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Phytochemical Analyses of Bioactive Compounds in the Roots of Cassia Alata Linn and the Anti-Angiogenic Evaluation of Rhein as a Therapeutic Agent against Breast Cancer Cells

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PHYTOCHEMICAL ANALYSES OF BIOACTIVE COMPOUNDS IN THE ROOTS OF *CASSIA ALATA* LINN AND THE ANTI-ANGIOGENIC EVALUATION OF RHEIN AS A THERAPEUTIC AGENT AGAINST BREAST CANCER CELLS

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DEDICATION

To my LORD and Savior, the Lover of my soul:
Jesus Christ

LORD GOD Almighty, all throughout my life and education, as I went through the valleys and hills, You have been always there for me. In the course of my studies, You lovingly strengthened and patiently carried me in Your arms, while gently comforting me and instilling your principles in me, as well as building my character. You gave me bountiful grace and mercy to run my challenging educational race with wisdom, perseverance, diligence, and endurance. Thank You for Your grace and mercy upon my life and for lifting my head up, especially during tough times. You have been Faithful!

To my dear parents:
Rudi Fernand and Elize Fernand-Buyne

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Andrea Jubithana-Fernand, Earl Fernand, and Mavis Fernand

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In the United States of America, Suriname, and the Netherlands

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The LORD GOD is my strength; He will make my feet like deer's feet,
And He will make me walk on my high hills. Habakkuk 3:19 (NKJV)

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AIDS	Acquired immune deficiency syndrome
AK-3	Adenylate kinase 3
ALDA	Aldolase-A
Ang-2	Angiopoietin
AP-1	Activator protein-1
APCI	Atmospheric pressure chemical ionization
ATM	Ataxia telangiectasia mutated gene
ATP	Adenosine tri-phosphate
BC	Before Christ
bFGF	Basic fibroblast growth factor
BIS-TRIS	2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol
Bcl-2	B-cell lymphoma 2
BRCA-1; 2	Breast cancer 1 and 2 gene
BSA	Bovine serum albumin
CA-9	Carbonic anhydrase 9
CAM	Cell adhesion molecules
CATDH	Cathepsin D
COX-2	Cyclooxygenase-2
Co-A	Co-enzyme A
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extracellular matrix

ECG	Epicatechin gallate
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
ELISA	Enzyme-linked immuno-sorbent assay
EMR	Electromagnetic radiation
EMS	Electromagnetic spectrum
ENG	Endoglin
ENO-1	Enolase-1
EPA	Eicosapentaenoic acid
EPO	Erythropoietin
ER^-; ER^+	Estrogen receptor-negative; positive
ESI	Electrospray ionization
FCC	Flash column chromatography
FTIR	Fourier transform infrared
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GLUT	Glucose transporter
GS	Growth signal
Her2/neu	Human epidermal growth factor receptor 2/ neuroglioblastoma
HETP	Height equivalent to a theoretical plate
HIF-1	Hypoxia-inducible factor-1
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRE	Hypoxic response elements

Hsp	Heat shock protein
HTS	High-throughput screening
IGFR-2	Insulin-like growth factor receptor-2
I-κB	Inhibitor of NF-κB
IL	Interleukin
IR	Infrared
LC	Liquid chromatography
LDH-A	Lactate dehydrogenase A
LEP	Leptin
MMPs	Matrix metalloproteinases
MS	Mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NCEs	New chemical entities
NCI	National cancer institute
NF-κB	Nuclear factor-kappaB
NMR	Nuclear magnetic resonance
NOS-2	Nitric oxide synthase-2
p53	tumor suppressor protein 53
PAI	Plasminogen activator-inhibitor
PBS	Phosphate buffered saline
PCR	Photosynthetic carbon reduction
PD-EGF	Platelet-derived endothelial growth factor
PDGF	Platelet-derived growth factor
PKC	Protein kinase C

PLC	Preparative layer chromatography
PVDF	Polyvinylidene difluoride
pRb	retinoblastoma tumor suppressor gene
PTK	Protein tyrosine kinases
pVHL	Von Hippel-Lindau tumor suppressor protein
QIT	Quadrupole ion trap
SEM	Standard error of mean
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	Selected ion monitoring
TCR	Tricarboxyl acid
TIC	Total ion chromatogram
TOF	Time-of-flight
TGF-β	Transforming growth factor-beta
Tie2	Angiopoietin receptors of angiopoietin
TIMP-1	Tissue inhibitor of metalloprotease
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-alpha
uPAR	Urokinase-type plasminogen activator receptor
UV-Vis	Ultraviolet-visible
VCAM	Vascular cell adhesion molecule
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
WHO	World health organization

TERMINOLOGY

Angiogenesis
The growth of new blood vessels from pre-existing ones

Angiogenic switch
Change in the balance of angiogenesis inducers and inhibitors

Anoxic
A decrease in the oxygen level (a complete deprivation of oxygen), an extreme form of hypoxia

Anthelmintic or Vermifuge
A substance that expels parasitic worms from the body, by either stunning or killing them

Cancer
A class of diseases that results from multiple abnormalities in genetic materials

Cathartic
A substance which accelerates defecation
A substance which eases defecation, usually by softening the stool; a substance can be both a laxative and a cathartic

Combinatorial chemistry
The generation of large collections, or 'libraries,' of compounds by synthesizing combinations of a set of smaller chemical structures

Crisis state
Massive cell death associate with end-to-end fusion of chromosomes

Cytostatic
A substance that suppresses the growth and multiplication of cells

Emetic
A therapeutic drug or natural substance that stimulates vomiting

Ethno-botany
The science that studies relationships between people and plants

Hypoxia
A pathological condition in which the body or tissue is deprived of adequate oxygen supply

Immortalized state
Ability to multiply without limit

Mammary dysplasia
Fibrocystic disease of the breast

Medicinal plants

Plants that are used or of medicinal properties in the treatment of human diseases or in the maintenance or improvement of health

Necrosis
Premature death of cells and living tissue in response to a disease or injury

New chemical entity
An approved medication by the FDA that contains a novel active ingredient

Pharmacophore
The ensemble of steric and electronic features that is necessary to ensure optimal interactions with a specific biological target structure and to trigger (or to block) its biological response

Phytochemical
Individual chemicals from which plant are made

Phytomedicine
Phytochemicals that are used in the treatment of human diseases and health maintenance

Plant natural product
Phytochemicals that are used in medicines, industry, insecticides, pesticides, vitamins, etc.

Probe
Antibody that is bound to a protein

Purgative or Laxative
An agent (foods, bioactive compounds, or drugs) taken to empty the bowls

Senescence
Loss of the ability to divide *in vitro*

Telomere
The end of a chromosome

Traditional medicine, folk medicine, or herbal medicine
Medicines derived from the extract of one or more plant species, mixed together and usually taken in the form of a tea

ABSTRACT

The research presented in this dissertation includes the novel phytochemical analysis of root extracts of *Cassia alata* L. and the anti-angiogenic evaluation of rhein in the treatment of breast cancer. To conduct these studies a combination of analytical techniques were employed, namely, chromatographic, spectroscopic, and bioanalytical. The first part of this research is focused on the phytochemical analysis of *C. alata* root extracts. A high performance liquid chromatography method was developed and validated for the determination of six phenolic compounds in the root extracts. The investigated compounds were identified by their ultraviolet spectra. Additionally, the presence of these phenolics was confirmed by on-line mass spectrometric detection using atmospheric pressure chemical ionization. Subsequently, the primary anthraquinone, rhein, was isolated by use of flash chromatography and purified using preparative thin layer chromatography. Lastly, characterization studies were performed by utilizing mass spectrometry, nuclear magnetic resonance, ultraviolet, and infrared spectroscopy.

The final part of this study is focused on assessing the effectiveness of rhein as an anti-angiogenic agent in the treatment of breast cancer. The *in vitro* anti-angiogenic activity of rhein was evaluated using human umbilical vein endothelial cells, non-invasive (MCF-7) and invasive (MDA-MB-435s) breast cancer cells. Rhein's mechanism of action was investigated by use of *in vitro* angiogenesis assays, enzyme-linked immunosorbent-assays, and Western Blotting. Rhein inhibited endothelial cell tube formation, MCF-7 and MDA-MB-435s cell viability, as well as MDA-MB-435s cell migration and invasion under normoxic and hypoxic conditions. In addition, rhein significantly reduced vascular endothelial growth factor levels in supernatant fractions and decreased hypoxia inducible factor-1 activities in nuclear extracts of both cell lines. These results indicate the novel use of rhein as an anti-angiogenic agent, which could have a vital impact in the prevention and treatment of breast cancer.

CHAPTER 1

INTRODUCTION

Part I Natural Products in Drug Discovery

1.1 Importance of Natural Products

Natural products have been a major source for medicines throughout human history. This is because extracts of chemicals from plants, animals, and microbes have been used in folk medicine since ancient times to maintain health and to treat a wide variety of ailments. Over the last two centuries an explosion of information has emerged regarding the biosynthesis of natural compounds, their biochemical properties, and their interaction with organisms.[1, 2] During this same time period, the first commercial drug, morphine, derived from an isoquinoline alkaloid isolated from opium poppy (*Papaver somniferum*) became part of Western medicine in 1806.[3] In 1897, the modern pharmaceutical industry was born with the synthesis of aspirin from salicylic acid in willow bark (*Salix* spp.). Subsequently, in 1928, the widely used antibiotic penicillin was discovered in mold.[4] Therefore, there is little doubt that the use of natural products as active ingredients in therapeutic drugs in the 18th and 19th century has set the foundation for modern drug development.[5, 6]

According to the World Health Organization (WHO), approximately 80% of the world's population still relies on traditional medicines, i.e., primarily medicinal plants, for primary health care.[7] In the remaining world's population, natural products have also proven to be of significant value in health care. For instance, it is estimated that 25% of all medicines dispensed in the United States of America (USA) are directly or indirectly derived from higher plants. In addition, about 74% of the 119 most vital drugs contain active ingredients from plants used in folk medicine.[7, 8] Furthermore, several studies that have been performed on the most prescribed drugs in the USA showed that the active ingredient of the majority is either a natural product or a

derivative of a natural product.[8, 9] Taken together, these investigations indicate that natural products have an significant role in drug discovery and its developmental process.

Drug discovery was until the 1970's more or less based on coincidence. Rational drug discovery was introduced with the dawn of molecular biology and computers. Therefore, in the period between 1970 and 1980 a peak was reached in the Western pharmaceutical industry with the investigation of natural products as a source of novel drugs. Additionally, between 1981 and 2006, about 63% of the small-molecules new chemical entities launched on the market were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural-product pharmacophores. It is noteworthy that between 2001 and 2005, a total of 23 natural-product-derived drugs were approved for the treatment of several ailments such as Alzheimer's disease, bacterial and fungal infections, cancer, and diabetes.[8, 10] Currently, around 100 natural-product-based drugs are in pre-clinical trials, and more than 100 similar investigations are in clinical trials. Most of these compounds originate from leads from plants and microbial sources and are mainly being studied as anti-neoplastics and anti-infectious agents (Figure 1.1).[8, 11]

Despite this success, natural product research in the pharmaceutical industry has experienced a significant decrease during the past decade. Between 2001 and 2008 there has been a 30% decline in natural product research projects.[11] One of the main reasons has been the development of combinatorial chemistry, which offers simpler and more drug-like screening libraries of thousands of compounds in a short period of time. Furthermore, the introduction of high-throughput screening (HTS) against defined molecular targets offers 'screen friendly' synthetic chemical libraries. Another vital factor that has caused many pharmaceutical companies to move from natural product research is the advancement in molecular biology, cellular biology, and genomics, leading to an increase in the number of molecular targets over a

shorter drug discovery time period. Moreover, uncertainties remain regarding collection of biomaterials as well as concerns about intellectual property rights.[6, 12, 13]

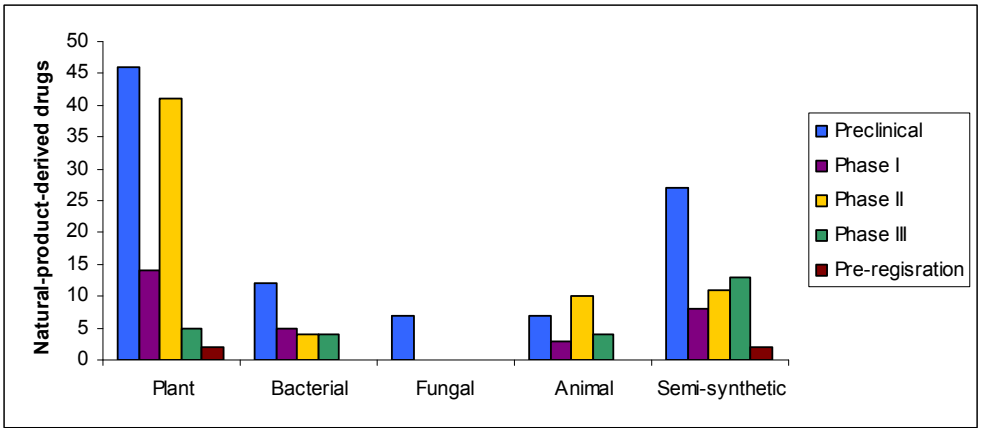
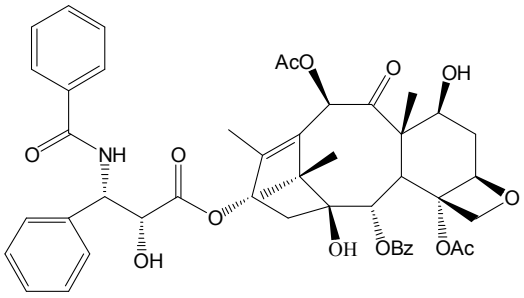


Figure 1.1 Natural-product-based drugs at various development stages (Adapted from Harvey)[11]

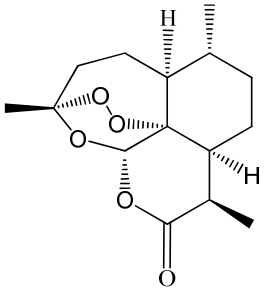
Nevertheless, Nature is still the best source of novel active compounds, because plants synthesize a great number of chemically diverse compounds with biochemical specificity. Furthermore, although natural product screening and isolation offers fewer compounds over a longer time period, this approach can produce novel molecular structures not predicted through combinatorial and synthetic chemistry. The exclusive ring system of paclitaxel (Taxol®) [**1.2a**] and the peroxy-bond of artemisinin [**1.2b**] are a few examples of how natural product structures differentiate themselves from libraries of synthetic and combinatorial compounds. Additionally, combinatorial chemistry libraries are generally based on natural product compounds and tend to lack originality.[10, 14]

According to the US Pharmaceutical Manufacturers Association (PhRMA) the cost of drug development, from the initial research stage to the successful marketing of a drug, is around US$1,318 billion (in year 2005 dollars) over a period of 10-15 years.[15] In an attempt to reduce the costs many pharmaceutical research and development (R&D) groups are contemplating "quantity" (such as combinatorial chemistry) versus "quality" (such as natural product

chemistry) strategies. Therefore, numerous pharmaceutical companies are currently pursuing a multidisciplinary approach to drug discovery, which includes natural product screening and isolation combined with combinatorial and synthetic methodologies. In this scenario, natural products are used as lead compounds for the design, synthesis, and development of novel drugs.



1.2a Paclitaxel



1.2b Artemisinin

Figure 1.2 Natural products

1.2. Medicinal Plants in Drug Discovery

Medicinal plants are defined as plants that are used for their medicinal properties in the treatment of human diseases, or in the preservation or improvement of health. Every plant is composed of phytochemicals, which are individual chemicals that can be divided into two groups: primary and secondary metabolites.[1] Primary metabolites, i.e carbohydrates, lipids, proteins, and nucleic acids, are organic compounds ubiquitous in all plant cells which are essential to sustain life, growth, and reproduction. These metabolites are synthesized by the plant through several metabolic pathways. For example, glucose and fructose are synthesized in the photosynthetic carbon reduction (PCR) cycle and adenosine triphosphate (ATP) is released in the tricarboxylic acid (TCA) or the Krebs cycle.[1, 16]

In drug discovery, the major secondary metabolites, i.e. terpenoids, alkaloids, and phenolics, are of potential medicinal interest. The production of secondary metabolites can be induced by biotic and abiotic factors. In addition, these metabolites are synthesized by the plant to deter predators and pathogens, attract and deter pollinators, attract symbionts, perform

allelopathic action, and protect against UV radiation. Furthermore, the plant synthesizes secondary metabolites to supply food for pollinators, symbionts, herbivores, pathogens and decomposers.[1, 16]

The major pathways involved in the biosynthesis of secondary metabolites are Acetate, Shikimate, and Mevalonate, with acetyl-CoA, shikimic acid, and mevalonic acid, as the building blocks, respectively. Figure 1.3 gives an overview of these major pathways, which demonstrate that primary and secondary metabolisms are closely linked to each other and share many intermediates.[1, 16] Some intermediate processes that are involved in natural product synthesis are photosynthesis, glycolysis, the Krebs cycle, and the pentose phosphate pathway.

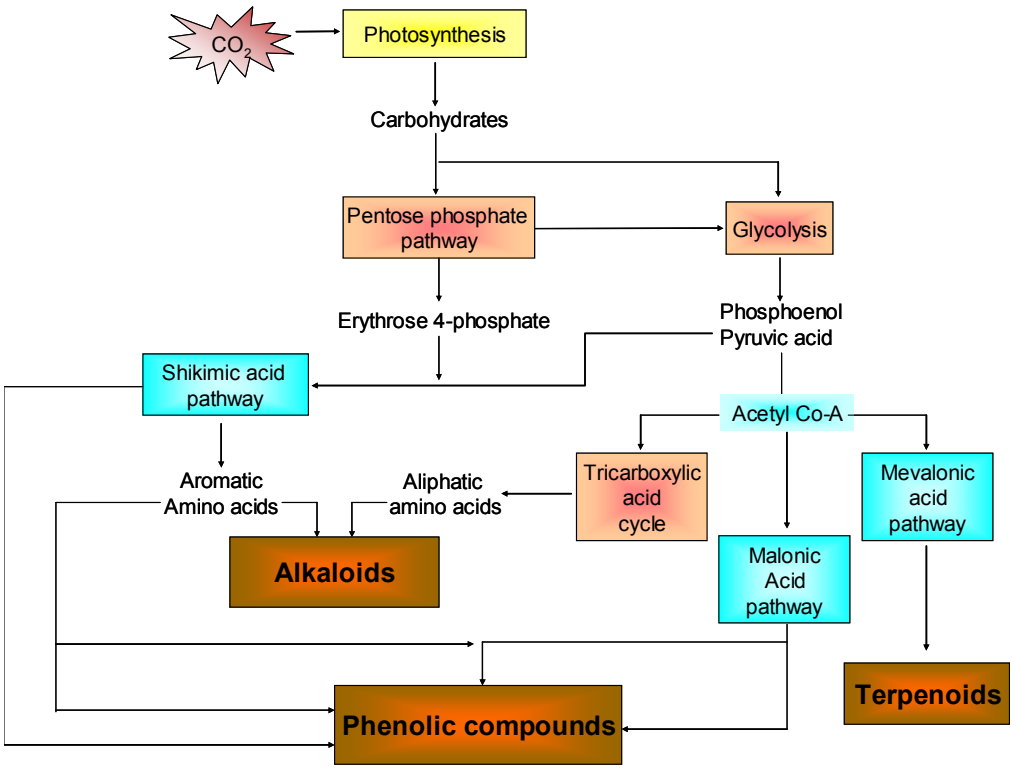


Figure 1.3 Major pathways involved in the biosynthesis of secondary metabolites[1, 16]

1.2.1 Principles of Secondary Metabolites

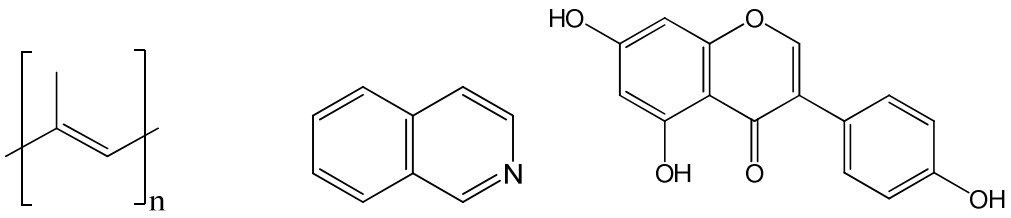
Terpenoids or terpenes are water insoluble compounds, with characteristic isoprene unit (C_5H_8) (Figure 1.4a). Terpenes are classified based on the number of isoprene units such as

hemiterpenes (one unit: C_5H_8), monoterpenes (two units: $C_{10}H_{16}$), sesquiterpenes (three units: $C_{15}H_{24}$), diterpenes (four units: $C_{20}H_{32}$), and triterpenes (six units: $C_{30}H_{48}$). The terpenoid biosynthesis pathways in the plant include acetate and mevalonate, and takes place in the cytoplasm and plastids of epidermal and parenchyma cells. Once synthesized, terpenoids are stored in the epidermal cells, bark, and wood. Some examples of terpenes used as phytomedicines are: paclitaxel (for treatment of ovarian and breast cancer), ginkgolide (for treatment of cerebral vascular disease and as a dietary supplement), gutta percha (to reduce hypertension), and aucubin (to lower blood-sugar levels and as antibiotic).[1, 16]

Alkaloids are a second major group of secondary metabolites which are used as phytomedicines. They are bitter-tasting nitrogenous organic compounds which are generally water soluble. Alkaloids are among the most important medicinally active compounds. The basic structure of an alkaloid is a nitrogen-containing heterocyclic ring (Figure 1.4b). Therefore, alkaloids are classified according to the nature of their basic structure, such as pyrrolidine, tropane, isoquinoline, and indole alkaloids. Although, alkaloids are synthesized during the Shikimate and Mevalonate pathway, their synthesis site is unclear. Upon synthesis, alkaloids are stored in the vacuoles of plant cells primarily in bark, leaves, roots, flowers, and fruits. Examples of alkaloids used in Western medicine include camptothecin (for treating breast and colon cancer), atropine (as a cardiac stimulant), morphine (as an analgesic and cough suppressant), theobromine (as a muscle relaxant in the relief of bronchial ashma), and quinine (as an anti-malaria agent).[1, 16]

Finally, the phenolics are the third group of major secondary metabolites that has significance to natural product chemistry. Phenolics are a broad range of compounds featured with a hydroxyl group attached to a benzene ring (Figure 1.4c), and can be divided into five main groups: anthocyanins, flavonoids, lignins, quinones, and tannins. The biosynthetic pathways

involved in the synthesis of phenolic compounds are Shikimate and Phenylpropanoid, which occur in the plastids and cytoplasm of plant cells. The storage sites vary depending on the type of phenolic compound; in general, they are stored in the vacuoles and cell wall of flowers, leaves, bark, and roots. Some examples of phenolics used as phytomedicines are: catechins (as antioxidants and anticancer agents), genistein (as an anti-angiogenic agent for treatment of cancer), kaempferol (as an anticancer agent), quercetin (as an antioxidant), and geraniin (as an anti-HIV agent).[1, 16]



a. Terpenoids (e.g., Isoprene unit)
b. Alkaloids (e.g., isoquinoline)
c. Phenolics (e.g., isoflavonoids)

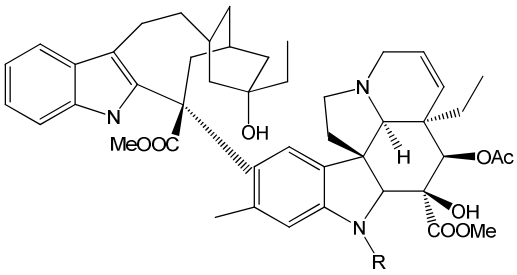
Figure 1.4 Basic structures of secondary metabolites

1.2.2 Secondary Metabolites as Antineoplastics

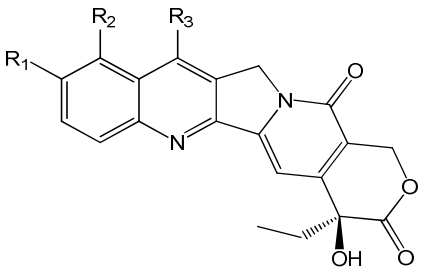
There are many secondary metabolites, either directly or indirectly derived from natural products, in clinical use as anticancer drugs. For example, the *Vinca* alkaloids, vinblastine (Velban®) [**1.5a**] and vincristine (Oncovin®) [**1.5b**] were isolated from the medicinal plant *Catharanthus roseus* and are currently used in the treatment of Hodgkin's disease and pediatric leukemia, respectively.[17] Their mechanism of action is based on inhibition of tubulin polymerization by binding to tubulin, which ultimately stops the cell reproduction. Because of the isolation of vinblastine, the five-year survival rate of Hodgkin's disease patients in 1970 increased from 5% to 98% in 1982. An additional example of an antineoplastic natural product is bleomycin [**1.5c**], a mold product, which directly damages DNA, and hence inhibits cell reproduction. Due to this drug, testicular cancer sufferers presently have over a 90% survival rate.[1, 18]

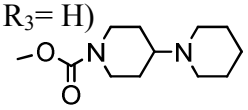
In 1958, an ambitious search for anticancer compounds from higher plants was started by the National Cancer Institute (NCI) which ended around 1991. During this period more than 120,000 plant extracts from 35,000 species were investigated. In the course of this mission, compounds such as paclitaxel (Taxol®) [**1.2a**] and camptothecin [**1.5d**] were isolated from the medicinal plants *Taxus brevifolia* and *Camptotheca acuminata*, respectively. The mechanism of action of paclitaxel is based on promoting tubulin polymerization, thus, preventing cell reproduction. On the other hand, camptothecin inhibits topoisomerase I, an enzyme responsible for the uncoiling of DNA, and ultimately helps prevent the rapid growth and reproduction of cancer cells.[1, 18]

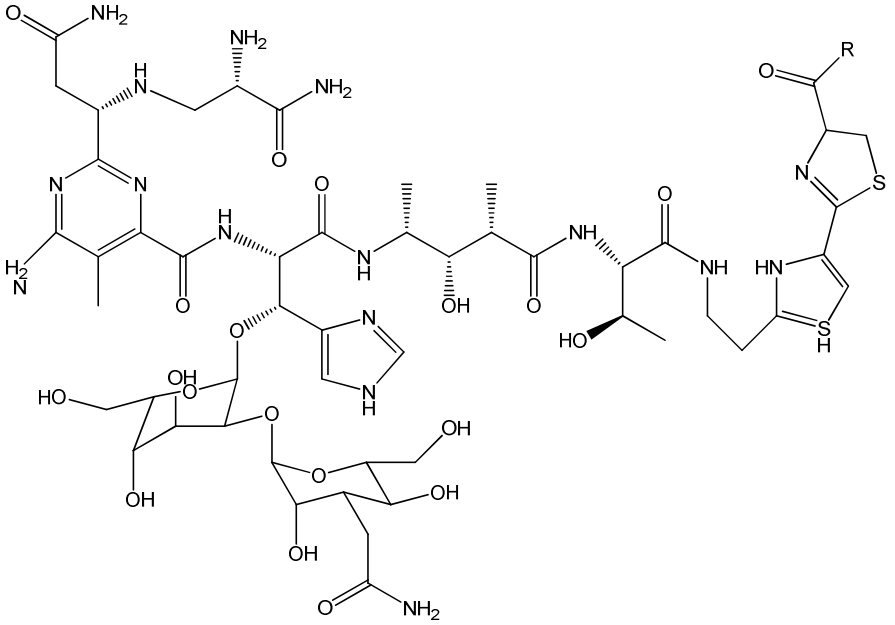
Although the use of natural products as antineoplastics has resulted in modern day oncologic drug discovery miracles, there are, some challenges facing this approach. The major problems are the solubility, toxicity, and supply of natural products. For instance, pharmaceutical companies have experienced in the past with the use of paclitaxel [**1.2a**] supply and solubility problems. Originally this compound was isolated from the bark of the very slow growing Pacific Yew (*Taxus brevifolia*), which eventually resulted in the death of the tree. Subsequently, the supply dilemma was resolved through its semi-synthetic production from renewable needles of a related tree, English Yew (*Taxus baccata*), and the solubility problem was managed by formulation. Another example is the chemotherapeutic agent camptotechin [**1.5d**]. This drug proved to be highly insoluble for clinical use and was initially administered as a sodium salt of the ring-opened lactone which showed to be inactive. The structure of this therapeutic agent was modified, resulting in two water soluble semi-synthetic drugs topotecan (Hycamtin®) [**1.5e**] and irinotecan (Camptosar®) [**1.5f**], for treatment of ovarian and colon cancer, respectively.[1]



1.5a Vinblastine (R= CH_3)
1.5b Vincristine (R= CHO)



1.5d Camptothecin (R_1= R_2= R_3= H)
1.5e Topotecan (R_1= OH, R_2= CH_2NMe_2, R_3= H)
1.5f Irrinotecan (R_1=  R_2= H, R_3= CH_2CH_3)



1.5c Bleomycins (R= various)

Figure 1.5 Secondary metabolites as anticancer drugs

1.2.3 Selection Criteria for Investigation of Plants

The selection criteria used for the investigation of plants can be based on traditional usage/knowledge, toxicity, chemical composition, biological activity, random sampling, or a combination of the aforementioned. Ethno-pharmacological knowledge has resulted in three indicator levels for drug discovery. These three levels include, (1) a general lead of non-specific bioactivity that is appropriate for a group of broad screens, (2) an exact indicator of bioactivity

suitable for certain high-resolution bioassays, and (3) a pointer of pharmacological activity aimed at designing a mechanism-based bioassay.[17]

In the past, ethno-botanical information has led to three types of drug discovery, (1) authentic natural products used in ethno-pharmacology which implied clinical efficacy (for example, digitalis), (2) unmodified natural products of which the ethno-botanical use only vaguely implied therapeutic efficacy (for example, vincristine), and (3) natural-product-derived drugs (semi-synthetic or synthetic) based on a natural product used in traditional medicine (for example, aspirin).[17]

In this dissertation, the investigated medicinal plant, *Cassia alata* L., was selected based on: (1) traditional medicinal information (ethno-pharmacological knowledge) from Suriname, (2) literature reports available on the biological activity of *C. alata* plant extracts, (3) chemical composition of the genus *Cassia* (mainly anthraquinones and polyphenols), and (4) scientific knowledge available on the biological activity of quinones. Therefore, in the following sections, §1.3 and §1.4, a general review is given on the medicinal plant *C. alata* and the physiology of quinones, respectively.

