fraction showed to completely inhibit the gelatinolytic activity of MMP-2 and no band corresponding to the molecular weight of MMP-9 was observed, suggesting that MCF-7 cells produce undetectable levels of MMP-9. The *n*-butanol fraction further showed to down-regulate the expression of a range of proteins such as MMP-9, uPA, VEGF, TGF-β1 implicated in metastasis and angiogenesis determined using the human angiogenesis antibody array kit. This study demonstrated that the fractions of *R. communis* extracts have the ability to inhibit major processes of the metastatic cascade by down-regulating the expression of proteins relevant to metastasis. Thus, the fractions can be considered as potential anti-metastatic agents functional in the regulation and/or treatment of malignant of cancers.

CHAPTER 1

1 Introduction

The burden of cancer and cancer-related diseases continues to rise, particularly in African countries, due to inefficient prevention strategies, poor prognosis following late diagnosis and scarcity of cancer treatments (Morhason-Bello *et al.,* 2013). An 85% increase in the occurrence of cancer in Africa is expected by 2030 (Bray *et al.,* 2012). The South African National Cancer Registry identifies breast cancer as the predominant cancer affecting women in South Africa as well as a common cause of mortality in women worldwide (Siegel *et al*., 2012). Cancer is defined as a neoplastic disease distinguished by the development of malignant tumour cells which have the ability to resist growth inhibition, proliferate indefinitely, escape programmed cell death (apoptosis), induce angiogenesis and subsequent tissue invasion and metastasis (Hanahan and Weinberg, 2011). There are two types of tumours, namely; benign and malignant. Benign tumours are usually more differentiated and grow slower than their malignant counterparts. These tumours are not considered cancerous or life-threatening since they lack the ability to spread and invade distant parts of the body and can be easily removed surgically (Lee *et al.,* 2011). On the other hand, malignant tumours (commonly called cancer) grow very rapidly and have the ability to spread and invade distant parts of the body such as the lungs, liver and brain. This process of spreading and invasion of distant body tissues by cancer cells is known as metastasis and has serious health consequences (Rendon *et al.*, 2007). Theveneau *et al.* (2013) reported that most cancer patients die from development of secondary tumours originating from malignant tumour cells in the primary site. Most of the currently used cancer treatment strategies are effective against cancer only when the tumour cells are confined to their primary site.

Most cancer drugs have been developed to specifically kill and/or reduce cancer cell division with a few having the ability to inhibit the movement of cancer cells (Gawecka *et al.,* 2012). As such, a number of cancer deaths are associated with the spread of malignant tumour cells rather than the primary tumour itself. Due to the spread of cancer cells to adjacent tissues and other parts of the body, disseminated tumour cells have shown resistance to existing therapeutic drugs (Hunter *et al.,* 2008) and as a consequence, the management of malignant tumours is a huge challenge.

Approximately 90% of all cancer-related deaths are attributed to the metastatic nature of malignant tumour cells (Spano and Zollo, 2012). Despite the high probability of positive colonisation and growth of cancer cells in the secondary site, metastasis is not a completely efficient process. An unsuccessful progression and regulation of a single step of the cascade can result in failure of primary tumour cells to migrate invade, proliferate and survive at the distant site (Hunter *et al.,* 2008). Shutting down the progression of any process of the metastatic cascade is one of the research prospect areas currently investigated as a way of preventing the development of metastasis and/or its subsequent health effects.

It is well known that in ancient times, people relied solely on medicinal plants as remedies to cure different diseases in the verge to increase their lifespan (Ingale and Hivral, 2010). Recently, the value of medicinal plants as promising sources of active drugs has gained more interest in modern system of medicine, mainly because of their availability, cost efficiency and improved bioactivity with fewer or no side effects. Most of the plants currently examined for their therapeutic potential as new drugs, e.g. anticancer drugs, have a long historical preference and traditional use in the treatment of a variety of illnesses (Yadav and Agarwala, 2011). Thus, this study was conducted to evaluate the anti-metastatic capabilities of *Ricinus communis* semi-purified extracts and their effects as potential sources of anti-metastatic agents.

1.1 Cancer metastasis

The initial steps of cancer metastasis are believed to involve the phenomenon of epithelial-mesenchymal transition (EMT) (figure 1.1). This morphological transition leads to the loss of cell-cell and cell-extracellular matrix (ECM) adhesion and subsequent acquisition of migratory abilities, cleavage and remodelling of the basement membrane or ECM resulting in the entry of tumour cells (intravasation) into the blood and/or lymphatic vessels and survival through adhesion to the endothelial lining (Paterlini-Brechot and Benali, 2007). Tumour cells are able to survive within the blood and/or lymphatic vessels, exit from the blood vessels (extravasation) and adhere to the lining of distant organs. This leads to reverse transition from mesenchymal-epithelial phenotype (MET), dormancy of tumour cells in the metastatic site and/or subsequent development of macro-metastasis. For growth and survival, both in the local and foreign microenvironment, tumour cells stimulate the formation of new blood vessels, a process known as angiogenesis (Valastyan and Weinberg,

2011). The newly formed blood vessels, as in normal cells, supply tumour cells with nutrients and oxygen needed for cell growth, proliferation and subsequent formation of a secondary tumour (Davis and Senger, 2005; Lee *et al.,* 2010).

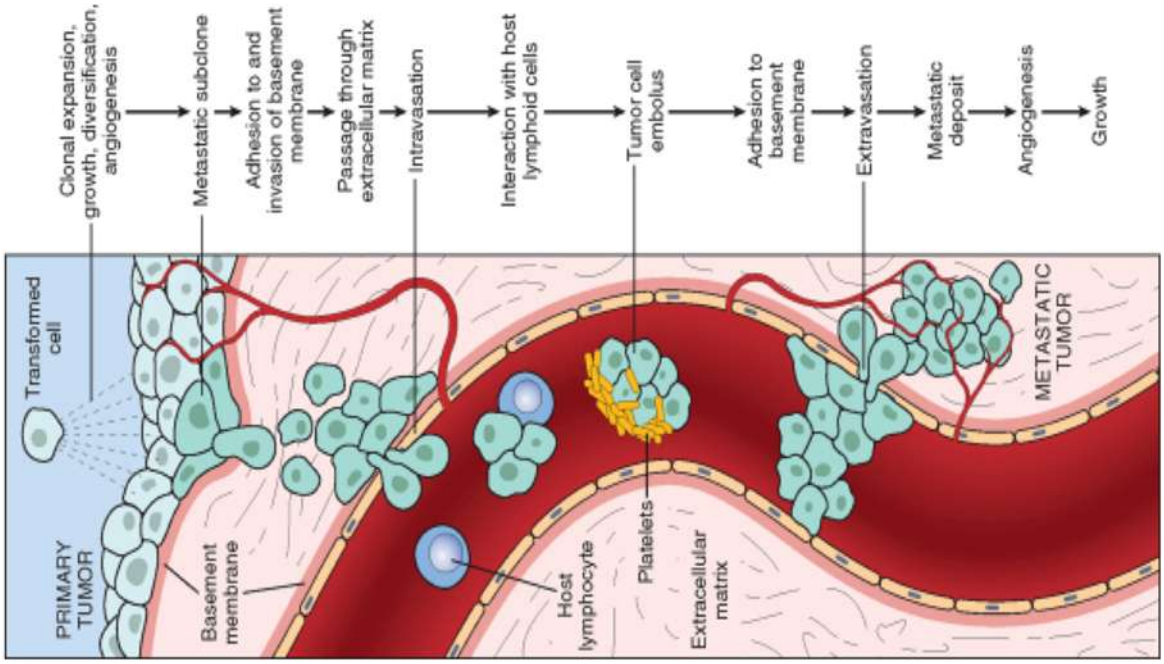

Figure 1.1: Sequential steps involved in the spread of tumour cells via the blood system. Morphologically transformed metastatic cells cleave the basement membrane and remodel the extracellular matrix to escape from the primary tumour and enter the blood vessels (intravasation). In the vasculature, tumour cells survive by association with immune cells (lymphocytes and platelets) and attachment to the endothelial lining. The cells finally exit the blood stream (extravasation) to the metastatic site where they induce the formation of new blood vessels for nutrient supply and grow to form a secondary tumour (Kumar *et al*., 2010).

1.1.1 Epithelial to mesenchymal transition

Epithelial–mesenchymal transition (EMT) is a morphological transition through which epithelial cells, which are the source of many solid tumours, undergo a series of biochemical events and differentiation into highly motile mesenchymal phenotype that contributes to cancer progression (Lamouille *et al*., 2014; Tamiya *et al*., 2010). Epithelial–mesenchymal transition is observed in adults during normal immunological responses such as wound healing and tissue inflammation. It involves several morphological changes which result in altered cellular function. These coordinated changes includes weakening of cell-cell adhesion, loss of cell polarisation (apical baso-lateral polarity of epithelial cells) and acquisition of cell motility permitted by the spindle shape of mesenchymal cells (Tsuji *et al.,* 2009). The morphological transition

has been shown to permit the occurrence of tumour cell invasion associated with elevated expression of N-cadherin resulting from the inhibition of E-cadherin expression. The interchange in expression levels of cadherin molecules, as epithelial and mesenchymal markers, regulates the EMT process (Kallergi *et al*., 2011). Furthermore, the up-regulation of these EMT markers in disseminating or circulating tumour cells has been associated with the development of resistance to conventional chemotherapeutic agents and treatment failure in cancer patients (Monteiro and Fodde, 2010). The development of EMT subsequently leads to acquisition of cell migratory power as well as the promotion of invasion and dissemination of tumour cells to distant location.

1.1.2 Extracellular matrix remodelling and matrix metalloproteinases

The extracellular matrix (ECM) is an essential structure composed of a large collection of distinct biochemical components including proteins, glycoproteins, proteoglycans and polysaccharides (Ozbek *et al.,* 2010; Whittaker *et al.,* 2006). These components describe the ECM's physical properties such as its rigidity, permeability, spatial topography and other features that determine how it supports and maintain the integral arrangement of cells in tissues. The ECM functions as a support system for cell attachment, a movement track and as a physical barrier, by interacting with the basement membrane (Lu *et al*., 2012). It is also involved in the regulation of many cellular processes such as cell proliferation, differentiation, migration and invasion (Finger and Giaccia, 2010). As such, it is highly regulated to ensure the maintenance of its molecular and structural dynamics which are collectively controlled by its production, degradation and remodelling. Abnormal alterations of these control mechanisms disorganise the architecture of the ECM leading to abnormal behaviour of residing cells and development of clinical diseases such as cancer (Cox and Erler, 2011; Page-McCaw *et al.,* 2007). Extracellular matrix cleaving or degrading enzymes such as matrix metalloproteinases (MMPs) expressed in several tumour cells have pathological consequences on tissues including loss of tissue architecture as an essential function of the ECM. Extracellular matrix remodelling enzymes are, therefore, highly regulated at the transcriptional, translational and posttranslational levels with the use of specific protease inhibitors that block their functions (Aitken and Bägli, 2009).

Matrix metalloproteinases (MMPs), a family of zinc-dependant endopeptidases, have been widely studied as proteases responsible for the remodelling and degradation of major components of the extracellular matrix (ECM) as well as the basement membrane. Of the major five classes of metalloproteinases, MMP-2 (gelatinase A and MMP-9 (gelatinase B) which are both type IV collagenases have gained a huge interest due to their involvement in cancer invasiveness and metastasis (Sato *et al*., 2004). Matrix metalloproteinases are produced as latent proteases and are secreted from the cell to the ECM where they may be activated. Zymogenic forms of MMPs (pro-MMPs) are activated by other proteases and several extracellular factors such as growth factors, cytokines and inducers to yield the activated enzyme with the ability to degrade the ECM/BM (Dong *et al*., 2001).

There are many regulatory points at which the activity of MMPs is controlled. These points include activity exhibition, inhibition by TIMPs, pro-MMPs activation, secretion, and transcription (Roomi *et al*., 2010). Matrix metalloproteinases have not only attracted great attention as promoters of tumour invasion but also as angiogenic factors (Wiranowska *et al*., 2015). The activity of MMPs expressed by invading tumour cells also assist in branching of angiogenesis-generated blood vessels by breaking the basement membrane (Lu *et al.,* 2012). The MMP inhibitors that have been discovered from natural products include resveratrol, genistein and curcumin, although their interaction with MMPs is not well understood. However, they are presumed to act as chelating agents due to the presence of hydroxyl and carbonyl rings in their structure which are capable of removing the zinc(II) ion from the active site of MMPs (Verma and Hansch, 2007). Clinical studies have correlated the aggressiveness of many tumours to the abnormally elevated expression levels of MMPs, particularly MMP-2 and MMP-9 (Roomi *et al*., 2010). In light of this, inhibitors of these proteases (MMPs) are now considered potential therapeutic targets. The role of matrix metalloproteinases is clearly a very important aspect in cancer metastasis because they digest the basement membrane and support branching of tumour vasculature that becomes the main route through which cancer cells can spread via the blood and/or lymphic circulatory system.

1.1.3 Tumour cell migration

Cell migration is a well regulated process for structural organisation, i.e., during embryogenesis and normal brain development. Incorrect neuron wiring and organisation of complex networks can lead to development of neurodegenerative diseases such as epilepsy and Ischemia (Cayre *et al*., 2009). Most cell types migrate individually or collectively as a group during normal embryonic development, immune response and in cancer cell metastasis (Theveneau, 2010). During embryonic development, undifferentiated cells migrate directionally and arrange themselves in regulated patterns to allow development of specialised tissues and organs (Jossin and Cooper, 2011). This is also observed during normal body development, whereby cells proliferate, migrate and coordinate their differentiation process which allows them to evolve from less-specialised cells into highly regulated more-specialised cells. Dysregulation of the migration mechanism employed by normal cells enable cancer cells to acquire migratory characteristics and invade adjacent tissues (Carmona-Fontaine *et al.,* 2008). A cell surface membrane protein, N-cadherin, known to function as a cellular stabiliser that allows proper organisation of tissues by forming tight bridges between adjacent cells, has been reported to assist in directional migration of cancerous cells to a specific foreign location in the body, subsequent to its up-regulation in tumour cells (Fred Hutchinson Cancer Research Center, 2011).

Cadherins, selectins and integrins are the most common large cell adhesion molecules, although there are over 50 cell adhesion molecules identified so far. Cadherin adhesion molecules are calcium dependent and are structurally composed of three different domains, viz, the extracellular domain, trans-membrane domain, and intracellular domain which allows them to pass communication signals between neighbouring cells (Bendas and Borsig, 2012). E-cadherin, the main component facilitating adherence in epithelial cells, is a trans-membrane glycoprotein that establishes adherent junctions and also creates a bridge connecting the actin cytoskeleton of neighbouring cells and maintains epithelium cell cohesion and shape (Voulgari and Pintzas, 2009). This molecule is involved in EMT through the process called ‘cadherin switch’ defined by the up-regulation of N-cadherin (which is a mesenchymal marker) and the associated decrease in the expression of epithelial adhesion molecule (E-cadherin) (Derksen *et al.,* 2006). Over-expression of N-cadherin weakens the cell adherence junctions, disrupting cell polarisation and a

subsequent transition to a mesenchymal phenotype. The down-regulation of E-cadherin is associated with cells resistance to anoikis even after losing adhesion to the ECM (Onder *et al*., 2008).

Anoikis is cell death induced by inappropriate or loss of cell adhesion. Normal endothelial and epithelial cells trigger anoikis response as soon as they lose their cell–cell and cell–matrix interactions, or if the adhesive substrate is inadequate. Malignant tumour cells, however, have the ability to escape or suppress anoikis. Anoikis suppression, therefore, is closely linked with successful migration through the bodys' circulatory systems of tumour cells to distant sites (Geiger and Peeper, 2009).

1.2 Cell adhesion molecules

Cell adhesion is a vital process required during cell growth, cell migration, cell differentiation and correct organisation of cells in mammalian systems. Selectins facilitate cell adhesion within the blood circulation system which is used by metastatic tumour cells as the main route of spreading, thereby, supporting their involvement in metastasis. The action of selectins is well demonstrated during inflammation and wound healing where leukocytes and platelets interact with the endothelium within the blood vessels (Ley *et al*., 2007). Integrins, the major cell surface and extracellular matrix receptor essential in receiving directional signals from outside the cell, consist of two covalently linked trans-membrane protein subunits. The protein binds to different ECM components, such as fibronectin, vitronectin, laminin, and collagen. These proteins regulate cell proliferation and angiogenesis conferring their involvement in various stages of the metastatic cascade (Bendas and Borsig, 2012).

1.3 Tumour cell microenvironment

Metastasis is said to be successful only when cancer cells have acquired the ability to survive in the foreign microenvironment encountered in the distant tissue (Valastyan and Weinberg, 2011). Tumours are not only composed of characterised cancer cells that proliferate and form masses of abnormal cells, but are also surrounded by different cell types collaborating together within the microenvironment. However, micro-environmental variations, such as the type of stromal cells, ECM constituents, cytokines and growth factors, between the primary site and the metastatic site greatly affect the adaptability of disseminated tumour cells to the new environment and thus formation of the secondary tumour (Psaila and Lyden, 2009).

Some of the disseminated tumour cells in the metastatic site remain dormant until they become well adapted to their new environment before they begin proliferating due to parameters associated with incompatibility (Chambers *et al*., 2002). It is said that tumour cells start by establishing the 'pre-metastatic niche' in the metastatic site to address poor compatibility with the new microenvironment (Psaila and Lyden, 2009). However, tumour cells that assume an EMT-like phenotype become poorly differentiated which allows them to quickly adapt and survive in the new metastatic microenvironment (Bendas and Borsig, 2012).

Angiogenesis is defined as the development of new blood vessels from pre-existing ones. Formation of these new blood vessels facilitates tumour growth and provides a pathway for the spread of tumour cells to new metastatic sites (Mumprecht and Detmar, 2009). Since cell invasion cannot take place as an independent process, the formation of blood and lymph vessels through angiogenesis has been considered a significant process in the invasiveness of tumour cells (Gomes *et al*., 2012). The role of angiogenesis in metastasis is also well established. Invasive cells as well as stromal cells in the tumour microenvironment produce many angiogenic signalling molecules to promote its formation (Dittz *et al*., 2015). Proliferation and migration of endothelial cells to form tubules is enhanced by several angiogenic factors with VEGF and endostatin being the key players (Kerbel, 2008). The angiogenic process is regulated by the action of many pro-angiogenic factors, of which the predominant ones include a family of vascular endothelial growth factor (VEGF), Transforming growth factor β (TGF-β1) and platelet-derived growth factor (PDGF) and strong angiogenic inhibitors (angiostatin, endostatin) (Bergers and Benjamin, 2003; Kalluri, 2003). The search for inhibitors of pro-angiogenic factors is one of the major research interests making promising developments.

1.4 Antioxidants and reactive oxygen species

The number of patients with suppressed immunity is rising above advances in the look for effective cancer treatment. Some of the cancer treatment strategies such as radiotherapy and chemotherapy may compromise the immune system of cancer patients increasing their risk factors for new dangerous infections (Klastersky, 2004; Klastersky *et al.,* 2000). The therapeutic effects of traditionally used plants adopted in modern medicine are attributed to their antioxidant compounds (Chanda *et al.,* 2011). Antioxidants play a vital role in the oxidant-antioxidant balance of the body.

Intolerable amounts of oxidants or free radicals that cannot be counter balanced by antioxidants leads to oxidative damage of cell constituents and therefore increase the risk of degenerative diseases attributed to oxidative stress (D'Archivio *et al.,* 2007).

The most common forms of free radicals, referred to as reactive oxygen species (ROS), include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen. Oxygen can be a potential free radical and a toxic element when it is metabolically converted to a more reactive form. This transformation is influenced by various metabolic pathways in living cells. The importance of antioxidants in living systems is valued due to their ability to counteract the dangerous effects of these free radicals and prevent damage of cellular organelles, by inhibiting processes such as lipid peroxidation (Kadri, 2011). Studies have strongly associated antioxidants with the prevention of cancer, diabetes mellitus, neurodegenerative and cardiovascular diseases which may arise due to damage of essential biomolecules such as nucleic acids and proteins by free radicals (Scalbert *et al*., 2005). It has been thus asserted that the consumption of antioxidant rich fruits and vegetables can be used in cancer prevention strategies (Marchioli *et al.,* 2001).

1.5 Medicinal Plants use

In ancient times, people relied solely on medicinal plants as remedies to cure different diseases and sustain their lifespan (Ingale and Hivral, 2010). Recently, the value of medicinal plants as promising sources of active drugs has gained more interest mainly because of various claims of therapeutic efficacy, safety, cost efficiency, and better bioactivity with less frequent side effects as compared to modern drugs (Ekor, 2014; Lahlou, 2013). Consequently, development and use of indigenous medicinal plants for treatment of various diseases prevalent today enclose some economic benefits due to the level of poverty and affordability of modern drugs (Azaizeh *et al.,* 2003). Most of the plants currently examined for therapeutic activity as new drugs, for example, anticancer drugs, have a long historical preference and traditional use in the treatment of a variety of illnesses (Yadav and Agarwala, 2011). Unavailability of health facilities especially in developing countries, has forced people from rural communities to rely on the traditional practice of medicine for treatment of common ailments (Muthu *et al.,* 2006). Over 80% of the world's population still relies on traditional medicine from plants for their primary health care needs and between 60-85% of anticancer drugs in use today are derived from natural products (Cragg and Newman, 2013).

It is well known that plants are a rich source of bioactive phytochemicals with a wide range of beneficial applications in medicine. Availability of these phytochemicals in plant products, usable as pure compounds or standardised crude extracts, provides unlimited opportunities for the discovery of new drugs (Cos *et al.,* 2006). Secondary metabolites such as alkaloids, flavonoids, tannins, terpenoids, phytosterols, steroids and phenolic compounds, are the most common group of bioactive compounds found in plants (Jasuja *et al.,* 2012; Koul and Walia, 2009). Most of these plant based compounds have shown to possess therapeutic effects such as anticancer (pro-apoptosis, anti-carcinogenic, anti-mutagenic, anti-angiogenesis), anti-bacterial and anti-inflammation (Han *et al.,* 2007; Ozgov´a, 2003).

Many plant derived drugs used in modern medicine were initially useful in traditional healing practices. The interest has now shifted to investigating the primary benefits of using plant products as medicines due to their traditionally suggested bioactivity. Plants are relatively safe and they may offer affordable and better therapeutic welfare than synthetic medicine (Ciocan and Bara, 2007; Panghal *et al.,* 2011). Extracts from plants can be obtained from different parts of the plant such as the barks, leaves, flowers, roots, fruits and seeds. Screening studies that examine phytochemical composition and fingerprint profiles of these plant extracts reveal diverse compounds belonging to several chemical classes all contributing to the effects of medicinal plants in treating many diseases (Criagg and David, 2001; Street and Prinsloo, 2012). In many cases, the plant chemical constituents responsible for the specific medicinal or biological properties have been isolated, purified, identified and characterised as pure defined compounds (Mughal, 2010). Separation of these compounds for identification and characterisation still remains a very long process (Amsath, 2013). Moreover, isolation of pure compounds from a mixture of various phytochemicals with defined biological effects may lead to loss of activity. This is explained by the synergic relationship of compounds whereby the activity of a particular compound is aided by its interaction with other compounds.

1.6 *Ricinus communis* (Castor oil plant)

Ricinus communis (Euphorbiaceae family), commonly known as castor oil plant, is a shrub-like soft wooden tree with 30–60 cm width smooth leaves (Pingale, 2010). The plant is indigenous to Eastern Africa, and India, but is also widely cultivated in the

world's tropical and warm temperature regions (Momoh *et al.,* 2012; Parekh and Chanda, 2007). Potentially, all the different components of this plant have therapeutic significance. The leaves, seed oil and roots are reported to have biological activity in the treatment of inflammation, diabetes, infertility, liver disorder and as a laxative (Iqbal *et al.,* 2012; Naz and Bano, 2012). This plant is also considered as one of the many natural crude remedies with the potential to combat different diseases in traditional medicine (Jena and Gupta, 2012).

Castor oil sourced from the seeds (consist of 40–60% of inedible oil rich in ricinolein, a triglyceride) has a variety of uses in cosmetics (Rao *et al.,* 2013). The leaves are applied to swellings and sores in the traditional practice of Indian medicine and also used to treat fever, headache and several skin infections (Pingale, 2010). Extracts from different parts of this plant have been evaluated for various biological activities such as antioxidant, anti-bacterial and anti-inflammatory activities (Iqbal *et al.,* 2012). Leaf extracts have been found to be cytotoxic to a number of human tumour cells in a dose-dependent manner (Darmanin *et al.,* 2009).

1.7 Rationale of the study

The ability of metastatic tumour cells to spread from the primary site to vital parts of the body reduces the effectiveness of currently available cancer treatments such as surgery, radiotherapy and chemotherapy. These treatments are also associated with a number of physiological side effects. Investigation of plant based compounds as anticancer agents have grown positively over the years and a considerable amount of modern drugs present on the market are derived from plants (Naz and Bano, 2012). Despite the substantial effort committed to the search and development of new anti-metastatic cancer drugs, deaths related to metastatic cancers continue to rise rapidly. Therefore, there is a need for further research contributions towards the discovery of novel anti-metastatic cancer agents that can inhibit the spread and invasion of tumour cells. Medicinal plants have shown great potential in the management of various diseases in both traditional and western medicine. These plants are recommended for their diverse phytochemicals (Cosa *et al.,* 2006) with therapeutic effects against a variety of pathologic diseases such as cancer, diabetes, inflammation.

This study responds to the global and national challenges affecting the lifespan of cancer patients through the search for novel anti-metastatic cancer agents from *R. communis* leaves. Knowledge of the activity of this plant extracts on the metastatic behaviour of malignant tumour cells may impact or substantiate the presently postulated underlying mechanisms of metastatic processes. Furthermore, a better approach of undertaking cancer metastasis as a challenge in prognosis and management of many cancer patients can be developed. Significant contributions in the knowledge of the causes and mechanisms governing progression and metastasis of breast cancer have already been made. However, new therapeutic products are required to serve as efficient metastatic cancer agents with the ability to augment the effectiveness of available cancer treatments and foremost address the high global mortality in women caused by this disease. Thus far, medicinal plants have shown to be the prime derivation of potential bioactive compounds. Hence, this study aims to investigate semi-purified extracts of *R. communis* leaves as a potential source of anti-metastatic cancer agents.

1.8 Hypothesis

Semi-purified extracts of *R. communis* leaves impede metastasis by inhibiting the migration, invasion (by reducing the activity of matrix metalloproteinases, MMP-2 and MMP-9) and adhesion of metastatic breast (MCF-7) cancer cells.

1.9 Aim

The principal aim of the study was to investigate potential anti-metastatic effect of semi-purified extracts of *Ricinus communis* using metastatic MCF-7 cancer cells.

1.10 Objectives

The objectives of the study were to:

i. Sub-fractionate the crude acetone extract of *R. communis* leaf material using solvent-solvent fractionation.
ii. Determine the fingerprint profile of the sub-fractions of *R. communis* leaf extract using thin-layer chromatography.
iii. Determine the presence of antioxidant constituents in the sub-fractions of *R. communis* leaf extract on TLC, using DPPH as an indicator.

iv. Determine the effect of sub-fractions of *R. communis* leaf extract on metastatic MCF-7 cancer and normal BUD-8 cell viability using the MTT assay.

v. Determine the effect of sub-fractions of *R. communis* leaf extract on the formation of reactive oxygen species (ROS) using the DCFH-DA assay.

vi. Assess the effect of sub-fractions of *R. communis* leaf extract on metastasis by determining their effect on:

   a) MCF-7 cell migration using the wound healing migration assay.
   b) Cell attachment using the cell adhesion assay.
   c) Cell invasion using the matrigel Boyden chamber invasion assay.

vii. Determine the effect of sub-fractions of *R. communis* leaf extract on the activity of MMPs using gelatin-zymography.

viii. Analyse the effect of sub-fractions of *R. communis* leaf extract on the relative levels of expression of angiogenesis and metastasis related proteins using proteome profiler antibody arrays.

CHAPTER 2

2 Material and Methods

2.1 Materials

2.1.1 Equipment

- Waring Commercial Blendor (Model 32BL79, Dynamics Corporation, New Hartfort, Connecticut, USA)
- Microtiter-Plate Multimode Detector (Model 550 and Model DTX 800, Bio-Rad Laboratories, California, USA)
- CO_2 incubator (NAPCO model, Instrulab cc, Johannesburg, RSA)
- New Brunswick G-25 Shaker Incubator (New Brunswick Scientific, New Jersey, USA)
- Centrifuge (Model GS-15R, Beckman Coulter, Germany)
- Inverted light and fluorescence microscope (Nikon, Japan)
- GloMax®-Multi+Detection System (Promega, Madison, USA)

2.1.2 Cells, culture media and chemicals

- Organic solvents, Acetone, Chloroform, n-butanol, n-hexane, Ethyl acetate, benzene, Methanol, Ethanol, Ammonium hydroxide, Formic acid (Rochelle Chemicals, SA)
- BUD-8 and MCF-7 cell lines (ATCC, Rockville, USA)
- Fetal bovine serum (FBS) (Hyclone, Cramlington, UK)
- DMEM and RPMI-1640 medium, PSN (penicillin, streptomycin and neomycin cocktail) (Gibco, Auckland, New Zealand)
- 0.25% Trypsin-EDTA (1X) (Gibco, Auckland, New Zealand)
- Dimethylsulfoxide (DMSO) (Merck Chemicals, (PTY) LTD, Darmstadt, Germany)
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), 2′,7′-Dichlorofluorescin diacetate(DCFH-DA) (Sigma-Aldrich, Saint Louis, Missouri, USA)

2.2 Methods

2.2.1 Plant collection and preparation of semi-purified leaf extracts by solvent-solvent fractionation

The leaves of *Ricinus communis* were harvested from within the premises of the University of Limpopo and air-dried at room temperature. The air-dried leaves were ground to fine powder using a commercial blender. Powdered leaves (20 g) were exhaustively extracted using 200 ml of acetone with continuous shaking at 150 RPM for 24 hours at room temperature using a rotary shaker (New Brunswick Scientific, New Jersey, USA). The resultant acetone extract was filtered using Whatman No. 2 filter paper and air-dried under a fan to evaporate the extraction solvent.

Sub-fractions of the acetone leaf extract of *R. communis* were obtained by solvent-solvent fractionation procedure as described by Suffness and Douros (1979). The procedure yields 6 fractions separated based on the polarity of extract components. Briefly, the dried residue of the crude acetone extracts was dissolved in a mixture of chloroform: water (1:1) and separated using a separating funnel. An equal volume of *n*-butanol was added to the water fraction to yield two fractions (H_2O and *n*-butanol). The water fraction was further mixed with equal volumes of ethyl acetate to yield water and ethyl acetate fractions. The chloroform fraction was air-dried, re-dissolved and separated in equal volumes of *n*-hexane and 90% (v/v) methanol mixture. The 90% methanol fraction was then diluted to 65% by adding water and mixed with chloroform to produce the chloroform and 65% methanol fractions. A schematic representation of the produce is outlined in figure 2.1. All the fractions were dried under the fan, dissolved in dimethyl sulphoxide (DMSO) to give stock solutions of 10 mg/ml and stored at -20^{o}C until required for cell based assays. The extract and resultant sub-fractions used in all assays are from a single extraction.

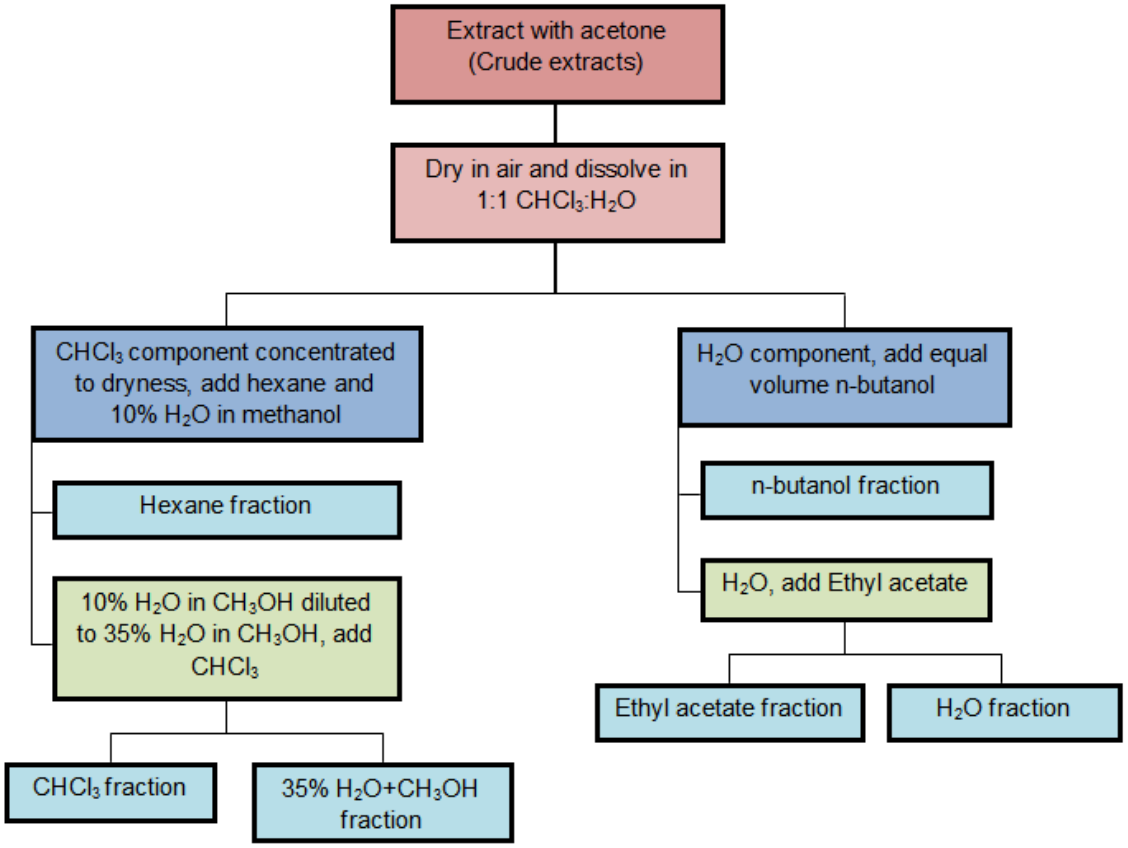

Figure 2.1: Solvent-solvent fractionation scheme of *R. communis* crude acetone extracts separated into 6 different fractions (Suffness and Douros, 1979).

2.2.2 Thin layer fingerprint profile of sub-fractions

Thin layer chromatography (TLC) was used to determine the fingerprint profile of *R. communis* sub-fractions. Dried residues of the 6 acquired fractions namely; *n*-butanol, ethyl acetate, H_2O, *n*-hexane, chloroform and 35% H_2O+CH_3OH were re-dissolved in acetone to prepare 10 mg/ml stock solutions and 10 µl of each was spotted on TLC plates. The TLC plates were allowed to dry and then developed in three solvent systems of varying polarity, namely, ethyl acetate/methanol/water [EMW] (10:1.3:1) (polar/neutral); chloroform/ethyl acetate/formic acid [CEF] (10:8:2) (intermediate polarity/acidic); benzene/ethanol/ammonium hydroxide [BEA] (18:2:0.2) (non-polar/basic). To visualise the phytochemical components present in the fractions, the plates were viewed under UV light for detection of fluorescent

compounds and then sprayed with vanillin (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) in the fume hood and heated at 110^{o}C to allow maximum development of colour.

2.2.3 Antioxidant activity

Thin layer chromatography plates were used to separate phytochemicals of sub-fractions of leaf extract of *R. communis* as described above (2.2.2). To detect the presence of compounds with antioxidant activity, chromatographs were sprayed with 0.2% (w/v) 2, 2-diphenyl-2-picrylhydrazyl (DPPH) in methanol. Yellow zones against the purple background of the chromatograms indicate free radical scavenging activity by compounds present in the sub-fractions (Masoko and Eloff, 2007).

2.2.4 Cell culture and treatment

Breast MCF-7 (ATCC® HTB-22™) cancer and normal skin BUD-8 (ATCC® CRL-1554™) cell lines were purchased from the American Type Culture Collection (ATCC®, Rockville, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA) and supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS) (Hyclone, USA) and 1% antibiotic mixture, Penicillin, Streptomycin and Neomycin, (PSN) (Gibco, New Zealand) at 37^{o}C in a humidified atmosphere of 5% CO_2. The *n*-hexane and *n*-butanol fractions were selected for evaluation based on their phytochemical composition and antioxidant activity, respectively. For the treatment of cells, the stock solution (10 mg/ml) of each fraction was diluted in serum-free media to obtain concentrations ranging between 0-500 µg/ml. The solutions were filter-sterilised before treatment. DMSO was used at 0.05% (v/v) in all assays as a solvent control.

2.2.5 Determination of *n*-hexane and *n*-butanol fractions' effect on cell viability

The effect of the *n*-hexane and *n*-butanol fractions on the viability of MCF-7 and BUD-8 cells was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (Sigma-Aldrich, USA). This is a colorimetric assay that measures the amount of formazan produced by mitochondrial enzymes within living cells. The absorbance of the solubilised formazan produced only by metabolically active cells is measured using the microtiter plate reader to indirectly quantify the amount of viable cells. Cells (2 x 10^5 cells/ml) were seeded in 96-well plates and allowed to attach overnight. Attached cells were then treated with

varying concentrations (0, 100, 200, 300, 400 and 500 µg/ml) of the fractions, 20 µM of curcumin (positive control) and incubated at 37^{o}C for 24 and 48 hours. After 22 and 46 hours of incubation, respectively, 25 µl of MTT (5 mg/ml) solution was added to the cells, followed by further incubation at 37^{o}C for 2 hours. After incubation, the cell culture media was carefully removed and 100 µl of DMSO was added to each well to solubilise the formazan crystals produced within the cells. The amount of formazan produced was quantified at 560 nm using the GloMax®-Multi+ Detection System (Promega, Madison, USA). Percentage cell viability of MCF-7 and BUD-8 cells after treatment with the fractions was calculated using the following formula:

$$\%\ Cell\ viability\ = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ control} \times 100\%$$

2.2.6 Hoechst 33342 nucleic acid fluorescent staining

The apoptotic effect of the *n*-hexane and *n*-butanol fractions on MCF-7 cells was analysed by Hoechst 33342 nucleic acid staining following the description by Danieland DeCoster, (2004) with some adjustments. Hoechst is a common DNA-binding stain used to identify nuclear morphological changes associated with apoptosis. Cells were grown to 90% confluency and then treated with (100 and 200 µg/ml) or without the fractions for 24 hours. Curcumin at 50 µM was used as a positive control. Treated and untreated cells were washed with 1x Phosphate buffered saline (PBS) (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4), fixed with 3.7% (w/v) paraformaldehyde and then stained with Hoechst 33342 dye at 20 µg/ml for 15 min. Fluorescence intensity and morphology of the stained nuclear DNA was observed and photographed under a fluorescence microscope at a maximum excitation/emission spectra of 350/461 nm.

2.2.7 ROS formation by DCFH-DA assay

The effect of *n*-hexane and *n*-butanol fractions on the formation of intracellular reactive oxygen species (ROS), e.g. hydroxyl and peroxyl species, by MCF-7 cells was determined by the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay as described by Hamel *et al.* (2008) with minor modifications. MCF-7 cells were seeded and grown overnight on microscope slide cover slips and treated with 100 and 200 µg/ml of the extracts for 24 hours. After treatment, cells were carefully washed three times with 1x PBS followed by addition of DCFH-DA at 10 µM (as a fluorescent probe

used to detect reactive oxygen species produced by the cells) and 50 µM of H_2O_2 (as a positive control). After incubation for 30 minutes at room temperature in the dark, cells were washed three times with 1x PBS, viewed and photographed under an inverted fluorescence microscope (Nikon, Japan).

2.2.8 Wound healing migration assay

To assess the effect of *n*-hexane and *n*-butanol fractions on the migration of MCF-7 cells, the wound healing migration assay was used as described by Chia *et al.* (2010) with slight modifications. MCF-7 cells were seeded in 24-well plates and allowed to grow overnight to form monolayers. The cell monolayers were then scratched with a pipette tip to create a wound and washed three times with serum-free medium to remove suspended cells and debris. The wounded cell monolayers were treated with various concentrations (100 and 200 µg/ml) of the fractions and 20 µM of curcumin for 6 and 24 hours. After 6 and 24 hours, the media was discarded and cell monolayers were gently washed three times with 1x PBS, fixed with 3.7% (w/v) paraformaldehyde and stained with 0.5% (w/v) crystal violet solution. Wound closure was monitored by taking photographs of the wounded monolayers at 0 hours and after each incubation time using an inverted light microscope (Nikon, Japan).

2.2.9 Cell adhesion assay

To investigate the effect of *n*-hexane and *n*-butanol fractions on the ability of MCF-7 cells to attach to the surface of cell culture plates, the cell attachment assay was performed as described previously (Yahayo *et al.,* 2013) with slight modification. MCF-7 cells (2×10^6 cells/ml) were treated with 100 and 200 µg/ml of the fractions and 20 µM of curcumin for 2 hours. Following treatment, cells were then plated in 24-well cell culture plates and incubated for 6 and 24 hours. Non-adherent cells were removed by gently washing the plate three times with 1x PBS. Attached cells were fixed with 3.7% (w/v) paraformaldehyde and stained with 0.5% (w/v) crystal violet solution. Photographs of stained cells were captured under an inverted light microscope (10X magnification) (Nikon, Japan). Percentage cell adhesion was determined by solubilising the crystal violet stained-cells with 10% (v/v) acetic acid and quantified at 560 nm using the GloMax®-Multi+Detection System (Promega, Madison, USA).

2.2.10 Boyden chamber invasion assay

To assess the effect of *n*-hexane and *n*-butanol fractions on the invasiveness of MCF-7 cells, the Boyden chamber invasion assay was used as previously reported by Zhang *et al*. (2013). MCF-7 cells at density of 1 x 10^6 cells/ml were harvested, centrifuged and re-suspended in cell culture media containing 0.1% (v/v) of FBS and different concentrations (100 and 200 µg/ml) of the fractions. Cells were then added into cell culture inserts coated with a matrigel matrix (BD Biosciences, California, USA). The inserts were then placed into 24-well cell culture plates containing cell culture media with 10% FBS (serving as a chemo-attractant) and incubated at 37°C for 6 and 24 hours. After incubation, non-invasive cells were removed from the inserts by wiping with a cotton swab. Cells that migrated to the bottom of the polyethylene terephthalate (PET) membrane of the cell culture inserts were fixed with 3.7% (w/v) paraformaldehyde and stained with 0.5% (w/v) crystal violet solution. Photographs of migrated cells were captured under an inverted light microscope (Nikon, Japan).