1.3 *Cassia alata* L.

1.3.1 Description and Ethnobotanical Use

Cassia alata L., a wild-growing shrub, often cultivated as an ornamental plant, is indigenous to northern South America but is now widely distributed in the tropics.[19-21] *C. alata* is locally abundant throughout Suriname along secondary vegetation or riverbanks.[20] Its local name in Suriname is slabriki, but in other parts of the world it is known as bajagua, candlebush, fluer palmiste, golden candle, mocoteno, ringworm shrub, and wild senna (Figure 1.6).[20, 21] *C. alata*, as a shrub or small tree, can reach up to 4 m in height with stout branches; and the leaves are 20 to 40 cm long, containing 12 to 24 leaflets, which are typically oblong, but the terminal pair is

elliptic and 5 to 17 cm long. The flowers are deep yellow, 2.5 cm wide, in erect, dense, cylindrical clusters which are approximately 15cm in length. The pod is 10 to 15 cm long, linear, with four longitudinal wings and the seeds are numerous, four-sided, flattened, and about 5 mm long.[21]

Figure 1.6 *Cassia alata* L.

Plants in the genus *Cassia* can be found in all tropical regions around the world, from tropical America to the West Indies, Africa, and Asia.[21] The genus *Cassia* belongs to the Fabaceae family and Caesalpiniaceae subfamily and has more than 600 species as herbs, shrubs, and trees. Approximately 26 species of this genus have been reported to have medicinal value, in which *C. alata* is one of the most important species.[21-23] The leaves of *C. alata* have been used for centuries in traditional medicine as a laxative as well as in the treatment of a variety of skin and respiratory diseases.[19-21, 24] In Suriname, the seeds of *C. alata* are used for their vermifugal and purgative action.[21] While the root extracts are used alone or in combination with *Phyllanthus amarus* L. to relieve uterine complaints, it is also used in combination with *Allamanda cathartica* L. as a medicine for malaria and swollen spleen.[20, 25] Additionally, in Panama a root decotion of *C. alata* is used to relieve rheumatism and stomachache.[21]

1.3.2 Pharmacological and Phytochemical Investigations

Cassia species are well-known for their exclusive ethno-pharmacological features; and hence, have been extensively studied for their anthracene derivatives.[23] Pharmacological investigations performed so far on leaf extracts of *C. alata* have shown that this herb has several biological activities, such as antimicrobial,[26, 27] antifungal,[28, 29] purgative,[30] analgesic,[29, 31] anti-inflammatory,[29, 32] anti-tumor,[33] and hypoglycemic.[29]

The leaves of *C. alata* have been qualitatively analyzed for the presence of five pharmacologically active anthraquinones: rhein, aloe-emodin, chrysophanol, emodin, and physcion, as well as the flavonoid kaempferol.[34, 35] The seeds are reported to contain chrysophanol, an anthracene derivative.[23] Rhein and chrysophanol are known to be present in the roots, in addition to two other quinone pigments.[36, 37] These anthraquinone derivatives are well known to exhibit a variety of biological activities,[38] such as antimicrobial,[23] antifungal,[39] antitumor,[40] antioxidant,[41] cytotoxic,[42] laxative,[43, 44] and hypoglycemic.[45] The flavonoid, kaempferol, has been reported to have anticancer properties.[46, 47] It is clear that there is a correlation between the ethno-pharmacological properties ascribed to this herbal medicine and the biological activities of these phenolic compounds.

1.4 Physiology of Quinones

1.4.1 Biosynthesis and Occurrence

Naturally occurring quinones are an important class of secondary metabolites produced by higher plants, fungi, and bacteria. They are synthesized primarily through the Shikimate or Polyketide pathways. Nevertheless, in humans quinones are synthesized mainly via the oxidative metabolism of catecholamines and estrogens, in addition to being an intermediate in the melanin synthesis pathway of human skin. Quinones are a class of aromatic compounds that are biologically significant and active as coenzymes or acceptors or vitamins.[38]

Quinones are ubiquitous in nature and typically highly reactive. They are colored compounds (ranging from pale yellow to almost black) and are responsible for the browning reaction in cut or injured fruits and vegetables.[38] In higher plants, quinone pigments make a relatively small contribution to color and are usually found in the bark, heartwood or roots, as well as in tissues (e.g., leaves) where their colors are covered by other pigments. On the other hand, however, in bacteria, fungi, and lichens they make significant contribution to the color.[2, 48]

Quinones have been used since ancient times as drugs and as pigments. The use of plant extracts as purgatives and emetics has been documented for more than 4000 years. Therefore, the pharmacological properties ascribed to naturally occurring quinones are well established and their distribution in higher plants has been primarily studied because of the cathartic/purgative action of certain anthraquinones. For instance, rhubarb (*Rheum palmatum*), containing a variety of anthraquinones as active compounds, is described in Chinese herbal medicine since ~2700 BC. In addition, the dried legumes of senna (*Cassia acutifolia*) were used by the Arabs as early as the ninth century for their laxative properties. Furthermore, some traditional cultures prepared pigments from henna (*Lawsonia inermis*) and madder (*Rubia tinctorum*) and used them as dyes and cosmetics. The active constituent of henna, lawsone (2-hydroxy-1,4-naphthoquinone), is now commercially available in USA supermarkets as a "natural" hair dye.[38, 49]

Quinones may contribute to oral hygiene because of their antibacterial effect. For instance, some traditional cultures used chewing sticks made from *Acacia*, *Cassia* or *Diospyros* plants (which contain quinones) for religious purposes to clean the mouth. Likewise, durable tropical woods are protected against fungal decay, wood-boring insects or marine-boring animals because of the presence of quinones. Finally, the toxicological properties of quinones have also been reported.[49] For example, walnut hulls and leaves contain a quinone, juglone (5-hydroxy-

1,4-naphthoquinone), which is extremely toxic and also acts as a weak tumor promoter in mouse skin.[50]

1.4.2 Chemical Structure and Classification

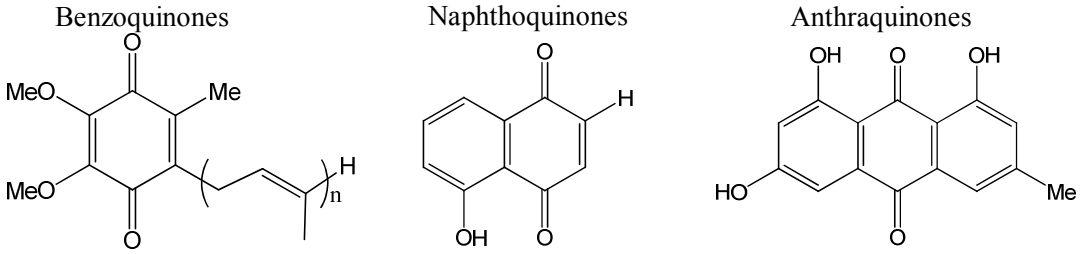
Numerous naturally occurring quinones (Figure 1.7) have been shown to possess a vital role in ecological and basic physiological processes of biological systems. In addition, the activity of quinones as therapeutic or toxic agents has contributed to substantial research on their biochemical and biophysical properties.[49, 51] Hence, quinones are extensively used as components of multivitamin formulation, dyes, platelet anti-aggregation, inhibitors of thromboxane A_2, and clinically as antitumor drugs and anti-allergic agents.[49, 52]

The biological activity of quinones can be subscribed to two main chemical properties, i.e. oxidants and electrophilic. The oxidation of diphenols (hydroquinones) yields readily diketones (quinones), and likewise dihydroxy compounds are formed by reduction of the corresponding quinones. Therefore, the quinone structure provides the basis for their catalytic function in electron transport in animals, plants, and micro-organisms.[49, 51]

Naturally occurring quinones exhibit great structural variation and currently more than 1200 quinones are known.[48] Their basic structural pattern, a *para* or an *ortho* substituted dione in conjugation with double bonds of a benzene or condensed aromatic ring system, provides the foundation for the systematic chemical recognition of naturally occurring quinones.[49] Hence, quinones can be classified, depending on the mono-, bi-, or tri-cyclic ring systems they possess, into three groups: benzo, naphtha, and anthraquinones, respectively (Figure 1.7).[2]

The benzoquinones are found in all types of living tissues.[51] Benzoquinones contain an aromatic ring with or without an aliphatic side chain. Only a few of them are pharmacologically active; for example, embelin, a dihydroxyquinone extracted from the dried fruits of *Embelia ribes* Burm. f., is an anthelmintic used against tapeworms and in the treatment of skin diseases.

In this group only the plastoquinones, ubiquinones, and tocopherols have specific physiological roles in electron transport systems. Plastoquinones are known to act as redox carriers in photosynthesis, ubiquinones (coenzyme-Q) as hydrogen carriers in the respiratory chain, and tocopherols as antioxidants or growth regulators.[2, 38]



a. Ubiquinones (n= 1 to 12) b. Juglone c. Emodin

Figure 1.7 Naturally occurring quinones

Naphthoquinones comprise the largest group of quinones.[38] They are widely distributed in micro-organisms (Vitamin K_2 type: multiprenyl menaquinones) and plants (Vitamin K_1 type: phylloquinones).[51] All these related compounds of Vitamin K have specific roles in electron transport systems. The majority of naphthoquinones are yellow or red pigments. Only a few (for example, chimaphilin, juglone, and eleutherin) are biologically active, namely, toxins or antimicrobial agents. A potent anticancer agent is arnebin, which is isolated from the roots of *Arnebia nobilis*.[2]

Anthraquinones are virtually as abundant as the naphthoquinones.[38] Approximately 50% are distributed in higher plants and the rest is found in fungi, marine animals, and insects.[2, 38] In higher plants, anthraquinones are mostly present in the Rubiaceae, Leguminosae, Rhamnaceae, and Polygonaceae families. This quinone group contributes to some important dye compounds and many purgatives.[2, 49] The role of anthraquinones in plants is not properly understood; however, it is assumed that they might have some function in redox reactions. In the plant anthraquinones generally occur in combined form with sugars as glycosides (hydroxylated

anthraquinones). During storage a slow hydrolysis of these glycosides takes place which results in the formation of pharmacologically active anthraquinones (anthranols). Sequentially, anthranols, over a period of time, are oxidized to anthraquinones. The purgative action of anthraquinones is the result of their conversion to anthranols by intestinal bacteria. It is worth mentioning that both naturally occurring and synthetic anthraquinones are extensively used as phytotherapeutic drugs.[2, 48]

Part II Background on Cancer

1.5 Characteristics of Cancer Cells

The word cancer has been derived from the Latin word for *crab*, since cancer cells affix themselves as a crab to tissues and organs. Cancer (malignant neoplasm) is a disease that results from multiple abnormalities in genetic materials.[53] In contrast to normal cells, which live in complete harmony with their environment, cancer cells tend to live toward chaos (highly disorganized with excessive branching) as they gradually proliferate in the body as a result of excess stimulation.[54, 55] The over-expression of stimuli in cancer cells is a result of mutations in deoxyribonucleic acid (DNA) sequencing which leads to amplification or increased expression of oncogenes and/or deletion of tumor suppressor genes. Oncogenes, typically normal genes, are programmed for cellular growth factor receptors, growth factors, or constituents of the growth machinery of the cancer cell. Activation of oncogenes in cancer cells leads to abnormal cell division and agitated growth. Tumor suppressor genes encode for regulatory proteins that usually suppress cellular proliferation, and induce controlled and programmed cell death. Therefore, inactivation of tumor suppressor genes in cancer cells results in abnormal cell function.[53, 56]

Carcinogenesis in humans is characterized as a multistep process which involves a succession of genetic changes that leads to the progressive conversion of normal human cells into malignant cells.[54] The specific cause of these genetic mutations remains unknown. However,

there tends to be a correlation between normal cell transformation into cancer cells and the environment (e.g., workplace and residence), chemical exposure (e.g., tobacco smoke, benzene, and asbestos), radiation exposure (e.g., ion and ultraviolet light), dietary factors, oncogenic viruses (e.g., human papilloma and Epstein-Barr), and oxidative damage to DNA due to endogenous metabolic reactions.[53, 57] In summary, carcinogenesis involves complex interactions between both endogenous (i.e. genetic, hormonal) and exogenous (e.g., life style and environmental exposure) factors.[54, 58] These interactions are accompanied by mainly three subsequent stages: initiation (DNA mutations), promotion (appearance of benign tumors), and progression (alteration of benign to malignant tumor).[55, 59]

According to Hanahan and Weinberg,[54] there are six changes in cell physiology that cooperatively play a significant role during carcinogenesis. These include self-reliance in growth signals (GS), insensitivity to anti-growth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, induction of angiogenesis, and invasion and metastasis. The first change in cell physiology involves the production of growth signals by tumor cells, which reduces their dependence upon stimulus from normal tissue growth microenvironment. The molecular strategies for attaining GS autonomy include alteration of extracellular growth signals, cell surface growth receptors, and signal transduction.[60] During the second physiological modification, cancer cells avoid anti-proliferative signals by evading cytostatic antigrowth signals.[60, 61] The third cell physiological change includes the apoptotic resistance of cancer cells through activation of oncogenes (such as *Bcl-2* and *c-myc*) and inactivation of the p53 protein, a product of the *p53* tumor suppressor gene, a proapoptotic regulator.[62, 63]

The fourth physiological alteration of cancer cell involves the avoidance of senescence by disabling pRb and *p53* tumor suppressor proteins, which in turn leads to a crisis state and ultimately to the immortalized state. In addition, these malignant cells maintain telomeres at a

length above critical threshold, which allows limitless multiplication of successor cells.[64, 65] The fifth physiological modification of cancer cells includes the induction of angiogenesis during development via an "angiogenic switch" from vascular serenity.[66, 67] Finally, the sixth physiological change of cancer cells involves their invasion and metastatic capability, which is mediated through cell adhesion molecules (CAMs) that activate cell-to-cell communication and integrins that connect cells to extracellular matrix substrates. Malignant cells are able to migrate to distant tissues and organs via newly formed blood vessels and lymph circulation.[59, 68]

All these characteristics of cancer cells lead to their independent functioning where they lose contact via gap junctions with normal cells, aberrant proliferation, invasion and metastasis in the body. Figure 1.8 gives a schematic overview of acquired capabilities of cancer cells. Inhibition of these six hallmarks of cancer singularly or combined is an imperative target for therapy. The research presented in the bioanalytical part of this dissertation is primarily focused on the inhibition of tumor angiogenesis.

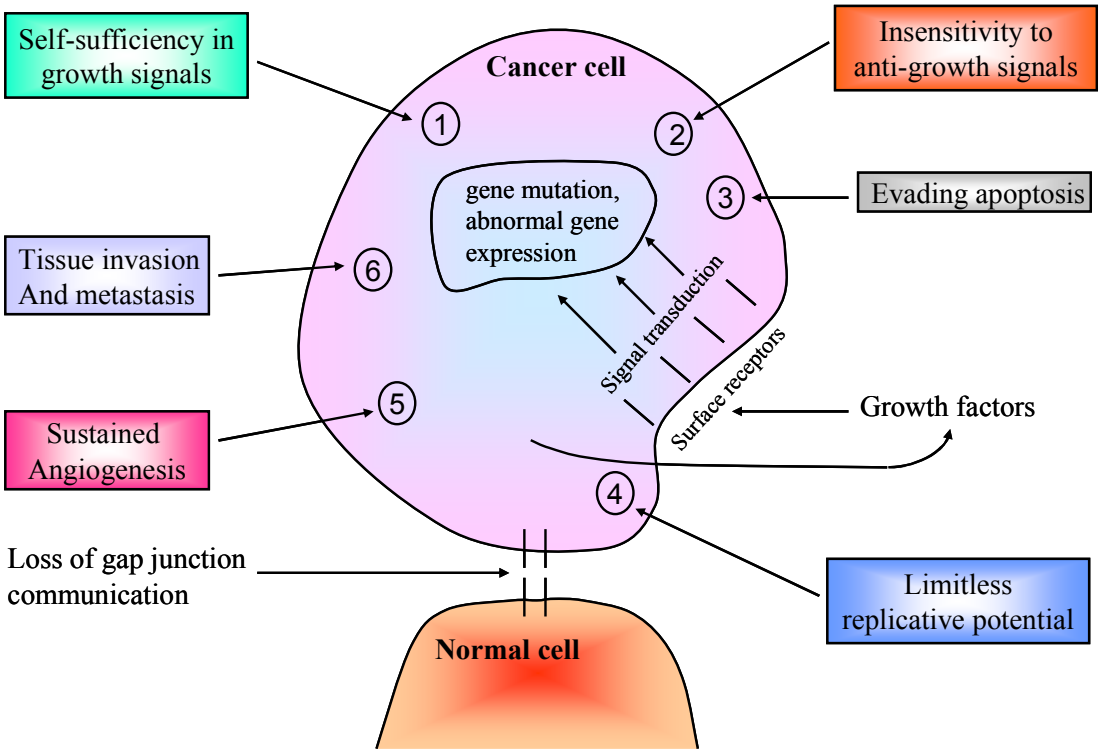


Figure 1.8 Functional Characteristics of Cancer Cells[54, 59]

1.6 Breast Cancer

1.6.1 Incidence and Risk Factors

Breast cancer has been for centuries one of the most devastating diseases in the world. It is the second leading cause of cancer death, after lung cancer, among women in the US and occurs with 100-fold greater frequency in females than males.[69, 70] In 2008, breast cancer was expected to account for 26% (182,460) of all new cancer cases in women and for 15% (40,480) of all female cancer deaths in the US.[70] Although the incidence of breast cancer is higher in Caucasian than in African American women, the death rate in African American women is higher due to the lack of access to high-quality regular screening and timely diagnosis as well as treatment.[70, 71]

There is an increased risk of developing breast cancer all through life, as well in women with a family and/or personal history of breast cancer or some types of mammary dysplasia. However, early age of menarche (under age 12), late age of first full-term pregnancy (after age 35), late natural menopause (after age 50) are also associated with a slightly increased risk of developing breast cancer.[69, 71] Furthermore, dietary factors (particularly increased fat content), and alcohol consumption may increase the risk of breast cancer.[57, 58, 71]

Hitherto, the etiology of breast cancer is largely unknown. Nevertheless, it can be stated, in general, that there are three major factors that increase the probability of developing breast cancer, i.e. genetic, hormonal, and environmental.[71] In addition, there is profound evidence that only 5 to 10% of breast cancer cases are genetically related; however, this percentage is higher among younger women.[72, 73] Mutations in BRCA1, BRCA2, p53, and ATM genes have been implicated as being fundamental in susceptibility to breast cancer.[74-78] Furthermore, epidemiological studies indicate that about 90 to 95% of breast cancer occurs spontaneously and that breast cancer development is strongly related to environmental factors in Western

countries.[79-81] This latter statement is based on the fact that the incidence of breast cancer is about 5-fold higher in Europe and North America than in Asia or Japan.[80, 82]

1.6.2 History and Pathologic Types

Hippocrates, born around 460 BC on a tiny island of Cos, is believed to be the founder of rational medicine. In his days, he reported breast cancer as a carcinoma of the breast that had a bloody expulsion from the nipple. Throughout Antiquity, the Middle Ages, Renaissance, Eighteenth and Nineteenth Century there have been several reports on the occurrence, diagnosis, and therapy of breast cancer,[83] which signifies the devastating effect that breast cancer has had through human history and still has to date.

Breast cancer is defined as a heterogeneous disease and is characterized by the activation of multiple signal pathways that stimulate tumor growth and proliferation, inhibit apoptosis, promote the formation of new blood vessels, as well as invasion and metastasis.[56, 84] The majority of breast cancer cases evolves either from the lining cells of the ducts (ductal) or from the secretory cells at the terminal ducts of the lobules (lobular). In Figure 1.9 is shown a schematic representation of a normal breast profile. Once breast cancer develops, it can be non-invasive (in situ) or invasive (infiltrating).[71]

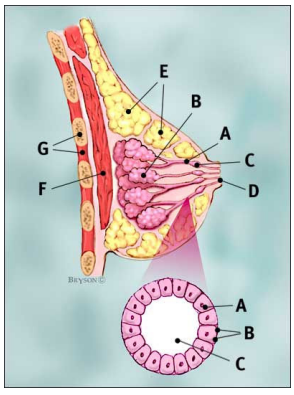


Figure 1.9 Normal breast profile with an enlarged cross-section of normal duct. **(A)** Ducts; **(B)** Lobules; **(C)** Dilated section of duct to hold milk; **(D)** Nipple; **(E)** Fat; **(F)** Pectoralis major muscle; **(G)** Chest wall/rib cage. Enlargement: **(A)** Normal duct cells; **(B)** Basement membrane; **(C)** Lumen: center of duct.[85]

Non-invasive breast cancer cells restrict themselves to the ducts (ductal carcinoma in situ) or lobules (lobular carcinoma in situ) and do not spread to surrounding tissues. Usually, ductal carcinoma in situ is unilateral and will almost certainly progress to invasive cancer in the same breast. However, a woman with lobular carcinoma in situ has a greater chance of developing invasive breast cancer in both breasts. It is estimated that 20% of women with lobular carcinoma in situ ultimately develop invasive cancer.[71]

Invasive cancers (i.e. hormone-independent, ER^-) are much more acute than non-invasive cancers (i.e. hormone-dependent, ER^+), because of their capability of disseminating outside the milk duct (e.g., lymph nodes) and growing into normal tissue within the breast. In addition, invasive cancer cells are capable of traveling through the bloodstream and lymphatic system to different parts of the body, especially: bone, liver, and lung. Invasive cancers are also more resistant to standard therapy.[71] Table 1.1 gives an overview of the various pathologic subgroups of breast cancer based on their histologic appearance.

Table 1.1 Histologic types of breast cancer[71]

Type	Percent Occurrence
Infiltrating ductal (not otherwise specified)	70-80
Medullary	5-8
Colloid (mucinous)	2-4
Tubular	1-2
Papillary	1-2
Invasive lobular	6-8
Noninvasive	4-6
Intraductal	2-3
Lobular in situ	2-3
Rare cancers	<1
Juvenile (secretory)	
Adenoid cystic	
Epidermoid	
Sudoriferous	

1.7 Role of Angiogenesis in Cancer

1.7.1 Physiological and Pathological Angiogenesis

Angiogenesis, the development of new blood vessels from existing vasculature, is an intricate process that is essential for tissue development and repair. During angiogenesis, capillary endothelial cells proliferate at aberrant speed (7-10 days), while under normal conditions vascular cells divide once every 7 years. Angiogenesis is a fundamental part in both physiological (e.g., wound healing, inflammation, ovulation, menarche, and embryogenesis) and pathological (e.g., tumor growth, rheumatoid arthritis, psoriasis, pulmonary hypertension, and atherosclerosis) processes.[59, 86, 87]

The major pro-angiogenic factors are vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Other angiogenic stimulators are angiopoietin (Ang-2), epidermal growth factor (EGF), platelet-derived endothelial growth factor (PD-EGF), platelet-derived growth factor (PDGF), interleukin-8 (IL-8), hypoxia-inducible factor-1α (HIF-1α), and tumor necrosis factor-alpha (TNF-α). Pro-angiogenic factors are also critical to angiogenesis matrix proteins (such as collagen and integrins), cathepsin, urokinase plasminogen activator (uPA), and transforming growth factor-β (TGF-β). Angiogenesis can be physiologically suppressed by endogenous inhibitors, such as angiostatin, endostatin, interferon α/β, platelet factor-4, prolactin (16kD fragment), thrombospondin, and tissue inhibitors of metalloproteinase.[59, 88] The list of angiogenic activators and suppressers increases annually, Table 1.2 illustrates the most common.

Recapitulating angiogenesis can be regulated both by inducers and inhibitors of endothelial cell proliferation and migration. In physiological angiogenesis there is a controlled balance between stimulators and endogenous tissue inhibitors.[59] During this condition, endothelial cells line all blood vessels and form virtually all the capillaries. In order to form new

blood vessels, endothelial cells first break through the basement membrane. Subsequently, the endothelial cells migrate toward an angiogenic stimulus that can be released from e.g., wound-associated macrophages or activated lymphocytes. Through proliferation the migrated endothelial cells provide the necessary number of cells to form a new vessel. This is followed by formation of lumen (canalization) in the endothelial array/sprout and formation of branches and loops by convergence of sprouts to allow blood flow.[89, 90]

Table 1.2 Stimulators and inhibitors of angiogenesis[59, 88]

	Angiogenic Stimulators	**Angiogenic Inhibitors**
Growth factors:	Angiogenin Angiotropin Epidermal growth factor Fibroblast growth factor: acidic and basic Platelet-derived endothelial growth factor Platelet-derived growth factor-BB Tumor necrosis factor-alpha Vascular endothelial growth factor	
Oncogenes	c-myc c-jun c-fos	p53 Rb
Hormones	Estrogen Progesterone Androgen	
Enzymes	Aspartic proteinases (Cathepsin D) Cysteine proteinases (Cathepsin B) Cyclooxygenase-2 Metalloproteinases Urokinase-plasminogen activator	Tissue inhibitor of metalloprotease (TIMP-1, TIMP-2) Plasminogen activator-inhibitor (PAI-1)
Signal Transduction Enzymes	Farnesyltransferase Thymidine phosphorylase	Farnesol
Cytokines	Interleukin-1 Interleukin-6 Interleukin-8	Interleukin-10; Interleukin-12
Endogenous Modulators	Angiopoietin-1 Erythropoietin Hypoxia Nitric oxide synthase Platelet-activating factor Prostaglandin E Thrombopoietin	Angiostatin Endostatin Interferon-α/β Isoflavones Platelet factor-4 Prolactin (16kD) Thrombospondin-1
Trace elements	Copper	Zinc

The mechanism of physiological angiogenesis is characterized by the following steps: (a) pericyte removal from endothelium and angiopoietin-2 (Ang-2)-catalyzed conversion of

endothelial cells from a steady to a proliferative phenotype, (b) growth factors, VEGF and VE cadherin catalyze vessel hyper-permeability and matrix remodeling by the action of serine and matrix metalloproteinase, (c) endothelial cell proliferation catalyzed by growth factors VEGF, FGF, and EGF, (d) endothelial cell migration catalyzed by $\alpha_v\beta_3$ integrin, and the growth factors VEGF and FGF, (e) cell-cell signaling catalyzed by VE cadherin and ephrin B2/ephrin B4, (f) FGF, PDGF, TNF-α, and Eph-2A catalyze tube formation as blood channels, (g) PDGF- and Ang1/Tie2-catalyze proliferation and migration of mesenchymal cells along the new vessels and TGF-β catalyze pericyte differentiation into mature pericytes, and (h) Ang1/Tie2, PDGF, VE cadherins, and TGF-β catalyze vessel stabilization.[88, 91]

In contrast to physiological angiogenesis, pathological angiogenesis exhibits an imbalance between angiogenesis inducers and inhibitors, also known as the angiogenic switch.[66, 88, 92] The external and internal microenvironment, i.e. metabolic and mechanical stress, immune/inflammatory response, and genetic mutations have a significant influence in regulating the molecular mechanism of the angiogenic switch."[88] The angiogenic switch is turned "off" when the balance between proangiogenic and antiangiogenic signaling favors vasculature. In addition, when the switch is in the "off" position, there is a deficiency of angiogenic stimulators, which is identified as insufficient angiogenesis. This stage can lead, for example, to a dormant nonangiogenic tumor that is usually less than 0.5 to 1 mm in diameter ("in situ" carcinoma). However, when proangiogenic conditions thrive, the angiogenic switch is turned "on" which can lead, for example, to tumor vascularization and metastatic growth. When the angiogenic switch is in the "on" position, this is known as excessive angiogenesis.[66, 92]

Excessive angiogenesis is characterized by (a) withdrawal of pericytes from the abluminal surface of the capillary, (b) release of proteases from the activated endothelial cells, (c) protease-catalyzed dissolution of the basement membrane adjacent to the pre-existing vessels,

(d) endothelial cell migration toward an angiogenic stimulus and their proliferation, (e) tube formation, (f) fusion of blood vessels, and (g) initiation of blood flow. The mechanism of insufficient angiogenesis on the other hand involves: (a) insufficient vascularization, (b) delayed formation of granulation tissue, (c) decreased collagen content, (d) loss of vascular tone, (e) higher concentration of oxidized lipids, (f) absence of microtubular structures, (g) impaired collateral vessel formation, and (h) impaired blood vessels.[88, 91] A partial list of human diseases associated with or caused by insufficient and excessive angiogenesis is presented in Table 1.3.