2.2.11 Gelatin-zymography assay

The effect of the *n*-butanol fraction on the activity of matrix metalloproteinases (MMP-2 and MMP-9) was assayed using gelatin-zymography as previously described by Kupai (2010) with minor modification. MCF-7 cells grown in serum-free media were incubated with 100 and 200 µg/ml of the fraction and 20 µM of curcumin for 6 and 24 hours. After incubation, cell culture media from untreated and treated cells was collected and protein concentration quantified using the BCA protein assay kit (Thermo Scientific, Rockford, USA). Thirty micrograms of protein was mixed with SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 1% (v/v) SDS, 10% (v/v) Glycerol and 0.01% (w/v) Bromophenol blue]. The samples were then electrophoresed on 10% sodium dodecyl sulphate polyacrylamide gels containing 0.1% (w/v) gelatin. Following gel electrophoresis, the gels were washed with 2.5% (v/v) Triton X-100 solution for 40 min at room temperature to remove SDS and allow renaturation of the gelatinases (MMP-2 and MMP-9). The gels were incubated overnight at 37°C in a developing buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM $CaCl_2$] to allow enzymatic reaction. The gels were then stained with Coomassie Brilliant Blue staining solution [0.05% (w/v) Coomassie Brilliant Blue, 50% (v/v) methanol and 10% (v/v) glacial acetic acid] for 1 hour and destained for 2 hours using a destaining solution containing 10% (v/v) glacial acetic acid and 50% (v/v) methanol.

Photographs of the stained gels were captured using the ChemiDoc XRS image analyser (Bio-Rad laboratories, California, USA).

2.2.12 Analysis of relative protein expression

The effect of *n*-butanol fraction on the expression levels of angiogenesis and metastasis related proteins was analysed using the Proteome Profiler™ human angiogenesis antibody array kit (R&D systems, USA) following the manufacturer's instructions. Briefly, the assay uses nitrocellulose membranes each spotted with 55 different antibodies specific to proteins involved in angiogenesis and metastasis. Prepared MCF-7 cell lysate samples (with 300 µg of protein) were mixed with 15 µl of the biotinylated detection antibody cocktail for 1 hour at room temperature. The sample/antibody mixture was added to the nitrocellulose membranes and incubation overnight at 4°C on a rocking platform shaker. After a wash, streptavidin-horseradish peroxidase was added onto the membranes followed by exposure to the chemiluminescence reagent mix. Array data was developed using the ChemiDoc XRS image analyser (Bio-Rad laboratories, California, USA). The pixel density in each spot of the array was analysed using the image analysis software. Signal values were exported to a Microsoft Excel template and the mean signal (pixel density) of the duplicate spots representing each protein was determined. Average value of the background signal was subtracted from each spot. The corresponding signals on different arrays were compared to determine the relative change in angiogenesis and metastasis proteins between samples.

2.2.13 Statistical analysis

Statistically significant levels were calculated using the GraphPad InStat 3 Software by one-way ANOVA followed by Dunnett`s comparison test. The data were expressed as mean ± standard deviation (SD) of three independent experiments. *P* value ≤ 0.05 was considered significant.

CHAPTER 3

3 Results

3.1 Phytochemical analysis of *R. communis* semi-purified extracts

The fingerprint profile of constituents of *R. communis* sub-fractions were qualitatively determined and detected using thin layer chromatography (TLC). The phytochemical profile showed a diversity of compounds represented by the different colours after reacting with vanillin (figure 3.1). The *n*-hexane fraction showed abundant of phytochemicals that were well separated in the intermediate polar/acidic CEF and non-polar/basic BEA solvent systems. This demonstrated that the fraction had a high quantity of non-polar compounds as compared to the other fractions. *n*-butanol, Ethyl acetate, CH_3OH+H_2O and H_2O fractions were shown to contain compounds that are yellow in colour on reaction with vanillin, on the TLC plate, which suggested the presence of antioxidant compounds. A discrete separation of these compounds was observed in the EMW mobile phase more than in other solvent systems. Due to diverse phytochemicals shown to be present in both *n*-butanol and *n*-hexane fractions, the potential anti-metastatic effects of these fractions were evaluated using metastatic breast (MCF-7) cancer cells.

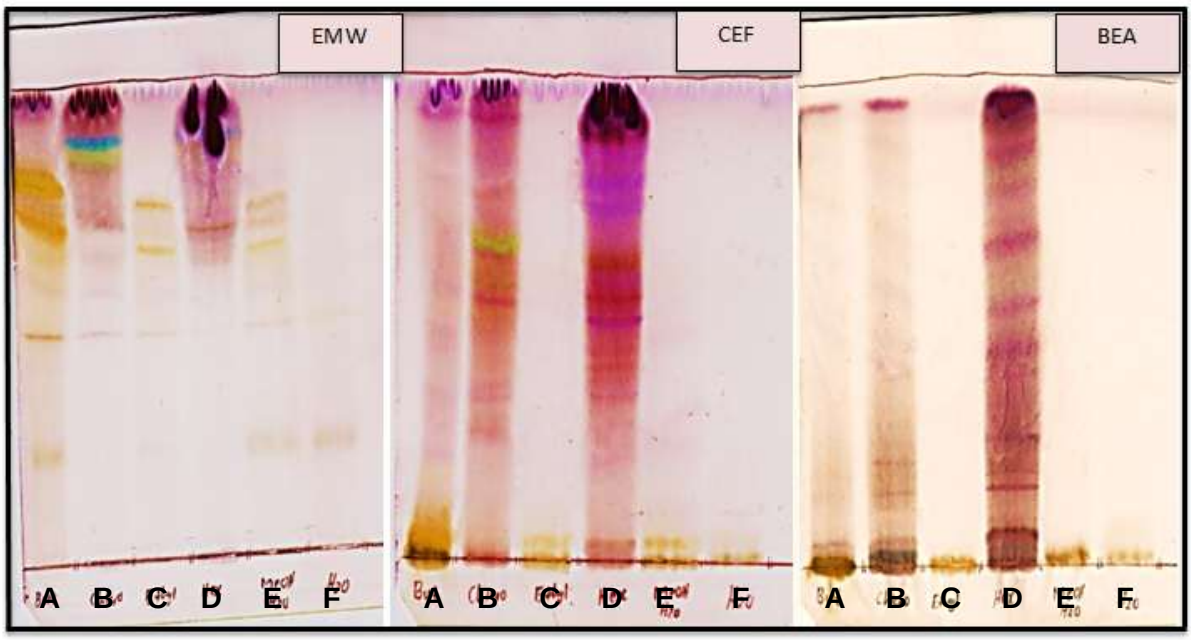

Figure 3.1: Thin layer chromatograms of *R. communis* sub-fractions. The TLC plates were developed in EMW (10:1.3:1), CEF (10:8:2), and BEA (18:2:0.2), sprayed with vanillin in sulphuric acid and heated at 110^{o}C for maximum colour development. A: *n*-Butanol, B: Chloroform, C: Ethyl acetate, D: *n*-Hexane, E: Methanol + H_2O, F: H_2O.Different colours represent the diverse phytochemicals present in all individual fractions.

3.2 Antioxidant activity of *R. communis* semi-purified extracts

The qualitative antioxidant potential of separated phytochemical compounds of the fractions was assessed using TLC-DPPH free radical scavenging assay. The chloroform and *n*-hexane fractions were shown to contain compounds with antioxidant scavenging activity, although the intensity of the bands was faint. However, all the other fractions (*n*-butanol, Ethyl acetate, CH_3OH+H_2O and H_2O) were shown to contain compounds with high scavenging activity as indicated by an intense yellow colour on a purple background on TLC plates (figure 3.2). This activity was seen to be relative to the amount of antioxidant compounds present in each fraction. Comparatively, the *n*-butanol fraction was shown to contain more phytochemicals with antioxidant activity. Furthermore, the migration distance of these compounds corresponded to that of the yellow compounds observed on the chromatograms sprayed with vanillin in figure 3.1 though the respective Rf values were not calculated. These antioxidant compounds were better separated in the polar EMW mobile system and no separation was observed in the non-polar (BEA) mobile phase, suggesting that most of these compounds are polar in nature. The *n*-butanol fraction was further evaluated for its potential as an anti-metastatic agent using metastatic breast (MCF-7) cancer cells due to its high content of compounds with antioxidant activity.

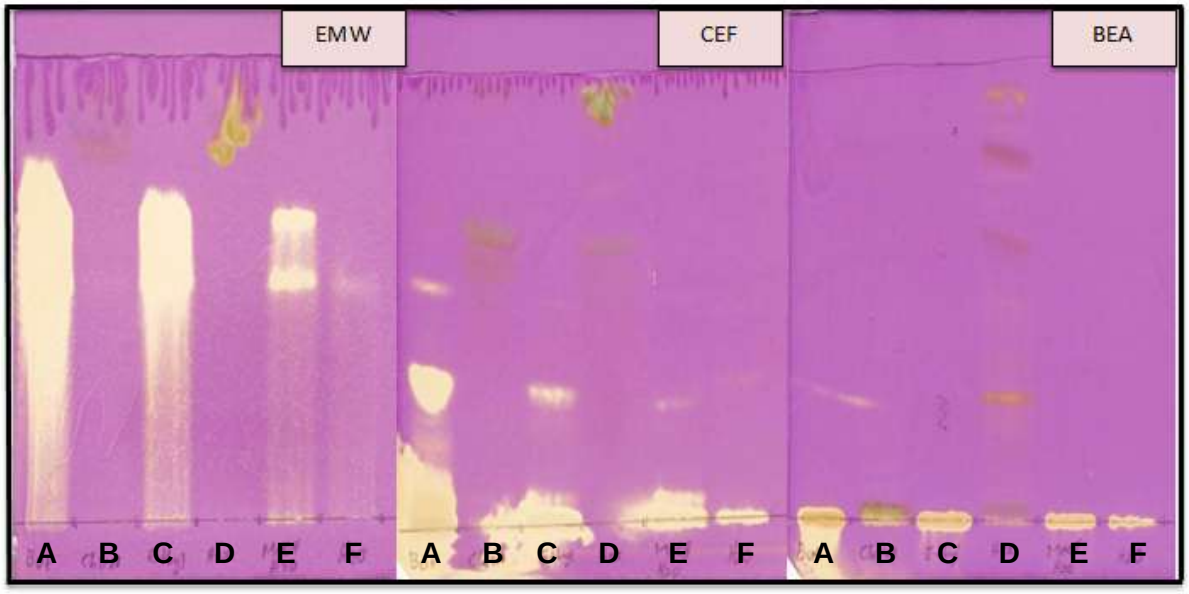

Figure 3.2: Thin layer chromatograms of *R. communis* sub-fractions. The TLC plates were developed in EMW (10:1.3:1), CEF (10:8:2), and BEA (18:2:0.2). Plates were sprayed with 0.2% DPPH in methanol. A: *n*-Butanol, B: Chloroform, C: Ethyl acetate, D: *n*-Hexane, E: Methanol + H_2O, F: H_2O. The presence of antioxidant compounds is indicated by the yellow bands against the purple background.

3.3 Effect of *n*-butanol and *n*-hexane fractions of *R. communis* on BUD-8 and MCF-7 cell viability

The effect of *n*-butanol and *n*-hexane fractions on the viability of MCF-7 and BUD-8 cells was determined by the MTT assay. The *n*-butanol fraction was shown to significantly decrease the viability of BUD-8 cells to between 50–80% at concentrations of 300–500 µg/ml after 24 and 48 hours of exposure (figure 3.3). At concentrations of 100 and 200 µg/ml, the extracts did not cause a significant decrease in cell viability. However, BUD-8 cells (figure 3.4) were shown to be more sensitive to the *n*-hexane fraction with the percentage viability of less than 60% at 400 and 500 µg/ml of the extract after 24 and 48 hours of incubation. At extract concentrations between 100–300 µg/ml the viability of BUD-8 cells was between 70–90% after 48 hours of exposure, which is a significant decrease relative to the control.

n-butanol fraction, as shown in figure 3.5, did not cause a significant decrease in MCF-7 cell viability except at concentrations above 300 µg/ml after 48 hours of exposure. As shown in figure 3.6, the viability of MCF-7 cells was significantly ($p<0.05$) reduced after being exposed to 300–500 µg/ml of the *n*-hexane fraction for 48 hours. Cell viability was maintained at above 70–96% after exposure to 100–500 µg/ml of extract concentrations for 24 hours. MCF-7 cells appeared to be more sensitive to the non-polar compounds of *n*-hexane fraction compared to the polar compounds of the *n*-butanol fraction. However, over 50% of the cells were still viable after 48 hours of treatment. The *n*-butanol and *n*-hexane fractions of *R. communis* leaf extracts were shown to reduce the viability of MCF-7 cells in a time- and concentration-dependant manner. Hence for further investigations, 100 and 200 µg/ml of both fractions were selected as the working concentrations based on the percentage viability of cancerous and normal cells

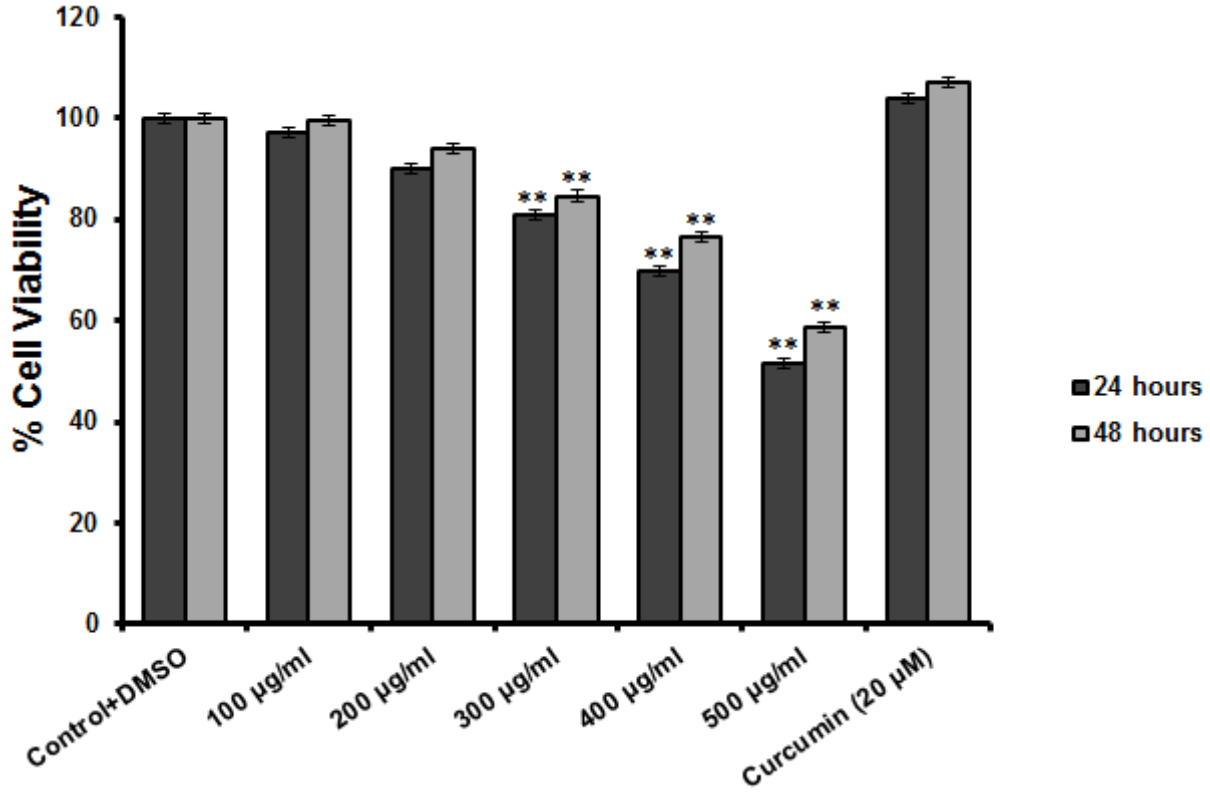

Figure 3.3: Effect of the *n*-butanol fraction of *R. communis* extracts on BUD-8 cell viability. Cells were treated with various concentrations (0–500 µg/ml) of the *n*-butanol fraction and 20 µM curcumin (positive control) for 24 and 48 hours. The % cell viability was assessed by the MTT assay. The data shows the mean±S.D of three independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, indicate a significant difference to the control.

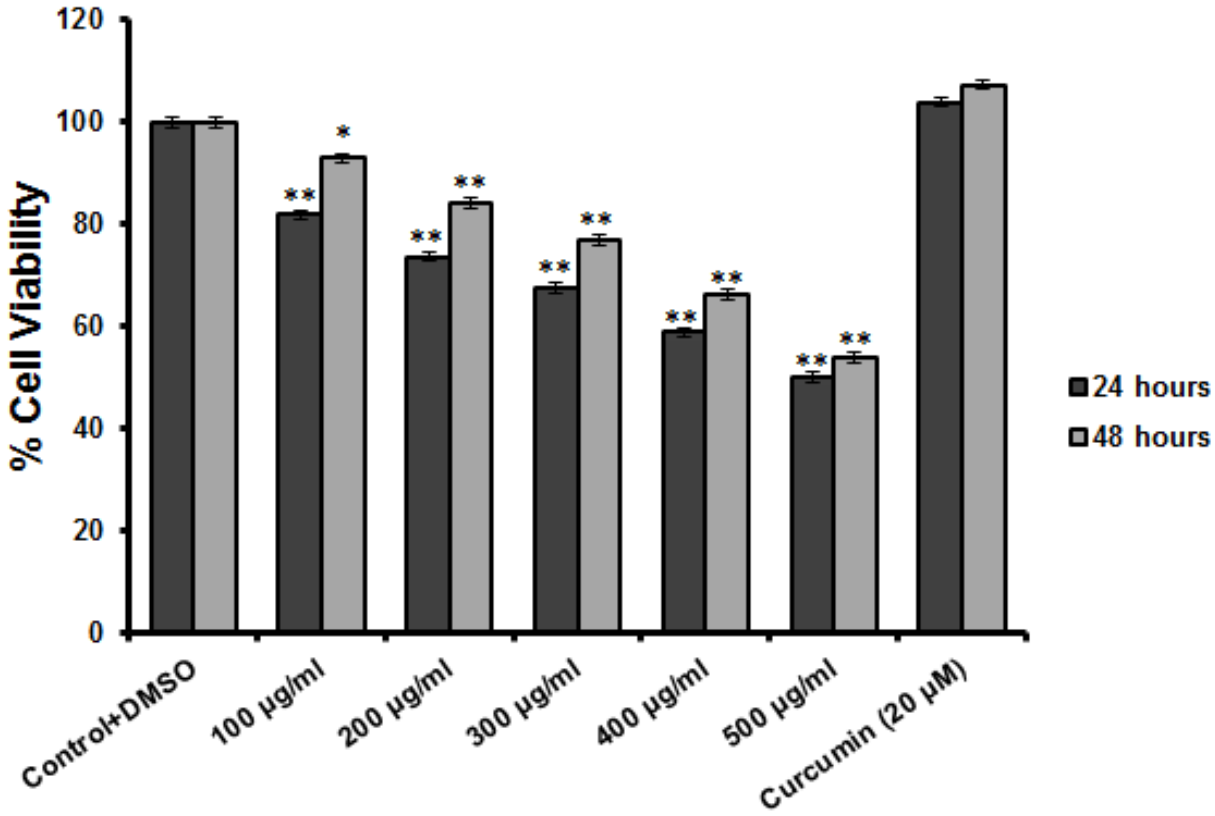

Figure 3.4: Effect of the *n*-hexane fraction of *R. communis* extracts on BUD-8 cell viability. Cells were treated with various concentrations (0–500 µg/ml) of the *n*-hexane fraction and 20 µM curcumin (positive control) for 24 and 48 hours. The % cell viability was assessed by the MTT assay. The data shows the mean±S.D of three independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, indicate a significant difference to the control.