Table 1.3 Diseases characterized or caused by pathological angiogenesis[88, 93]

Insufficient	Excessive
• Chronic non-healing ulcer • Coronary heart disease • Delayed wound healing • Diabetic neuropathy • Hair loss • Heart disease • Impaired collateral vessel formation • Impaired healing of bone fracture • Infertility • Ischemia (myocardial, peripheral, cerebral) • Lymphoedema • Osteoporosis • Placental insufficiency • Scleroderma • Stroke • Systematic sclerosis • Vascular dementia	• Age-related macular degeneration • AIDS complications • Alzheimer's • Bone/cartilage destruction • Diabetes retinopathy • Hematological malignancies • Hepatitis • Multiple sclerosis • Obesity • Ocular neovascularization • Osteoporosis • Parkinson's • Psoriasis • Pulmonary and systematic hypertension • Retinopathy of prematurity • Rheumatoid arthritis • Synovitis • Thyroid enlargement • Tumor growth and metastasis

1.7.2 Hypoxia Tumor Angiogenesis

The fast and highly disorganized growth of solid tumors results in the formation of large cell masses in the central tumor cells which ultimately leads to a heterogeneous distribution of hypoxic (micro-) regions, sometimes anoxic or even necrotic areas, within a tumor.[94] This rapid growth of tumors is accompanied by an increase in oxygen demand and simultaneous decrease in oxygen delivery to central areas of the tumor, primarily because of insufficient blood supply and increased diffusion distance between the blood vessels and the oxygen consuming tumor cells.

Hence, hypoxia, O_2 partial pressure < 2.5 mmHg, has been recognized as a hallmark of locally advanced solid tumors and is identified as the primary pathophysiological stimulus of tumor angiogenesis.[86, 95-98]

It is, therefore, worth mentioning that solid tumors beyond a size of 1 to 2 mm are dependent on angiogenesis for growth (oxygen and nutrients) and metastasis (migration to a different location in the body).[86] Due to the hypoxic environment, tumor cells are capable of expressing angiogenic growth factors such as VEGF, bFGF, IL-8, and TGF-β which in turn trigger the differentiation of the tumor endothelium into mature vessels. Nevertheless, the resulting chaotic tumor microvasculature is seldom complete. Tumor blood vessels are structurally and functionally aberrant (e.g., tortuous, dilated, permeable with lethargic and irregular blood flow) and contain hypoxic regions between the vessels (Figure 1.10).[59, 99, 100]

In 1936, Mottram demonstrated that hypoxic tumor cells are more resistant to radiation therapy, mainly because of a decrease in free radical generation in hypoxic tissue after irradiation.[94] In addition, Gray and colleagues reported in the 1950s the vital role of oxygen concentration during radiation therapy. They found that intratumoral hypoxia reduces the effectiveness of radiation therapy and that exposure of cancer cells to hypoxia before radiation diminished the radiosensitivity.[95] Moreover, studies performed over the past two decades have proven that tumor hypoxia reduces both the efficacy of radiation therapy and of most cytotoxic drugs.[101-105] This insensitivity of tumor cells to chemotherapy can be attributed to (a) hypoxic cells that are not sufficiently exposed to the anticancer drug, because of their distance from the blood vessel,[106, 107] (b) the cytotoxic efficiency of some anti-cancer drugs is increased in the presence of oxygen (producing DNA damage),[101, 108] (c) hypoxic cells are insensitive to p53-mediated apoptosis, which might lead to a diminished effect of some anticancer drugs,[109] and (d) hypoxia stimulates the transcription of genes involved in drug resistance.[102, 103] Finally, it has to

be stated that hypoxic tumor cells are (1) resistant to apoptosis, (2) more invasive and metastatic, and (3) genetically unstable. Hence, they can develop to a more malignant phenotype.[95, 102, 110]

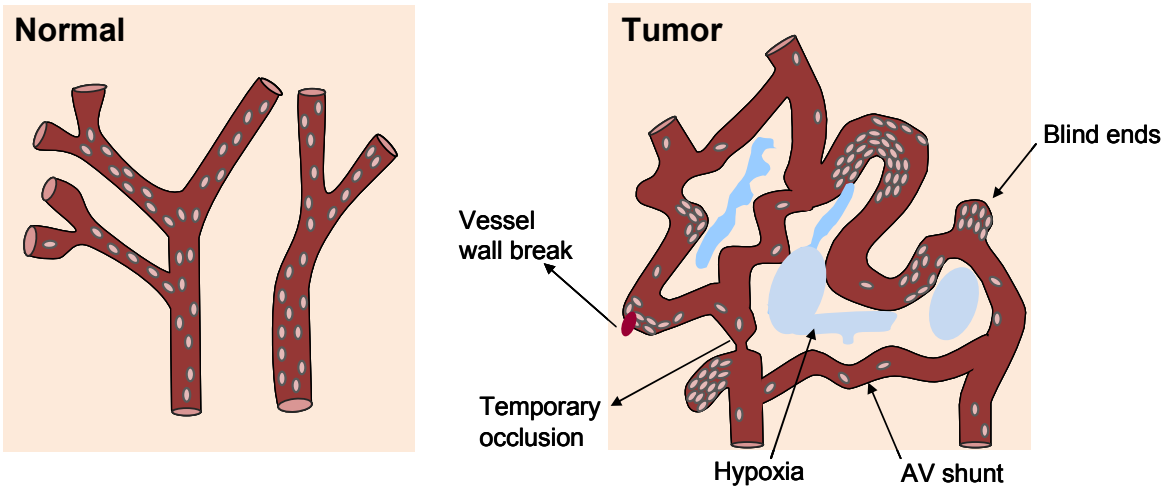


Figure 1.10 Typical differences between normal and malignant vasculatures (Adapted from Brown).[100] In contrast to normal tissue, which has fairly uniform vasculatures that adequately oxygenate the normal cells, tumor vasculatures seldom have complete walls and are structurally and functionally aberrant with hypoxic regions between the vessels

Most cells adapt to hypoxia in three major ways. The first response is by production of angiogenic cytokines such as VEGF which support neovascularization leading to increased tissue oxygenation.[92, 111-113] The second major response is the metabolic switch of glucose metabolism from the oxygen-dependent tricarboxylic acid cycle (yield of 38 ATP molecules) to glycolysis, the oxygen-independent pathway (yield of two ATP molecules). Glycolysis is used by hypoxic tumor cells as a primary mechanism of ATP production.[114-116] The third response to hypoxia is growth adaptation, which is identified by an increase in cell proliferation and survival. Hypoxia may induce in normal cells p53-dependent apoptosis, due to an increased level of p53, or p53-independent apoptosis (e.g., by genes of the Bcl-2 family).[117] However, on the other hand, hypoxic tumor cells can evade hypoxia-induced apoptosis, owing to their capability of inactivating tumor suppressor genes and activating oncogenes.[114, 117, 118]

1.7.2.1 Hypoxia-Inducible Factor-1

The transcription factor hypoxia-inducible factor-1 (HIF-1), a heterodimer consisting of HIF-1α and HIF-1β subunits, is responsible for the regulation of all the genes that encode for proteins in the above-mentioned processes in section 1.7.2.[112, 114, 118, 119] The subunit HIF-1β, a nuclear protein, is constitutively expressed and independent of oxygen, while the subunit HIF-1α, a cytoplasmic protein, is very responsive to oxygen.[120] Under normoxic conditions, HIF-1α is constantly degraded by the ubiquitin-proteasome pathway. During this process oxygen-dependent prolyl hydroxylation of HIF-1α occurs, which then interacts with the von Hippel-Lindau tumor suppressor protein (pVHL), a recognition element of an E3 ubiquitin ligase, ultimately leading to the proteasomal degradation of HIF-1α. However, under hypoxic conditions, the degradation of HIF-1α subunits is suppressed, and these subunits move to the nucleus where they heterodimerize with HIF-1β subunits leading to formation of active HIF-1 proteins.[113, 121, 122]

Additionally, HIF-1 binds exclusively to hypoxic response elements (HRE) present in target genes which finally activate their transcription. Accordingly, HIF-1 regulates expression of more than 30 genes encoding for proteins involved in many processes crucial to cell adaptation, such as: angiogenesis (e.g., VEGF), oxygen delivery (e.g. erythropoietin), glucose and energy metabolism (e.g., glucose transporters and glycolytic enzymes), pH regulation (e.g., carbonic anhydrase-9), cell proliferation and survival (e.g., insulin-like growth factor-2 [IGF-2] and epidermal growth factor [EGF]), and apoptosis (e.g., *Bcl-2* family and *p53* gene).[119, 123] Figure 1.11 gives a schematic overview of the role of HIF-1 in human cancers. Thus far, research has shown that HIF-1α is over-expressed in many solid tumors including bladder, brain, breast, colon, ovarian, pancreatic, prostate, and renal.[114, 124, 125] In addition, its over-expression has proven to be a biomarker for the malignant progression of hypoxia tumor angiogenesis and has

been correlated with poor prognosis and treatment failure in several cancers such as breast, lung, ovarian, and oropharyngeal.[105, 125-128]

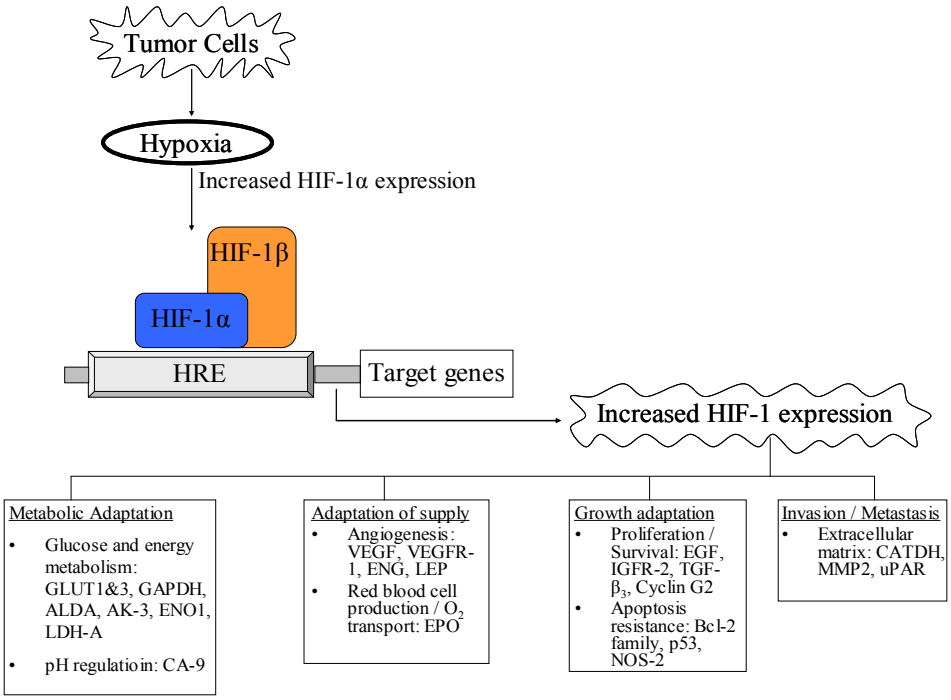


Figure 1.11 Mechanisms and transcriptional activity of HIF-1α in human cancers.[129-131] Increased HIF-1 activity results in up-regulation of genes involved in tumor progression, metabolic adaptation, and aggressiveness, and may lead to increased resistance to standard therapy.

1.7.2.2 Nuclear Factor-κB

Another transcription factor that is also activated in a hypoxic tumor microenvironment is the nuclear factor-κB (NF-κB).[132] Generally, NF-κB is capable of controlling the expression and function of several genes involved in cell cycle, apoptosis, cell growth, tissue invasiveness, and inflammation. Hence, NF-κB has been found to be a key factor competent of inducing various aspects of cellular homeostasis, and thus, can lead to the initiation of cancer.[133, 134]

The transcription factor NF-κB, is a heterodimeric complex of Rel family proteins, mainly composed of p50 and p65 subunits. In normal/quiescent cells NF-κB is present in the cytoplasm where it is inactivated by binding to inhibitory proteins, I-κB (inhibitor-of NF-κB). Degradation of I-κB proteins can occur through the ubiquitin-proteasome pathway upon

exposure of cells to growth factors (e.g., EGF) or receptors (e.g., Her-2/neu), cytokines (e.g., interleukin-1 [IL-1] and tumor necrosis factor-α [TNF-α]), and hypoxic conditions. This leads to translocation of NF-κB to the nucleus, where it binds to response elements of target genes and activates their transcription.[133-136]

Constitutive activation of NF-κB leads to up-regulation of genes that encode for cell proliferation (e.g., cyclin D1 and IL-6), survival (e.g., TNF receptor associated factor and members of *Bcl*-2 family), cell adhesion (e.g., VCAM-1 and ICAM-1), angiogenesis (e.g., VEGF), and tissue invasion and metastasis (e.g., matrix metalloproteinases [MMPs], urokinase-type plasminogen activator [uPA], and IL-8).[59, 133, 136] Elevated levels of NF-κB have been found in many types of human tumors, such as breast, colon, melanomas, ovarian, pancreatic, prostate, and thyroid carcinomas.[137-142]

Altogether, NF-κB has a crucial role in tumorigenesis. Therefore, when the constitutive activation of NF-κB in tumor cells is blocked this may lead to (1) a decrease of their elevated proliferative rate, (2) increased sensitivity to apoptosis during standard therapy, and (3) inhibition of angiogenesis due to a decrease in their tissue invasiveness and metastatic capability. In addition, the over-expression of NF-κB can be blocked at different levels such as through the Iκ-B inhibitory protein, the p65 subunit of the transcriptional active heterodimer, and the proteasome.[133, 143]

1.7.3 Angiogenesis and Breast Cancer

It has been well documented that angiogenesis has an essential role in the growth, progression, and metastasis of breast cancer.[111, 144-146] Almost 50% of locally developed breast cancers have been identified with heterogeneously disseminated hypoxic and/or anoxic regions within the tumor mass. The treatment of hypoxic tumor cells still remains a serious challenge since hypoxia bestow, directly or indirectly, resistance to standard therapy, mainly radiotherapy

and chemotherapy.[100, 147, 148] Hence, understanding the fundamental mechanism and the exact consequences of tumor hypoxia is of uttermost importance in oncologic drug discovery.

Several key factors are known to play an imperative role in the development of breast cancer. Most of these biomarkers are associated with the hypoxia/HIF-1 system, which explains the over-expression of HIF-1α in pre-invasive as well invasive breast cancer cells.[146, 149] It has been found that high levels of HIF-1α are correlated with over-expression of VEGF,[149] estrogen receptor (ER),[149] HER-2/neu,[127] bFGF,[146] PDGF-BB,[146] and EGFR[146] in breast cancer cells. Other biomarkers that have been found to be extremely elevated during the development and progression of breast cancer include *c-myc,*[150] HER-1/EGFR,[151] p53,[152] *Bcl-2,*[153] telomerase activity,[154] and interleukin (IL-12).[155]

Activation of NF-κB has also been associated with progression from hormone dependent (non-invasive) to hormone-independent (invasive) breast cancer.[137] The most significant biomarkers associated with the aggressiveness of breast cancer are matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9),[156, 157] cathepsin D,[158] NF-κB,[159, 160] heat shock protein of 90kDa [Hsp90],[161, 162] cyclooxygenase-2 [COX-2],[163, 164] and uPA.[165] Taken together, the majority of breast cancer biomarkers has been identified in the proliferative and invasive stage and is correlated to cell proliferation, migration, invasion, hormone-dependence, apoptosis, and metastasis.[56, 145, 166]

1.8 Inhibition of Tumor Angiogenesis

1.8.1. Angiogenesis as Therapeutic Target

Understanding tumor angiogenesis and its mechanism is of extreme significance in providing insight into various ways to disrupt the process. One essential biomedical approach is the development/discovery of antiangiogenic agents that can restore the angiogenic switch to the "off" position.[66, 86, 92] Therefore, several angiogenic inhibitors, with respect to their target and

function, are currently in both pre-clinical and clinical trials. Angiogenesis inhibitors are extremely specific therapeutic agents since they can be aimed towards pathological angiogenesis, while causing low toxicity and few side effects. Recall that physiological angiogenesis only occurs during certain processes in the human body, for example, wound healing, embryogenesis, and menarche.[167, 168]

Antiangiogenic agents can be categorized, based upon their mechanism of action, into two groups: direct and indirect. Direct angiogenesis inhibitors (e.g., thalidomide, angiozyme, and endostatin) target vascular endothelial cells (ECs) and prevent them from proliferating, migrating, or evading cell death by decreasing/blocking growth factors (e.g., VEGF, bFGF, and IGF). This antiangiogenic therapy is very promising, because direct antiangiogenic agents are very selective inhibitors due to the specific physiology of ECs. In addition, proliferating tumor-derived endothelial cells are more sensitive to antiangiogenic agents than quiescent endothelial cells. Finally, endothelial cells are genetically stable, and therefore, cannot develop drug resistance.[167, 169, 170]

In contrast to direct antiangiogenic agents, indirect angiogenesis inhibitors interfere with tumor-cell proteins and block their expression/activity that stimulates angiogenesis or its receptors.[170] Numerous of these tumor proteins are known to be the products of oncogenes and some of tumor suppressor genes, which control the angiogenic switch.[171, 172] Therefore, indirect inhibitors not only prevent tumor-cell proliferation and stimulate cell death, but also diminish or stop the production of angiogenic factors. However, the use of indirect anti-angiogenic agents might be discontinued in an early stage, due to acquired drug resistance which is a result of increased tumor angiogenesis.[167, 170] As an alternative, an anti-angiogenic cocktail can be used, for example, by adding a second inhibitor to the therapeutic treatment.[168, 173]

Angiogenesis inhibitors can also be classified according to their function into various groups such as (1) drugs that obstruct vascular endothelial activation, (2) compounds that block vascular endothelial migration, proliferation, and survival, (3) agents that prevent degradation of extracellular matrix, and (4) compounds that impede for example, integrin activation.[168, 174]

1.8.2 HIF-1α and NF-κB as Therapeutic Target

A more fundamental mechanism-based antiangiogenic approach is the inhibition of HIF-1α. The HIF-1 transcription factor (reviewed in §1.7.2.1) has been identified as one of the most significant mediators of cell response to low oxygen levels. Several investigations have proven that HIF-1 activity, which depends on HIF-1α availability, contributes to turning the angiogenic switch "on" in many tumors such as breast, ovarian, and brain cancer.[124, 125, 149] Furthermore, HIF-1 activity drives tumor angiogenesis, promotes cell survival, tissue invasiveness and metastasis. Evidently, elevated levels of HIF-1α are correlated with increased formation of pro-angiogenic factors (e.g., VEGF), aggressive tumor growth, and poor prognosis.[124, 146, 149] Therefore, the use of anti-HIF-1 agents can lead to tumor starvation (i.e. decreased tumor growth, vascularization, and energy metabolism) and ultimately inhibit tumor growth.[105, 175, 176]

The transcription factor NF-κB also has an imperative role in tumor angiogenesis. Hence, suppression/inactivation of NF-κB has been found to be a promising therapeutic method in the treatment of cancer.[133, 143, 177] For example, inhibition of NF-κB, through I-κB, can make chemoresistant tumors sensitive to chemotherapy, which will result in tumor regression.[143] Hence, a unique approach would be to incorporate NF-κB inhibitors with standard therapy with the aim of preventing or overcoming radiation and chemo-resistance of solid tumors.[159, 178] Another approach is using proteasome inhibitors to inactivate NF-κB for treatment of hematological malignancies, such as multiple myeloma and adult T-cell leukemia.[179-181]

In summary, to selectively and effectively inhibit tumor angiogenesis a potential therapeutic approach could be combining anti-angiogenic therapy and anti-HIF-directed therapy. Using anti-HIF agents as part of the anti-angiogenic therapy has several advantages, such as (1) synergistic effect, (2) prevention of hypoxia resistance, (3) target mainly resistant cancer cells.[182] Furthermore, the addition of NF-κB inhibitors to standard therapy could enhance the efficiency of the treatment.[143] Hence, identifying novel compounds, with the aim to inhibit tumor angiogenesis from this prospective, is of extreme importance in oncologic drug discovery.

1.8.3 Natural Compounds as Antiangiogenic Agents against Cancer

As previously discussed, VEGF plays a vital role in angiogenesis since it stimulates endothelial cell proliferation and migration. VEGF is one of the most potent and vital inducers of vascular permeability, the rate-limiting step, in angiogenesis. Primarily, VEGF is produced within tumors in response to hypoxic conditions. Secondarily, it can be produced by macrophages in solid tumors as a reaction to elevated levels of other growth factors such as PDGF, EGF, TNF, and TGF-β.[54, 59] In addition, it is worth mentioning that the receptors for most growth factors are protein tyrosine kinases (PTK). Subsequently, the membrane receptor PTK activates protein kinase C (PKC) leading to aberrant high PKC activity in cancer cells. Both PTK and PKC are signal transduction proteins that play a role in angiogenesis.[59]

The production of VEGF can be reduced by natural compounds such as antioxidants (e.g., vitamin E) and inhibitors of PTK (e.g., epigallocatechin gallate), PKC (e.g., eicosapentaenoic acid), and NF-κB (e.g., resveratrol), as well as anti-copper compounds and vitamins A and D_3.[59, 183] Accordingly, these inhibitors are also capable of reducing the stimulatory effects of growth factors such as EGF and PDGF on VEGF production. For example, several investigations have been performed on the effect of PTK inhibitors, such as green tea catechins, genistein, and curcumin on EGF and PDGF signaling in various cancer cells.[184-187]

Flavonoids, for example luteolin and apigenin, have been reported to also inhibit angiogenesis through blocking VEGF expression by suppression of HIF-1α.[188, 189] In addition, several natural compounds/medicinal plants, such as anthocyanidins, butcher's broom, horse chestnut, and proanthocyanidins, have been examined on their inhibition effect on increased vascular permeability.[59] Finally, natural compounds can have multiple effects on the inhibition of several angiogenic factors, which can be of enormous benefit since the body uses multiple factors to ensure angiogenesis.[59, 190] Table 1.4 lists some naturally occurring bioactive compounds present in foods/medicinal plants and their mechanism of action on the inhibition of angiogenesis.

Table 1.4 Effect of natural products on the inhibition of angiogenesis[59, 190]

Natural Product	**Bioactive Compound**	**Mechanism of Inhibition**
Black seed	Thymoquinone	Apotosis[191]
Citrus	Apigenin	↓TNF, IL-1, inflammation, HIF-1α, MMPs; Inhibits VEGF and HIF-1α[189, 192]
Citrus, Onion	Quercetin	Inhibits inflammation, PKC, ↓VEGF [193, 194]
Ginger	6-gingerol, 6-shogaol	Inhibition of NF-κB activation, ↓VEGF and IL-8[183]
Ginseng	Ginsenosides	↓COX-2, stimulates wound healing, ↓VEGF and bFGF-induced angiogenesis, MMP-2, MMP-9[195]
Grapes, nuts	Resveratrol	Inhibits EC activation, FGF, VEGF, wound healing, COX-2, TNF-α, MMP-2, MMP-9, ↓NF-κB[196-198]
Green tea	Catechins (EGCG, ECG)	↓EC proliferation, MMP-2, MMP-9, NF-κB; Inhibits uPA, cell migration and invasion[199-201]
Mammalian milk	Vitamin D_3	↓EC proliferation, VEGF, EGF, MMPs, ↑TGF [202, 203]
Oilseeds, milk, succinate	Vitamin E	↓VEGF, TGF-α, IL-8, VE-cadherin[204, 205]
Omega-3 fatty acids	EPA	Inhibits PKC, ↓VEGF[206, 207]
Pepper	Capsaicin	Inhibits VEGF and COX-2 expression[208, 209]
Rhizome of *Rheum palmatum* L.	Emodin	Inhibits PTK, NF-κB (weak), cell proliferation and cell adhesion, induces apoptosis[210-212]
Soybean	Isoflavones (eg., genistein)	↓EC proliferation, block uPA, EC migration, and proliferation; ↓EGF, VEGF, NF-κB[213, 214]
Turmeric	Curcumin	Inhibits MMPs, HIF-1; ↓EC proliferation. Apoptosis; ↓COX-2, NF-κB[215, 216]

1.9 Scope of Dissertation

This dissertation is focused on the phytochemical investigation of root extracts of the medicinal plant *Cassia alata* L. and the anti-angiogenic biochemical analysis of its primary anthraquinone, rhein. Chapter 2 gives a review of the fundamental theory of the various analytical techniques used throughout this dissertation, including high performance liquid chromatography (HPLC), thin layer chromatography (TLC), flash column chromatography (FCC), mass spectrometry (MS), ultraviolet-visible (UV-Vis), infrared (IR), and nuclear magnetic resonance (NMR) spectroscopy. In addition, I reviewed the several bio-analytical methods used in the course of this study namely, *in vitro* angiogenesis assays, enzyme-linked immuno-adsorbent assay (ELISA), and Western Blot.

In chapter 3, a simple HPLC method was developed and validated for the determination of six phenolic compounds, five anthraquinones (rhein, aloe-emodin, emodin, chrysophanol and physcion) and a flavonoid (kaempferol), in root extracts from *C. alata*. The extracts were analyzed on a C_{18} column using an isocratic mobile phase. Identification of the analytes was performed by use of standards and on-line mass spectrometric detection using atmospheric pressure chemical ionization. The concentration of the phenolic compounds in the root extracts was determined using HPLC with ultraviolet detection at 260 nm. Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) was found to be the primary anthraquinone in the roots. In chapter 4, the isolation and characterization of rhein from the root extract is reported. Rhein was fractionated and isolated using FCC on a silica column with gradient elution, and purified by use of normal phase PLC plates. In addition, rhein was characterized by MS, ^{1}H-NMR, IR, and UV spectroscopy.

In chapter 5, the effectiveness of rhein as a potential inhibitor of hypoxia-induced tumor angiogenesis in non-invasive (MCF-7) and invasive (MDA-MB-435s) breast cancer cells is

explored. Several bioanalytical techniques were used during the anti-angiogenic evaluation of rhein including *in vitro* angiogenesis assays, ELISA, and Western Blot. The effectiveness of rhein as an anti-angiogenic agent was determined by assessing the *in vitro* angiogenesis activity of HUVECs, the cell viability of MCF and MDA-MB-435s cells, and the cell migration and invasion of MDA-MB-435s cells. In addition, the influence of rhein on VEGF and EGF levels of supernatant fractions in MCF-7 and MDA-MB-435s cells under normoxic and HIF-induced conditions was investigated. Furthermore, we examined cytoplasmic and nuclear extracts of both rhein-treated cell lines in relation to several other angiogenesis factors including HIF-1, NF-κB, HER-2, COX-2, and Hsp90α and β. Finally, chapter 6 recapitulates the studies in this dissertation and provides recommendations for future research.

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CHAPTER 2

ANALYTICAL TECHNIQUES USED IN THIS STUDY

2.1 Chromatographic Methods

One of the primary challenges in the analysis of natural product samples is the interference of phytochemicals with the one under investigation. Hence, the use of chromatographic techniques such as thin layer chromatography (TLC), gas chromatograpy (GC), and/or high performance liquid chromatography (HPLC) is of great significance during the analysis (i.e., separation, identification, and quantification) of phytochemicals. In addition, the utilization of flash column chromatography, preparative layer chromatography, and/or preparative-high performance liquid chromatography may be required to separate and purify a specific phytochemical. The review in this chapter is focused on the theoretical/instrumental aspects of the chromatographic techniques used in the course of this study.

2.1.1 History, Description, and Classification

The Russian botanist, Mikhail Tswett (1872-1919) is usually referred to as the *Father of Chromatography*. In 1906, he published his work on the separation of dyes from plant extracts by use of column chromatography. In this study, he used calcium carbonate as a stationary phase and petroleum ether as the mobile phase. After separation of the dyes, which appeared as colored bands on the glass column, he removed the calcium carbonate, fractionated it, and recovered the plant pigments by extraction. Tswett named this separation method "Chromatography" using the Greek words for *color* and *to write*.[1]

It was not until 1931 when this technique was used for biochemical separations that it became a common analytical method. The pioneering work of Martin and Synge, published in 1941, introduced liquid-liquid chromatography and the accompanying theory that became known as the plate theory. In 1952, both scientists were awarded the Nobel Prize in chemistry for their

outstanding work.[2] It is noteworthy that between 1937 and 1972 several Nobel Prizes (a total of 12) were awarded to scientists for their significant contribution to the chromatographic field. The Dutch chemical engineers van Deemter, Zuiderweg, and Klinkenberg published an imperative paper in 1956 that became the backbone of chromatographic theory. This manuscript explained the kinetics of a chromatographic process and examined the diffusion and mass transfer, which resulted in the popular van Deemter equation.[3]

Chromatographic analysis generally involves the separation, detection, and determination of analytes in a multi-component mixture. During the chromatographic process, separation is achieved through passing a mobile phase over a phase that remains fixed, or stationary. In addition, the sample is introduced into the mobile phase and as it moves with the mobile phase, its components partition themselves between the mobile and the stationary phase. Sample components whose distribution ratio favors the stationary phase travel slowly with the mobile phase (i.e. Q_m in Figure 2.1). On the contrary, components that have a high affinity for the mobile phase require a shorter pass through the column (i.e. R_m in Figure 2.1).[4, 5]

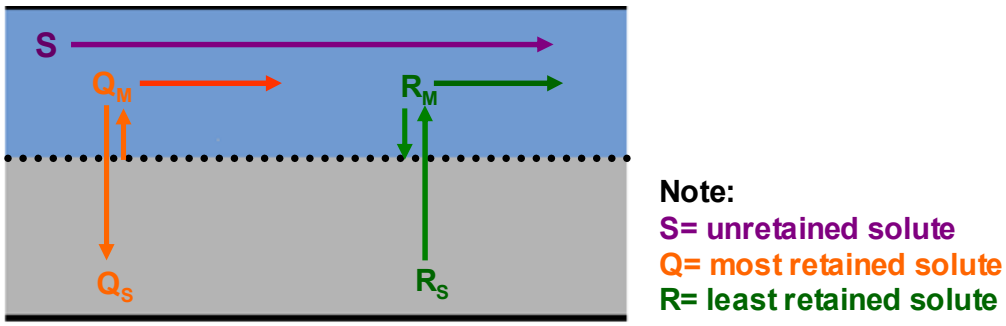


Figure 2.1 Features of retention in liquid chromatography[5]

A common classification of chromatographic methods is based upon the physical method of bringing the mobile and stationary phase into contact with each other, i.e. in a column or on a planar surface. In column chromatography, the stationary phase is situated within a column through which the mobile phase moves under the influence of pressure, vacuum, or gravity. In

planar chromatography, the stationary phase is coated on a flat sheet of glass, metal, plastic, or paper and the mobile phase moves by capillary action or gravity. It is instructive to note that the equilibria for these two chromatographic categories are the same. Accordingly, the developed theory for column chromatography is adapted to planar chromatography.[4, 6, 7]

The second classification is based upon the types of mobile and stationary phases. According to this classification there are three general categories of chromatography, namely, liquid, gas, and supercritical-fluid, which indicates that the mobile phase in these methods are liquids, gases, and supercritical fluids, respectively. Liquid chromatography forms an integral part of the research presented in this Dissertation. Therefore, the discussion in the following section is focused on the theory of column chromatography, primarily with respect to liquid chromatography. Figure 2.2 summarizes a complete classification scheme of liquid chromatography.[4, 6]

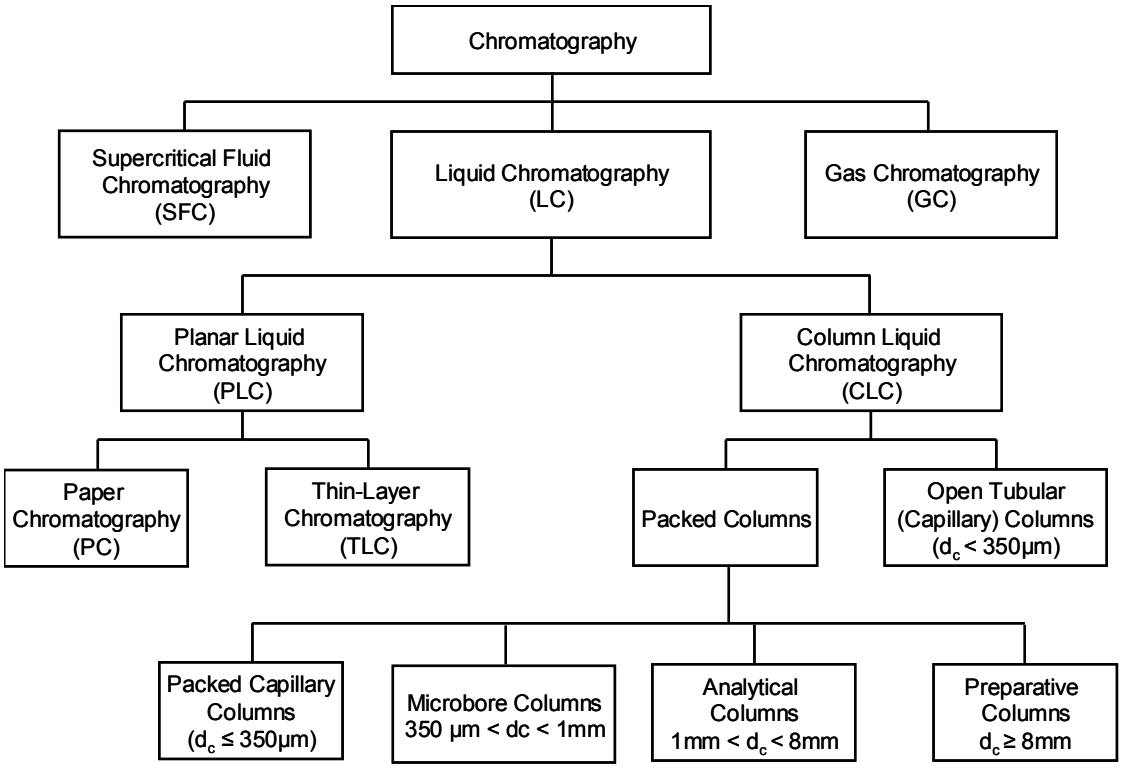


Figure 2.2 Classification of chromatographic methods[6]

2.1.2 Fundamental Theory and Terminology

In column chromatography the sample is injected as a narrow band into the mobile phase at the head of the column. While the sample travels through the column, its components start to separate and the single components begin to broaden and develop a Gaussian peak shape. The solutes will separate into individual zones if the strength of each component's interaction with the stationary phase (i.e. solute distribution constant) is significantly different from each other. These individual zones can be monitored with a detector, depending on the nature of the analytes being studied, and/or collected at the end of the column.[5, 7]

2.1.2.1 Retention Parameters

In order to accomplish isolation of the separated components, sufficient mobile phase must be passed through the column at a constant flow rate (F) or at a flow rate that is intentionally changed or programmed. A common way of presenting chromatographic data is by plotting the detector's signal as a function of time (t) or volume (V) of eluted mobile phase. This plot is called a *chromatogram* and is useful for both qualitative and quantitative analysis. The retention time is the time required for a component to elute from the column. Therefore, the symbols t and V are labeled with a subscript R:

$$V_R = t_R \times F \tag{1.1}$$

An example of a typical chromatogram is given in Figure 2.3. As can be seen each Gaussian peak can be identified by its retention time and width at the baseline. The width of the peak (w), measured in units of time or volume, is governed by kinetic effects related to the solute's migration between the stationary and mobile phase. In turn these effects are controlled by various factors jointly named "column efficiency" (*vide infra*). The baseline width can be determined by the intersection with the baseline and tangent lines drawn from the inflection points of the peaks. In addition, another chromatographic characteristic is the void time (or dead

time), t_M, which is defined as the time it takes for an unretained solute to pass through the column.[4]

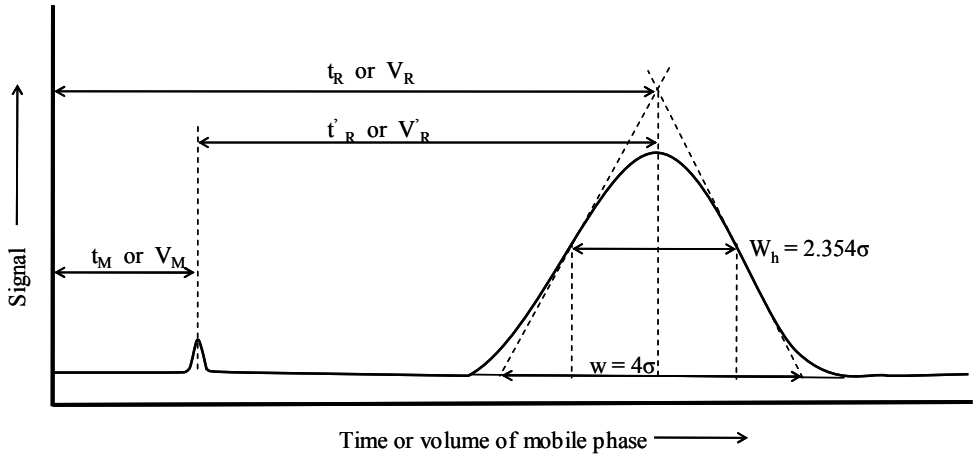


Figure 2.3 A typical chromatogram

During a chromatographic separation the analytes distribute themselves between the mobile and stationary phases. The interaction of a solute with these two phases is described by column efficiency. The distribution of an analyte A between the mobile and the stationary phase can be written as follows:

$$A_{mobile} \leftrightarrow A_{stationary}. \tag{1.2}$$

Additionally, the distribution constant or partition coefficient, K_c, for this reaction is defined as the ratio of the concentration of analyte A in the stationary phase to its concentration in the mobile phase.

$$K_c = \frac{[A]_S}{[A]_M} \tag{1.3}$$

This equation for the distribution constant applies to linear chromatography, which is characterized by symmetrical Gaussian peaks.[4, 7] Therefore, this review will be restricted to the theoretical discussion of linear chromatography.

An important factor describing the column selectivity is the retention factor or capacity factor, *k'*. The retention factor describes the elution rate of a solute from the stationary phase and can be determined using the following formula:

$$k' = \frac{t_R - t_M}{t_M} \quad \text{or} \quad k' = \frac{V_R - V_M}{V_M}. \tag{1.4}$$

Another way of expressing the retention factor is through its relationship with the partition coefficient (K_c) by considering the volumes of the solute in the stationary (V_S) and mobile (V_M) phases, using the equation:

$$k' = K_c\left(\frac{V_S}{V_M}\right) \tag{1.5}$$

In addition, the ratio of retention factors for two solutes A and B can be used to determine the selectivity factor, α, of a column and is defined by[4, 8, 9]

$$\alpha = \frac{k'_B}{k'_A}. \tag{1.6}$$

2.1.2.2 Plate Number

The plate number, plate count or number of theoretical plates (N) is the most common expression of the efficiency of a chromatographic column and is determined as follows:

$$N = \left(\frac{t_R}{\sigma}\right)^2. \tag{1.7}$$

The plate count is dimensionless when both parameters t_R and σ are measured in the same units. The parameter σ can be expressed for a Gaussian distribution by using the peak width:

$$\sigma = \left(\frac{w_h}{2.354}\right) = \left(\frac{w}{4}\right), \tag{1.8}$$

where w_h is the width of the peak at half its maximum height. In addition, by substituting equation 1.8 in equation 1.7, the plate number N is calculated as follow[4]

$$N = 5.54\left(\frac{t_R}{w_h}\right)^2 \qquad \text{or} \qquad N = 16\left(\frac{t_R}{w}\right)^2. \tag{1.9}$$

2.1.2.3 Band Broadening

An important factor that can influence column efficiency is band broadening. During this process, as the solute migrates through the column, continuous broadening of the initial analyte zone occurs. Martin and Synge described the theoretical aspects of chromatography by treating the column as though it contained numerous discrete adjacent segments, where partition of the solute occurred between phases. These layers or theoretical plates, quantitatively define column efficiency in terms of plate height (*H*) and number of theoretical plates (*N*). In fact, the plate height or *the height equivalent to a theoretical plate* (HETP) is referred to as the column length that contains one plate, which is obtained by:

$$H = \frac{L}{N}, \tag{1.10}$$

where L is the total length of the column, usually in centimeters. Optimum column efficiency is determined by a high plate number value and a small value for the plate height.[2, 4, 7]

There are several kinetic parameters affecting chromatographic zone broadening, which are best described by the van Deemter equation.

$$H = H_{TOT} = A + B/u + Cu$$

$$H_{TOT} = A + B/u + (C_S + C_M)u \tag{1.11}$$

In this equation H_{TOT} is the height equivalent to a theoretical plate in centimeters, u is the linear flow velocity in centimeters per second, and the coefficients A, B, and C represent the eddy diffusion, longitudinal diffusion, and resistance mass transfer, respectively. In addition, C_S and C_M are the resistance to mass transfer in the stationary and in the mobile phase, respectively.[4, 8]

In packed columns peak broadening can occur due to the fact that different solute molecules take multiple paths down the column over a period of time. Therefore, the A-term, also known as the multiple path term, is dependent on the packing factor, λ, and the particle size diameter, d_p. Equation 1.12 describes this relationship for the plate height contributable to eddy diffusion.[7, 8, 10]

$$H_E = 2\lambda d_p \tag{1.12}$$

The second cause of peak broadening in column chromatography is longitudinal diffusion. This process occurs when a concentrated band of solutes diffuses down the column over a certain time interval. Consequently, the longitudinal diffusion term (H_L) or B-term is inversely proportional to the linear flow velocity (u), i.e. at a high velocity there is less diffusing of the solute band. In addition, the B-term is directly proportional to the tortuosity factor, γ (for packed columns: γ = ~0.6), which implies that H_L is impeded by the packing. Furthermore, the longitudinal diffusion term is dependent on the diffusion coefficient of the mobile phase (D_M), which is a migration rate constant. In liquid chromatography (LC), the B-term usually approaches zero since the diffusion coefficients of liquids are very small. The mathematical relationship between H_L with the above mentioned factors is described by[7, 8]

$$H_L = \frac{2\gamma D_m}{u}. \tag{1.13}$$

The third coefficient that contributes to band broadening in column chromatography is the resistance to mass transfer term (C-term). The mass transfer in the mobile phase or stationary phase is not instantaneous. Hence, a non-equilibrium continuous process takes place as the solute migrates down a column, leading to peak broadening. The slower the solute migrates the more time it has to equilibrate. The stationary phase mass transfer term is based on the variance of time that analyte molecules reside in the stationary phase. For example, solute molecules that

diffuse deeper into the stationary phase will reside longer in this phase before returning to the mobile phase. Accordingly, the stationary phase mass transfer coefficient is directly proportional to the thickness of the stationary phase d_f and the linear velocity, while indirectly proportional to the diffusion coefficient D_S of the solute in the stationary phase.[8, 10, 11]

$$H_S = \frac{k' d_f^{\,2} u}{D_S (1+k')^2} \tag{1.14}$$

The mobile phase mass transfer term can be subdivided into two terms: the intra-particle and inter-particle stagnant mobile phase. The mass transfer between the stationary phase and mobile phase is due to stagnant pools in the intra-particle void volume, which leads to band broadening. The mathematical definition for the intra-particle stagnant mobile phase for packed LC columns is described as follows

$$H_{SM} = \frac{\gamma'(1-\phi+k')^2 d_p^{\,2} u}{30(1-\phi)(1+k')^2 \gamma D_M}, \tag{1.15}$$

where γ' is the intra-particle tortuosity factor and ϕ is the fraction of stagnant mobile phase relative to the terminal mobile phase in the column. The inter-particle stagnant mobile phase mass transfer H_M depends mainly on how well the column is packed. This is denoted by the constant, ω (~ 0.02 - 5.0), which takes into account the effect of the packing structure. The inter-particle mass transfer term for packed columns is determined as follows[8-10]

$$H_M = \frac{\omega d_p^{\,2} u}{D_M}. \tag{1.16}$$

Theoretically, H_M is very difficult to predict, since the exact flow profile of the mobile phase is not known and the diffusion between particles takes place slowly. Therefore, according to Giddings,[12] the eddy diffusion can be combined with the inter-particle mass transfer term (convective flow) since they are dependent on each other. Equation 1.17 describes this

relationship. It is instructive to note that only at high linear flow velocity and large particle size does the H_{ME} term significantly contribute to H_{TOT}.[10, 12]

$$H_{ME} = \frac{1}{\frac{1}{H_E} + \frac{1}{H_M}} = \frac{1}{\frac{1}{2\lambda d_p} + \frac{1}{\omega d_p{}^2 u / D_M}} \tag{1.17}$$

Finally, another contribution to band broadening arises from dispersion in different instrumental components such as the injector, the connecting tubing, and the detector. These extra-column effects (H_{EXT}) are often more obvious with peaks having low *k'* values. Recall that band broadening increases with retention (Equation 1.7). Extra-column effects can be diminished for LC columns by retaining the total extra-column volumes at less than 10% of the peak volume. Subsequently, the general plate height equation for packed LC columns can be denoted as follows

$$H_{TOT} = H_E + H_M + H_L + H_S + H_{SM} + H_{EXT} . \tag{1.18}$$

Recapitulating, the van Deempter equation (Equation 1.11) primarily describes the relationship between plate height and linear velocity taking into account several other factors which also contribute to optimum column efficiency. Figure 2.4 illustrates the van Deempter plot applied to liquid chromatography.[3, 6, 10]

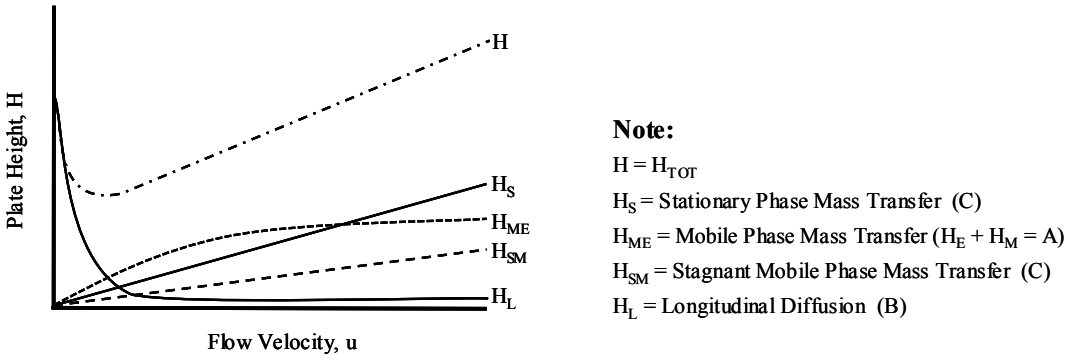


Figure 2.4 Van Deempter plot applied to liquid chromatography[6, 8]

2.1.2.4 Resolution

The aim of chromatography is to accomplish separation between a series of analytes. The resolution (R_S) of two peaks, A and B, is the evaluation of their separation and is determined as follows

$$RS = \frac{2[(t_R)_B - (t_R)_A]}{w_A + w_B}, \tag{1.19}$$

where w_A and w_B are the widths of peak A and B, respectively. An example of hypothetical separation is given in Figure 2.5. A resolution value of 1.5 or greater indicates complete separation between two peaks. An additional equation that has been derived for calculating the resolution is one that correlates with the selectivity factors of peaks A and B, the retention factor, and the number of theoretical plates.[8, 10]

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1 + k'}\right) \tag{1.20}$$

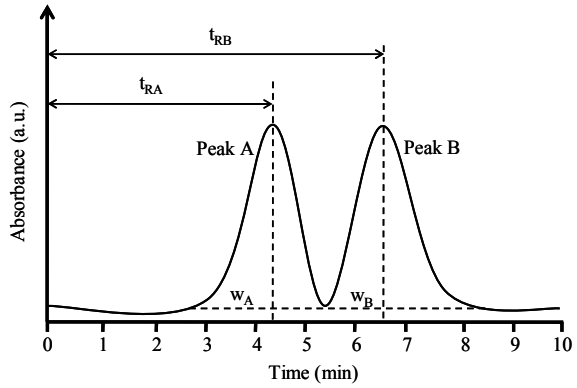


Figure 2.5 A hypothetical separation

2.1.3 High Performance Liquid Chromatography

High performance liquid chromatography **(**HPLC), also known as high speed or high pressure liquid chromatography is an analytical technique that is extensively used in various industries such as biochemical, environmental, food, petrochemical, and pharmaceutical. The wide range of applications of HPLC is due to the fact that both the stationary and mobile phase

interaction can be functional in the separation of analytes; therefore, the resulting selectivity is higher. Moreover, HPLC has the largest number of applications in the analysis of biological extracts, mostly because it allows direct analysis of aqueous extracts. An HPLC chromatogram can provide both qualitative and quantitative information. This is due to the fact that each peak in a chromatogram has a specific elution time, as well as an area and height which are both proportional to the amount of analyte present in the sample. In addition, HPLC can be used both as an analytical and preparative technique. Most of the review in this section is devoted to HPLC as an analytical procedure. [4, 13]

High performance liquid chromatography is primarily characterized by high operating pressure (i.e. up to 5000 – 6000 psi) and small particle sizes (i.e. ~ 2 to 10 μm) of the stationary phase. This leads to reduced H (because of a decrease in C_m term) and sequentially permits higher flow rates (ranging from 0.1 to 10 mL/min) to be used. The separation of analytes from a complex mixture can be achieved not only by using the most suitable column (i.e. effectively adjusting α) for the compounds being separated, but also by using the correct mobile phase combined with the most suitable elution system, which can influence both k' and α. At this point please recall equation 1.20.[12, 13]

Accordingly, the elution system is named isocratic when the mobile phase composition is constant during the separation process. On the other hand, if the composition of the mobile phase is intentionally changed or programmed during the separation process, the elution method is called gradient. Gradient elution is very useful, particularly, when there is a wide variation between the retention times of the analytes being separated (i.e., wide range in analyte polarity). In this case, the range of the retention times can be decreased by gradually increasing the solvent strength depending on the separation mode being used. Nevertheless, the use of gradient elution has some drawbacks; for instance, it is very time consuming since the column needs to be

reconditioned before each run. Furthermore, during gradient elution a baseline drift can occur due to the difference in UV absorbance between solvents. In addition, spurious peaks can appear as a result of solvent impurities and flow rate fluctuations can occur because of changes in liquid compressibility.[9, 13]

Another chromatographic technique utilized in this study was flash column chromatography (FCC). This preparative technique was used in the separation and isolation of secondary metabolites. The theoretical aspects for HPLC and FCC are the same; however, the experimental conditions are very diverse. In FCC, the pressure in the column is much lower and the particle size of the stationary phase is much larger (~ d_p > 10 μm) than in HPLC. Flash column chromatography provides, in comparison with HPLC less resolution, while requiring less complicated and inexpensive equipment.[12, 13]

2.1.3.1 Separation Modes

During a chromatographic separation both the stationary and mobile phases interact with the analytes. As a result, separation occurs on a column between, for example, analyte A and B, based on their different retention factors. In turn the retention factor depends on the distribution ratio of the analyte in the stationary and mobile phase. It is worth mentioning that the eluent and analyte molecules vie for active sites on the stationary phase. Therefore, one of the most significant parameters governing the separation is the interaction between the analyte molecules and the stationary phase. The selectivity factor α of a column is another vital parameter during the separation since it determines the column efficiency.[4, 8, 13]

In liquid chromatography, various stationary phases can be used, such as active adsorbent surfaces, polymer-coated supports, and chemically bonded surfaces resulting in a diversity of separation modes. These modes include: normal-phase, reversed-phase, ion-exchange, ion-pair partition, size-exclusion, and affinity chromatography. The most commonly used separation

modes in HPLC and TLC are normal-phase and reversed-phase chromatography, which are part of partition chromatography.[9, 13]

The supports for these two phases can be attached on the surface of the packing as a liquid stationary phase (by physical adsorption) or as a chemically bonded-phase. The majority of bonded-phase coatings consist of a silica support with an organic group attached to it via a silicon oxygen-silicon carbon covalent bonding system. It is noteworthy that most functional bonded-phase coatings are siloxanes (Figure 2.6). Currently, in partition chromatography, the use of the bonded-phase stationary method is preferred over the liquid stationary phase method, because it is more stable as well as more universal. The discussion in the remainder of this section is focused on bonded-phase partition chromatography, in relation to normal-phase and reversed-phase chromatography.[7, 9, 13]

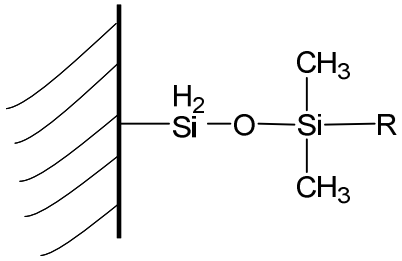


Figure 2.6 Siloxane bonded-phase structure

Normal-phase bonded packings have a highly polar stationary phase due to the polar R group, such as amino, diol, or cyano, in the siloxane structure. Separation of fairly polar organic molecules occurs as they migrate down the adsorbent bed with a relatively non-polar mobile phase (e.g. ethyl ether, chloroform, dichloromethane, *n*-hexane). In normal phase chromatography the most polar compound is eluted last, since it is more strongly retained by the adsorbent surface. Therefore, increasing the polarity of the mobile phase will decrease the elution time of solutes from a normal phase column.[7, 13]

Presently, approximately 80% of all HPLC separations are performed in columns with reversed-phase phase packings. The stationary phase in these packings is non-polar; hence, it is

suitable for the separation of non-polar molecules using a polar mobile phase such as an aqueous mixture of acetonitrile and/or methanol. Generally, in commercial reversed-phase bonded packings, the R group (Figure 2.6) in the siloxane structure is a C_8 chain (n-octyl) or a C_{18} chain (n-octyldecyl). It is obvious that in reversed-phase chromatography polar molecules will have a low affinity for the hydrophobic support, and therefore, will be eluted first by the polar mobile phase. Additionally, in contrast to normal phase, increasing the polarity of the mobile phase will increase the elution time.[7, 9, 13]

2.1.3.2 Instrumentation

The high pressures (up to 6000 psi) which are required to pump the eluent at reasonable flow rates (~ 0.1 – 10 mL/min) through the column packing make the HPLC equipment more complicated and expensive than other chromatography types. Therefore, when selecting an HPLC system from a manufacturer or assembling it from modules it is advisable to take into consideration several factors such as the sample complexity (isocratic versus gradient elution), sample size (analytical versus preparative), sample matrix to be analyzed (detector type), and number of analyses to be performed (manual versus automatic injector).[7, 8, 13]

In general, every modern high performance liquid chromatograph is equipped with the following main components: (a) solvent reservoir, (b) degasser/filter system, (c) pump, (d) pressure gauge, (e) injector, (f) column, (g) detector, and (h) data recorder (Figure 2.7). During a single HPLC run the eluent is filtered, degassed, pressurized, and pumped through the column. The pump is often referred to as the core of an HPLC apparatus, since it provides the mobility of the eluent as well as compels it through the column at a constant and robust flow rate (or pressure). The pump of an HPLC system can be very expensive due to the high-performance devices required to produce high pressures and stable flows.[7, 13]

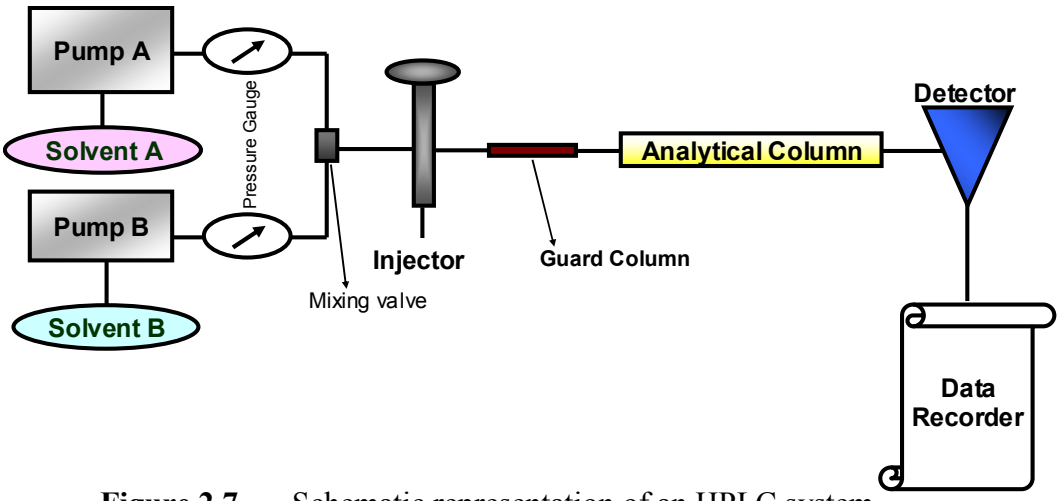


Figure 2.7 Schematic representation of an HPLC system

There are three types of pumps available, namely, reciprocating or constant flow pumps, pneumatic or constant pressure pumps, and syringe or displacement-type pumps. Currently, reciprocating pumps are the most commonly used pumps in commercially available HPLC systems. This is simply because of their small internal volume (35 – 400 μL), high pressure output (up to 10,000 psi), and flexibility to gradient elution. In addition, these pumps are very popular owing to their constant flow rates that tend to be independent of solvent viscosity and column back pressure. In brief, this pump usually operates electrically and drives a reciprocating piston that pressurizes the eleunt from the solvent reservoir via a one-way valve. The drawback of a single piston reciprocating pump is that it produces pulsed flow, which causes significant baseline noise. Therefore, the use of a pulse damper in combination with this pump is a major requirement. Another solution that greatly reduces baseline noise is the use of a dual piston pump, where the pistons are mounted 180° out of phase (one piston is filling while the other pumps).[7, 8, 13]