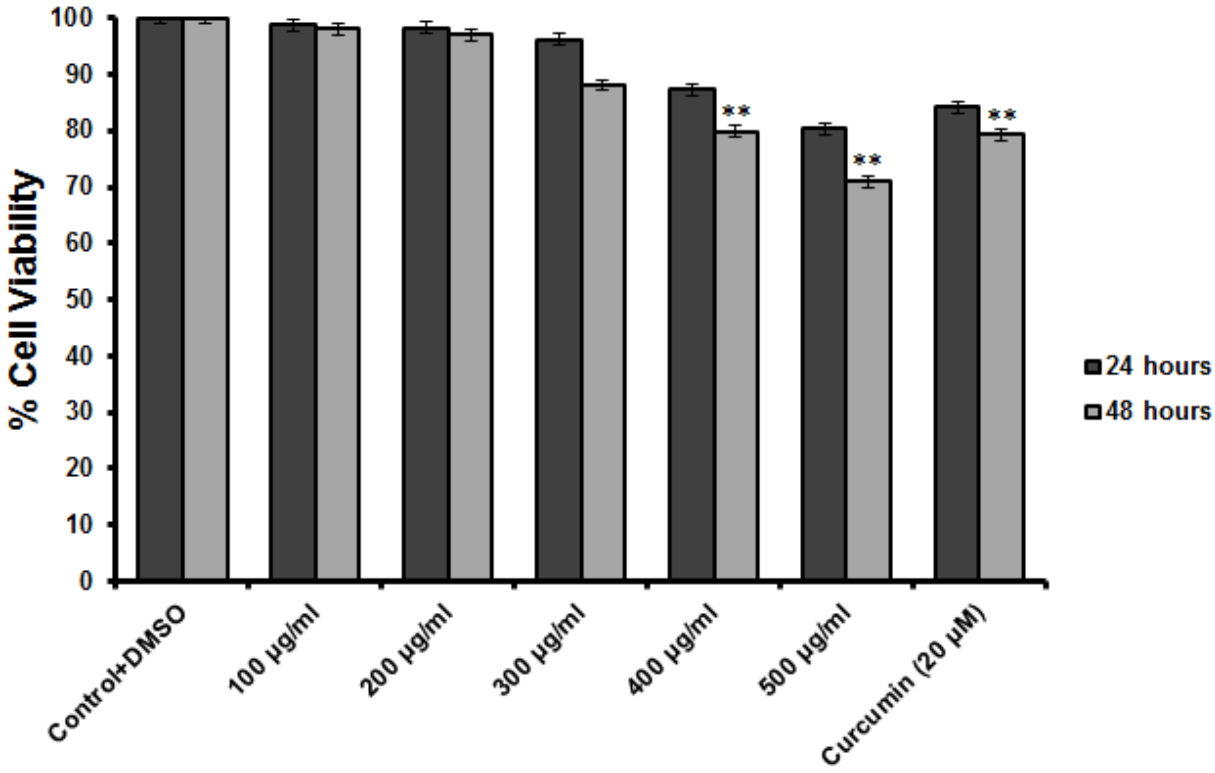

Figure 3.5: Effect of the*n*-butanol fraction of *R. communis* extracts on MCF-7 cell viability. Cells were treated with various concentrations (0–500 µg/ml) of the *n*-butanol fraction and 20 µM curcumin (positive control) for 24 and 48 hours. The % cell viability was assessed by the MTT assay. The data shows the mean±S.D of three independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, indicate a significant difference to the control.

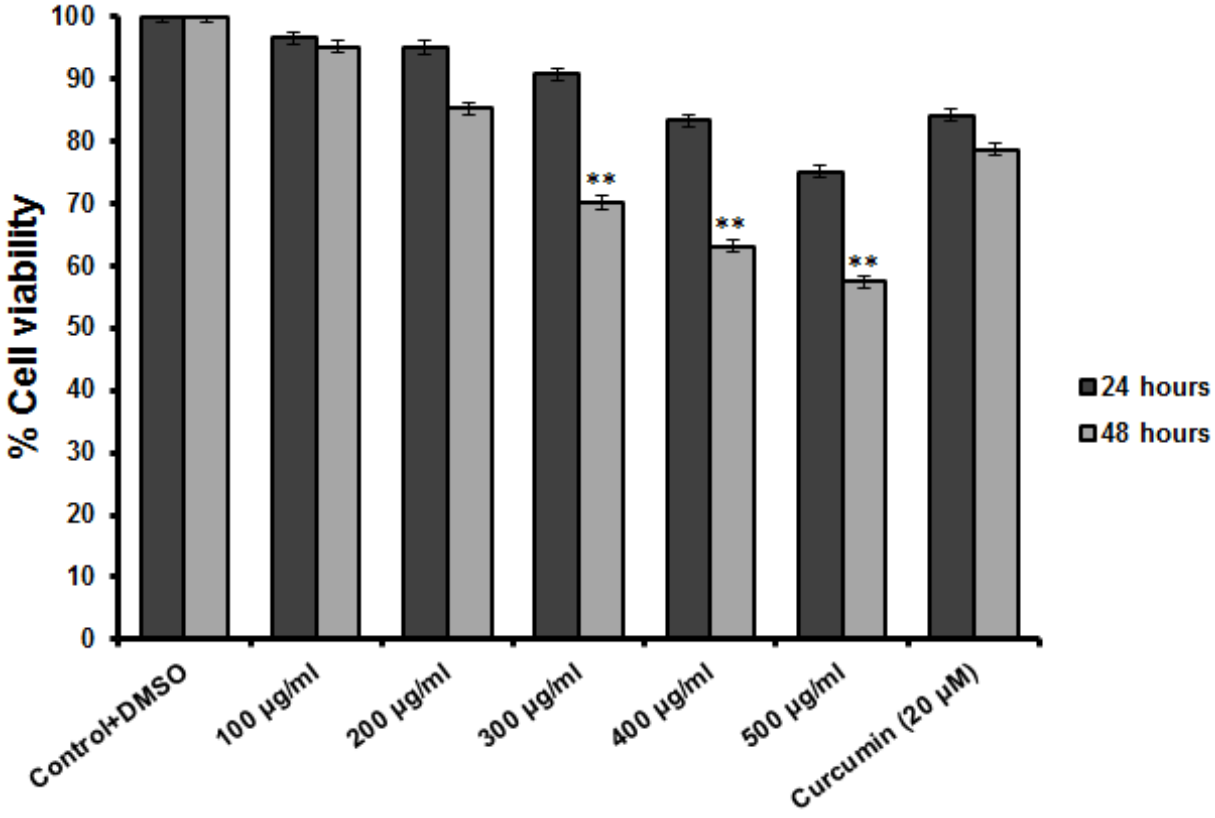

Figure 3.6: Effect of the *n*-hexane fraction of *R. communis* extract on MCF-7 cell viability. Cells were treated with various concentrations (0–500 µg/ml) of the *n*-hexane fraction and 20 µM curcumin (positive control) for 24 and 48 hours. The % cell viability was assessed by the MTT assay. The data shows the mean±S.D of three independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, indicate a significant difference to the control.

3.4 Effect of *n*-butanol and *n*-hexane fractions of *R. communis* on nuclear morphology

The MTT assay was used to indirectly quantify the amount of live and/or dead cells inferred through the mitochondrial activity of viable cells to produce formazan crystals that can be solubilised and its absorbance measured spectrophotometrically. To verify the cytotoxic effects of the *n*-butanol and *n*-hexane fractions on MCF-7 human breast cancer cells exposed to 100 and 200 µg/ml of the extracts, Hoechst staining was employed. The viability assay showed that MCF-7cellstreated with test concentrations (100 and 200 µg/ml) of the fractions for 24 hours maintained their viability above 90% (figure 3.5 and 3.6). Statistical analysis also confirmed that the number of cells undergoing apoptosis were not significant to render these concentrations unusable for metastatic studies. Under the fluorescence microscope, only a small number of cells (exposed to 100 and 200 µg/ml of the fractions) showed nuclear morphological features associated with apoptosis (figure 3.7). Cells undergoing apoptosis were identified by the intense blue colour as compared to control cells with a less bright blue colour. Few of the indicative nuclear features such as chromatin condensation and blebbing as well as formation of apoptotic bodies were also observed. The observation revealed that at concentrations of 100 and 200 µg/ml, both fractions did not show to strongly affect the integrity of the cell nuclei, confirming the insignificant cytotoxicity effects shown by the MTT assay (figure 3.5 and 3.6).

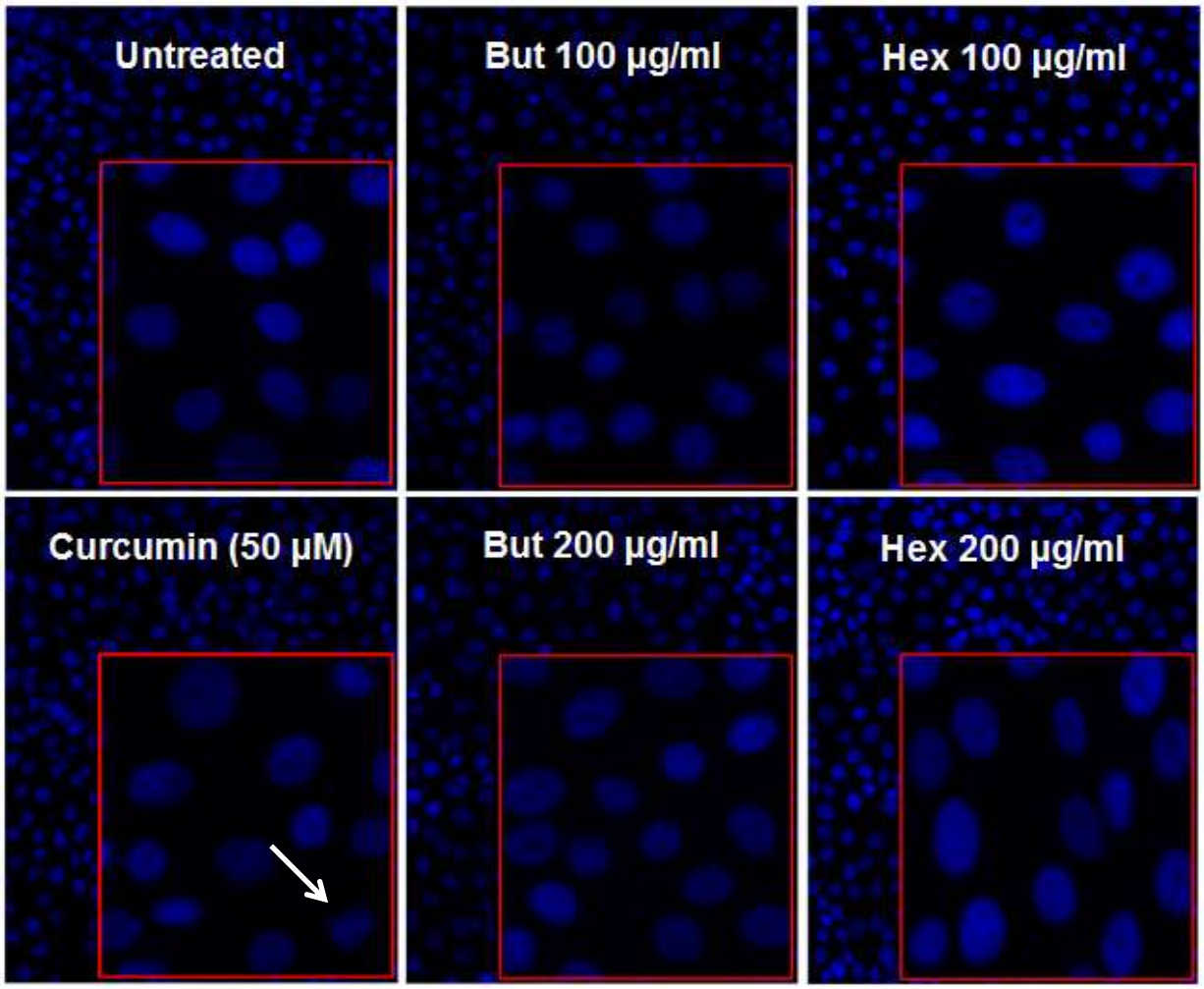

Figure 3.7: Hoechst 33342 fluorescent staining of nuclear DNA in human breast MCF-7 cancer cells. Cells were exposed to 100 and 200 µg/ml of *n*-butanol and n-hexane fraction for 24 hours. Cell nuclei were stained with Hoechst 33342 and examined under a fluorescence microscope. Brightly stained nuclei would indicate cells with apoptotic manifestations such as chromatin condensation, formation of apoptotic bodies and total fragmentation of the nucleus. The white arrow identifies nuclear blebbing as one of the apoptotic features. But = *n*-butanol, Hex = *n*-hexane.

3.5 Effect of *n*-butanol and *n*-hexane fractions of *R. communis* extracts on the formation of intracellular ROS.

The balance between antioxidant defence and formation of free radicals is very important for the survival and normal functioning of the cell as well as disease prevention. Secondary metabolites such as flavonoids and polyphenols are largely responsible for the antioxidant potential or free radical scavenging activity seen in many plant extracts. Cancer cells have been shown to have abnormal levels of reactive oxygen species (hydroxyl, peroxyl and other ROS). Free radicals (reactive oxygen and nitrogen species) have been associated with the aggressiveness of malignant tumour cells, including breast cancer, through their function as activators of inactive forms of membrane degrading proteins (MMPs) (Gu, 2011; Ríos-Arrabal *et al.,* 2013). The effect of the *n*-butanol and *n*-hexane fractions on cellular production of ROS was assessed by fluorescent DCFH-DA assay. Production of ROS is proportional to the fluorescence intensity of DCF produced through deacetylation by cellular esterases following oxidation by ROS. Cells treated with *n*-butanol and *n*-hexane fractions (figure 3.8) exhibited very low fluorescence intensity as compared to the untreated and H_2O_2-treated cells. The finding suggests the ability of the extracts to strongly reduce the amounts of cellular oxidative species in a concentration-dependent manner.

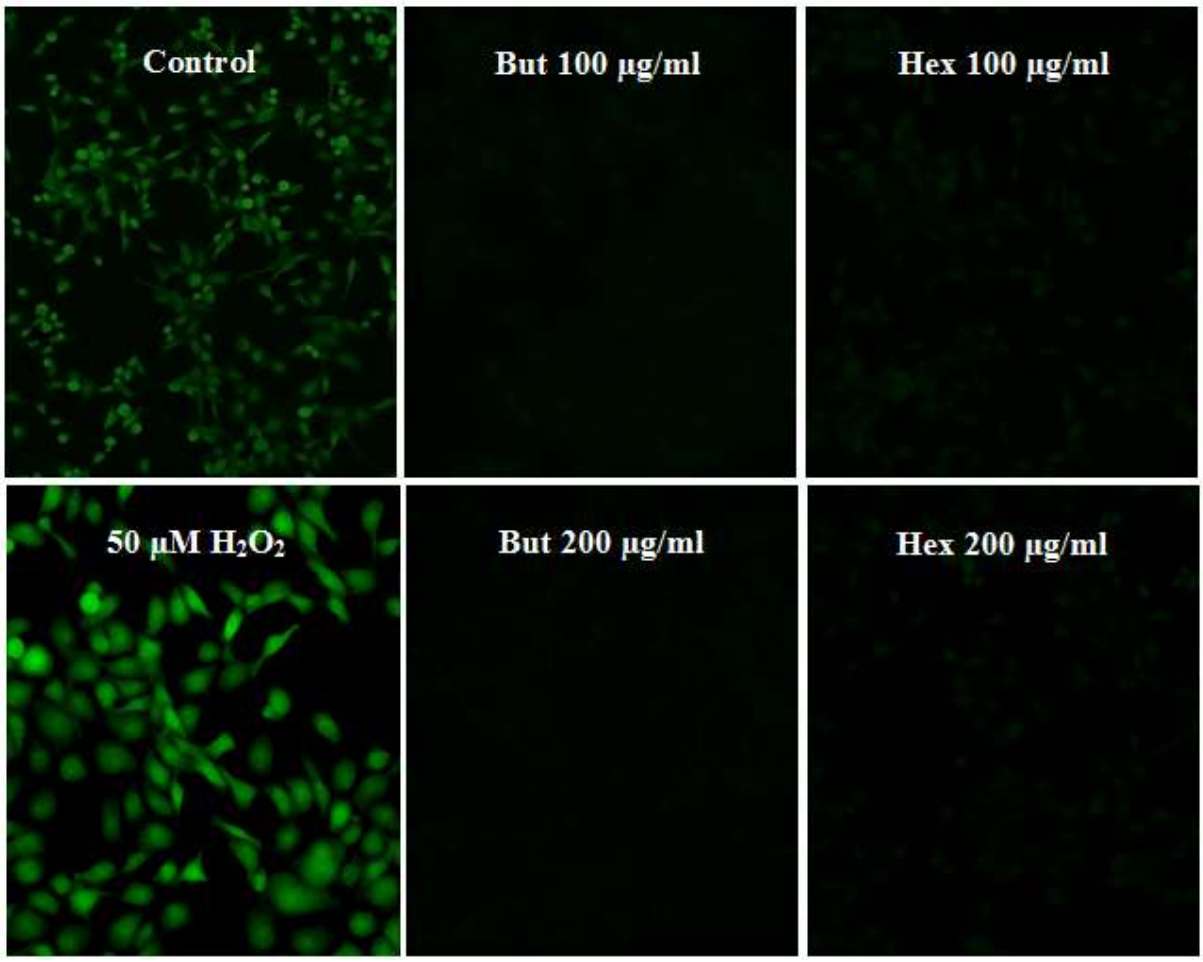

Figure 3.8: Effects of *n*-butanol and *n*-hexane fractions of *R. communis* extracts on the production of intracellular reactive oxygen species. Cells were treated with different extract concentrations and 50 µM H_2O_2 (as positive control). A fluorogenic dye, 2',7'–dichlorofluorescindiacetate (DCFH-DA), was added onto the cells after 24 hours of incubation. The fluorescence intensity relative to the amount of ROS produced within the cells was observed and pictures were taken under a fluorescence microscope with maximum excitation/emission spectra of 495/529 nm. But= *n*-butanol, Hex= *n*-hexane.

3.6 Effect of *n*-butanol and *n*-hexane fractions of *R. communis* on MCF-7 cell migration

The effect of *n*-butanol and *n*-hexane fractions on the migration of MCF-7 cells was determined by wound healing migration assay. The untreated MCF-7 cells exhibited the ability to partially close the scratched wound after 6 hours and to completely close the wound after 24 hours (figure 3.9 and 3.10). In contrast, cells treated with both *n*-butanol (figure 3.9) and *n*-hexane (figure 3.10) fractions showed partial wound closure after incubation for 6 and 24 hours. The inhibition of MCF-7 cell migration was seen to be in a time- and concentration-dependant manner. Furthermore, the fractions exhibited higher inhibition potential at 200 µg/ml than curcumin as the positive control. *n*-butanol fraction was shown to be more effective than *n*-hexane fraction at both 100 and 200 µg/ml. These findings suggest that the phytochemical compounds in the *n*-butanol and *n*-hexane fractions possess migration inhibitory activity against metastatic MCF-7 cells.

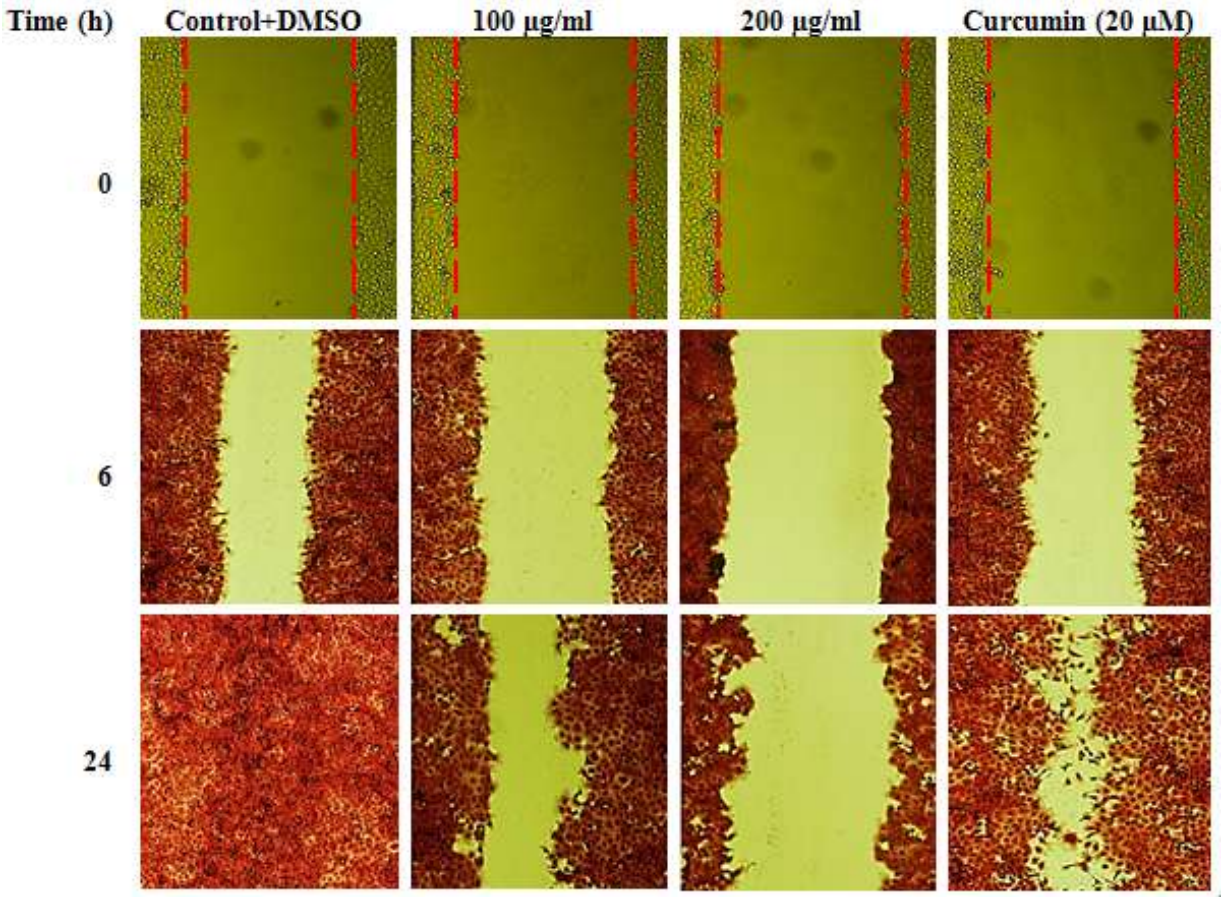

Figure 3.9: Effect of *n*-butanol fraction of *R. communis* extracts on MCF-7 cell migration. Wounded MCF-7 cell monolayers were exposed to 0, 100 and 200 µg/ml of *n*-butanol fraction and 20 µM of curcumin, as a positive control. Cell monolayers were stained with 0.5% crystal violet solution at 6 and 24 hours. Photographs of the wound were captured under an inverted light microscope (10X) after 0, 6 and 24 hours of incubation. The data are representative of four independent experiments done in duplicates.