As a sample matrix is injected at the head of a column, it is separated into single components upon passing through the column. An HPLC column is usually 10 to 30 cm long and has a 0.3 to 10 mm internal diameter (for analytical 1 to 8 mm). Often upstream of the head of an analytical column, a guard column is attached to increase its life time. The column is typically

enclosed in a thermostat in order to perform temperature controlled separations. Lastly, the components are "visualized" using a detector and registered as chromatographic peaks by a recorder.[7, 13]

Hitherto, there has been no universal liquid chromatographic (LC) detector yet developed that is capable of responding to all components with equal sensitivity. However, every LC detector should mainly have (1) adequate sensitivity, (2) high precision, (3) good stability and reproducibility, (4) minimum internal volume, (5) wide linear range, (6) rapid response time, and (7) no substantial effect on the separation process. There are two categories of HPLC detectors, namely, bulk property detectors and solute property detectors. The former type of detectors (for example, refractive index and dielectric constant detectors) compares a property of the eluent with the similar property of the solute. The latter type or selective detectors (for example, UV-Vis, fluorescence, and electrochemical detectors) respond to some specific property of the solute.[8, 13, 14]

The most commonly used detector in HPLC is the UV-Vis because of its high sensitivity (around 10^{-8} or 10^{-9} g/ml), wide dynamic range, as well as insensitivity to flow rate and temperature changes. These detectors operate by measuring the change in absorbance of UV or visible radiation of compounds that absorb in this range such as alkenes and aromatics. When the solute, transported by a UV transparent eluent, leaves the column it goes through a small flow cell, which is situated in the UV-Vis radiation path. Single-wavelength detectors utilize a 254-nm mercury line to permit detection of solute molecules that absorb at this wavelength. Conversely, the constant emission from a deuterium lamp can be employed in combination with a monochromator to offer an adjustable wavelength detector. Variable-wavelength detectors are very effective in acquiring increased sensitivity in complex analyses as well as minimizing the background absorbance of the eluent when needed.[7, 13, 14]

It is noteworthy that many of the absorbance detectors used in HPLC systems are multi-wavelength detectors, most often double-beam devices. During the absorbance measurement with a double-beam device one beam goes through the flow cell and the other via a filter, which reduces its intensity. The intensities of the two beams are then compared by use of matched photoelectric detectors. Another multi-wavelength detector is the diode array, which is the most powerful ultraviolet absorbance detector. Diode array detectors are used to monitor polychromatic radiation that has passed through a flow cell. The released light is then dispersed by a diffraction grating onto a photodiode array surface. Consequently, each photodiode will receive a different wavelength. The major advantage of the diode array detector is that it can simultaneously detect numerous wavelengths.[7, 13, 14]

2.1.4 Thin Layer Chromatography

Thin layer chromatography (TLC) is a mode of planar chromatography, which is part of liquid chromatography. This technique can be used both as an analytical and a preparative method. Thin layer chromatography can provide separations in the milligram up to nanogram range.[15] In this study, analytical TLC was utilized as a preliminary method for the qualitative analysis of *C. alata* root extracts by use of HPLC and FCC. In addition, preparative layer chromatography (PLC) was employed to separate and purify sample compounds with the aim to perform characterization studies. Most of the review in this section is devoted to TLC as an analytical method.

In TLC, the sample matrix is applied as a small spot or zone, with a capillary tube (1 to 10 μL) to the beginning of a thin layer which is coated on a glass plate, polyester, or aluminum sheet. The sorbent layer (i.e. stationary phase) can be composed of aluminum oxides, cellulose, chemically bonded polar and non-polar phases, ion exchangers, polyamides, and silica gel. Around 90% of TLC separations are performed on normal phase silica gel sorbent layers, which

are attached to a glass support. Thin-layer plates are commercially available as high-performance and conventional plates in several sizes such as 5×20, 10×20, and 20×20 cm. The former plates, in comparison with the latter, provide better resolution in a shorter time period due to their relatively thin film thicknesses (~ 100 μm) and small particle sizes (≤ 5 μm). Conventional plates, on the other hand, have thicker layers (200 to 250 μm) and particle diameters of 20 μm or greater.[7, 15]

Small and concentrated spots can be acquired by spotting a sample multiple times in one place and allowing each spot to dry in between. Following the application of the sample, the TLC plate is placed in a saturated development chamber, containing the mobile phase or solvent system. During development of the plate, the mobile phase moves through the sorbent layer by capillary action, at times assisted by gravity or pressure. As in HPLC, the separation of sample components occurs based on their interaction with the stationary phase. In TLC the mobile phase is composed of a single solvent or a mixture of aqueous and/or organic solvents. When the mobile phase has moved about 75% beyond the origin, the plate is removed from the chamber and the solvent front is marked. The mobile phase is then evaporated from the plate in open air or by applying heat.[15]

After drying the plate, the positions of sample substances can be determined in several ways. Colored components are detected in daylight without any treatment. On the contrary, colorless compounds are visualized under ultraviolet light, in case the substances show self-absorption in the short-wave UV region (254 nm), or by use of a detection reagent such as a chromogenic or a fluorogenic. An additional detection method is the incorporation of fluorescent substances into the stationary phase. Subsequent to development the plate is observed under UV light. As a result, the whole plate will fluoresce while the sample substances will quench the fluorescing stationary phase.[7, 15]

Upon detection of a single solute the retardation factor or R_f factor can be determined. The R_f value in TLC is defined as the ratio of the distance migrated by the solute to the distance migrated by the solvent front. Recall that the *k'* value in HPLC determines the ratio of the solute's time present in the stationary phase to the time present in the mobile phase (equation 1.5). Therefore, the R_f value in TLC and the *k'* value in HPLC are related to each other as follows:

$$R_f = \frac{1}{1+k'} \quad \text{or} \quad k' = \frac{1}{R_f} - 1. \tag{1.21}$$

The positions of the substances on the plate are used for qualitative identification. In addition, quantification of the sample components can be achieved by determining the size and/or intensity of the zones.[7, 13, 15] Figure 2.8 shows a schematic representation of a TLC separation process.

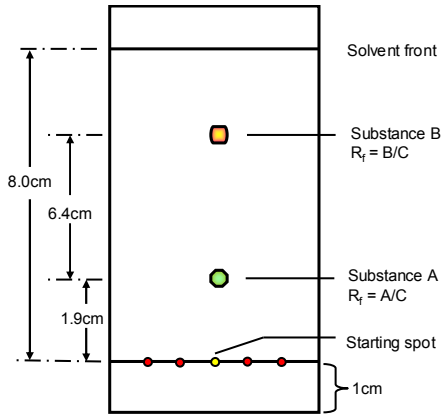


Figure 2.8 Representation of a thin layer chromatogram

2.2 Mass Spectrometry

The coupling of a liquid chromatograph to a mass spectrometer is referred to as liquid chromatography-mass spectrometry (LC-MS), which is a very specific way of identifying organic compounds. The use of LC-MS in the analysis of natural products has proven to be very successful, because of its capability to provide preliminary structural information on extract constituents during a chemical screening. Moreover, this technique provides the vital elements

required for natural product analysis such as small sample size, as well as high separation efficiency and accurate peak identification of crude extract constituents.[16, 17]

Generally, mass spectrometry (MS) is considered as one of the most versatile and comprehensive analytical techniques for the structure elucidation of organic compounds. The mass spectrometer provides extremely high detection sensitivity (detection in the attomole and zeptomole ranges are common) and can also identify the molecular mass and structure of fragment ions. The uniqueness of this analytical technique is that it can be utilized for a diversity of analytes, namely, volatile or non-volatile, polar or non-polar, low or high molecular weight, as well as solid, liquid, or gaseous compounds. Conversely, MS is a destructive analysis method, i.e. after mass spectral analysis the sample cannot be recovered.[16, 18]

Mass spectrometry involves the production of gaseous molecular ions, which are consequently separated in proportion to their mass-to-charge (m/z) ratio, and detected. The ensuing mass spectrum is constructed by plotting the signal intensity versus the m/z-ratio. Basically, a mass spectrometer consists of five parts, namely, inlet or sample introduction system, ion source, mass analyzer, detector, and data system (Figure 2.9). The ion source, mass analyzer, and detector are contained in an ultraclean housing and kept under continuous moderately high vacuum (~10^{-3} to10^{-10} torr). High vacuum assures that the ions, on their way to the detector, do not collide with each other since this could lead to further fragmentation or even divert them from their original path. In addition, high vacuum shelters the metal and oxide surfaces of the above-mentioned MS components from corrosion by water-vapor and air.[16, 18]

The sample introduction system includes controlled leaks for introducing a sample vapor from a reservoir, conjunctions with several chromatographic systems (such as HPLC and GC), and/or a variety of direct insertion probes for introduction of solid materials into the MS. After the sample is introduced into the ion source, the solvent is evaporated, and individual solute

molecules are converted into gaseous ions. Fragmentation of molecular ions can also occur due to the energy that is generated during the ionization process.[16, 18]

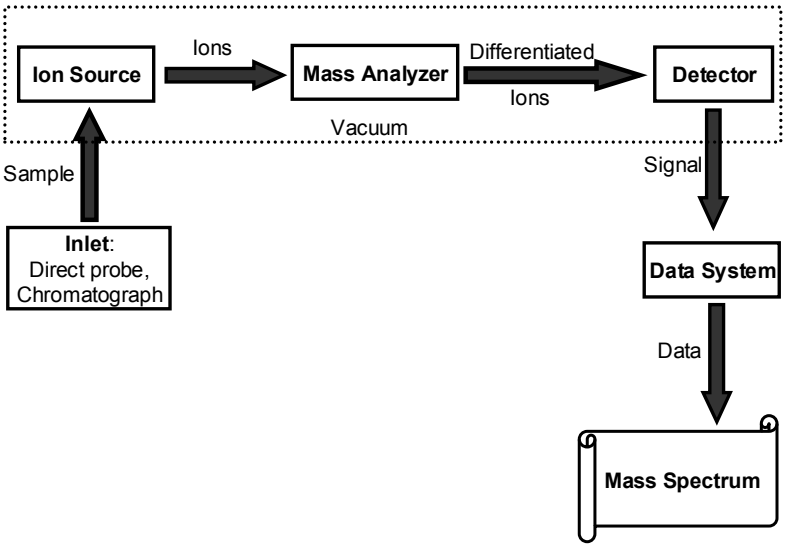
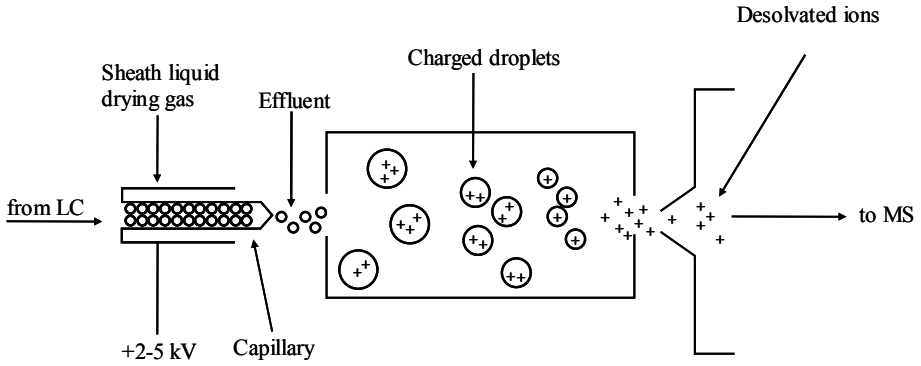


Figure 2.9 Basic diagram for a mass spectrometer

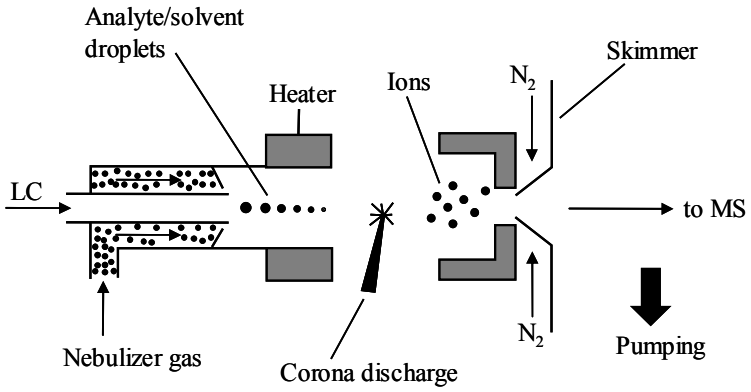
There are two soft ionization techniques frequently used in combination with LC-MS, namely, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). In an ESI interface a strong electric field is applied to an eluent moving through a capillary tube, which allows the formation of solution phase ions that are electrically charged droplets on a solvent spray. Dispersion of the spray is limited through injection of a gas coaxially at a low flow rate. In addition, to remove the last solvent molecules, the charged droplets pass through a curtain of heated inert gas (usually nitrogen). As a result, gas phase ions are generated from the electrospray under atmospheric pressure. This ionization method is suitable for the analysis and characterization of high molecular weight polar molecules such as proteins and peptides (~ larger than 1500 Da). The principle governing an ESI interface is illustrated in Figure 2.10a.[18, 19]

The second ionization technique, APCI, produces primary gaseous ions by corona discharges on a solvent spray at atmospheric pressure. As the eleunt enters the APCI probe it is directly converted by a high-speed nitrogen beam into a thin fog. In the vaporization chamber droplets are then displaced by the gas flow, in which vaporization of the mobile phase and the

solute occurs. The analyte molecules are ionized, subsequent to desolvation, along a corona discharge electrode. This ionization method is typically used for the analysis of less polar or neutral molecules, as well as ionic compounds with moderate molecular weight (up to ~1500 Da). Figure 2.10b shows a generic diagram of an APCI interface. Both probes, ESI and APCI, can be either ran in the positive or negative ionization mode depending on the analytes.[18, 19]



a. Diagram of an ESI source



b. Diagram of an APCI source

Figure 2.10 Schematic diagrams of two soft ionization techniques used in LC-MS[19]

After ionization, the solute molecules enter the mass spectrometer through the curved desolvation line (CDL), which ensures that all of the solvent molecules are vaporized. The charged ions are then propelled into the gas phase through the octapole lense toward the mass analyzer. Subsequently, the mass analyzer separates the ions according to their m/z ratio by use of an electric or magnetic field. There are several types of mass analyzers, namely, quadrupole

mass filters, time-of-flight (TOF) and magnetic sector analyzers, quadrupole ion trap (QIT), and ion cyclotron resonance.[16, 18, 19]

The most commonly used mass analyzer in an LC-MS system is the quadrupole mass filter, which separates analyte masses based on electric field parameters. The quadrupole filter, for a given DC and AC potential, only allows ions of a certain m/z ratio to pass through. The quadrupole consists of four parallel rods, wherein an electric field is created by linking opposite pairs. One pair is utilized for selecting ions with a mass higher than a threshold, while the second pair is used to select ions with molecular weight lower than a particular threshold. Accordingly, ions that do not have the correct frequency are thrown out of their original path and crash onto the quadrupole rods, never reaching the detector. Generally, the quadrupole mass analyzer serves as a narrow band filter of m/z. Additionally, the quadrupole's mass range is usually between 3000 to 4000 Da.[18-20]

Subsequent to mass analysis the detection of ions occurs. The detector consists of a conversion dynode and electron multipliers. Molecular ion detection is performed by the electron multiplier upon conversion of the ion current to an electrical signal. The signal of the multiplier is further amplified and processed by a pre-amplifier, and then sent to the data system. Finally, a mass spectrum, a plot of the (relative) intensity of the ions as a function of the m/z ratio, is obtained.[16, 18, 19]

The mass data can also be recorded in a total ion chromatogram (TIC), wherein the total ion current is plotted versus the time or number of scans. The total ion current is obtained when all the signal intensities (i.e. all mass peaks) for one scan are combined together. When presenting data as a TIC, the mass spectrometer is employed as a general detector, i.e. is operated in the scan mode. On the other hand, the mass spectrometer can be operated in the selected ion monitoring (SIM) mode. In this scenario, a small mass window is monitored resulting in a single

ion mass chromatogram, which denotes the detection of a specific mass. Collecting data in the SIM mode increases the selectivity and the signal-to-noise (S/N) ratio. Therefore, the SIM mode can be very useful for detecting low concentrations of substances in samples.[16, 18]

2.3 Spectrochemical Methods

The structural elucidation of secondary metabolites is generally a challenge in natural product chemistry. Therefore, the use of spectroscopic techniques during the characterization of phytochemicals is a focal point in modern structural analysis. Frequently used spectroscopic methods for structure elucidation of natural products include nuclear magnetic resonance (NMR) spectroscopy and MS. Additionally, UV-Vis and infrared (IR) spectroscopy may be useful in the characterization process.[20]

The spectroscopic techniques such as UV-Vis, IR, and NMR are also known as spectrochemical analysis methods since they involve the interaction of chemical species with electromagnetic radiation (EMR). The spectrum obtained during such an analysis can provide structural information and/or can be used for quantification purposes of the investigated matter. This spectrum records the intensity of absorbed, emitted, or scattered light of the chemical substance versus a quantity associated to photon energy, such as wavelength or frequency. During the interaction of EMR with matter, several electronic transitions occur that propagate certain discrete packets of energies, namely, photons within the electromagnetic spectrum (EMS). The energy released by a photon during electronic transitions in the EMS can be related to its frequency and wavelength by

$$E = h\nu = \frac{hc}{\lambda} \tag{1.22}$$

where E is the energy (J), h is the Planck's constant (6.6261×10^{-34} Js), ν is the frequency (Hz or s^{-1}), c is the speed of light (2.9979×10^{8} m/s, and λ is the wavelength (m).[21] This chapter

provides a brief introduction to the spectrochemical methods employed in the course of this study.

2.3.1 Ultraviolet-Visible Spectroscopy

The UV region of the EMS is within the wavelength range from 10 to 350 nm, whereas the visible region is between 350 and 770 nm. UV-Vis absorption spectroscopy is a molecular characterization technique that measures the amount of ultraviolet or visible radiation (photons) absorbed by a molecule as a function of the wavelength. Upon photon absorption the electrons in the molecule are elevated from the ground energy state to an excited energy state. The absorption of radiation in the UV-Vis region causes electronic transitions at different wavelengths within molecules.[21, 22]

An absorption spectrum is the result of the absorbed light of a molecule at different wavelengths and is obtained by plotting the absorbance versus wavelength. In turn at a specific wavelength there is a linear relationship between the absorbance and concentration of the absorbing molecules. The Beer-Lambert's law denotes this relationship as follows:

$$A = \log(I_o / I) = \varepsilon l c \qquad (1.23)$$

where A is the absorbance, I_o is the intensity of light incident upon the sample cell, I is the intensity of light leaving the sample cell, ε is the molar absorptivity constant ($Lmol^{-1}cm^{-1}$), l is the path length of the sample cell (cm), and c is the molar concentration of the sample (mol/l). By means of this equation a calibration curve, at maximum absorption wavelength (λ_{max}), can be constructed (from a series of known standard concentrations of the absorbing specie) to determine the concentration of the unknown.[21, 22]

In general, a spectrophotometer consists of a radiation source, wavelength selector, a sample section, a detector, and a readout device. A basic diagram of a spectrophotometer is illustrated in Figure 2.11. The wavelength range for a typical spectrometer is between 190 and

800nm. During a spectroscopic measurement, a light source (e.g., deuterium-, hydrogen-, or tungsten lamp) capable of emitting polychromatic radiation in the UV and/or visible EMR region is focused towards the wavelength selector to produce monochromatic light. Typically, the monochromator utilizes a diffraction grating to separate the beam of light into distinct wavelengths. The desired wavelength is then isolated by use of a slit and consequently passed through the sample cell. The transmitted light by the sample is generally detected using a photomultiplier tube or photodiode. These detectors are capable of converting the EMR signal into an electrical signal, which in turn is directly proportional to the light intensity reaching the detector. An absorption spectrometer can be either a single beam or a double beam instrument, and can operate by single or multi-channel detectors.[21, 22]

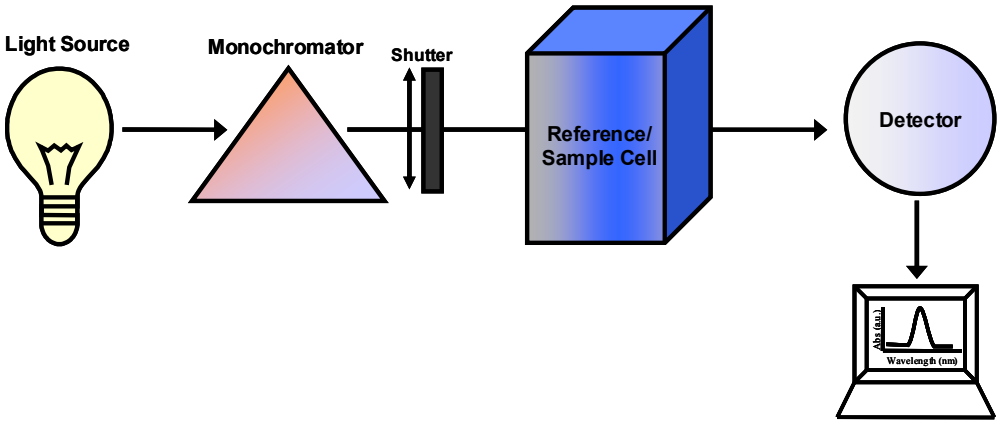


Figure 2.11 Block diagram of a single-beam UV-Vis absorption spectrometer

In this study, off-line UV-Vis measurements were performed for characterization purposes of the isolated anthraquinones. The absorption characteristic of these quinones is based on the presence of certain chromophores that are aromatic and have one or two double bonds. In addition, on-line UV-Vis spectroscopy was employed during the detection of phenolic compounds in HPLC separation and in enzyme-linked-immuno-absorbent assay measurements. On the whole, the use of UV-Vis spectroscopy in the structural elucidation process of natural products is limited.

2.3.2 Infrared Spectroscopy

Infrared (IR) absorption spectroscopy is an analytical technique that deals with the interaction of IR radiation with material. Typically, the IR region of the EMS is considered within the wavelength range of 770 nm to 1000 μm, which in turn is subdivided into near-IR (770 – 2500 nm or 12,900 – 4000 cm^{-1}), mid-IR (2.5 – 50 μm or 4,000 – 200 cm^{-1}), and far-IR (50 – 1000 μm or 200 – 10 cm^{-1}). The infra-red spectrum can be used for molecules to provide chemical (characteristic fingerprint regions) and structural information (determination of functional groups). Infrared spectroscopy is useful for structural analysis of components and confirming their identity. This technique finds its application in both organic and inorganic chemistry.[20, 21]

Upon absorption of IR radiation the corresponding energies promote vibrational frequencies (i.e. stretching and bending) of the bonds in nearly all covalent molecules. Only molecules which have a dipole moment absorb IR radiation, namely, are IR active. Generally, for IR measurements the wavelength is reported in terms of wavenumbers, ϋ (cm^{-1}). Quantitative analysis of components, by applying the Lambert-Beer's Law, can also be performed with an IR spectrometer. However, this is uncommon, because of deviations from Beer's Law; for example, at high concentrations and the sample cell requirements in IR. Additionally, in IR spectrometry the detection limits are higher than in UV-Vis spectrometry, due to weak IR radiation sources and inferior detectors (*vida infra*).[7, 21]

An IR spectroscopic instrument has relatively the same basic design as the UV-Vis absorption instrument (see Figure 2.11). The majority of IR light sources are black body emitters, which are solid materials heated electrically to extremely high temperatures. Infrared sources are moderately weak sources that exhibit low light levels. Some examples of these thermal devices emitting IR radiation include the Nernst glower (1200 to 2200 K), the Globar

source (1300 to 1500 K), the incandescent wire (up to ~1100 K), and the tungsten filament lamp (up to ~2700 K). Based on the fact that most solvents absorb in the IR region, many IR spectra are often collected as "neat" in a thin sample cell (10 μm to 1 mm). A general requirement for an IR sample cell is that it should be IR transparent. Hence, some commonly used sample containers are salt plates, for example NaCl, KBr.[7, 21]

Infrared spectrometers both employ monochromators and filters. In addition, when using IR radiation special optical devices are required such as gratings, lenses, and mirrors. It is noteworthy that in IR spectroscopy, due to weak sources, the loss of light through optics creates a more serious problem than in UV-Vis. In IR instruments transducers are employed to convert photons to an electrical signal. Basically, there are three types of infrared transducers: (1) thermal transducers, which determine temperature increases as a function of absorbed light, (2) pyroelectric transducers, also known as specialized thermal transducers, and (3) photoconducting transducers, which are semiconductors that absorb IR light while displaying less resistance. Likewise, the same type of IR detectors can also be distinguished. Photoconductive detectors, for example, mercury/cadmium telluride, are very sensitive detectors that operate at a high speed and are commonly used in Fourier transform infrared (FTIR) spectrometers (*vida infra*).[7]

Three distinct types of IR absorption spectrometers are known. The first type is the dispersive grating instrument, which is employed in the mid-IR region for spectral scanning and quantitative analysis. The second kind of IR spectrometers is the FTIR spectrometer, which is also the primary instrument used for IR measurements in the far-IR and mid-IR region. The majority of these instruments are based on the Michelson interferometer (Figure 1.12).

Briefly, the interferogram has a wave-like pattern containing all the frequencies of the IR region. In an interferogram the light intensity is plotted versus the optical path difference, which is denoted in time (time-domain spectrum). The Fourier transform is a mathematical operation

that converts the time-domain spectrum into the frequency-domain spectrum. The primary advantage of FTIR instruments is that they afford high speed and great sensitivity. In addition, these types of spectrometers do not utilize filters and monochromators; therefore, the loss of light is decreased significantly. It is worth-mentioning that most computer-interfaced FT-IR spectrometers function in a single beam mode. The third group of IR absorption spectrometers is the non-dispersive instruments that employ filters for wavelength selection.[7, 21]

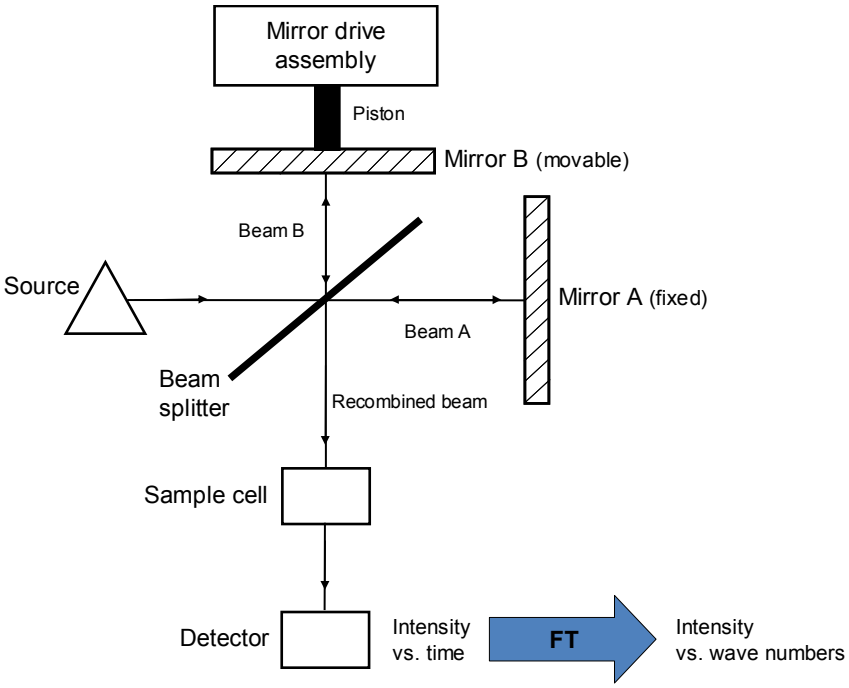


Figure 2.12 Single beam FTIR spectrometer with Michelson interferometer.[21, 23] The interferometer consists of an IR source radiation, a fixed mirror, and a movable mirror. The beam splitter transmits half of the beam from the IR source radiation to the fixed mirror and half is reflected to the movable mirror. Subsequent to reflection the beams are recombined by the beam splitter and sent through the sample to the detector. The recombined beam is the difference in optical path lengths between the two beams.