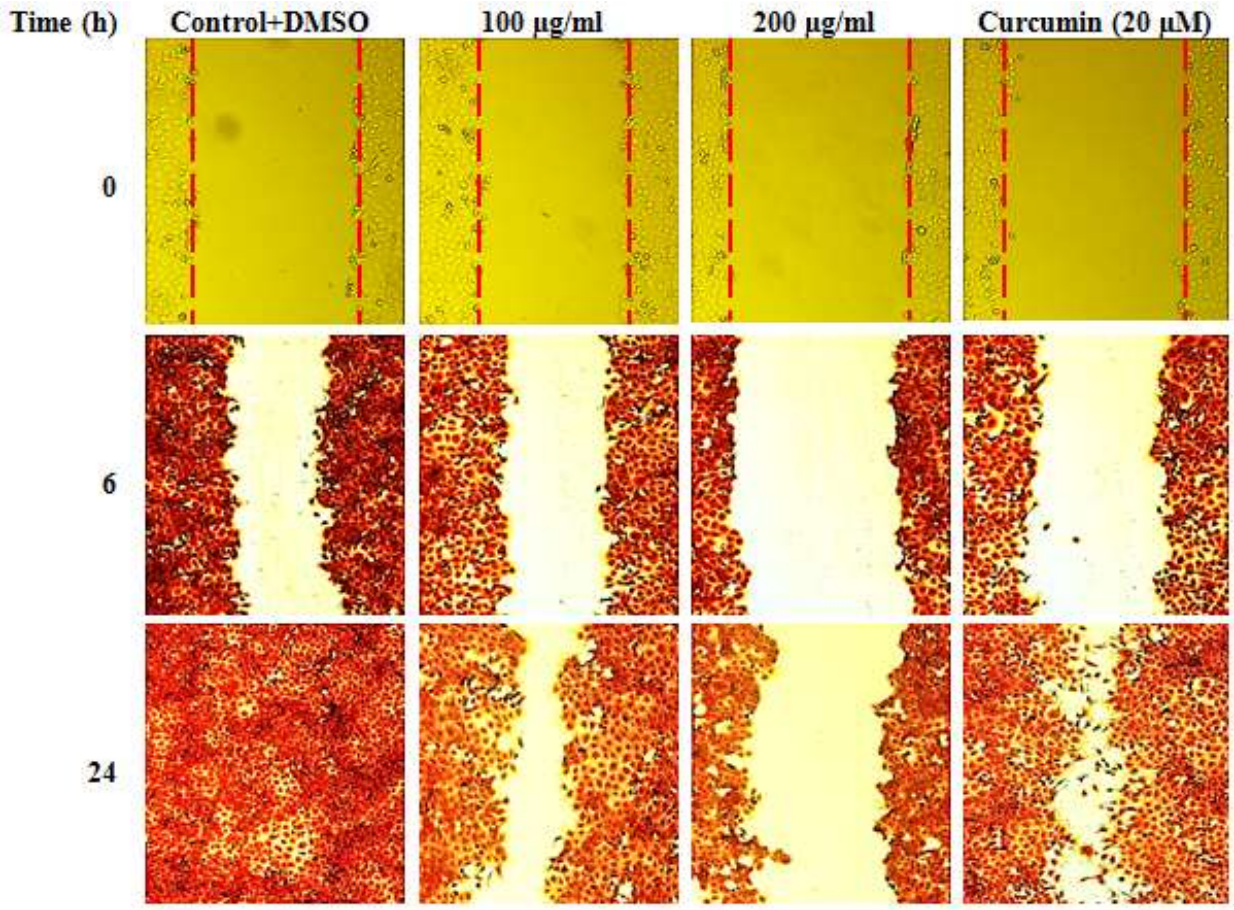

Figure 3.10: Effect of the *n*-hexane fraction of *R. communis* extracts on MCF-7 cell migration. Wounded MCF-7 cell monolayers were exposed to 0, 100 and 200 µg/ml of *n*-hexane fraction and 20 µM of curcumin, as a positive control. Cell monolayers were stained with 0.5% crystal violet solution at 6 and 24 hours. Photographs of the wound were captured under an inverted light microscope (10X) after 0, 6 and 24 hours of incubation. The data are representative of four independent experiments done in duplicates.

3.7 Effect of *n*-butanol and *n*-hexane fractions on the adhesion of MCF-7 cells

The effect of *n*-butanol and *n*-hexane fractions on the ability of MCF-7 cells to adhere was determined by the attachment assay. The results revealed that untreated cells were able to attach to the surface of the cell culture plate within 6 hours and propagated to confluency within 24 hours of incubation (figures 3.11A and 3.12A). The exposure of MCF-7 cells to 100 and 200 µg/ml of the *n*-butanol (figure 3.11) fraction reduced the ability of the cells to attach to the surface of the cell culture plate, which was demonstrated by small number of attached cells compared to the control after 6 and 24 hours of incubation. Even though the cells showed to slightly proliferate after 24 hours reflected as an increase in the number of attached cells the percentage cell adhesion was still significantly lower relative to the control (figure 3.11). On the other hand, the exposure of MCF-7 cells to 100 and 200 µg/ml of the *n*-hexane fraction led to a decreased ability of the cells to attach at both 6 and 24 hours of incubation (figure 3.12). The cell adhesion assay demonstrated that the *n*-butanol and *n*-hexane fractions retard the adhesive capability of MCF-7 cells in a time- and concentration-dependant manner.

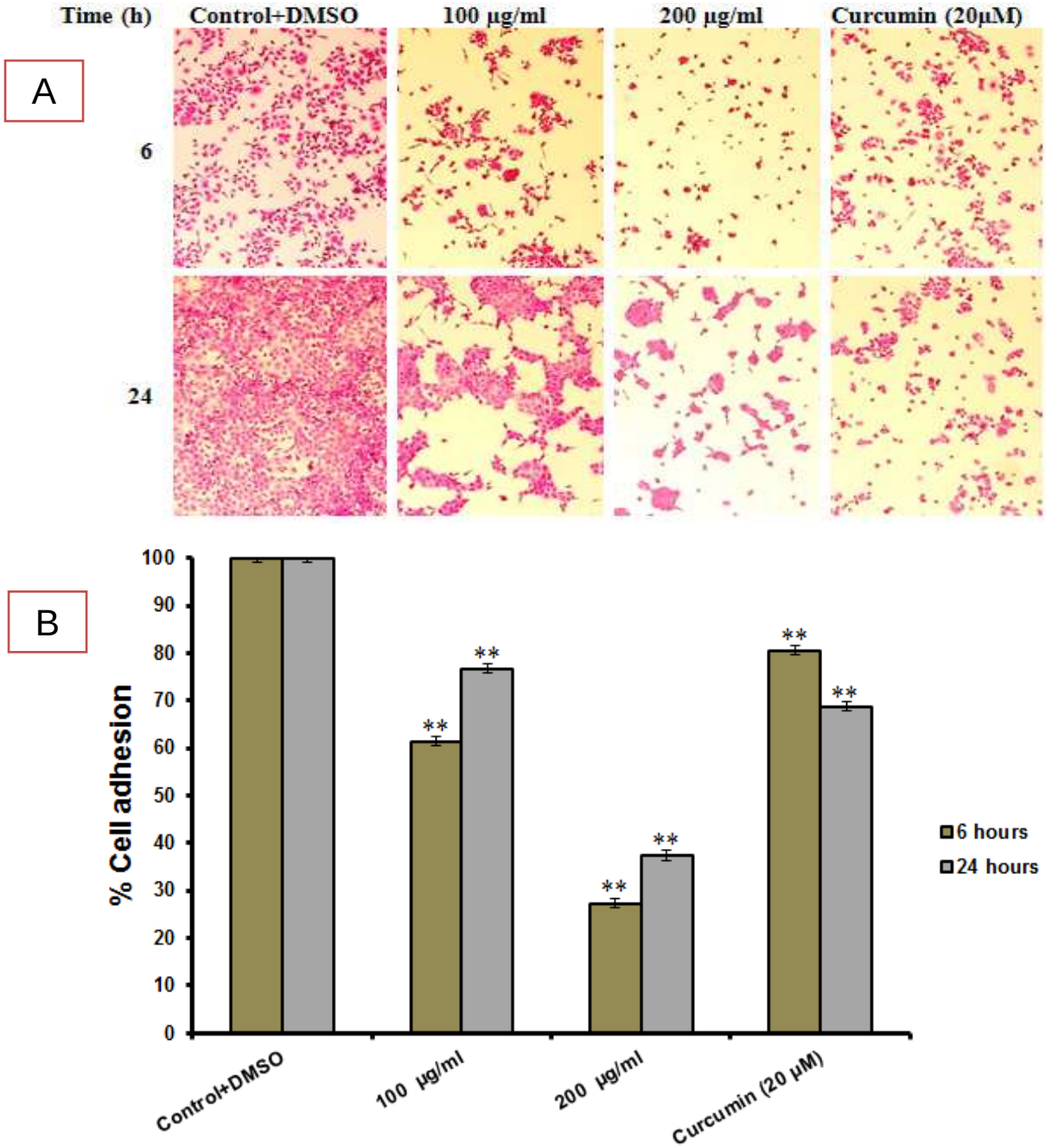

Figure 3.11: Effect of *n*-butanol fraction of *R. communis* extract on MCF-7 cell adhesion. Cells were pre-treated with the *n*-butanol fraction for 2 hours and then allowed to attach on a 6-well plate for 6 and 24 hours. Attached cells were fixed and stained with 0.5% crystal violet solution. Photographs of attached cells were captured under an inverted light microscope (10X). Percentage number of attached cells was estimated by solubilising the crystal violet stained-cells with 10% acetic acid and reading the absorbance at 560 nm using a microtiter plate reader. The data represent the mean±S.D of three independent experiments done in triplicates. *$p \leq 0.05$, **$p \leq 0.01$, indicate a significant difference to the control.

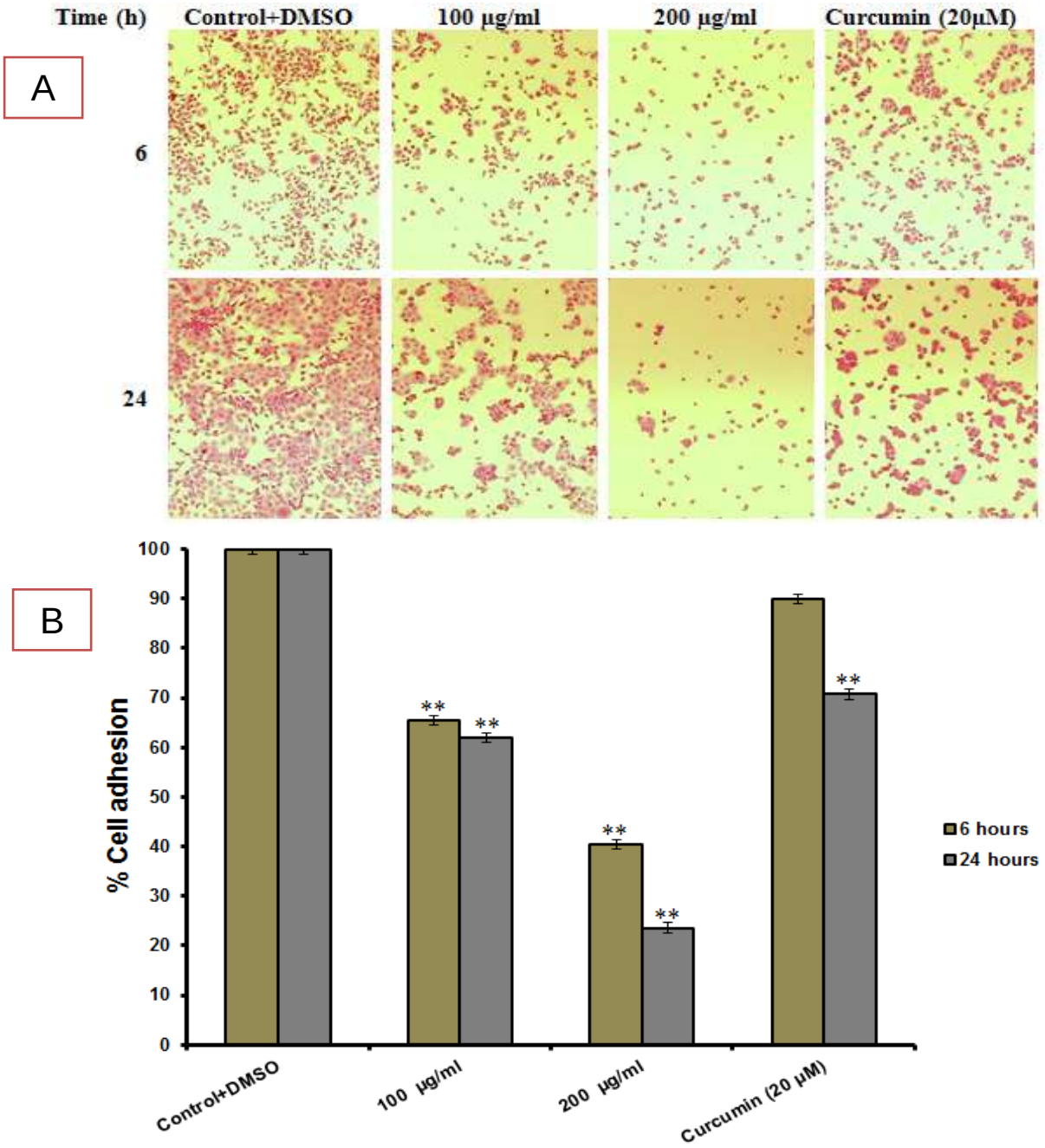

Figure 3.12: Effect of *n*-hexane fraction of *R. communis* extract on MCF-7 cell adhesion. Cells were pre-treated with the *n*-hexane fraction and then allowed to attach on a 6-well plate for 6 and 24 hours. Attached cells were fixed and stained with 0.5 % crystal violet solution. Photographs of attached cells were captured under an inverted light microscope (10X). Percentage number of attached cells was estimated by solubilising the crystal violet stained-cells with 10% acetic acid and reading the absorbance at 560 nm using a microtiter plate reader. The data represent the mean±S.D of three independent experiments done in triplicates. *$p \leq 0.05$, **$p \leq 0.01$, indicate a significant difference to the control.

3.8 Effect of *n*-butanol and *n*-hexane fractions on the invasion of MCF-7 cells

Matrigel matrix coated trans-well inserts were used to assess the effect of the *n*-butanol and *n*-hexane fractions on the invasiveness of MCF-7 cells. As represented in figure 3.14, the *n*-hexane fraction showed little or no evidence of inhibitory activity on the invasiveness of MCF-7 cells at both extract concentrations (100 and 200 µg/ml). On the other hand, the *n*-butanol fraction (figure 3.13) was shown to strongly inhibit the invasiveness of these cells in a dose-dependent manner. After 24 hours of exposure, curcumin (positive control) was shown to inhibit cell invasion comparable to the *n*-butanol fraction at 200 µg/ml. The assay revealed that compounds of *n*-butanol fraction contain anti-invasive activity against MCF-7 cancer cells. This fraction was, therefore, further evaluated for its effect on the activity of MMP-2/-9 and the expression of metastasis related proteins.

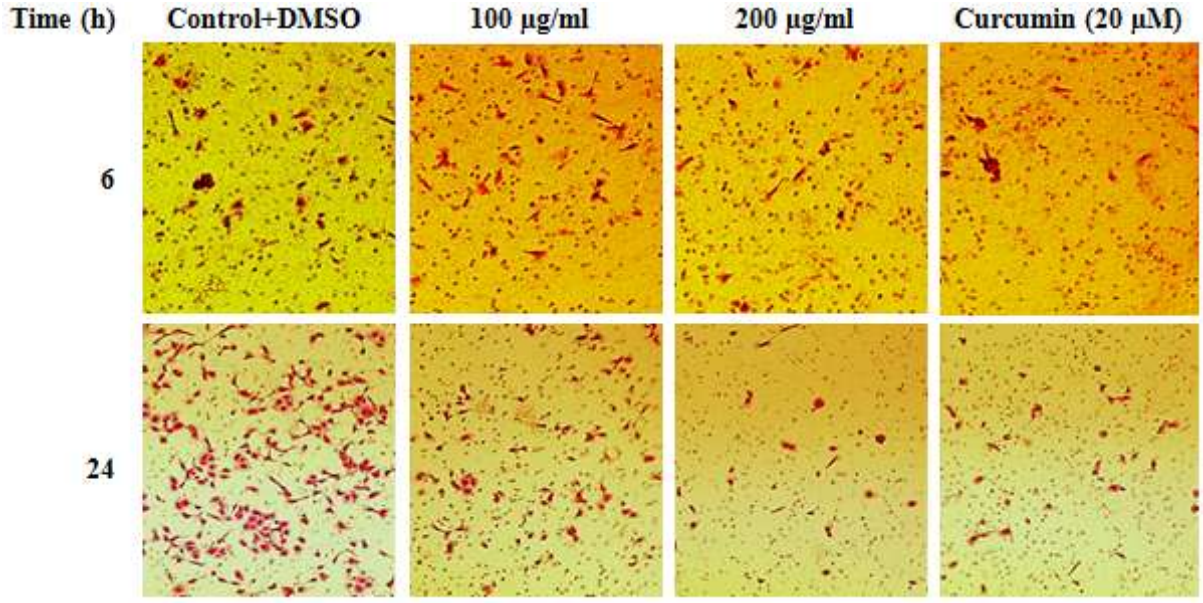

Figure 3.13: Effect of *n*-butanol fraction of *R. communis* extracts on MCF-7 cell invasion. Cells were plated in trans-well inserts coated with matrigel matrix and treated with 100 and 200 µg/ml of *n*-butanol fraction for 6 and 24 hours. Cells that invaded the matrigel matrix and migrated to the bottom of the membrane were fixed and stained with 0.5% crystal violet solution. Photographs were captured under an inverted light microscope (10X). The data are representative of four independent experiments done in duplicates.

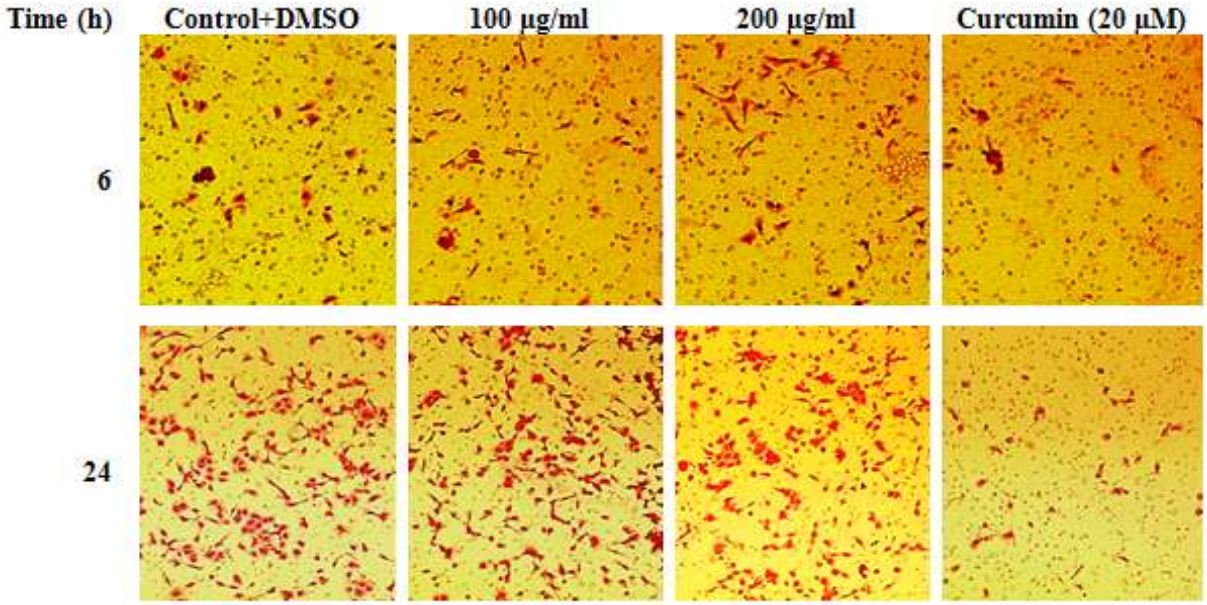

Figure 3.14: Effect of *n*-hexane fraction of *R. communis* extracts on MCF-7 cell invasion. Cells were plated in trans-well inserts coated with matrigel matrix and treated with 100 and 200 µg/ml of *n*-butanol fraction for 6 and 24 hours. Cells that invaded the matrigel matrix and migrated to the bottom of the membrane were fixed and stained with 0.5% crystal violet solution. Photographs were captured under an inverted light microscope (10X). The data are representative of four independent experiments done in duplicates.

3.9 Effect of *n*-butanol fraction on the enzymatic activity of MMP-2 and MMP-9

To assess the effect of *n*-butanol fraction of *R. communis* on the activity of MMPs, gelatin-zymography was employed. Figure 3.15, shows that untreated MCF-7 cells produced MMP-2 (72 kDa) with detectable gelatinolytic activity. Conversely, no detectable gelatinolytic activity of MMP-2 was observed in MCF-7 cells treated with concentrations (100 and 200 µg/ml) of the *n*-butanol fraction. The constitutively secreted MMP-2 activity was completely inhibited to undetectable levels by the *n*-butanol fraction. Furthermore, MCF-7 cells were not shown to produce active MMP-9 that could be observable through gelatin-zymography with or without treatment as seen by the lack of activity corresponding to the molecular weight of MMP-9 (92 kDa).

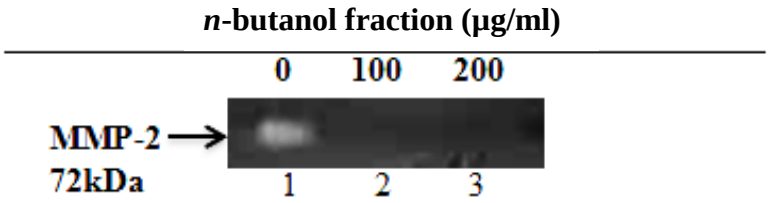

Figure 3.15: Effect of *n*-butanol fraction of *R. communis* extracts on the activity of MMP-2 and MMP-9 produced by MCF-7 cells. Cells cultured in serum free media were treated with or without the extract (100 and 200 µg/ml) for 24 hours. Following exposure, media was collected and subjected to gelatin-zymography. Photographs of the zymogram were captured using ChemiDoc XRS imager (Bio-Rad Laboratories, USA). Gelatinolytic activity is indicated by the clear band against the dark background.

3.10 Effect of *n*-butanol fraction on the expression levels of angiogenesis and metastasis related proteins.

The human angiogenesis antibody array kit was used to assess the effects of *n*-butanol fraction of *R. communis* on the expression of pro-metastatic proteins, pro-angiogenic proteins and factors that inhibit angiogenesis and metastasis. As shown in figure 3.16, cells treated with the extract were observed to express reduced levels of membrane degrading proteins (MMP-8/-9) as well as urokinase-type plasminogen activator (uPA), responsible for activation of latent proteases and growth factors implicated in cancer progression. Furthermore, the *n*-butanol fraction was seen to slightly inhibit the multifunctional protein that binds to matrix proteins important in adhesion, cell motility, angiogenesis and inflammation (thrombospondin-1). Both treated and untreated MCF-7 cells were shown to produce elevated levels of thrombospondin-1 (TSP-1) as compared to other proteins, although a decrease in expression levels was seen in treated cells. Figure 3.17, shows that the fraction also down-regulated the expression of cytokines or growth factors (angiogenin, CXCL8, FGF-1, PIGF, TGF-β1, VEGF) that play a key role in angiogenesis in a concentration-dependent manner. On the contrary, the expression of endostatin (Figure 3.18), a strong inhibitor of angiogenesis and tumour growth, was seen to be up-regulated in cells treated with the *n*-butanol fraction. Furthermore, an increase in the expression of TIMP-1 and -4, however slightly on TIMP-4, inhibitors of matrix remodelling and basement membrane degrading enzymes (MMPs), was observed (figure 3.18). The *n*-butanol fraction was demonstrated to selectively regulate the expression of pro-angiogenesis/metastasis and anti-angiogenesis/metastasis-related proteins.

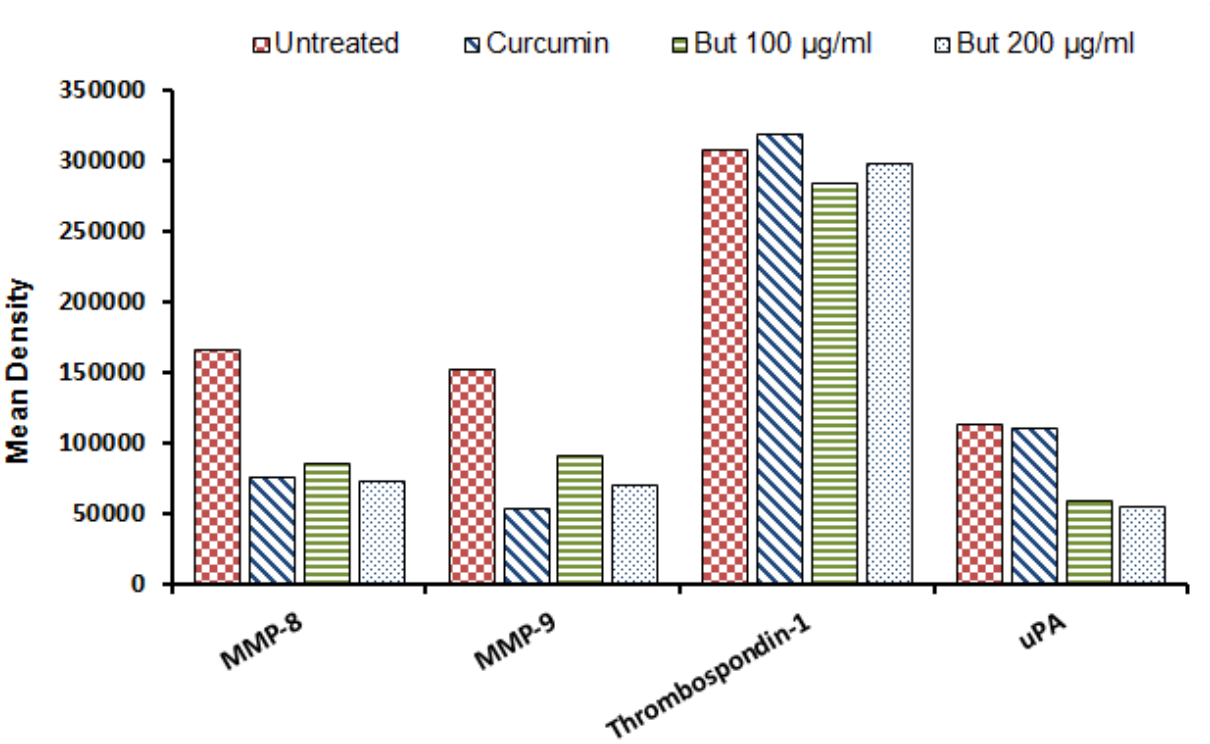

Figure 3.16: Effect of *n*-butanol fraction of *R. communis* extracts on expression levels of pro-metastatic proteins involved in degradation of the basement membrane and extracellular matrix during cancer metastasis. MCF-7 cancer cells were treated with (100 and 200 µg/ml) or without the *n*-butanol fraction for 24 hours. Protein expression levels were determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised and the signal density of each spot representing the proteins of interest was determined using ChemiDoc XRS imager. Data shows the mean signal density of each representative pair of duplicate spots on the array.

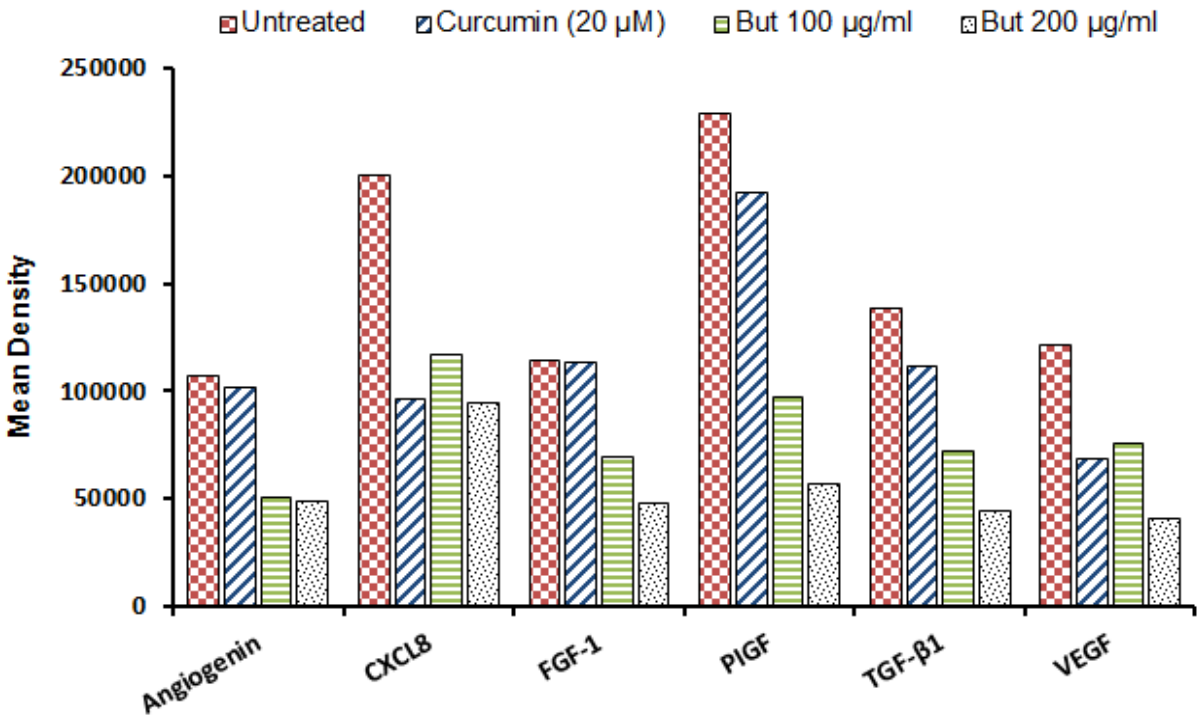

Figure 3.17: Effect of *n*-butanol fraction of *R. communis* extracts on expression levels of pro-angiogenic growth factors involved in proliferation, differentiation and migration of tumour cells during metastasis. MCF-7 cancer cells were treated with (100 and 200 µg/ml) or without the *n*-butanol fraction for 24 hours. Protein expression levels were determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised and the signal density of each spot representing the proteins of interest was determined using ChemiDoc XRS imager. Data shows the mean signal density of each representative pair of duplicate spots on the array.

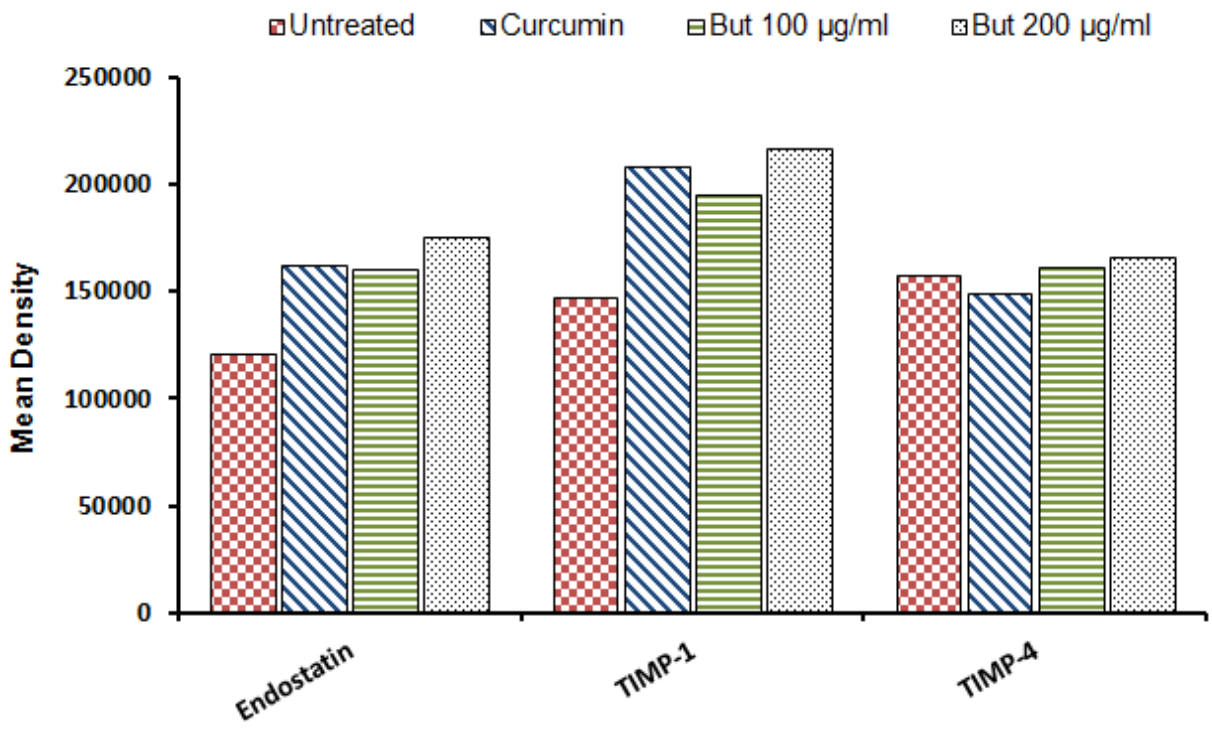

Figure 3.18: Effect of *n*-butanol fraction of *R. communis* extracts on expression levels of the antiangiogenic factor and inhibitors of MMPs. MCF-7 cancer cells were treated with (100 and 200 µg/ml) or without the *n*-butanol fraction for 24 hours. Protein expression levels were determined using the human angiogenesis antibody array kit. After the array procedure, membranes were visualised and the signal density of each spot representing the proteins of interest was determined using ChemiDoc XRS imager. Data shows the mean signal density of each representative pair of duplicate spots on the array.

CHAPTER 4

4 DISCUSSION AND CONCLUSION

Cancer stands as one of the major global health problems (Siegel *et al.,* 2012). The invasiveness of cancer is poorly treated by apoptosis inducing conventional anticancer drugs, leading to increased mortality in cancer patients (Weber, 2008). In addition to drugs that limit tumour growth by blocking cell cycle progression, treatments that lessen the spread and inhibit invasiveness of tumour cells as well as prevent them from colonising healthy tissues are required to promote patient survival and prevent cancer recurrence (Condeelis and Segall, 2003; Liotta *et al.,* 1991). Plants have always served as a good source of medicinal remedies and are continuing to play a crucial role in the management of many health conditions. At present, about 50% of all drugs used in the modern medical system are derived from plants (Mahesh and Salish, 2008; Mishra *et al.,* 2013). The pharmacological actions of most plant extracts are attributed to the nature of their phytochemical constituents, particularly secondary metabolites such as phytosterols, tannins, coumarins, alkaloids, flavonoids, etc. In addition, phenolic compounds with antioxidant potential have been widely assessed for their ability to counteract the progression of diseases such as inflammation, diabetes and cancer (Alhakmani *et al.,* 2013). The phytochemical properties and bioactivity of plant extracts as potential chemotherapeutics should be studied in order to define the molecular mechanisms by which they facilitate their disease prevention and/or treatment actions. In this study, fractions of *Ricinus communis* leaf extracts were evaluated for their anti-metastatic potential.

The phytochemical composition and antioxidant activity of six fractions (*n*-butanol, Chloroform, Ethyl-acetate, *n*-hexane, Methanol + H_2O and H_2O) of *R. communis* leaf extracts were determined by spraying TLC plates with vanillin-sulphuric acid and 0.2% DPPH, respectively. The non-polar fractions, *n*-hexane in particular, was shown to contain a variety of phytochemical compounds represented by their separation in the BEA solvent system (figure 3.1). However, not much diversity was observed in constituents of the polar fractions, evident by the presence of only yellow compounds in all the four polar fractions separated in EMW (figure 3.1). These compounds are assumed to be similar in chemical properties by virtue of their migration at the same distance within the eluent system. The polar fractions showed very good antioxidant

activity when compared to the non-polar ones (figure 3.2). The *n*-butanol fraction showed a high content of the compounds liable for the observed antioxidant activity. This activity implied that the fractions have free radical scavenging ability or can donate hydrogen to oxidants responsible for oxidative damage. A study conducted by Singh *et al.* (2009) revealed that of the numerous phytochemicals present in extracts of *R. communis* leaves, the phenolic compounds with antioxidant activity include mainly gallic acid, quercetin, gentisic acid, rutin, epicatechin and ellagic acid. Most of these compounds are largely isolated by polar alcoholic solvents. These compounds might be the ones responsible for the antioxidant activity of the fractions of *R. communis* extracts observed in this study.

The *n*-butanol and *n*-hexane fractions of *R. communis* extracts were evaluated for their effect on the viability of normal fibroblast skin cells (BUD-8) and invasive breast epithelial cancer cells (MCF-7). BUD-8 cell was shown to be very sensitive to the *n*-hexane fraction as represented by a significant decrease in the viability of these cells at low concentrations of the fraction. The *n*-butanol fraction induced a decrease in BUD-8 cell viability only at high concentrations. Of interest, the cells were shown to regain their proliferative ability at longer incubation time (after 24 hours) even in the presence of the fractions. This suggests that the observed decrease in BUD-8 cell viability may not be a consequence of the fractions' cytotoxicity but their cytostatic effects or longer exposure time may have allowed the cells to develop the capacity to resist the growth inhibitory effect of the fractions. In addition, the extracts could be losing their inhibitory potential in a time-dependant manner, allowing the cells to regain resistance and proliferative capacity. These properties were not observed in MCF-7 cells, suggesting that the fractions could be selectively inducing cytostatic activities on BUD-8 cells for a defined period. Microscopic observations of these cells also showed no change that was evident in cellular morphology that could be related to apoptotic cell death (data not shown). The fractions were also shown to reduce the viability of MCF-7 cells in a time- and concentration-dependant manner. Furthermore, the fractions were not shown to significantly decrease cell viability at concentrations of 100 and 200 µg/ml with the exception of *n*-hexane fraction that was shown to reduce the viability of BUD-8 cells by up to 30% and 20% after 24 and 48 hours, respectively.

Hoechst stain was used to assess changes in the nuclear morphology of MCF-7 cells in the presence and absence of *n*-butanol and *n*-hexane fractions at non-cytotoxic concentrations of 100 and 200 µg/ml for 24 hours. The study was done to investigate whether the extent of the fractions' toxicity observed through MTT assay would correlate to the amount of cells showing apoptotic manifestations. Cells dying through apoptosis are identified by morphological characteristics such as chromatin condensation, formation of apoptotic bodies and blebbing of cell membrane. As demonstrated by the Hoechst staining assay a small percentage of cells were shown to have apoptotic features suggesting cell death observed from the nuclei of cells treated with 100 and 200 µg/ml of the fractions. Cells treated with the fractions displayed apoptotic nuclear morphological changes to a comparable extent as the untreated cells, since cells undergo spontaneous apoptosis as a normal regulatory process. It was, therefore, confirmed by both MTT and Hoechst staining assays that at 100 and 200 µg/ml, the *n*-butanol and *n*-hexane fractions induce negligible levels of cytotoxicity and cell death of MCF-7 cells. These sub-toxic concentrations (100 and 200 µg/ml) were therefore used in subsequent assays to avoid false positive results due to the cytotoxic effects of the fractions at high concentrations.

Fractions (*n*-butanol and *n*-hexane) of *R. communis* extracts were further tested for their effects on the production of intracellular reactive oxygen species by MCF-7 cancer cells using peroxide sensitive fluorescent probe DCFH-DA. The anticancer properties of phytochemical antioxidants are contributed to their capacity to reduce oxidative stress through free radical scavenging activity or by inducing endogenous antioxidant systems (Du *et al*., 2007; Gupta and Massague, 2006). The fractions were shown to inhibit the production of intracellular ROS, evident by the very low fluorescence intensity of -treated cells as compared to that of the untreated and H_2O_2 treated cells. Although the amount of ROS produced by the cells were not quantified in this assay, the fractions illustrated good free radical scavenging activities, with *n*-butanol showing the greatest activity, possibly due to the greater number of antioxidant compounds in the fraction. Production of cellular reactive oxygen species has been shown to regulate the proteolytic activity of MMPs (Gupta *et al*., 2014), suggesting one of the possible mechanisms by which the *n*-butanol fraction may have inhibited the activity of MMP-2. The fraction may indirectly influence the regulation of the activity of MMPs through its regulatory action on the production of ROS. Saari *et al*. (1992) reported H_2O_2 and other oxides to activate pro-MMPs in

neutrophils. In addition, Zhang *et al*. (2002) also demonstrated the relationship between ROS production and the activation of MMP-2 with emphasis that antioxidant compounds with direct or indirect inhibitory effects on the levels of intracellular ROS formation, particularly peroxides, can play a crucial role in the activation of MMP-2 and therefore its enzymatic activity. This is in agreement with the ability of the fractions to inhibit metastatic activities of MCF-7 cells through the regulation of MMPs at the enzyme activity level.