2.3.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) is a spectrochemical analysis method that measures the amount of absorption of EMR, by atomic nuclei, in the radio frequency region, approximately between 4 and 900 MHz. This technique has been for the past four decades the most important and powerful tool for the determination of molecular structures of biological-,

chemical-, geological-, medicinal-, and material science compounds. As a whole it enables a chemist to determine the numbers and sorts of nuclei present in a molecule, to study their particular chemical environments within the molecule, as well as their interconnectivity.[17, 24]

The principle of NMR is based on the investigation of the magnetic properties of nuclei, which are induced by their spin states. In order for a nucleus to be magnetically active it must have an isotope, i.e. its spin quantum number (I) should be nonzero (for example, I = 1/2, 3/2, 5/2). Each spinning nuclei has an angular momentum (J) that induces a magnetic moment (μ):

$$\mu = \gamma J \,, \tag{1.24}$$

where γ is the magnetogyric ratio, a constant specific for each nucleus that determines its magnetic strength. The angular momentum and magnetic moment are both vectors; thus, each one has a magnitude and direction. Hence, once nuclei are placed in an exterior magnetic field (B_o), their magnetic moments align either parallel or anti-parallel to the field due to absorption of energy from the radio frequency range. Altogether, the frequency (ν) at which a specific nucleus absorbs energy is dependent on its magnetogyric ratio and the external magnetic field into which it is positioned. The Larmor equation denotes this relationship mathematically by:[17, 24]

$$\nu = \frac{\gamma B_o}{2\pi} \,. \tag{1.25}$$

This process wherein the nucleus changes its spin state upon absorption of a quantum of energy is known as nuclear magnetic resonance. For the spin change to occur it is required that the frequency of the absorbed energy matches the Larmor precession frequency in order to cause resonance. Recall that this energy is applied as a radiofrequency:

$$\Delta E = h\nu = \frac{h\gamma Bo}{2\pi} \tag{1.26}$$

Therefore, the coupling of the external magnetic field with the radiofrequency field causes a spin change due to energy transmission to the nucleus.[24]

In natural product chemistry, where NMR spectroscopy is used for the structural analysis of phytochemicals, the nuclei of proton (^{1}H) and carbon (^{13}C) are of major interest. These nuclei have both a spin quantum number of 1/2. The nuclei of an isotope with I =1/2 can reside in a magnetic field in any of two energy states, i.e. higher and lower. Nuclei in the higher energy state "precess" in opposite direction of the magnetic field, whereas those in the lower energy state have the same orientation. These nuclei in the lower energy state are in a small population excess. The extent of this prevalence is described by the Boltzmann distribution:

$$\frac{N_j}{N_o} = e^{\left(\frac{-\Delta E}{kT}\right)} \tag{1.27}$$

where N_j and N_o indicate the number of nuclei in the higher and lower energy state, respectively, k is the Boltzmann constant (1.38×10^{-23} JK^{-1}), and T is the absolute temperature. The small energy difference between the parallel and antiparallel orientation leads to the net absorption of energy once the nuclei are irradiated with the proper radiofrequency. Recall that the absorbed energy is different for each nucleus and linearly dependent on the external magnetic field. Hence, various nuclei can be detected at different radiofrequencies. In this way, for example, ^{1}H or ^{13}C can be observed by choosing the correct frequency for each nuclei.[17, 24]

The frequencies of the abovementioned transitions are, besides the external magnetic field, also dependent on the chemical environment of the nucleus under investigation. Every nucleus is shielded from the external magnetic field by an electron density, unique to each nucleus. Therefore, within a molecule different nuclei resonate at different frequencies. This effect is known as the chemical shift since the frequency at which a nucleus absorbs energy is distinctive to the chemical environment of the specific nucleus. The chemical shift of ^{1}H nuclei has a range of 15 ppm, whereas ^{13}C nuclei resonate over a range of approximately 220 ppm.[17, 24]

In addition, a nucleus is also influenced by adjacent fields produced by other nuclei. Hence, the coupling constant is another factor that influences an NMR spectrum. However, the effect of nearby nuclei is smaller than the effect coming from electrons. In one-dimensional NMR spectra, for example ^{1}H and ^{13}C spectra, the frequency is plotted versus the signal intensity. Similarly, two-dimensional NMR spectroscopy engages the use of two frequencies versus signal intensity and provides information concerning through-bond coupling, through-space coupling as well as chemical exchange. During the characterization of natural products a common procedure in obtaining structural information includes the determination of chemical shifts and coupling constants in one- and two-dimensional NMR spectra.[17, 24]

Subsequently, over a period of time the absorbed energy is lost to the surroundings (lattice). This process is known as relaxation and is typified by the spin-lattice relaxation time. The spin-lattice relaxation times for ^{1}H and ^{13}C nuclei are a few seconds and tens of seconds, respectively. Additionally, the natural abundances of ^{1}H nuclei are 99.98%, while that of ^{13}C nuclei are 1.11%. Therefore, ^{13}C-NMR is less sensitive than ^{1}H-NMR. On top of this, the overall disadvantage of NMR spectroscopy is that it has an extremely low sensitivity in comparison with other spectroscopic methods.[17]

An NMR spectrometer is an extremely complicated and expensive instrument. In brief, it consists of three main parts: the magnet (producer), the console (controller), and the probe (source and detector). The magnet is superconducting and contains an extremely strong, stable, and homogeneous magnetic field (for example, between 200 and 700 MHz). The console is the computer system that controls several units such as the radio frequency transmitter, the receiver, the digitizer, the Fourier transform, the operation of temperature devices, and printers. The probe is the most specialized part of the spectrometer. In the probe, the NMR signal is generated and detected. Some major components that an NMR probe consists of include radiofrequency

circuits, capacitors (matching and tuning), channels (decoupling, lock, and observe), and coils (decoupling and observe).[24]

2.4 Bio-analytical Methods

Upon separation, isolation, and characterization of a particular secondary metabolite the use of bioassays is vital in determining the compound's biological activity. Generally, the selection of assays relies on the bioactivity of interest and can be either specific (e.g., mechanism-based assays) or non-specific (e.g., broad screen assays). Moreover, in drug discovery the use of sensitive and selective bio-analytical methods for the evaluation of potential phytochemicals is a foremost requirement. In this dissertation, *in vitro* angiogenesis assays were employed in assessing the effectiveness of rhein as an anti-angiogenic agent. Furthermore, enzyme-linked immunosorbent-assays and Western Blotting were utilized in determining the inhibition effect of rhein on various proteins (such as HIF-1, VEGF, EGF, and NF-κB) that significantly contribute to the progression of hypoxia tumor angiogenesis. This chapter gives a concise overview on the different bio-analytical methods used throughout this study.

2.4.1 *In vitro* Angiogenesis Assays

Tube formation, the configuration of endothelial cells into a three-dimensional tubular structure, is one of the most specific tests for evaluating the *in vitro* anti-angiogenic activity of a compound. Endothelial cells are capable of forming tubules *in vitro* through extra-cellular matrix components. In general, endothelial cells are cultured on matrices containing fibrin, collagen, and Matrigel. These substances stimulate the attachment, migration, and differentiation of endothelial cells into tubules in a way similar to *in vivo* conditions.[25-27]

Cell proliferation is another type of assay that is relevant in determining the effectiveness of an anti-angiogenic agent. These assays can be categorized into two major classes according to their analysis method, namely, (1) net cell number determination, and (2) cell-cycle kinetic

evaluation. Usually, a haemocytometer or an electronic counter (e.g., Coulter counter) is employed to determine the net cell number. In addition, staining methods can be used in determining the net number. For example, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) gives a purple formazan product upon cleavage by active mitochondria. The second method in evaluating the cell proliferation is based on cell-cycle analysis, wherein DNA binding molecules (i.e. bromodeoxyuridine, BrdU) are coupled with flow cytometry examination.[25, 27]

In order to assess *in vitro* migration and invasion capacity of cells in response to a particular bioactive compound, the modified Boyden chamber or blind-well chemotaxis assay can be utilized. The Boyden chamber contains two parts, i.e. an upper and a lower compartment. The cells are added to the upper (migration) chamber containing a porous filter that is cell permeable. In addition, cell-conditioned medium is situated in the lower chamber. After four hours, the filter is removed from the upper chamber, inverted, and the cells are stained and counted. Finally, the cells that migrate across the filter are determined through cell enumeration. Basically, the cell migration and invasion assays are performed in the same manner with the only difference being that in cell invasion more cells are used.[25, 26]

2.4.2 Enzyme-Linked Immuno-Sorbent Assay

Enzyme-linked immuno-sorbent assay (ELISA) is the most frequently used immunoassay in diagnostic medicine and plant pathology. The principle of this bio-analytical technique is based on the use of strong and selective affinity interactions of antibodies to detect proteins (antigens) in tissues and cell cultures. In general, ELISA involves the immobilization of an antigen—either via adsorption or bio-conjugation with a specific capture antibody (sandwich ELISA)—to a solid surface, usually a 96-well polysterene microtiter plate. Subsequent to immobilization of the antigen, an antigen-specific (detection) antibody is added whereupon an

antigen-antibody complex is formed. In this scenario, the detection antibody can be either covalently linked to an enzyme (direct ELISA) or detected itself by a secondary antibody that is enzyme labeled (indirect ELISA). The support is usually rinsed with a wash buffer between sample/standard and antibody application to remove unbound proteins. Finally, the plate is visualized by addition of an enzymatic substrate solution, which produces a signal proportional to the amount of antigen present in the sample/standard.[27, 28] A schematic representation of an ELISA is shown in Figure 2.13.

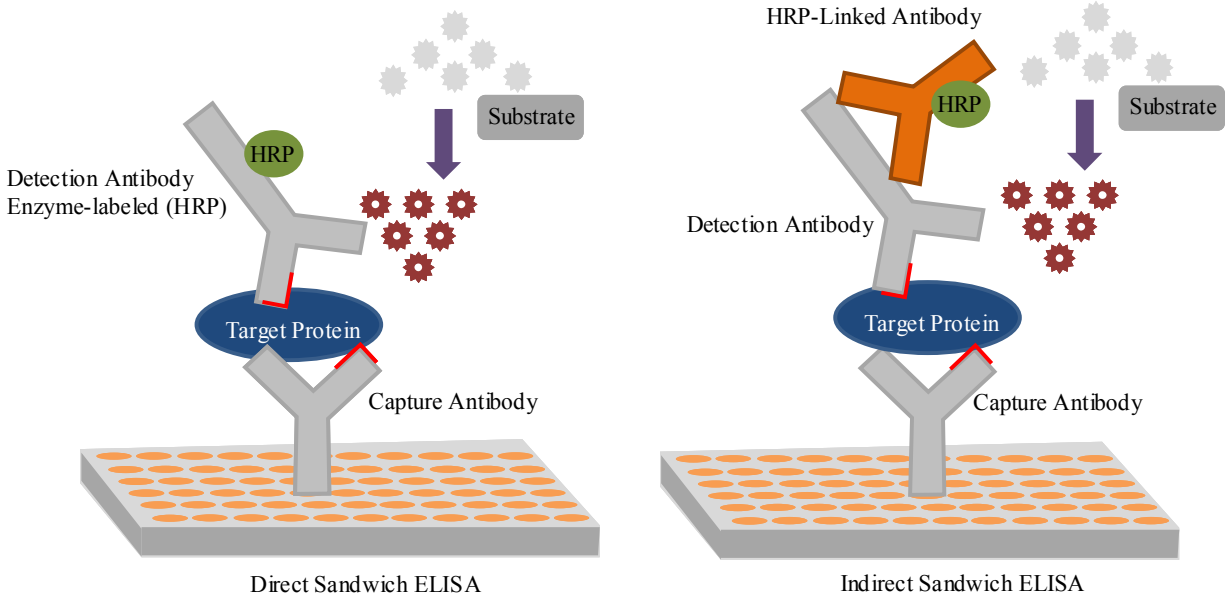


Figure 2.13 A comparative representation of the ELISA format[29]

Briefly, an ELISA experiment consists of four steps, namely, coating, blocking, washing, and detection. The coating procedure, the adsorption of a capture antibody to a plate, is usually completed by an overnight incubation at room temperature. The incubation period for antigens and antibodies is anywhere between one to two hours and one to three hours, respectively. In order to reduce background activity due to nonspecific adsorbed enzyme-labeled antibodies and/or antigen molecules, the free protein binding sites on the solid surface need to be blocked. Frequently used blocking buffers include phosphate buffered saline (PBS) solutions with various proteins such as bovine serum albumin (BSA), casein, and gelatin. After one hour incubation

with the blocking buffer, the microplate is rinsed. Several washing buffers can be used, such as PBS, saline, or PBS with a detergent. The incubation time for the substrate solution can be anywhere between 20 to 45 minutes. Readings are taken within 30 minutes subsequent to addition of a stop solution. The detection step includes the use of detectable substrates, namely, chromogenic, fluorogenic, or luminogenic to determine the enzymatic activity.[27, 28]

2.4.3 Western Blot

Western Blot, also known as immunoblot, is an analytical method that is employed to detect the presence and/or relative abundance of proteins in a cell extract or tissue homogenate. The proteins are separated by use of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on their individual sizes. Subsequent to electrophoresis the proteins are transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane, where they are detected by using a monoclonal or polyclonal antibody unique to that protein. Western Blot analysis is used in biology-related disciplines such as molecular biology, biochemistry, and immunogenetics.[27]

There are several steps involved in a Western Blot analysis, namely, the sample preparation, gel electrophoresis, transfer, blocking, immune detection, and visualization. Whole tissue and cell extract samples can be obtained by use of a blender/homogenizer and by cell lysis, respectively. Subsequently the extracted proteins are denatured by a strong reducing agent, which will permit their separation by molecular weight (usually in kDa). These protein samples are then loaded into small wells of a polyacrylamide gel and separated through electrophoresis by use of a buffer containing sodium dodecyl sulfate (SDS). The SDS buffer retains proteins in a denatured state. The principle of gel electrophoresis is based on applying an electric current along the gel and allowing the negatively charged polypeptide-surfactant complex to travel through the acrylamide matrix to the positively charged electrode. The proteins migrate through

the gel at different rates (i.e. electrophoretic mobilities), according to their size. For example, smaller proteins will travel faster. Due to the differences in electrophoretic mobilities between proteins, separate bands occur within each lane. Generally, the proteins are identified by use of a molecular weight marker, which has specific protein sizes.[27]

Following the electrophoretic separation, the proteins are transferred onto a PVDF or nitrocellulose membrane. The transfer stack is prepared by placing on the cathode successively: a packing sponge, wet filter paper, the gel, the membrane, wet filter paper, and two packing sponges. The transfer stack assembly is then filled with, for example, a BIS-TRIS buffer and inserted into an electrophoresis unit. Upon applying an electric current, perpendicular to the gel, the negatively charged polypeptides travel out from the gel, toward the positive electrode (anode), and then get accumulated on the membrane. The proteins are bound and immobilized on the membrane through hydrophobic and charged interactions. This process, in which the separated proteins in a gel are transferred to the surface of a thin membrane for detection (*vide infra*), is called Western Blotting.[27] Figure 2.14 gives an illustration of a blotting procedure.

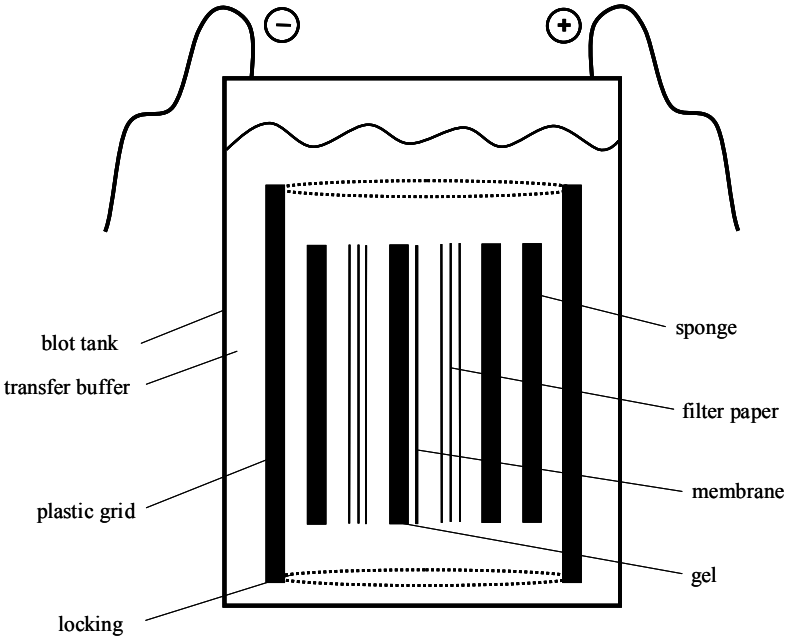


Figure 2.14 Schematic illustration of a Blotting procedure

Based on the fact that not only proteins from the gel can bind to the membrane but also other proteins such as detection antibodies, it is extremely necessary to block the non-specific protein binding sites on the membrane. Therefore, to prevent protein interactions between the membrane and detection antibody, the membrane is incubated with some irrelevant proteins; for example, a dilute solution of BSA or non-fat dry milk. Blocking of the non-specific sites reduces the background (noise), and hence increases the sensitivity of the Western Blot.[27]

The principle that governs the immune detection in Western Blotting is similar to that of ELISA. In fact, a Western Blot is an ELISA performed on a membrane. Briefly, the membrane is incubated (under gentle shaking) with a specific primary antibody that is diluted in the appropriate blocking solution. The incubation period can be anywhere from one hour to overnight. Subsequent to this incubation the membrane is rinsed to remove any unbound primary antibody. The secondary antibody, usually conjugated with biotin or a reporter enzyme, is incubated with the membrane under similar conditions for one to two hours. After rinsing off the unbound secondary antibodies from the membrane, the Western Blot is visualized.[27]

Detection of the probes can be performed in various ways, depending on the detection reagent conjugated to the secondary antibody. The detection can be either directly visible (e.g., radioactive-labeled or fluorescence-labeled secondary metabolites) or indirectly visible (e.g., alkaline phosphatase or horseradish peroxidase). To visualize the protein binding, a certain concentration of a substrate solution is added according to manufacturer's instructions. Bands appear as dark regions on the developed blot within 2 to 20 minutes, during which time the color reaction is stopped by removing the soluble dye. Additionally, after rinsing the membrane with de-ionized water, it is dried in open air and imaged. Finally, by use of densitometry or spectrometry the protein levels are evaluated, wherein the relative intensity of the bands is proportional to the amount of antibody bound to the protein.[27]

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CHAPTER 3*

DETERMINATION OF PHARMACOLOGICALLY ACTIVE COMPOUNDS IN ROOT EXTRACTS OF *CASSIA ALATA* L. BY USE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.1 Introduction

Determining the concentration of bioactive constituents in a natural product extract is of great significance in the drug discovery process. Therefore, developing an analytical separation method for the analysis of complex natural product extracts is of utmost importance. The results obtained from such a study could aid in determining the natural products' active component(s). It is obvious that the first phase in drug discovery, the separation and determination of potential bioactive constituents, is an essential and very critical step. In this chapter, an analytical method for the simultaneous analysis and identification of bioactive compounds in the roots of *C. alata* was developed.

In previous reports *C. alata* extracts have been analyzed for the presence of kaempferol-3-*O*-gentiobioside in various parts of the plant (leaves, flowers, rachis, stem and seed) using HPLC.[1] Several analytical methods such as thin-layer chromatography,[2] micellar electrokinetic capillary chromatography,[3] capillary zone electrophoresis,[4] capillary electrophoresis-mass spectrometry (CE-MS),[5] gas chromatography,[6] and gas chromatography-mass spectrometry (GC-MS)[7] have been used for the determination of anthraquinones in various medicinal plants and natural products. In addition, analytical methods involving the use of HPLC[6-10] and LC-MS[11-14] have also been used for anthraquinone analysis in several natural products.

Although CE, GC, and HPLC methods can afford sufficient sensitivity for the analysis of anthraquinones in natural products, they all lack accurate peak identification in comparison with

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CE-MS, GC-MS, and LC-MS methods. Generally, the use of LC-MS is advantageous over the use of CE-MS and GC-MS because it affords high separation efficiency and accurate identification of analytes over a wide range of molecular structures, chemical reactivities, polarities, and volatilities. Hence, separation of analytes as well as mass identification and confirmation of analytes can be achieved simultaneously in one experimental run using LC-MS. For example, LC-MS using ESI[11, 14] and APCI[12, 13] has been used for accurate identification of anthraquinones in plant extracts. In comparison with APCI-MS detection, the use of ESI-MS detection for anthraquinone identification is less effective, because the latter requires mobile phase additives other than the buffer to enhance analyte ionization.[11, 14] Moreover, aside from eluent additives, the efficiency of the mass detection of anthraquinones using an ESI interface depends on the pK_a value of the analyte.[11]

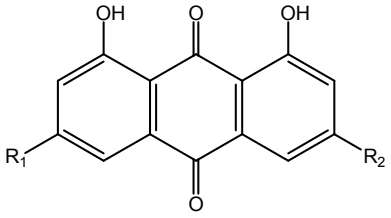
The aim of this study was to develop an HPLC-UV detection method for quantitative analysis of rhein **(1)**, kaempferol **(2)**, aloe-emodin **(3)**, emodin **(4)**, chrysophanol **(5)**, and physcion **(6)** in root extracts of *C. alata*. The structures of these analytes are shown in Figure 3.1. The root extracts were purified by use of solid-phase extraction, and separation of the phenolic compounds was performed by use of reversed-phase HPLC using isocratic elution. In addition, on-line mass spectrometric detection using APCI in the negative ionization mode was used to confirm the presence of these phenolics in the root extract. To the best of our knowledge, this study represents the first investigation of the simultaneous analysis and identification of these six phenolic compounds in root extracts of *C. alata* using LC-UV-APCI-MS.

3.2 Experimental

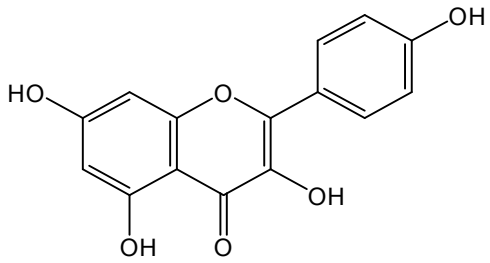
3.2.1 Chemicals and Materials

Rhein, kaempferol, aloe-emodin, emodin, and chrysophanol standards were purchased from Sigma Aldrich (St. Louis, MO), while the physcion standard was purchased from Micro

Source Discovery System (Gaylordsville, CT). Ammonium acetate (NH_4Ac) buffer was purchased from Fluka (Amsterdam, the Netherlands). HPLC grade acetonitrile, ethanol, methanol, water, and Whatman no. 1 filter paper were obtained from Fisher Scientific (Houston, TX). Doubly-distilled deionized water, used throughout the study, was obtained by use of an Elga Genetic Ultra-Pure water polishing system from US Filter (Lowell, MA). Maxi-clean RP solid-phase extraction (SPE) C_{18} cartridges and 0.45 μm filters were purchased from Altech (Deerfield, IL). *C. alata* root samples were collected by the Center of Agricultural Research, Suriname, and identified at the National Herbarium of the University of Suriname, Paramaribo, Suriname (Reference number: Jansen Jacobs 5027).[15]



Compound	R_1	R_2	MW
Rhein (**1**)	H	COOH	284
Aloe-emodin (**3**)	H	CH_2OH	270
Emodin (**4**)	OH	CH_3	270
Chrysophanol (**5**)	H	CH_3	254
Physcion (**6**)	OCH_3	CH_3	284



Kaempferol (**2**), MW= 286

Figure 3.1 Chemical structures of six phenolic compounds in *C. alata* root extract

3.2.2 Preparation of Standard Solutions

Standard stock solutions of compounds **2-6** were prepared in ethanol at a concentration of 0.5 mg/mL, while rhein **(1)** was prepared at 0.25 mg/mL. Standard mixture solutions were prepared in ethanol at various concentration levels in the range of 5 - 250 ppm. All solutions were filtered prior to analysis through a 0.45 μm syringe filter and injected four times into the

HPLC. The calibration curve for each compound was constructed by plotting the peak area as a function of the standard analyte concentration.

3.2.3 Sample Preparation

The *C. alata* root samples were oven dried at 40°C for five days. The dried roots were ground by use of a Wiley Mill grinder (Standard Model No. 3) to particle sizes of 6 mm or smaller. Ten grams of ground roots were extracted with 100 mL ethanol on an orbital shaker for 12 hours at room temperature. The extraction procedure was repeated two times, after which the two extracts were combined and filtered using Whatman no. 1 filter paper. The extraction solvent was removed by use of rotary evaporation, and the residue was reconstituted in 10 mL ethanol and diluted with water (1:1 v/v).

Solid phase extraction (SPE) was used to remove unwanted interfering phytochemicals from the root extract. The SPE procedure was performed on an Altech extraction manifold system. SPE C_{18} cartridges (4 mL; 600 mg) were first conditioned with 4 mL methanol, followed with 4 mL water. Following the conditioning step, 4 mL of the diluted root extract was loaded onto the cartridge. After sample loading, the interfering compounds were removed with 2 mL of 10% aqueous ethanol. Finally, the fraction containing compounds **1-6** were eluted with 2 mL of hot ethanol (60°C). The vacuum pressure was kept at 10 mm Hg during the pre-conditioning step and was held constant at 2 mm Hg during the loading and eluting steps. Four replicate SPE extracts were collected. Each eluate was diluted to 5 mL with ethanol. The diluted SPE root extract, the eluate, was then filtered through a 0.45 μm syringe filter and injected into the HPLC. Each diluted SPE root extract was injected into the HPLC five times, and the average peak area was reported and used for analyte quantification.

3.2.4 HPLC Analysis

Separation and quantitative analyses of compounds **1-6** were performed on a Shimadzu HPLC system (Kyoto, Japan) consisting of an SCL-10A system controller, two LC-10AD pumps, a DGU-14A degasser, an SIL-10AD auto injector and an SPD-10AV UV-VIS detector (λ = 260 nm). Separation of the analytes was performed at 40°C on a Phenomenex Luna C_{18} (2) column, 100 Å pore size, 5 μm particle size, 250 × 4.6 mm ID column containing a guard column (Phenomenex, Torrance, CA, USA). The analytes were eluted isocratically at a flow rate of 0.4 mL/min using an acetonitrile/methanol/buffer (25: 55: 20, v/v), where the buffer was 10 mM ammonium acetate (NH_4Ac) at pH 6.8. The injection volume was 10 μL.

3.2.5 LC-APCI-MS Analysis

Analyte identification was performed by use of a Shimadzu LCMS-2010 system (Kyoto, Japan). Operating conditions for the HPLC were as described in the previous section. The mass spectrometer used for the identification of the analytes consisted of a Q-array-octapole-quadrupole mass analyzer with an APCI interface used in the negative ionization mode and coupled to the Phenomenex Luna C_{18} (2) column described above. The APCI probe was operated at a temperature of 400°C, while the CDL and block temperatures were operated at 200°C. The detector voltage was 1.5 kV and the probe was operated in the negative ionization mode with a voltage of -4.0 kV. The nebulizing gas was nitrogen at a flow rate of 2.5 L/min. The optimum operating conditions for the LC-APCI-MS were determined for the separation and identification of compounds **1-6** in the scan mode with minimum fragmentation of the analytes. The scan rate of the mass analyzer was 1s/scan within the mass range of *m/z* 100-1000 Da.

3.2.6 Method Validation

Precision (reproducibility) of the method was obtained by calculating the relative standard deviation (R.S.D.) from repeated injections of the standard mixture solutions at 15, 45,

and 75 ppm for all analytes, except for kaempferol **(2)** that was determined at 30, 90, and 150 ppm. The intra-day precision was determined by six replicate injections, while the inter-day precision was determined by six injections for six days, for both the retention times and peak-areas.[16]

A recovery study was performed to validate the accuracy of the developed method. Hence, root samples (10 grams) were spiked with standard stock solutions of the analytes in triplicate at two different concentrations. The spiked root samples were extracted with 100 mL ethanol following the procedure for sample preparation as described in section 3.2.3. Finally, the spiked samples were analyzed using the same experimental and instrumental conditions as previously described for the analysis of the un-spiked roots. The recovery was determined by comparing the amount of analyte added to the root sample and the amount of analyte detected during HPLC analysis.[17]

3.3 Results and Discussion

3.3.1 HPLC Optimization

Several preliminary studies were conducted to develop an HPLC method for the separation of compounds **1-6** in the *C. alata* root extract. The LC separation conditions for the analytes were optimized by systematically adjusting the methanol and acetonitrile content in the mobile phase with the addition of different buffers, such as trifluoroacetic acid, formic acid, and ammonium acetate to obtain better resolution of the phenolic compounds. The retention times of the analytes decreased with an increase in the amount of methanol in the eluent. This observation was in agreement with a previous report by Ding *et al.*[9] An increase in the amount of acetonitrile in the eluent also resulted in a decrease in retention time of compounds **1-6**. The addition of 10 mM NH_4Ac buffer to the mobile phase resulted in the optimum peak resolution of compounds **1-**

6. The addition of NH_4Ac buffer to the mobile phase not only improved the resolution, but also resulted in complete deprotonation of compounds **1-6** $[M-H]^-$.