Dissemination of cancer cells involves complex changes in the structure and function of tumour cells contributing to architectural modifications of the extracellular matrix (ECM) and an exchange in cell-cell as well as cell-ECM adhesive interactions (Bogenrieder and Herlyn, 2003; Condeelis *et al*., 2005; Jeanes *et al.,* 2008). These events allow individual or collective tumour cells to migrate towards and past the basement membrane through the blood vessels to distant parts of the body (Polyak, 2010). The wound healing migration assay was used to observe the effects of *n*-butanol and *n*-hexane fractions on the migration of MCF-7 cells. Cell migration is acquired by the cell's ability to form lamellipodial extensions, actin rich projections at the leading edge of the cell, and preserve attachment to the endothelial lining within the blood vessels (Berrier and Yamada, 2007). The *n*-butanol fraction was seen to impede the rate of cell migration in a concentration- and time-dependant manner. Similar manifestations were observed in cells exposed to concentrations of the *n*-hexane fraction. During cell migration, metastatic cells must carefully maintain their attachment to the ECM and within the circulatory system apart from the development of anchorage independent survival (Simpson *et al.,* 2008). Inability to maintain adherence could lead to cell-detachment induced apoptosis, known as anoikis. Cell migration can also be suppressed through inhibition of cell adhesion by moderating the expression of critical adhesion proteins such as integrins, cadherins and selectins. Both fractions demonstrated to possess compounds with the ability to prevent the migration of MCF-7 cells.

As described by Simpson *et al.* (2008), adhesion to protein components of the ECM is essential for cell survival. Adhesion of migrating cells to the endothelial cell receptors is another key process during intravasation and spread of cancer cells into the circulatory or lymphatic system (Berrier and Yamada, 2007). The *in vivo* presentation of cell-ECM interactions is defined by interactions of cell surface

receptors (integrins) with fibronectin, laminin, collagen, glycoproteins and growth factors as constituents of the ECM. In this study, polystyrene (as a sort of matrix of cell attachment) treated cell culture plates were used to determine the effect of fractions of *R. communis* leaf extracts on the ability of MCF-7 cells to adhere to a support matrix. Findings revealed the *n*-butanol fraction to significantly decrease cell-matrix adhesion. However the proliferative ability of the attached cells was not affected as was observed by an increase in cell number after 24 hours of exposure. The *n*-hexane fraction was shown to be a more potent inhibitor of adhesion as well as cell proliferation. The findings show the *n*-butanol and *n*-hexane fractions of *R. communis* to reduce the adhesive capabilities of MCF-7 cells in a concentration-dependent manner. Cell adhesion may be regulated by the dynamics of the ECM. The ECM is essentially remodelled by MMP-2/-9 to suit the interactions of exchanged cell adhesion molecules to protein components of the ECM. Gangly *et al.* (2012) showed that MMP-9 expression and activity play a crucial role in the regulation of cell adhesion. This suggests that these fractions may be inhibiting the adhesive ability of MCF-7 cells by regulating adhesion proteins that are also substrates of MMPs.

Invasive tumour cells are characterised by their ability to induce proteolysis of the surrounding ECM as well as degradation of the basement membrane. This process allows cells to break out of the primary tumour and disseminate to target sites in the body (Zhang *et al.,* 2013). One of the widely explored factors that relate to the invasiveness of tumour cells is secretion of proteases. Over-expression of matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9, have been shown in several types of human cancers including breast, lung and ovarian cancers (Ala-aho and Kothari, 2005; Fisher *et al.,* 2009; Quan *et al.,* 2009). To assess the effect of *n*-butanol and *n*-hexane fractions on the invasiveness of MCF-7 cells, matrigel matrix-coated trans-well inserts were used. It was revealed that the *n*-butanol fraction inhibited the invasiveness of MCF-7 cells in a concentration-dependant manner. This was demonstrated by the reduced amount of cells that transited through the matrigel matrix barrier (as the basement membrane) over to the bottom of the trans-well. On the other hand, cells maintained the ability to permeate through the matrigel matrix coating even in the presence of concentrations of the *n*-hexane fraction that was effective in the previous assays. These findings suggest that the *n*-butanol fraction inhibited the invasiveness of MCF-7 cells by suppressing the expression or activities of MMP-2 and/or MMP-9. Since the *n*-butanol fraction was shown to be less toxic to

normal BUD-8 cells and exhibited strong inhibitory actions in preceding assay, i.e. MCF-7 cell migration, adhesion and invasion as well as intracellular ROS production, the fraction was selected for further evaluation in the subsequent assays over the *n*-hexane fraction which was not shown to impede the invasiveness of MCF-7 cells.

Despite the normal physiological functions of MMPs, they are also implicated in the progression of cellular processes that lead to development of many pathological conditions such as angiogenesis and metastasis (Attolino *et al.,* 2010). During cancer invasion or metastasis, MMPs degrade collagen, laminin, fibronectin, etc which are major protein components of the extracellular matrix and basement membrane (Wang *et al.,* 2012). Hence substances with the ability to suppress activities of MMPs are regarded as potential anti-metastatic agents. To investigate the mechanism by which the invasiveness of MCF-7 cells was suppressed, the effect of *n*-butanol fraction on activities of MMP-2/-9 was assessed through gelatin-zymography. Findings revealed the presence of a band that corresponded to the molecular weight of MMP-2 (72 kDa) only in the control conditioned media, while no band was comparable to the molecular weight of MMP-9 (92 kDa). From this, it is evident that the gelatinolyic activity of MMP-2 was inhibited in cells exposed to concentrations of *n*-butanol fraction. Furthermore, the fraction may have down-regulated the expression level of MMP-2 corresponding to the reflected reduction in activity (figure 3.15), as the fraction was also shown to reduce the expression levels of related pro-metastatic proteins responsible for ECM and basement membrane degradation in figure 3.16. However, the lack of MMP-9 activity on the zymogram suggests that MCF-7 cells may be secreting very low basal levels of MMP-9. Cancer cell lines do not secrete the same levels of MMPs and can as well be classified based on the amount of MMPs they express. The expression of MMP-9 is highly controlled by growth factors, cytokines and signal transduction pathways. A study by Roomi *et al* .(2009), in which PMA was used to stimulate the expression of MMPs, reported ovarian cancer (SK-OV-3) cell line to express between 0–2% basal MMP-9 levels. The study further demonstrated that breast cancer (MCF-7) cell line only shows the expression of MMP-9 after PMA stimulation. Thus, no band correlating to both MMP-2 and MMP-9 is observed in media without PMA. It was also demonstrated that MMP-2 is constitutively expressed in many cell lines and its secretion is not affected by inducers, while MMP-9 is typically not expressed at substantial levels though inducible (Roomi *et al.,* 2009). Compounds of natural origin have not only been

presented as inhibitors of the enzymatic activities of MMPs but also as suppressors of their expression in tumour cells (Wang *et al.,* 2012). It is still a question as to how the *n*-butanol fraction counteracts the function of MMP-2. Several compounds are contained in the fraction and they could be targeting different regulatory points to suppress the function of MMP-2 as shown by other studies that bioactive compounds in plant extracts exhibit their anticancer effects as a complex mixture through complementary and intersecting mechanisms (Fernandez-Panchon *et al*., 2008; Liu, 2004). The expression of MMPs activities is regulated at many levels, i.e. pro-enzyme activation, protease inhibition, secretion, translation and transcription (Gupta *et al*., 2014). One of the critical steps in defining the effects of *n*-butanol fraction on the activity of MMPs, particularly MMP-2, is determining the point of regulation at which the fraction acts on the protease(s). Therefore, it may be concluded that constituents of *n*-butanol fraction hindered the activity of MMP-2 by inhibiting its enzymatic activities or down-regulation of the protease's expression.

Various cytokines, growth factors (TGF-β, VEGF, FGF-1, etc.) and inhibitors have a defined involvement in metastasis and angiogenesis; for example, they have been shown to have regulatory properties on the expression MMP-2 and MMP-9 (Roomi *et al*., 2010). The human angiogenesis array kit was used to assess the effects of the *n*-butanol fraction on the expression levels of pro-metastatic proteins, pro-angiogenic cytokines and growth factors, and inhibitors of angiogenesis and metastasis. The fraction was shown to decrease the expression levels of promoters of metastatic processes including urokinase plasminogen activator (uPA). Several studies have reported uPA to initiate the activation of latent MMP-2 and MMP-9, supporting its function as a system that catalyses the transformation of basement membrane and extracellular matrix degrading proteases (Sasaki *et al*., 2015; Tang and Han, 2013) and thereby showing its direct involvement in the promotion of cell migration and invasion in many cancers. The expression and activity of MMPs, was shown to decrease in MCF-7 cells exposed to the *n*-butanol fraction. This suggests that the observed inhibition of cell invasiveness and MMP-2 activity may be due to the down-regulation of uPA. Thrombospondin-1 (TSP-1), a major activator of transforming growth factor-β1 (TGF-β1), binds to a number of cell membrane adhesion molecules and ECM proteins. Its down-regulation is associated with a decrease in the activity of TGFβ1 and a consequent inhibition of cell invasion, migration and EMT transition (Lopez-Dee *et al*., 2011).

Several studies have reported TGF-β1 as one cytokine known to promote morphological transition (EMT) in cancer cells and as well implicated in tumour cell migration and invasion (Bierie and Moses, 2010; Katz and Chen, 2013; Padua and Massagu´e, 2009; Sheen *et al*., 2013). Furthermore, a study by Tan *et al*. (2009) showed that breast cancer patients produce increased plasma levels of TGF-β1. The *n*-butanol fraction was shown to decrease the expression of all the growth factors investigated in this study. The inhibitory effects of the fraction on migration of MCF-7 cells may be related to its ability to reduce the expression of these angiogenic factors. The fraction's ability to down-regulate the expression levels of TGF-β1, VEGF, angiogenin and other pro-angiogenic factors suggests the fraction's potential as a source of antitumor agents. On the contrary, inhibitors of angiogenesis and metastasis were shown to be up-regulated in cells treated with the *n*-butanol fraction. It may be concluded that the *n*-butanol fraction selectively suppresses the expression of factors that promote metastasis and a great focus should be given to illustrate the molecular network induced by the fraction between the selected metastatic and angiogenic proteins.

Studies have analysed the anti-breast cancer properties of numerous extracts from different plant sources in their crude, fractionated and purified forms (Levitsky and Dembitsky, 2015). Other extracts have been further studied to determine their activity against defined metastatic processes using bioassays described in this study. A study by Khan and Mukhtar, (2010) reported that reduction in invasion and migration of prostate cancer PC-3 cells treated with (−)-epigallocatechin-3-gallate, polyphenolic compound in green tea, is facilitated through inhibition in the expression of VEGF, uPA. Root extract of *Withania somnifera* displayed a dose-dependent inhibition of human MDA-MB-231 and mouse 4T1 cancer cell migration determined through the wound healing assay (Yang *et al.,* 2013). *Lophatheri Herba* ethanol extracts were demonstrated to supress transwell invasion and migration of triple negative breast MDA-MB231 cancer cells (Kim *et al.,* 2016). The anti-cancer activity of *R. communis* extracts are well known, however, this study has reported particularly on the anti-metastatic effects of *R. communis* semi-purified leaf extracts at non-cytotoxic doses using MCF-7 breast cancer cells as a model.

In conclusion, the potential of *R. communis* fractions as effective source of anti-metastatic agents has been demonstrated in this study. Antioxidant compounds, as

some of the major constituents contained in the evaluated fractions, are deemed responsible for the decrease in generation of ROS by MCF-7 cells. However, the mechanisms of ROS scavenging activity by the fractions must be further studied to explain whether the activity is by induction of endogenous antioxidant systems or by direct action of the phytochemical antioxidants contained in the fractions. Relative to the *n*-hexane fraction, the *n*-butanol fraction is suggested to exhibit a higher free radical scavenging activity and should, therefore, be particularly investigated for its potential in the treatment of diseases such as inflammation and malignant cancers triggered by oxidative stress. Following that, the complete shutdown of the activity of MMP-2 can be linked to action of the fractions on inhibition of ROS (known to activate pro-MMPs) formation. These findings are in agreement with many other studies that proved the modulation of MMP-2 activity by cell produced reactive oxygen species. On the other hand, the MCF-7 cell line used in this study was not shown to produce detectable amounts of MMP-9, suggesting that the production of MMP-2 and MMP-9 is regulated through different pathways. This study also provides sufficient evidence to conclude that the inhibitory activity of *n*-butanol fraction on invasion of MCF-7 cells is subsequent to the presence of antioxidant active compounds. To successfully migrate, cancer cells must be able to maintain their adherence to surrounding matrices (ECM and vascular endothelium). The inhibitory effects of the fractions (*n*-butanol and *n*-hexane) on migration of MCF-7 cells can hence be attributed to the ability of the fractions to reduce the adhesive capabilities of the cells. This study has extensively revealed that fraction of *R. communis* leaves impede metastasis by inhibiting the migration, invasion (through the repressed expression of MMP-2, known to participate in the promotion of cancer invasiveness) and adhesion of metastatic breast (MCF-7) cancer cells. This is the first time that sub-fractions of *R. communis* leaf extract were reported to inhibit the migration and invasion and attachment of MCF-7 cells. Metastasis and angiogenesis linked proteins investigated in this study participate in many signaling pathways applicable to cancer. Further studies should be undertaken to determine the interconnectivity of these pathways. The ability of *R. communis* semi-purified extracts to down-regulate the expression levels of cancer associated proteins suggests that they can block or limit the progress of pathways implicated in metastasis. Studying this relationship may possibly define the mechanisms by which the extracts function as anti-metastatic agents, as fundamentally characterised in this study.

CHAPTER 5

5 References

Aitken, K.J. and Bägli, D.J. 2009. The bladder extracellular matrix, Part I: Architecture, development and disease. *Nature Reviews Urology,* **6**: 596–611.

Ala-aho, R. and Kahari, V. 2005. Collagenases in cancer. *Biochimie*, **87**: 273–286.

Alhakmani, F., Kumar, S. and Khan, S.A. 2013. Estimation of total phenolic content, *in-vitro* antioxidant and anti-inflammatory activity of flowers of *Moringa oleifera*. *Asian Pacific Journal of Tropical Biomedicine*, **3**(8): 623–627.

Amsath, A. 2013. Isolation techniques of bioactive molecules from plants and their applications. *International journal of current tropical medicinal plants and health research*, **1**(1): 001–004.

Attolino, E., Calderone, V., Dragoni, E., Fragai, M., Richichi, B., Luchinat, C. and Nativi, C. 2010. Structure-based approach to nanomolar, water soluble matrix metalloproteinases inhibitors (MMPIs). *European Journal of Medicinal Chemistry*, **45**: 5919–5925.

Azaizeh, H., Fulder, S., Khalil, K. and Said, O. 2003. Ethnomedicinal knowledge of local Arab practitioners in the Middle East Region. *Fitoterapia*, **74**: 98–108.

Bendas, G. and Borsig, L. 2012. Cancer cell adhesion and metastasis: selectins, integrins, and the inhibitory potential of heparins. *International Journal of Cell Biology,* **2012**: 1–10.

Bergers, G. and Benjamin, L.E. 2003. Tumorigenesis and the angiogenic switch. *Nature Reviews Cancer*, **3**: 401–410.

Berrier, A.L. and Yamada, K.M. 2007. Cell–matrix adhesion. *Journal of Cellular Physiology*, **213**(3): 565–573.

Bierie, B. and Moses, H.L. 2010. “Transforming growth factor beta (TGF-β) and inflammation in cancer.” *Cytokine and Growth Factor Reviews*, **21**(1): 49–59.

Bogenrieder, T. and Herlyn, M. 2003. Axis of evil: molecular mechanisms of cancer metastasis. *Oncogene*, **22**(42): 6524–6536.

Bray, F., Jemal, A., Grey, N., Ferlay, J. and Forman, D. 2012. Global cancer transitions according to the human development index (2008–2030): A population-based study. *Lancet Oncology*, **13**: 790–801.

Carmona-Fontaine, C., Matthews, H. and Mayor, R. 2008. Directional cell migration *in vivo*. *Landes Bioscience,* **2**(4): 240–242.

Cayre, M., Canoll, P. and Goldman, J.E. 2009. Cell migration in the normal and pathological postnatal mammalian brain. *Progress in Neurobiology,* **88**(1): 41–63.

Chambers, A.F., Groom, A.C. and MacDonald, I.C. 2002. Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews Cancer,* **2**(8): 563–72.

Chanda, S., Dave, R. and Kaneria, M. 2011. *In vitro* antioxidant property of some Indian medicinal plants. *Research Journal of Medicinal Plants,* **5**(2): 169–179.

Chia, J., Du, J., Hsu, W., Sun, A., Chiang, C. and Wang, W. 2010. Inhibition of metastasis, angiogenesis, and tumour growth by Chinese herbal cocktail Tien-Hsien liquid. *BMC cancer*, **10**(175): 1–16.

Ciocan, I.D. and Bara, I.I. 2007. Plant products as antimicrobial agents. *Analele Ştiinţifice ale Universităţii, Alexandruloan Cuza, Secţiunea Geneticăşi Biologie Moleculară*, **8**: 151–156.

Condeelis, J, Singer, R.H. and Segall, J.E. 2005. The great escape: when cancer cells hijack the genes for chemotaxis and motility. *Annual Review of Cell and Developmental Biology*, **21**: 695–718.

Condeelis, J. and Segall, J.E. 2003. Intravital imaging of cell movement in tumours. *Nature Reviews Cancer*, **3**(12): 921–30.

Cosa, P., Vlietinck, A.J., Berghe, D.V. and Maes, L. 2006. Anti-infective potential of natural products: How to develop a stronger *in vitro* 'proof-of-concept'. *Journal of Ethnopharmacology,* **106**: 290–302.

Cox, T.R. and Erler, J.E. 2011. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. *Disease Models and Mechanisms*, **4**(2): 165–178.

Cragg, G.M. and Newman, D.J. 2013. Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta*, **1830**: 3670–95.

Criagg, G.M. and David, J.N. 2001. Natural product drug discovery in the next millennium. *Journal of Pharmaceutical Biology,* **39**: 8–17.

D'Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C. and Masella, R. 2007. Polyphenols, dietary sources and bioavailability. *Annali Dell Istituto Superiore Di Sanita,* **43**(4): 348–361.

Daniel, B. and DeCoster, M.A. 2004. Quantification of sPLA2-induced early and late apoptosis changes in neuronal cell cultures using combined TUNEL and DAPI staining. *Brain Research Protocols*, **13**:144–150.

Darmanin, S., Wismayer, P.S., Podesta, M.T.C., Micallef, M.J. and Buhagiar, J.A. 2009. An extract from *Ricinus communis L.* leaves possesses cytotoxic properties and induces apoptosis in SK-MEL-28 human melanoma cells. *Natural Product Research,* **23**(6): 561–571.

Davis, G.E. and Senger, D.R. 2005. Endothelial extracellular matrix: Biosynthesis, remodelling, and functions during vascular morphogenesis and neovessel stabilization. *Circulation Research,* **97**: 1093–1107.

Derksen, P.W., Liu, X., Saridin, F., Van der Gulden, H., Zevenhoven, J., Evers, B., Van Beijnum, J.R., Griffioen, A.W., Vink, J. and Krimpenfort, P. 2006. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell,* **10**: 437–449.

Dittz, D., Figueiredo, C., Lemos, F.O., Viana, C.T.R., Andrade, S.P., Souza-Fagundes, E.M., Fujiwara, R.T., Salas, C.E. and Miriam, T.P. Lopes. 2015. Antiangiogenesis, loss of cell adhesion and apoptosis are involved in the antitumoral activity of proteases from *V. cundinamarcensis* (*C. candamarcensis*) in murine melanoma B16F1. *International Journal Molecular Science*, **16**: 7027–7044.

Dong, Z., Nemeth, J.A., Cher, M.L., Palmer, K.C., Bright, R.C. and Fridman, R. 2001. Differential regulation of matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1 (timp-1) and timp-2 expression in co-cultures of prostate cancer and stromal cells. *International Journal of Cancer*, **93**: 507–515.

Du, Y., Guo, H. and Lou, H. 2007. Grape seed polyphenols protect cardiac cells from apoptosis via induction of endogenous antioxidant enzymes. *Journal of Agricultural and Food Chemistry*, **55**(5): 1695–1701.

Ekor, M. 2014. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology,* **4**(177): 1–10.

Fernandez-Panchon, M.S., Villano, D., Troncoso, A.M. and Garcia-Parrilla, M.C. 2008. Antioxidant activity of phenolic compounds: From *in vitro* results to *in vivo* evidence. *Critical Reviews in Food Science and Nutrition*, **48**: 649–671.

Finger, E.C. and Giaccia, A.J. 2010. Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. *Cancer and Metastasis Reviews*, **29**: 285–293.

Fisher, G.J., Quan, T., Purohit, T., Shao, Y., Cho, M.K., He, T., Varani, J., Kang, S. and Voorhee, J.J. 2009. Collagen fragmentation promotes oxidative stress and elevates matrixmetalloproteinase-1 in fibroblasts in aged human skin. *American Journal of Pathology*, **74**(1): 101–114.

Fred Hutchinson Cancer Research Center. 2011. Brain cell migration during normal development may offer insight on how cancer cells spread. *ScienceDaily.* http://www.sciencedaily.com/releases/2011/04/110424152453.htm (Retrieved 26 June 2013).

Ganguly, K.K., Sen, T., Pal, S., Biswas, J. and Chatterjee, A. 2012. Studies on focal adhesion kinase in human breast cancer cell MDA-MB-231. *Advances in Biological Chemistry*, **2**: 29-42.

Gawecka, J.E., Young-Robbins, S.S., Sulzmaier, F.J., Caliva, M.J., Heikkila, M.M., Matter, M.L. and Ramos, J.W. 2012. RSK2 protein suppresses integrin activation and fibronectin matrix assembly and promotes cell migration. *Journal of Biological Chemistry,* **287**(52): 43–44.

Geiger, T.R. and Peeper, D.S. 2009. Metastasis mechanisms. *Biochemica et Biophysica Acta*, **1796**(2): 293–308.

Gomes, C.A., Junior, C.S., do Valle, J.A., de Assis Lopes, C.T., Barra, A., Gomes, C.C., do Carmo, T.H.L. and Gomes, F.C. 2012. Cancer metastasis. *Cell and Developmental Biology,* **1**(5): 1–10.

Gu, Y., Dee, C.M. and Shen, J. 2011. Interaction of free radicals, matrix metalloproteinases and caveolin-1 impacts blood-brain barrier permeability. *Frontiers in Bioscience (Scholar Edition),* **1**(3): 1216–1231.

Gupta, A., Kaur, C.D., Jangdey, M. and Saraf, S. 2014. Matrix metalloproteinase enzymes and their naturally derived inhibitors: Novel targets in photocarcinoma therapy. *Ageing Research Reviews*, **13**: 65–74.

Gupta, G.P. and Massague, J. 2006. Cancer metastasis: building a framework. *Cell,* **127**: 679-695.

Hamel, P., Abed, E., B., Brissette, L. and Moreau, R. 2008. Characterization of oxidized low-density lipoprotein-induced hormesis-like effects in osteoblastic cells. *American Journal of Physiology Cell Physiology,* **294**: 1021–1033.

Han, X., Shen, T. and Lou, H. 2007. Dietry polyphenols and their biological significance. *International Journal of Molecular Sciences,* **8**: 950–988.

Hanahan, D. and Weinberg, R.A. 2011. Hallmarks of cancer: the next generation. *Cell*, **144**: 646–674.

Hunter, K.W., Crawford, N.P.S. and Alsarraj, J. 2008. Mechanisms of metastasis. *Breast Cancer Research*, **10**(1): 1–10.

Ingale, A.G. and Hivral, A.U. 2010. Pharmacological studies of *Passiflora sp.* and their bioactive compounds. *African Journal of Plant Science,* **4**: 417–426.

Iqbal, J., Zaib, S., Farooq, U., Khan, A., Bibi, I. and Suleman, S. 2012. Antioxidant, antimicrobial, and free radical scavenging potential of aerial parts of *Periplocaaphylla* and *Ricinus communis*. *ISRN Pharmacology,* **2012**: 1–6.

Jasuja, N.D., Choudhary, J., Sharama, P., Sharma, N. and Joshi, S.C. 2012. A review on bioactive compounds and medicinal uses of *Commiphoramukul*. *International Journal of Plant Sciences,* **7**(4): 113–137.

Jeanes, A., Gottardi, C.J. and Yap, A.S. 2008. Cadherins and cancer: how does cadherin dysfunction promote tumour progression? *Oncogene*, **27**(55): 6920–6929.

Jena, J. and Gupta, A.G. *Ricinus communis linn:* a phytopharmacological review. 2012. *International Journal of Pharmacy and Pharmaceutical Sciences,* **4**(4): 25–29.

Jossin, Y. and Cooper, Y.A. 2011. Reelin, Rap1 and N-cadherin orient the migration of multipolar neurons in the developing neocortex. *Nature Neuroscience,* **14**: 697–703.

Kadri, A., Gharsallah, N., Damak, M. and Gdoura, R. 2011. Chemical composition and *in vitro* antioxidant properties of essential oil of *Ricinus communis L. Journal of Medicinal Plants Research,* **5**(8): 1466–1470.

Kallergi, G., Papadaki, M.A., Politaki, E., Mavroudis, D., Georgoulias, V. and Agelaki, S. 2011. Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. *Breast Cancer Research,* **13**(59): 1–11.

Kalluri, R. 2003. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nature Reviews Cancer*, **3**: 422–433.

Katz, L.H., Li, Y. and Chen, J.S. 2013. “Targeting the TGF-β signalling in cancer”. *Expert Opinion on Therapeutic Targets*, **17**(7)743–760.

Kerbel, R.S. 2008. Tumor angiogenesis. *New England Journal of Medicine*, **358**: 2039–2049.

Khan, N. and Mukhtar, H. 2010. Cancer and metastasis: prevention and treatment by green tea. *Cancer Metastasis Reviews,* **29**(3): 435–445.

Kim, A., Im, M., Gu, M.J. and Ma, J.Y. 2016. Ethanol extract of *Lophatheri Herba* exhibits anti-cancer activity in human cancer cells by suppression of metastatic and angiogenic potential. *Scientific Reports,* **6**: 1–14.

Klastersky, J. 2004. Management of fever in neutropenic patients with different risks of complications. *Clinical Infectious Diseases*, **39**: 32–37.

Klastersky, J., Paesmans, M. and Rubenstein, E. 2000. The MASCC Risk Index: a multinational scoring system to predict low-risk febrile neutropenic cancer patients. *Journal Clinical Oncology*, **18**: 3038–3051.

Koul, O. and S. Walia, S. 2009. Comparing impacts of plant extracts and pure allelochemicals and implications for pest control. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources,* **4**: 1–30.

Kumar, V., Abbas, A.K., Fausto, N. and Aster, J.C. 2010. Robbins and Cotran pathologic basis of disease. 8th edition. Philadelphia, USA: *Saunders*. Pp. 265.

Kupai, K. 2010. Matrix metalloproteinase activity assays: Importance of zymography. *Journal of Pharmacological and Toxicological Methods,* **61**(2): 205–209.

Lahlou, M. 2013. The success of natural products in drug discovery. *Pharmacology and Pharmacy,* **4**: 17–31.

Lamouille, S., Xu, J. and Derynck, R. 2014. Molecular mechanisms of epithelial–mesenchymal transition. *Nature Reviews Molecular Cell Biology*, **15**(3): 178–196.

Lee, S.H., Jaganath, I.B., Wang, S.M. and Sekaran, S.D. 2011. Anti-metastatic effects of *Phyllanthus* on human lung (A549) and breast (MCF-7) cancer cell lines. *Public Library of Science,* **6**(6): 1–14.

Lee, Y.C., Lin, H.H., Hsu, C.H., Wang, C.J. and Chiang, T.A. 2010. Inhibitory effects of andrographolide on migration and invasion in human non-small cell lung cancer A549 cells via downregulation of PI3K/Akt signaling pathway. *European Journal of Pharmacology,* **632**: 23–32.

Levitsky, D.O. and Dembitsky, V.M. 2015. Anti-breast Cancer Agents Derived from Plants. *Natural Products and Bioprospecting,* **5**:1–16.

Ley, K., Laudanna, C., Cybulsky, M.I. and Nourshargh, S. 2007. “Getting to the site of inflammation: the leukocyte adhesion cascade updated”. *Nature Reviews Immunology,* **7**(9): 678–689.

Liotta, L.A., Steeg, P.S. and Stetler-Stevenson, W.G. 1991. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell*, **64**(2): 327–336.

Liu, R.H. 2004. Potential Synergy of phytochemicals in cancer prevention: Mechanism of action. *Journal of Nutrition*, **134**: 3479–3485.

Lopez-Dee, Z., Pidcock, K. And Gutierrez, L.S. 2011. Thrombospondin-1: Multiple paths to inflammation. *Mediators of Inflammation*, **2011**: 1–10.

Lu, P., Weaver, V.M. and Werb, Z. 2012. The extracellular matrix: A dynamic niche in cancer progression. *Journal of Cell Biology,* **196**(4): 395–406.

Mahesh, B. and Satish, S. 2008. Antimicrobial activity of some important medicinal plant against plant and human pathogens. *World Journal of Agricultural Sciences*, **4**: 839–843.

Marchioli, R., Schweiger, C., Levantesi, G., Tavazzi, L. and Valagussa, F. 2001. Antioxident vitamins and prevention of cardiovascular diseases: epidemiological and clinical trial data. *Lipids*, **36**: 53–63.

Masoko, P. and Eloff, J.N. 2007. Screening of twenty-four South African *Combretum* and six *Terminalia* species (Combretaceae) for antioxidant activites. *African Journal of Traditional, Complementary and Alternative Medicines,* **4**(2): 231–239.

Mishra, P.K., Singh, R.K., Gupta, A., Chaturvedi, A., Pandey, R., Tiwari, S.P. and Mohapatra, T.M. 2013. Antibacterial activity of *Andrographis paniculata* (Burm. f.) Wall ex Nees leaves against clinical pathogens. *Journal of Pharmacy Research*, **7**: 456–462.

Momoh, A.O., Oladunmoye, M.K. and Adebolu, T.T. 2012. Evaluation of the antimicrobial and phytochemical properties of oil from castor seeds (*Ricinus communis Linn*). *Bulletin of Environment, Pharmacology and Life Sciences,* **1**(10): 21–27.

Monteiro, J. and Fodde, R. 2010. Cancer stemness and metastasis: therapeutic consequences and perspectives. *European Journal of Cancer,* **46**: 1198–1203.

Morhason-Bello, I.O., Odedina, F., Rebbeck, T.R., Harford, J., Dangou, J., Denny, L. and Adewole, I.F. 2013. Cancer control in Africa 1: Challenges and opportunities in cancer control in Africa: A perspective from the African organisation for research and training in cancer. *Lancet Oncology,* **14**: 142–151.

Mughal, T., Mamona, A., Saddiuqe, Z., Qureshi, S. and Mehboob, S. 2010. Phytochemical and pharmacognostical evaluation of *euphorbiaceae* species from Lahore region, Pakistan. *Journal of Applied Pharmacy,* **3**(2): 79–85.

Mumprecht, V. And Detmar, M. 2009. Lymph angiogenesis and cancer metastasis. *Journal of Cellular and Molecular Medicine*, **13**(8): 1405–1416.

Muthu, C., Ayyanar, M., Raja, N. and Ignacimuthu, S. 2006. Medicinal plants used by traditional healers in Kancheepuram District of Tamil Nadu, India. *Journal of Ethnobiology and Ethnomedicine*, **2**: 43.

Naz, R. and Bano, A. 2012. Antimicrobial potential of *Ricinus communis* leaf extracts in different solvents against pathogenic bacterial and fungal strains. *Asian Pacific Journal of Tropical Biomedicine,* **2**(12): 944–947.

Onder, T.T., Gupta, P.B., Mani, S.A., Yang, J., Lander, E.S. and Weinberg, R.A. 2008. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Research*, **68**(10): 3645–54.

Ozbek, S., Balasubramanian, P.G., Chiquet-Ehrismann, R., Tucker, R.P. and Adams, J.C. 2010. The evolution of extracellular matrix. *Molecular Biology of the Cell,* **21**: 4300–4305.

Ozgov´a, S., Hermanek, J. and Gut, I. 2003. Different antioxidant effects of polyphenols on lipid peroxidation and hydroxyl radicals in the NADPH-, Fe-ascorbate- and Fe-microsomal systems. *Biochemical Pharmacology,* **66**(7): 1127–1137.

Padua, D. and Massagu´e, J. 2009. “Roles of TGFβ in metastasis.” *Cell Research*, **19**: 89–102.

Page-McCaw, A., Ewald, A.J.andWerb, Z. 2007. Matrix metalloproteinases and the regulation of tissue remodelling. *Nature Reviews Molecular Cell Biology*, **8**(3): 221–33.

Panghal, M., Kaushal, V. and Yadav, J.P. 2011. *In vitro* antimicrobial activity of ten medicinal plants against clinical isolates of oral cancer cases. *Annals of Clinical Microbiology and Antimicrobials,* **10**(21): 1–11.

Parekh, J. and Chanda, S. 2007. *In vitro* antimicrobial activity and phytochemical analysis of some Indian medicinal plants. *Turkish Journal of Biology,* **31**: 53–58.

Paterlini-Brechot, P. and Benali, N.L. 2007. Circulating tumour cells (CTC) detection: clinical impact and future directions. *Cancer Letters,* **253**: 180–204.

Pingale, S.S. 2010. Hepatosuppression by *Ricinus communis* against CCl4 induced liver toxicity in rats. *Journal of Pharmacy Research,* **3**(1): 39–42.

Polyak, K. 2010. Molecular markers for the diagnosis and management of ductal carcinoma *in situ*. *Journal of the National Cancer Institute*, **2010**(41): 210–213.

Psaila, B. and Lyden, D. 2009. The metastatic niche: adapting the foreign soil. *Nature Reviews Cancer*, **9**(4): 285–93.

Quan, T., Qin, Z., Xia, W., Shao, Y., Voorhees, J.J. and Fisher, G.J. 2009. Matrix-degrading metalloproteinases in photoaging. *Journal of Investigative Dermatology,* **14**(1): 20–24.

Rao, N., Mittal, S., Sudhanshu, and Menghani, E. 2013. Assessment of phytochemical screening, antioxidant and antibacterial potential of the methanolic extract of *Ricinus communis L. Asian Journal of Pharmacy and Technology,* **3**(1): 20–25.

Rendon, B.E., Roger, T., Teneng, I., Zhao, M. and Al-abed, Y. 2007. Regulation of human lung adenocarcinoma cell migration and invasion by macrophage migration inhibitory factor. *Journal of Biological Chemistry,* **282**: 29910–29918.

Ríos-Arrabal, S., Artacho-Cordón, F., León, J., Román-Marinetto, E., Salinas-Asensio, M., Calvente, I. and Núñez, M.I. 2013. Involvement of free radicals in breast cancer. *SpringerPlus,* **2**: 404.

Roomi, M.W., Monterrey, J.C., Kalinovsky, T., Rath, M. and Niedzwiecki, A. 2009. Patterns of MMP-2 and MMP-9 expression in human cancer cell lines. *Oncology Reports*, **21**: 1323–1333.

Roomi, M.W., Monterrey, J.C., Kalinovsky, T., Rath, M. and Niedzwiecki, A. 2010. *In vitro* modulation of MMP-2 and MMP-9 in human cervical and ovarian cancer cell lines by cytokines, inducers and inhibitors .*Oncology Reports,* **23**: 605–614.

Saari, H., Sorsa, T., Lindy, O., Suomalainen, K., Halinen, S., Konttinen, Y.T., 1992.Reactive oxygen species as regulators of human neutrophil and fibroblast interstitial collagenases. *International Journal of Tissue Reactions*, **14**: 113–120.

Sasaki, H., Klotz, L.H., Sugar, L.M., Kiss, A. and, Venkateswaran, V. 2015. A combination of desmopressin and docetaxel inhibit cell proliferation and invasion mediated by urokinase-type plasminogen activator (uPA) in human prostate cancer cells. *Biochemical and Biophysical Research Communications*, **464**: 848–854.

Sato, T., Sakai, T., Noguchi, Y., Takta, M., Hirakawa, S. and Ito, A. 2004. Tumor-stromal cell contact promotes invasion of human uterine cervical carcinoma cells by augmenting the expression and activation of stromal matrix metalloproteinases. *Journal of Gynecologic Oncology*, **92**: 47–56.

Scalbert, A., Manach, C., Morand C., Remesy, C. and Jimenez, L. 2005. Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition,* **45**: 287–306.

Sheen, Y.Y., Kim, M.J., Park, S.A. and Nam, J.S. 2013. “Targeting the transforming growth factor-β signalling in cancer therapy.” *Biomolecules and Therapeutics*, **21**(5): 323–331.

Siegel, R., Naishadham, D. and Jemal, A. 2012. Cancer Statistics. *Ca Cancer Journal of Clinicians*, **62**(1): 10–29.

Simpson, C.D., Anyiwe, K. and Schimmer, A.D. 2008. Anoikis resistance and tumour metastasis. *Cancer Letters*, **72**: 177–185.

Singh, PP., Ambika and Chauhan, SMS. 2009. Activity guided isolation of antioxidants from the leaves of *Ricinus communis* L. *Food Chemistry*, **114**(3): 1069–1072.

Spano, D. and Zollo, M. 2012. Tumour microenvironment: a main actor in the metastasis process. *Clinical and Experimental Metastasis,* **29**: 381–395.

Steeg, P.S. 2006. Tumour metastasis: mechanistic insights and clinical challenges. *Nature Medicine,* **12**: 895–904.

Street, R.A. and Prinsloo, G. 2012. Commercially important medicinal plants of South Africa: A Review. *Journal of Chemistry,* **2013**: 1–16.

Suffness, M. and Douros, J. 1979. Drugs of plant origin. *Methods in Cancer Research*, **26**: 73–126.

Tamiya, S., Liu, L.H. and Kaplan, H.J. 2010.Epithelial-Mesenchymal Transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell-cell contact. *Investigative Ophthalmology and Visual Science*, **51**(5): 2755–2763.

Tan, A.R., Alexe, G. and Reiss, M. 2009. “Transforming growth factor-β signaling: emerging stem cell target in metastatic breast cancer?” *Breast Cancer Research and Treatment*, **115**(3): 453–495.

Tang, L. and Han, X. 2013. The urokinase plasminogen activator system in breast cancer invasion and metastasis. *Biomedicine and Pharmacotherapy,* **67**: 179–182.

Theveneau, E., Marchant, L., Kuriyama, S., Gull, M., Moepps, B., Parsons, M. and Mayor, R. 2010. Collective chemotaxis requires contact-dependent cell polarity. *Developmental Cell,* **19**(1): 39–53.

Theveneau, E., Steventon, B., Scarpa, E., Garcia, S., Trepat, X., Streit, A. and Mayor, R. 2013. Chase-and-run between adjacent cell populations promotes directional collective migration. *Nature Cell Biology,* **15**: 763–772.

Tsuji, T., Ibaragi, S. and Hu, G. 2009. Epithelial-mesenchymal transition and cell cooperativity in metastasis. *Cancer Research,* **69**(18): 7135–7139.

Valastyan, S. and Weinberg, R.A. 2011. Tumour metastasis: molecular insights and evolving paradigms. *Cell,* **147**: 275–292.

Verma, R.P. and Hansch, C. 2007. Matrix metalloproteinases (MMPs): Chemical–biological functions and (Q) SARs. *Bioorganic and Medicinal Chemistry*, **15**: 2223–2268.

Voulgari, A. and Pintzas, A. 2009. Epithelial-mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic. *Biochimica et Biophysica Acta,* **17**(96): 75–90.

Wang, L., Li, X., Zhang, S., Lu, W., Liao, S., Liu, X., Shan, L., Shen, X., Jiang, H., Zhang, W., Huang, J. and Li, H. 2012. Natural products as a gold mine for selective matrix metalloproteinases inhibitors. *Bioorganic and Medicinal Chemistry*, **20**: 4164–4171.

Weber, G.F. 2008. Molecular mechanisms of metastasis. *Cancer Letters*, **270**(2): 181–190.

Whittaker, C.A., Bergeron, K.F., Whittle, J., Brandhorst, B.P., Burke, R.D. and Hynes, R.O. 2006. The echinoderm adhesome. *Developmental Biology,* **300**: 252–266.

Wiranowska, M., Rojiani, A.M. and Rojiani, M.V. 2015. Matrix metalloproteinases-modulating the tumor microenvironment. *Journal of Carcinogenesis and Mutagenesis*, **6**: 2157–2518.

Yadav, R.N.S. and Agarwala, M. 2011. Phytochemical analysis of some medicinal plants. *Journal of Phytology,* **3**(12): 10–14.

Yahayo, W., Supabphol, A. and Supabphol, R. 2013. Suppression of human fibrosarcoma cell metastasis by *Phyllanthus emblica* extract *in vitro*. *Asian Pacific Journal of Cancer Prevention*, **14**(11): 6863–6867.

Yang, Z., Garcia, A., Xu, S., Powell, D.R., Vertino, P.M., Singh, S. and Marcus, A.I. 2013. *Withania somnifera* root extract inhibits mammary cancer metastasis and epithelial to mesenchymal transition. *PLoS ONE,* **8**(9): 1–17.

Zhang, C-Y., Zhang, L., Yu, H-Y., Bao, J-D., Sun, Z. and Lu, R-R. 2013. Curcumin inhibits invasion and metastasis in K1 papillary thyroid cancer cells. *Food Chemistry*, **139**: 1021–1028.

Zhang, H.J., Zhao, W., Venkataraman, S., Robbins, M.E.C., Buettner, G.R., Kregel, K.C. and Oberley, L.W. 2002. Activation of matrix metalloproteinase-2 by overexpression of manganese superoxide dismutase in human breast cancer MCF-7 cells involves reactive oxygen species. *The Journal of Biological Chemistry*, **277**(23): 20919–20926.

Zhang, L., Ridgway, L.D., Wetzel, M.D., Ngo, J., Yin, W., Kumar, D., Goodman, J.C., Groves, M.D. and Marchetti, D. 2013. The identification and characterization of breast cancer CTCs competent for brain metastasis. *Science Translational Medicine*, **5**(180): 180–148.