The pH of the mobile phase was also optimized to obtain better resolution of compounds **1-6**. Separation at pH 4.8 using NH_4Ac buffer resulted in co-elution of rhein **(1)** and kaempferol **(2)**. Therefore, resolution of only compounds **3-6** could be obtained. At pH 8.8 (adjusted with NH_4OH) compounds **1-3** co-eluted. Complete separation of compounds **1-6** were only achieved at pH 6.8 using NH_4Ac buffer. The flow rate of the eluent was also optimized at 0.4 mL/min for best resolution and MS detection. The use of flow rates greater than 0.4 mL/min resulted in overloading of the mass spectrometer detector.

Optimal separation of the analytes was obtained within 45 minutes for standard mixtures and the *C. alata* root extract by use of an isocratic mobile phase of ACN/MeOH/NH_4Ac buffer at pH 6.8 (25: 55: 20, v/v). The retention times of the analytes were optimized using a gradient elution system containing ACN/MeOH/NH_4Ac buffer at pH 6.8 (25: 55: 20, v/v) for solvent A and ACN/MeOH/NH_4Ac buffer at pH 6.8 (25: 65: 10, v/v) for solvent B, allowing successful separation of all analytes within 30.0 minutes. However, we did not use the gradient elution system for quantification of the analytes because it was not reproducible.

The phenolic compounds **1-6** were identified in the *C. alata* root extract by spiking the extracts with the respective standards. Prior to this procedure, all standards were run separately to determine the retention time of each analyte. The chromatographic separation of compounds **1-6** is shown in Figure 3.2A for the standard mixture at 30 ppm as an example, and in Figure 3.2B for the root extract (without spiking). In addition to the analyte peaks obtained in Figure 3.2B, an unidentified first eluting peak (at 6.83 min) was also observed. This unknown peak was isolated using flash column chromatography followed by purification using preparative HPLC. However, after performing spectroscopic studies (UV, IR, MS, and NMR), we concluded that

this unknown peak was an impurity composed of a mixture of compounds. No further analysis of this peak was attempted.

3.3.2 LC-MS Analysis

Simultaneous separation and identification of phenolic compounds **1-6** in the *C. alata* root extracts were performed by use of LC-APCI-MS detection. Identification of the peaks was achieved by comparison of the retention times, UV spectra, as well as MS data of the separated compounds with the respective standards. The total ion chromatograms (TIC) of analytes **1-6** in the standard mixture and root extract were recorded in the scan mode, and are shown in Figure 3.3A and 3.3B, respectively. As seen in Figure 3.3B, the peak intensities for aloe-emodin **(3)** and physcion **(6)** were very low, as a result of their low concentration (*vide infra*) in the root extract as determined by HPLC in this study.

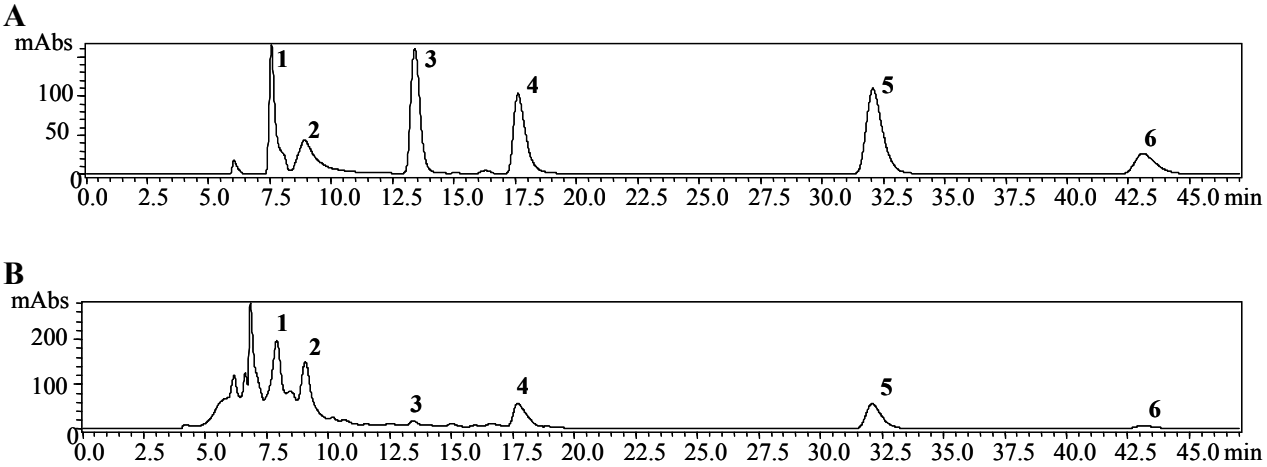


Figure 3.2 **(A)** HPLC chromatogram of the phenolic standard mixture (30 ppm), λ = 260 nm. Rhein (**1**, 7.57 min); Kaempferol (**2**, 8.92 min); Aloe-emodin (**3**, 13.41 min); Emodin (**4**, 17.63 min); Chrysophanol (**5**, 32.06 min); Physcion (**6**, 43.07 min). **(B)** HPLC chromatogram of *C. alata* root extract, λ = 260 nm. Rhein (**1**, 7.91 min); Kaempferol (**2**, 9.05 min); Aloe-emodin (**3**, 13.43 min); Emodin (**4**, 17.69 min); Chrysophanol (**5**, 32.04 min); Physcion (**6**, 43.01 min).

The mass spectra of the phenolic compounds **1-6** in the root extract are presented in Figure 3.4. The presence of each analyte in the root extract was confirmed by its respective $[M-H]^-$ *m/z*. In addition to the ions at $[M-H]^-$ of compounds **1-6**, the ion at *m/z* 239 was registered in

the mass spectrum of rhein **(1)** and aloe-emodin **(3)** due to fragmentation of molecular ions of the analyte resulting in $[M\text{-}COOH]^-$ and $[M\text{-}CH_2OH]^-$, respectively. The ions at *m/z* 253 and 271 were also recorded in the mass spectrum of rhein **(1)** which was assumed to be a fragment derived from the molecular ion resulting in $[M\text{-}CH_2OH]^-$ and an adduct formation between the ion at *m/z* 239 and methanol ($[M\text{-}COOH + MeOH]^-$), respectively. The ion at *m/z* 253 obtained in the mass spectrum of aloe-emodin **(3)** was due to the loss of a hydroxyl group, resulting in $[M\text{-}OH]^-$. The ions at *m/z* 255 and 269 observed in the mass spectrum of kaempferol **(2)** are possibly due to fragmentation of molecular ion resulting in $[M\text{-}CH_2OH]^-$ and $[M\text{-}OH]^-$, respectively. Finally, the ion at *m/z* 269 recorded in the mass spectrum of physcion (**6)** was due to the loss of a methyl group leading to $[M\text{-}CH_3]^-$.

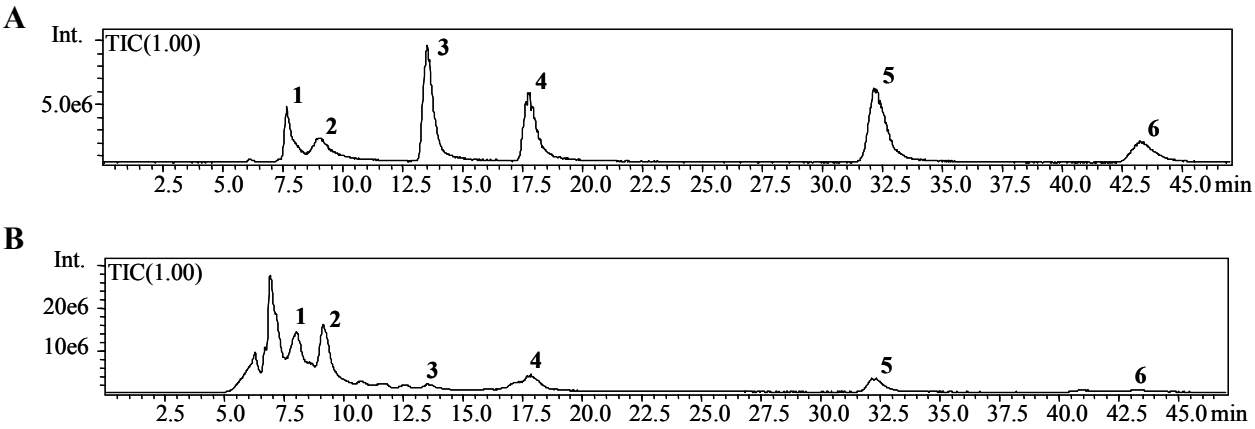


Figure 3.3 Total ion chromatograms of **(A)** Standard mixture (30 ppm);
(B) *C. alata* root extract

3.3.3. Calibration Curves

In this study as well as in previous reports,[11, 13] the analysis of anthraquinones using on-line mass spectrometric detection was found to be less sensitive than on-line UV detection. Therefore, HPLC-UV was chosen for the determination of compounds **1-6**. The investigated compounds in the root extract were quantified by integration of the peak areas at 260 nm using an external calibration method.

Calibration curves were constructed for each analyte by using a series of standard mixture solutions. Linear least-squares regression was used to determine the calibration parameters for each of the six standards. A summary of the calibration studies for the six analytes is presented in Table 3.1. The linearity of all calibration curves was determined by calculating the correlation coefficients, which varied from 0.9971 for kaempferol to 0.9999 for emodin and physcion.

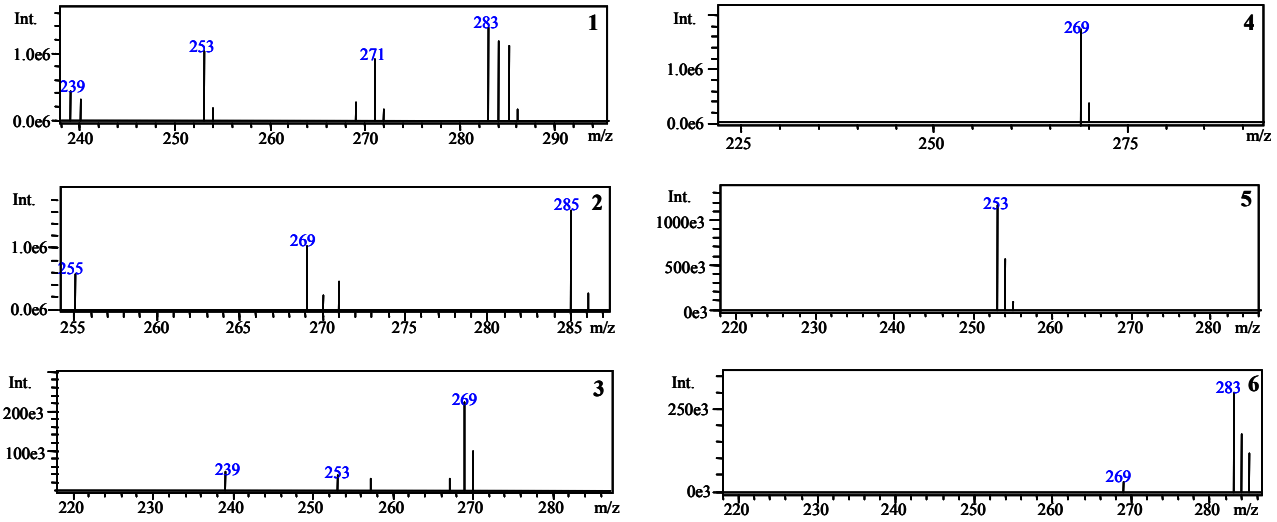


Figure 3.4 Mass spectra of Rhein **(1),** Kaempferol **(2),** Aloe-emodin **(3),** Emodin **(4),** Chrysophanol **(5),** and Physcion **(6)** from the *C. alata* root extract

The limit of detection (LOD), defined as the lowest detectable concentration of an analyte, was calculated using the formula $LOD = (b + 3\ \sigma_b)/a$, where a is the slope of the calibration curve; b is the intercept; and σ_b is the standard deviation associated with the intercept. The LOD for the analytes were in the range of 0.23 - 4.61 ppm. In addition, the limit of quantification (LOQ), defined as the lowest measurable analyte concentration, was determined according to the formula $LOQ = (b + 10\ \sigma_b)/a$, where all parameters are as defined for the LOD. The LOQ is reported in Table 3.1 as the lower limit from the linear range.

Table 3.1 Calibration parameters of HPLC analysis for phenolic compounds **1-6**

Compound	a	b	r	Linear range (ppm)	LOD (ppm)
Rhein (**1**)	145321	69934	0.9985	13.1 - 115	4.61
Kaempferol (**2**)	60862	-26171	0.9971	5.27 - 230	0.98
Aloe-emodin (**3**)	160496	-43698	0.9998	3.06 - 145	0.54
Emodin (**4**)	98680	-29660	0.9999	2.18 - 115	0.23
Chrysophanol (**5**)	170751	46224	0.9998	6.04 - 115	2.19
Physcion (**6**)	142579	-43473	0.9999	3.26 - 105	0.55

Note: The relationship between peak area and analyte concentration is expressed as linear regression lines ($y = ax + b$), where y is the peak area measured by UV detector, x is the concentration (ppm) of the analytes, and a and b are the respective slope and intercept of the calibration curve. The correlation coefficient is r.

3.3.4. Method Validation and Quantification

The method was validated by determining the intra- and inter-day precision. The relative standard deviation (R.S.D.) values for the retention times and peak-areas for the intra-day precision were 0.07-0.15% and 0.27-1.63% (n = 6), respectively. The R.S.D. values obtained for the inter-day precision study for the retention times and peak-areas were 0.07-0.16% and 0.39-2.10 (n = 6), respectively (Table 3.2).

Table 3.2 Relative standard deviation values for intra- and inter-day precision for compounds **1-6**

Compound	Intra-day precision (n = 6)		Inter-day precision (n = 6)	
	Retention time (R.S.D., %)	Peak area (R.S.D., %)	Retention time (R.S.D., %)	Peak area (R.S.D., %)
Rhein (**1**)	0.08	1.25	0.07	1.33
Kaempferol (**2**)	0.07	1.63	0.07	2.10
Aloe-emodin (**3**)	0.08	0.32	0.08	0.45
Emodin (**4**)	0.09	0.28	0.11	0.46
Chrysophanol (**5**)	0.12	0.27	0.13	0.46
Physcion (**6**)	0.15	0.31	0.16	0.39

In order to determine the accuracy of the method, the mean recovery of the phenolic compounds **1-6** was calculated at two concentration levels as reported in Table 3.3 for each

analyte. The overall mean recovery ranged between 81.2 ± 4.3% for aloe-emodin and 106 ± 2% for emodin (n = 3).

The concentration of each investigated compound in the root extract was determined by substituting its peak area for *y* in the equation listed in Table 3.1. The concentrations of compounds **1-6** detected in the root extract are given in Table 3.3. Among the six analytes determined in the root extracts, the concentrations of rhein (68.4 ± 1.6 ppm) and kaempferol (122 ± 7 ppm) were found to be highest.

Table 3.3 Concentrations and recoveries of phenolic compounds **1-6** in the root extract of *C. alata*

Compound	Root concentration (ppm)	Spiked concentration (ppm)	Total concentration detected (ppm)	Recovery (%) (mean ± S.D.)
Rhein (**1**)	68.4 ± 1.6	45.4 90.8	121 ± 1 151 ± 10	106 ± 1 90.4 ± 6.1
Kaempferol (**2**)	122 ± 7	65.0 130	192 ± 2 257 ± 1	103 ±1 102 ± 1
Aloe-emodin(**3**)	4.02 ± 0.37	3.30 6.61	6.43 ± 0.27 8.64 ± 0.46	87.7 ± 3.7 81.2 ± 4.3
Emodin (**4**)	26.5 ± 5.0	13.8 27.6	42.7 ± 0.9 52.5 ± 2.9	106 ± 2 97.0 ± 5.3
Chrysophanol (**5**)	21.6 ± 6.5	11.3 22.5	30.1 ± 0.6 37.1 ± 1.5	91.4 ± 1.8 84.1 ± 3.4
Physcion (**6**)	4.45 ± 0.71	2.62 5.24	7.37 ± 0.12 8.78 ± 0.20	104 ± 2 90.6 ± 2.0

3.4 Conclusions

A simple and reliable LC-UV-APCI-MS method was developed for the simultaneous analysis and identification of six pharmacologically active compounds in root extracts of *C. alata*. The extracts were purified by SPE and separated on a C_{18}-HPLC column using isocratic elution. The investigated phenolic compounds in the root extract were identified by their UV spectra and MS data. The compounds were ionized using an APCI interface and their molecular

masses as well as their fragmentation patterns were determined. Finally, HPLC-UV was used to determine the amount of these phenolic compounds in the root extract.

3.5 References

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CHAPTER 4

ISOLATION AND CHARACTERIZATION OF RHEIN, THE PRIMARY ANTHRAQUINONE IN THE ROOTS OF *CASSIA ALATA* L.

4.1. Introduction

A systematic and rational approach in drug discovery after analysis of a natural product extract is the separation, isolation, and purification of the primary compound, with the ultimate aim to examine a particular biological activity known to be exhibited by the compound's class and/or medicinal plant family. This methodology is a specific and a 'less' time consuming way of determining the certain biological activity of the compound. Another approach is bioassay-guided fractionation, wherein crude extracts are submitted to various bioassays for a rapid screening of their biological activity. Subsequently, the extracts of interest are fractionated by use of different chromatographic techniques. As denoted by the name, the bioassays serve as a guide for the discovery of novel bioactive compounds during the isolation process. In addition, all fractions that show biological activity are further isolated and purified until pure substances are acquired. Finally, the structures of the isolated compounds of interest are elucidated, along with in-depth biological and toxicological testing. Although this procedure is tedious, more expensive, and extremely time consuming, it is more comprehensive and eliminates the possibility of omitting a certain biological activity possessed by a compound.[1] In the execution of this study the former method was used (Figure 4.1).

The secondary metabolite, rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is the primary anthraquinone in the roots of *C. alata*.[2] Rhein is also the active compound in many laxative herbal drugs.[3-5] It relieves pain and fever, inhibits inflammation,[6, 7] and has been shown to have anti-tumor activity.[8-12] In addition, Lee *et al*. performed pharmokinetic studies on rhein in *Rheum undulatum* L. and demonstrated that this compound was the only anthraquinone (tested among emodin, aloe-emodin, and chrysophanol) absorbed by the human body.[13] Based on the

aforementioned characteristics of rhein as well as the ethno-pharmacological properties ascribed to *C. alata*, this study aims to isolate and characterize rhein from the roots of *C. alata*. This chapter describes the isolation of rhein from an ethanolic root extract using flash column chromatography (FCC) on a silica column with increasing polarity of the eluate. In addition, rhein was purified by use of preparative thin layer chromatography (PLC) and characterized using mass spectrometry (MS), nuclear magnetic resonance (NMR), ultraviolet (UV), and infrared (IR) spectroscopy.

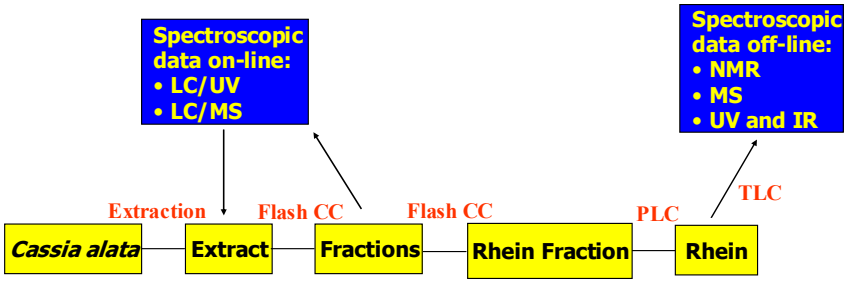

Figure 4.1 General procedure used for the isolation of rhein from *C. alata*

4.2 Experimental

4.2.1 Chemicals and Materials

Rhein standard and DMSO-d_6 (99.96 atom %D) were purchased from Sigma Aldrich (St. Louis, MO). Spectroscopic grade acetonitrile, ethanol, ethyl acetate, hexane, methanol, and Whatman no. 1 filter paper were obtained from Fisher Scientific (Houston, TX). Doubly-distilled deionized water, used during this study, was acquired using an Elga Genetic Ultra-Pure water polishing system from US Filter (Lowell, MA). Silica (particle size 0.04-0.063 mm) was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Chromatographic columns (45.7 × 6.3 cm and 30.5 × 1.3 cm) were obtained from Chemglass Life Sciences (Vineland, NJ). Normal phase thin layer chromatography (TLC) plates (Silica XHL TLC plates, w/UV254, glass backed, 250 µm, 5×10 and 10×10 cm) and normal phase PLC plates (Silica G Prep TLC plates, w/UV254, glass backed, 1000 µm, 20×20 cm) were purchased from Sorbent Technologies (Atlanta, GA). Disposable micro-pipettes (Drummond Microcaps of 3, 5, and 10 µL) were

obtained from VWR (Suwanee, GA). Spray reagent bottles were purchased from Kimble/Kontes (Vineland, NJ). In addition, NMR tubes (5 mm, 7 inch) were obtained from Norell (Landisville, NJ). Nylon membrane syringe filters (13 mm in diameter, pore size 0.45 μm) were purchased from Altech (Deerfield, IL).

4.2.2 Plant Material Extraction

Roots from the medicinal plant *C. alata* were collected from Paramaribo, Suriname. The plants were identified by the National Herbarium of the University of Suriname, Suriname (Reference number: Jansen Jacobs 5027).[14] Additionally, the root samples were oven dried at 40°C for five days. The dried roots were ground using a Wiley Mill grinder (Standard Model No. 3) to particle sizes of 6 mm or smaller. The ground roots were stored in the refrigerator at 4°C until further analysis.

Aliquots of the ground roots (75 g) were extracted with ethanol (750 mL) on an orbital shaker for 24 hours at room temperature. The extraction procedure was repeated one time. The two extracts were then combined and filtered through Whatman no. 1 filter paper. Subsequently, the extraction solvent was removed by use of rotary evaporation and the bulk residue was reconstituted in 75 mL ethanol. Prior to column chromatography, the root extract was monitored by normal phase TLC, wherein the optimal mobile phase for the elution of rhein from the column was obtained. After TLC monitoring, 2 g of silica was added to 5 mL of the crude extract and the excess ethanol was evaporated.

4.2.3 Isolation and Purification

The gummy residue of the ethanol extract was subjected to FCC on a silica column (45.7 × 6.3cm) and eluted with hexane/ethylacetate, ethylacetate/methanol, and methanol in increasing polarity. Fifty-nine fractions of approximately 75 mL were acquired. Following FCC, each fraction was monitored by normal phase TLC using various ratios of the solvent systems

hexane/ethylacetate, ethylacetate/methanol, and ethylacetate/methanol/water. After TLC monitoring, the fractions containing rhein (56 to 59) were combined and analyzed by LC-MS. Subsequently, the rhein fractions were concentrated and applied to a silica packed column (30.5 × 1.3 cm) using as eluent ethylacetate/methanol and methanol in increasing polarity. Sixty one fractions (containing approximately 25 mL) were obtained, wherein rhein was isolated using ethylacetate: methanol (1: 2) as mobile phase. In addition, each fraction was monitored by normal phase TLC using ethylacetate: methanol: water (6: 1.2: 1, v/v) as solvent system. The fractions containing rhein (21 to 43) were combined and concentrated. Finally, the isolated fraction was further purified using normal phase PLC plates with the solvent system ethylacetate/ methanol (5: 1, v/v) as mobile phase.

4.2.4 Characterization

The molecular weight of rhein was determined by use of the aforementioned Shimadzu LCMS-2010 system in section 3.2.5. The operating conditions for the HPLC instrument were similar to those described in section 3.2.4. In addition, ^{1}H-NMR spectra of rhein were collected in d_6-DMSO on a Bruker DRX400 spectrometer at 400MHz and IR spectra on a Bruker Tensor 27 spectrometer. Furthermore, UV spectra of the purified rhein were collected using a Shimadzu UV-3101 PC spectrometer. Figure 4.2 gives a schematic overview of the experimental design utilized for the extraction, isolation, and purification of rhein from root extract of *C. alata*.

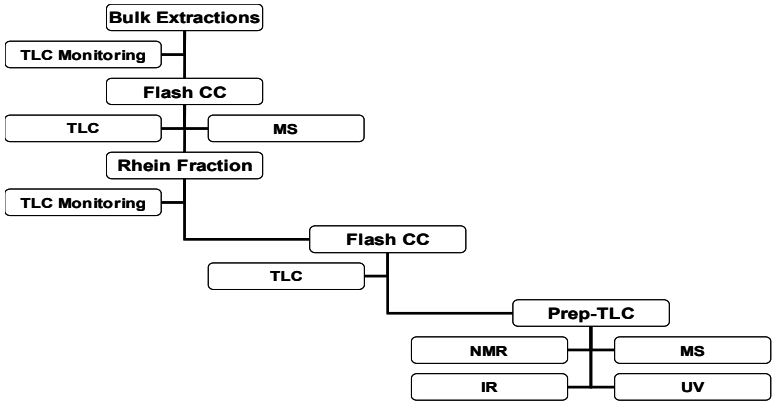

Figure 4.2 Summary of the employed experimental design

4.3 Results and Discussion

4.3.1 Isolation and Purification

Monitoring of the root extract by use of TLC (Figure 4.3) resulted in the complete separation of rhein (spot 1) and emodin (spot 4). However, the spot of rhein on the TLC plate is pinkish-orange instead of pink, indicating the interference of pigments with rhein. In addition, aloe-emodin was not detected by TLC, due to its low concentration in the root extract (see Table 3.3). As seen in Figure 4.3, no optimal separation was obtained between the standards of chrysophanol and physcion. Hence, taking into account that the concentration of physcion in the root extract is extremely low (see Table 3.3), it can be assumed that spot 5 from the root extract is chrysophanol.

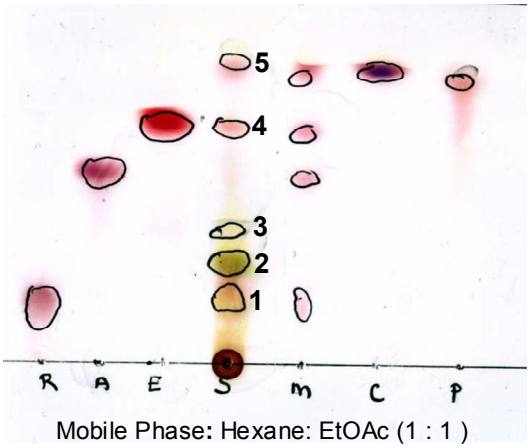

Figure 4.3 TLC monitoring of *C. alata* root extract. A potassium hydroxide (KOH) reagent in 10% ethanol solution was used to visualize the anthraquinones

Numerous fractions were acquired during the separation of the root extract by use of FCC. Rhein was separated from the root extract with methanol. In addition, the fractions containing rhein (56 to 59) were monitored by LC-MS. As the results demonstrate in Figure 4.4, the rhein fractions contained an impurity, most probably a red pigment (*vida infra*). Subsequently, rhein was isolated and purified using FCC and PLC, respectively. In Figure 4.5 the result of the purification of rhein is shown. Additionally, preliminary identification of the isolated rhein was

performed utilizing TLC, in which its R_f value (0.69) was compared with the R_f values of the rhein standard (0.71) and standard mixture (0.69). A poor extraction yield for rhein (between 34 and 40%) was obtained, which not only indicates the necessity of using a more polar extraction solvent, but also the possible loss of rhein during the fractionation, isolation and purification.

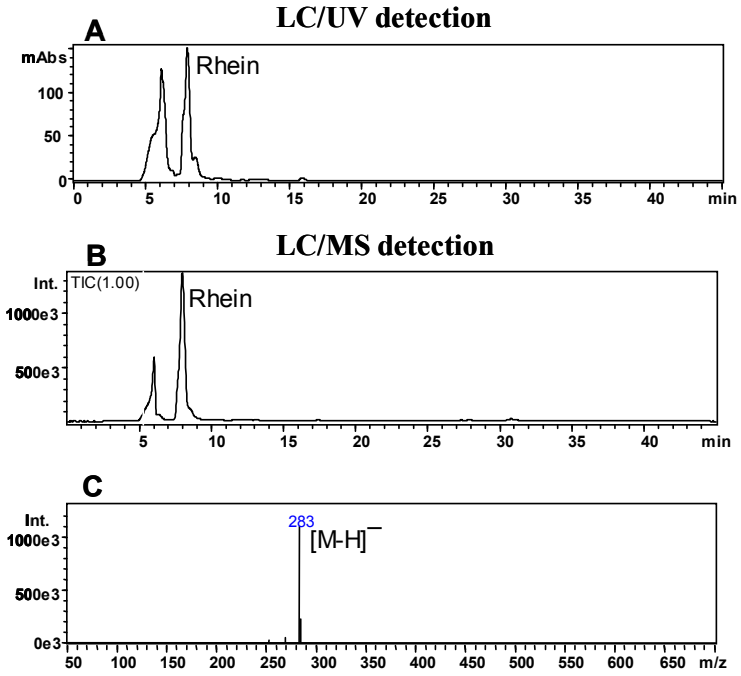

Figure 4.4 LC-MS anlaysis of rhein fraction. **(A)** HPLC chromatogram of *C. alata* root extract, λ = 260 nm, rhein (8.01 min); **(B)** Total ion chromatogram of rhein fraction; **(C)** Mass spectrum of rhein.

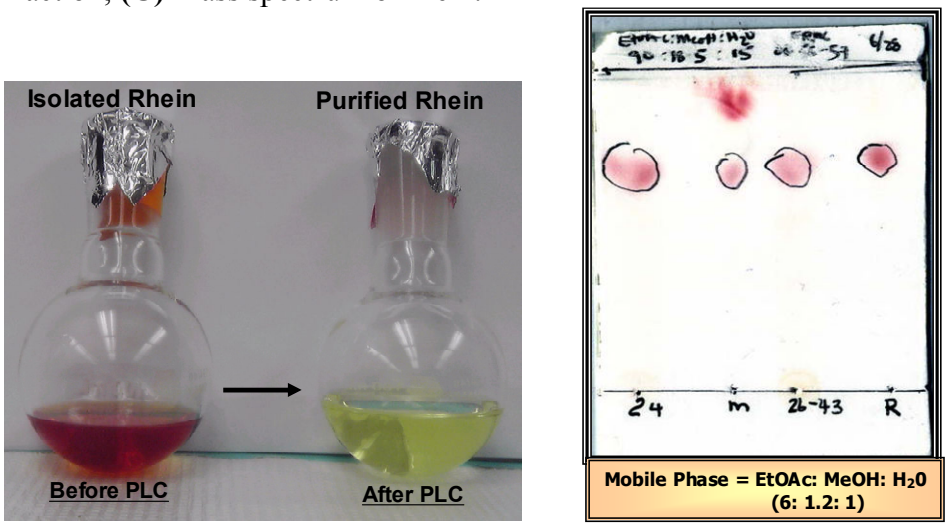

Figure 4.5 Picture of the isolated and purified rhein and thin layer chromatogram of the isolated rhein. Note: R = Rhein standard; M = Standard mixture

4.3.2 Characterization of Rhein

The structural characterization of the isolated rhein was performed by use of MS, NMR, IR, and UV as well as by comparison with a rhein standard. Rhein was identified on the basis of the following evidence: APCI-MS: *m/z* 284. IR (neat) EtOH: ν_{max} 3318, 2973, 2927, 2881, 1926 cm^{-1}. UV (EtOH) λ_{max} (nm): 260, 425. ^{1}H-NMR (400MHz, d_6 DMSO) (δ ppm) 11.91(s, 1H), 8.14 (d, J = 2.4Hz, 1H), 7.78-7.76 (m, 3H), 7.41(d, J = 13.2Hz, 1H), 2.49 (d, J = 2.4Hz, 1H). The obtained purity of the isolated rhein was 70%; therefore, ^{13}C-NMR studies could not be performed successfully.

4.4 Conclusions

The objective of this work was to isolate and characterize rhein, the primary anthraquinone, from the roots of *C. alata* for the use in bioassay studies. However, the poor extraction yield and relative low purity did not allow the use of the isolated rhein in bioassay analyses. The poor extraction yield of rhein can be resolved by using a more polar extraction solvent; for example, methanol and water. Nevertheless, utilizing methanol as an extraction solvent is often not supported because of its high toxicity. Although the use of water as an extraction solvent of polar substances in natural products is safe, these extracts do not contain long-term activity. Therefore, in this scenario, the use of an aqueous ethanolic solvent is advisable to increase the extraction efficiency, while conserving the active ingredients on the other hand. Furthermore, the obtained purity of the isolated rhein could be improved by using chromatographic techniques with a higher sensitivity, such as preparative-HPLC. In the remainder of this study, commercially available rhein (Sigma-Aldrich, St. Louis, MO) was used to conduct bioassay analyses.

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CHAPTER 5

RHEIN INHIBITS HYPOXIA-INDUCED TUMOR ANGIOGENESIS IN HUMAN BREAST CANCER CELLS

5.1 Introduction

After chemical analysis of a natural product and the isolation of its primary component, the assessment of the bioactivity of the isolated substance is the third significant factor in the drug discovery process. The biochemical examination of a promising phytochemical requires in-depth bioassay analysis to determine its mechanism of action. It is therefore, no doubt that the chemical, biochemical, and biological evaluation of a natural product is crucial during drug development. In this chapter, the effectiveness of rhein as an anti-angiogenic agent against non-invasive and invasive breast cancer was assessed.

Although the survival rate of breast cancer has been increasing since 1990 due to earlier detection and more effective chemotherapy,[1, 2] there is a need to treat hypoxic tumor cells which are more resistant towards standard therapy.[3-5] Tumor hypoxia in breast cancer patients can lead to advanced disease stages with poor prognosis and ultimately to painful death.[6, 7] Most breast tumors develop from non-invasive to invasive due to metastasis with the invasive cells being more aggressive and resistant to standard therapy.[8, 9] Bos et al. has shown that HIF-1α is over-expressed in pre-invasive as well as invasive breast cancer.[10, 11]

Hypoxia is the major physiological stimulus of HIF-1, which regulates the expression of genes involved in tumor development, progression, and aggressiveness. The hypoxia/HIF-1 system controls several angiogenic factors such as VEGF, EGF, and various glycolytic enzymes.[12-15] Another factor activated by hypoxia is the nuclear factor-kappa B (NF-κB). Stimulation of NF-κB leads to transcription of target genes that encodes for various proteins such as COX-2, VEGF, and MMPs. The protein, COX-2, has growth-stimulatory and angiogenic

properties. As well, it can activate some genes that are associated with tumor invasiveness, such as urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-2 (MMP-2).[16]

Recall that HIF-1 is over-expressed in many human tumor cells[17, 18] and that levels of its activity are a biomarker for the progression of hypoxia tumor angiogenesis.[17, 19] Therefore, in oncologic drug discovery, inhibition of the hypoxia/HIF-1system has become a potential therapeutic aim, since it can ultimately block tumor angiogenesis.[17, 20-22] One of the efforts to make this goal attainable is the use of bioreductive alkylating agents, such as quinones, against hypoxic tumor cells.[23, 24] Quinones can be reduced enzymatically by reductase under hypoxic conditions to generate quinone radicals, which have shown to be effective against hypoxic malignant cells.[25-27]

Several studies have been performed on the inhibition effect of quinones on tumor angiogenesis.[28-30] For instance, emodin, one of the most investigated quinones, inhibited *in vitro* the proliferation of a variety of cancer cell lines in cervical, breast, leukemia, lung, multiple myeloma, and prostate cancer.[31-33] In addition, *in vivo* studies that have been conducted with emodin indicated inhibition of tumor growth in mice transplanted with leukemia, melanoma, and breast cancer cells.[34-36] The potential anticancer actions of emodin include inhibition of PTK, PKC, NF-κB activity (weak), collagenase effects, and platelet aggregation. Furthermore, emodin has shown synergistic activity with chemotherapeutic drugs.[37]

Although rhein is one of the most ubiquitous anthraquinones present in medicinal plants, its role in tumor angiogenesis has not been established. However, recent studies have shown that rhein inhibits *in vitro* cell proliferation in human breast, central nervous system, colon, and lung cancer cells.[38] Additionally rhein induced apoptosis in human leukemia cells (HL-60) via a ROS (reactive oxygen species)-independent mitochondrial death pathway.[39] Another study showed that rhein inhibited the proliferation and induced apoptosis of human hepatoblastoma G2 (Hep

G2) cells partly through the p53-dependent apoptotic pathway.[40] Moreover, Floridi and co-workers performed several *in vivo* studies on the effect of rhein on the glucose metabolism of tumor cells. They found that rhein inhibited the glycolysis of cancer cells by reducing their ATP availability or glucose uptake.[41-43]

Altogether these fundamental results suggest that the anti-tumor effects of rhein are mainly mediated through the hypoxia/HIF-1 system. Therefore, it is hypothesized in this study that rhein is capable of inhibiting hypoxia tumor angiogenesis based on the fact that the transcription factor HIF-1 activates genes that encode the afore-mentioned processes. Furthermore, as it is well known, both hypoxia and angiogenesis play a vital role in the development, progression, and metastasis of human breast cancer.[15, 44] Hence, in this study, rhein was evaluated *in vitro* as a potential inhibitor of hypoxia-induced tumor angiogenesis in non-invasive (MCF-7) and invasive (MDA-MB-435s) breast cancer cells. In order to test the hypothesis, various biochemical and cytochemical *in vitro* systems were utilized. Our results show that rhein significantly inhibited (*in vitro*) the main factors involved in hypoxia tumor angiogenesis (i.e. HIF-1α, VEGF, EGF, and NF-κB) in both breast cancer cell lines. Thus, rhein is a promising anti-angiogenic quinone for inhibiting the growth of breast cancer cells.

5.2 Experimental

5.2.1 Materials and Reagents

Rhein and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of 10 and 20 mM rhein were prepared in DMSO and stored at -20°C for up to one month. In addition, for *in vitro* use, the rhein stock solutions were diluted with culture media to appropriate concentrations. Bovine serum albumin (BSA) standard was obtained from Bio-Rad (Hercules, CA). Recombinant human $VEGF_{165}$ standard was purchased from PeproTech Inc. (Rocky Hill, NJ). Gelatinase zymography standard human matrix metalloproteinase (MMP)-2,-9 was obtained

from Chemicon International Inc. (Temecula, CA). Magic Mark XP Protein and Sea Blue Pre-stained standards were purchased from Invitrogen (Carlsbad, CA).

5.2.2 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clontech (Mountain View, CA). The HUVECs were cultured in gelatin-coated plates with Cascade media 200 (Invitrogen, Carlsbad, CA) containing sodium heparin (0.1 mg/ml; Sigma-Aldrich, St. Louis, MO), endothelial cell growth supplement (0.03 mg/ml; Sigma-Aldrich, St. Louis, MO) and 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grant Island, NY), and incubated at 37°C in 5% CO_2 in air. Human breast cancer cells, MCF-7 and MDA-MB-435s, and normal human breast cells Hs578 Bst were purchased from ATCC (Rockville, MD) and cultured in serum-containing medium as recommended by the provider. Hypoxic conditions were generated for cell cultures by treating the cells with $CoCl_2$ (100 μM; Sigma-Aldrich, St. Louis, MO).

5.2.3 Endothelial Tube Formation Assay

The endothelial tube formation assay was performed with an *in vitro* angiogenesis assay kit from Cell Biolabs, Inc. (San Diego, CA). The ECM gel matrix was solidified in a 96-well microplate according to the manufacture's instructions. Human umbilical vein endothelial cells (1.0×10^4 cells in 100 μL) were seeded onto the gel matrix alone or mixed with rhein (10 to 100 μM) under normoxic or hypoxic conditions in the presence of 10 ng/ml of VEGF. The assay plate was incubated at 37°C, 5% CO_2 for six hours. After incubation, tube formation in each well was examined and photographed using a Leitz phase contrast inverted microscope.

5.2.4 Cell Viability Assay

Cell viability was determined by using the CellTiter96® AQueous One Solution Cell Proliferation Assay, an MTS bio-reduction method (Promega, Madison, WI). Normal and cancer cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated overnight at

37°C in a humidified 5% CO_2 atmosphere. Subsequently, cells were treated with various concentrations of rhein (50, 100, or 200 μM) or vehicle (< 1% DMSO) in triplicate under normoxic and hypoxic conditions. Replicate plates were incubated for 24, 48, or 72 hours in a humidified incubator at 37°C in 5% CO_2. After incubation, 20 μL of MTS tetrazolium compound (Owen's reagent) was added to each well and the plate was incubated for one hour at 37°C in a humidified, 5% CO_2 incubator. The absorbance of soluble MTS formazan product was measured at 490 nm using a Bio-Rad Model 680 microplate plate reader (Hercules, CA). The percentage of viable cells was determined by normalizing cell viability to the levels of the control.

5.2.5 Cell Migration and Invasion Assay

Cell migration and invasion assays were each performed by using the Quantitative Cell migration QCM™ assay (Chemicon, Temecula, CA) and CytoSelect™ 96-well cell invasion assay (Cell Biolabs, Inc. San Diego, CA) following the manufacturer's instructions, respectively. Aliquots of MDA-MB-435s cancer cells (1.0×10^6 cells in 100 μL for cell migration and 2×10^6 cells in 100 μL for invasion) were placed in the migration chamber and treated with rhein (0 and 50 μM) under *in vitro* normoxic and hypoxic conditions. Serum free media (150 μL) alone, as control, or in the presence of a chemo-attractant was added to the wells of the lower chamber. The plate was covered and incubated for four hours at 37 °C in a 5% CO_2 incubator. After a 15 minutes incubation at room temperature, 150 μL of the mixture was transferred to a 96-well plate suitable for fluorescence measurement and the fluorescence was read using a Perkin Elmer LS 50B using 480/520 nm filter set.

5.2.6 Cell Death ELISA

The Cell Death Detection ELISA kit from Roche Applied Science (Indianapolis, IN) was used to determine the amount of cytoplasmic histone-related DNA fragments (nucleosomes) in MCF-7 or MDA-MB-435s cells, after inducted cell death. In brief, 1×10^6 cells/well were plated

in a 12-well plate, treated with rhein for 48 hours and lysed to produce nucleosomes. The assay, a one-step sandwich ELISA procedure, was performed according to the manufacturer's instructions. Apoptotic cell death was detected in the sample by measuring the amount of nucleosomes bound to the anti-histone antibody, using a Benchmark Plus microplate reader at 405 nm, with a reference wavelength at 490 nm (Bio-Rad, Hercules, CA).

5.2.7 Protein Extraction

Human breast cancer cells (MCF-7 or MDA-MB-435s) were seeded in T-25 flasks (1×10^6 cells/flask in 5 mL) overnight at 37°C in 5% CO_2 and then treated with 50, 100, or 200 μM rhein or vehicle (< 1% DMSO) under normoxic and hypoxic conditions, in triplicate. The flasks were incubated in a humidified atmosphere at 37°C in 5% CO_2 for 48 hours. After incubation the supernatant was collected for the determination of VEGF, EGF, MMP-2, and MMP-9. Cytoplasmic and nuclear fractions were isolated from both cell lines using the commercially available Nuclear Extraction kit (Active Motif, Carlsbad, CA). The protein concentration in cytoplasmic and nuclear extracts was determined using the DC Protein Assay, Bradford method with BSA as standard (Bio-Rad, Hercules, CA).

5.2.8 Immunoprecipitation

Immunoprecipitation of MCF-7 or MDA-MB-435s cytoplasmic extracts was performed using the immunoprecipitation protocol from Santa Cruz Biotechnology (Santa Cruz, CA). In brief, 200 μg of cytoplasmic extract was pre-cleared and then incubated with 2 μg/ml of anti-HER-2, anti-COX-2, anti-Hsp90α, or anti-Hsp90β antibody for one hour at 4°C. Following incubation, lysates were immunoprecipitated with protein A/G PLUS-Agarose on a rotating device overnight. Finally, the resulting pellet was washed three times with PBS buffer, resuspended with 1×SDS sample buffer, and heated to 97°C for 3 minutes. The supernatant was then analyzed by Western Blotting.

5.2.9 Western Blot Analysis

Protein separation was performed using SDS-PAGE gel electrophoresis on a 4–12% Bis-Tris gel, followed by electro-transfer onto PVDF membranes (Invitrogen, Carlsbad, CA). Detection of the specific proteins was achieved with a Westernbreeze® Immunodetection Kit as per manufacturer instructions (Invitrogen, Carlsbad, CA). Concisely, after blocking, blots were probed with the antibody of interest: mouse monoclonal antibodies against Hsp 90β (1: 50 dilution; Invitrogen, Carlsbad, CA), COX-2 (1: 1,000 dilution; Cayman Chemical Company, Ann Arbor, MI), and HER-2 (1: 200 dilution; Thermo Fisher Scientific, Fremont, CA); rabbit polyclonal antibody against Hsp 90α (1: 1,000; Stressgen Bioreagents, Ann Arbor, MI). Membranes were re-probed with HRP conjugated anti-mouse or anti-rabbit secondary antibodies (1: 1,000 dilution). Afterward, bound antibodies were detected with a BCIP chromogenic detection reagent. Proteins of interest on the blots were identified using controls and molecular protein markers. The acquired blots were imaged with a Kodak Gel Logic 200 Imaging System and the relative pixel density was measured by use of Kodak Molecular Imaging Software (version 4).

5.2.10 Determination of HIF-1α

The level of HIF-1α in normalized nuclear extracts of control and rhein-treated breast cancer cells were analyzed using the TransAM HIF-1 kit (Active Motif, Carlsbad, CA). The standard ELISA test was used in determining the HIF-1α level as per manufacturer's instructions.

5.2.11 Determination of I-κB and NF-κB p50, p65

Following the 48 hour incubation of breast cancer cells with rhein (50, 100, or 200 μM) or vehicle (< 1% DMSO) under normoxic and hypoxic conditions in the presence of 10 ng/ml of TNF-α, whole cell extracts were isolated from the cells according to the Function ELISA I-κB

kit (Active Motif, Carlsbad, CA). The protein concentrations in these extracts were determined using the Bradford method with BSA as standard. Levels of Iκ-B in normalized whole cell extracts of control and rhein-treated MCF-7 or MDA-MB-435s cells were determined by use of ELISA.

The nuclear factor-κB (NF-κB) concentration in normalized nuclear extracts of control and rhein-treated MCF-7 or MDA-MB-435s cells (under normoxic and hypoxic conditions), in the presence of 10 ng/ml of TNF-α, were determined by use of the TransAM NF-κB p50 and p65 kit (Active Motif, Carlsbad, CA). Nuclear samples were analyzed using ELISA according to provider's procedures.

5.2.12 Quantification of VEGF and EGF

The levels of VEGF secreted by MCF-7 or MDA-MB-435s cells, untreated or treated with rhein under normoxic and hypoxic conditions, were measured using the human VEGF Sandwich ELISA kit from Chemicon International (Temecula, CA). Absorbance was measured at 490 nm on a Bio-Rad Model 680 Microplate Reader. The amount of VEGF in the supernatant of control and rhein treated samples was determined by use of a VEGF standard curve.

The amount of EGF in the supernatant of rhein-treated and control MCF-7 or MDA-MB-435s cells (under normoxic and hypoxic conditions) was determined by use of the Quantikine human EGF ELISA kit from R&D Systems (Minneapolis, MN). In order to determine the concentration of EGF in the samples, a serial dilution of human recombinant EGF was included in each assay to construct a standard curve. Absorbance was measured using a Benchmark Plus reader at 450 nm (reference wavelength at 540 nm).

5.2.13 Detection of MMP-2 and MMP-9 by Gelatin Zymography

The activities of electrophoretic separated MMP-2 (gelatinase A) and MMP-9 (gelatinase B) were determined in supernatants of control and rhein-treated breast cancer cells by gelatin

zymography. Equal volumes of supernatant and 2× SDS sample buffer were incubated at room temperature for 10 minutes. Zymography standard mixture of human pro-MMP-2 and MMP-9 was diluted 1: 100 with 2× SDS sample buffer. Subsequently, 10 μL of each sample was loaded into the wells of 10% polyacrylamide tris-glycine gels containing 0.1% gelatin substrate (Invitrogen, Carlsbad, CA). Gel electrophoresis was performed under non-denaturing conditions at 125 V for 90 minutes using a mini vertical electrophoretic system (Hoefer Pharmacia Biotech Inc., Piscataway, NY). Afterward, gels were incubated with zymogram renaturing buffer (Invitrogen, Carlsbad, CA) for 30 minutes at room temperature on an orbital shaker. The zymograms were then equilibrated with zymography developing buffer (Invitrogen, Carlsbad, CA) for 30 minutes at room temperature with gentle agitation, followed by a second incubation with fresh zymography developing buffer at 37°C for 20 hours. In addition, gels were stained using the Colloidal Blue Staining kit from Invitrogen (Carlsbad, CA) for 7 hours and destained with deionized water. Areas of enzymatic activities were detected as clear bands against a blue background. Zymogram gels were photographed using a digital camera.

5.2.14 Statistical Analysis

The obtained data was analyzed with Microsoft Excel software (version 5.0; Microsoft Corporation). Statistical analysis was performed using one-way analysis of variance (ANOVA). The data is presented as mean ± S.E.M.; and $p < 0.05$ is considered to be statistically significant for all tests.

5.3 Results and Discussion

5.3.1 Rhein Inhibits Endothelial Cell Tube Formation

The configuration of endothelial cells into a three-dimensional tubular structure is an attribute that is essential to angiogenesis. Therefore, an efficient approach to evaluate the effect of an agent on the inhibition of angiogenesis is to examine the capability of endothelial cells to

form tubes after treatment with the drug. Treatment of HUVECs with various concentrations of rhein resulted in a dose-dependent inhibition of VEGF-induced tube formation. Our results demonstrate that both under normoxic and hypoxic conditions, the VEGF-stimulated tube formation of HUVECs were disrupted completely at 50 μM rhein within six hours (Figure 5.1); hence, rhein is a potential angiogenic inhibitor.

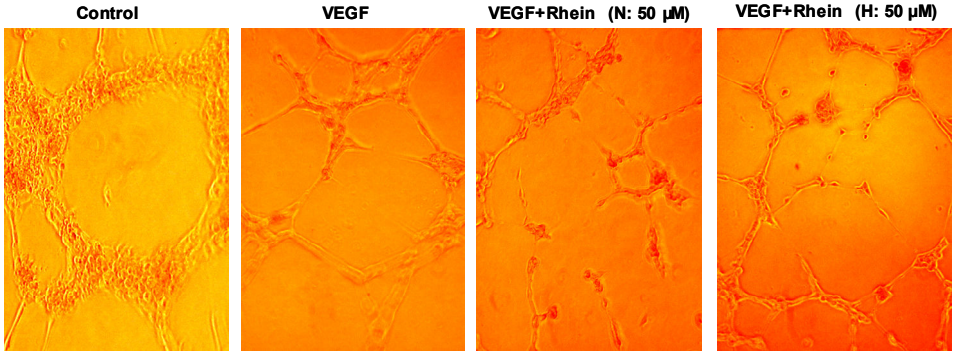


Figure 5.1 Rhein inhibits VEGF-induced tube formation of HUVECs under normoxic (N) and hypoxic (H) conditions. Cells were treated with rhein (10 to 100 μM) in the absence (control) or presence of VEGF (10 ng/ml). After six hours, disruption of endothelial tube formation was detected at 50 μM rhein (N and H) and microphotographs were taken (×10).

5.3.2 Effect of Rhein on Cell Morphology and Viability

Normal breast and breast cancer cells were treated with rhein (0-200 μM) for 24, 48, or 72 hours. Cell viablity was determined by cell morphology and cell viability assay. Our data shows that MCF-7 (Figure 5.2) and MDA-MB-435s (Figure 5.3) breast cancer cells died dose-dependently within 48 hours. According to the morphological results, the MCF-7 cells were more susceptible to the treatment of rhein than MDA-MB-435s, both under normoxic and hypoxic conditions. This data also indicates that there was no significant difference for MCF-7 cells in the treatment of rhein between normoxic and hypoxic conditions. However, for MDA-MB-435s, more cells died at 100 μM rhein (> 80% dead) under normoxic conditions than under hypoxic conditions (> 50% dead). In addition, at 200 μM rhein under normoxic conditions less cells died (> 90% dead) than under hypoxic conditions (> 95% dead). These results suggest that the viability of MDA-MB-435s cells is sensitive to an oxygen (i.e. normoxic or hypoxic)

environment. To attain better understanding of the cell viability of the breast cancer cells after treatment with rhein (over a certain time period at different concentrations) under normoxic or hypoxic conditions, a cell viability assay was performed.

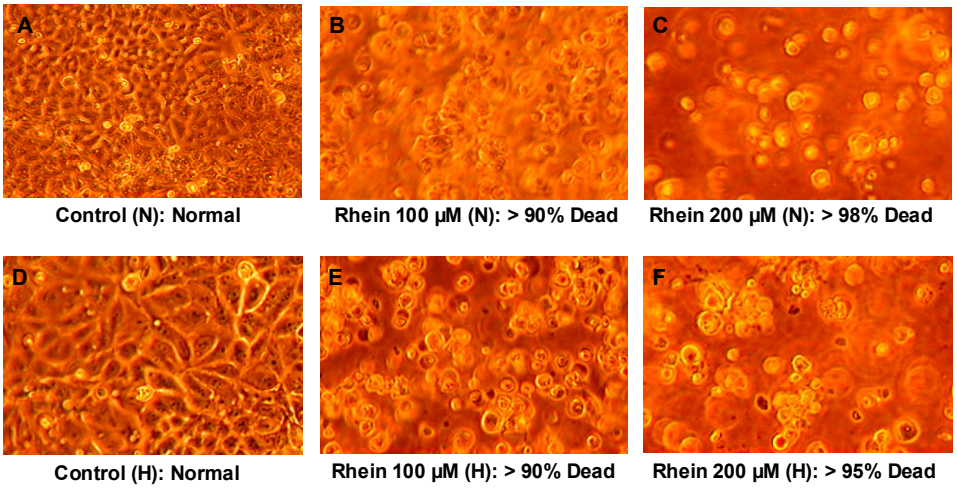


Figure 5.2 Morphological changes of MCF-7 human breast cancer cells after 48 hours incubation with rhein. Representative microphotographs, inverted microscope (×10): **(A)** Control (N): cells are normal; **(B)** Rhein 100 µM (N): > 90% dead; **(C)** Rhein 200 µM (N): > 98% dead; **(D)** Control (H): cells are normal; **(E)** Rhein 100 µM (H): > 90% dead; **(F)** Rhein 200 µM (H): > 95% dead.

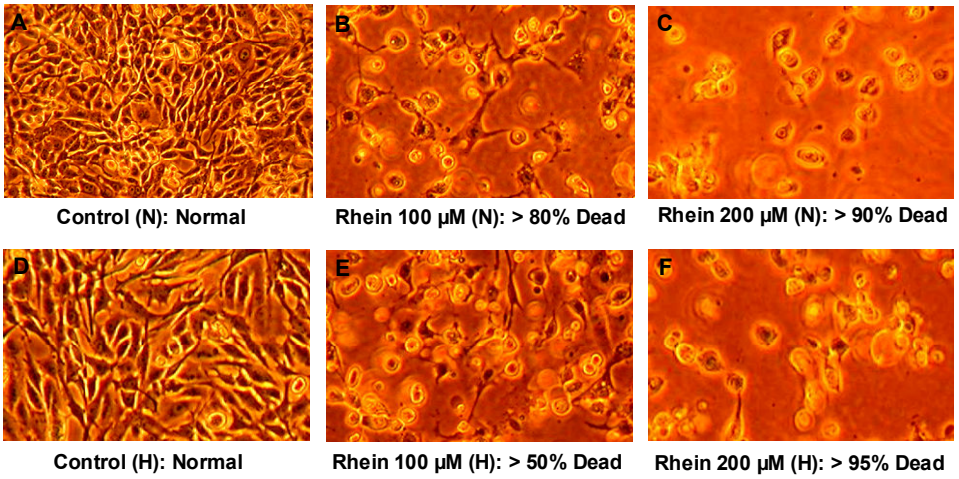


Figure 5.3 Morphological changes of MDA-MB-435s human breast cancer cells after 48 hours incubation with rhein. Representative microphotographs, inverted microscope (×10): **(A)** Control (N): cells are normal; **(B)** Rhein 100 µM (N): > 80% dead; **(C)** Rhein 200 µM (N): > 90% dead; **(D)** Control (H): cells are normal; **(E)** Rhein 100 µM (H): > 50% dead; **(F)** Rhein 200 µM (H): > 95% dead.

The effect of rhein on the cell viability was determined by measuring the ATP levels in normal breast and breast cancer cells. The cell viability was normalized to levels in untreated

control cells to determine the percentage of viable cells. As can be seen in Figure 5.4 there was no imperative suppression on the viability of the normal breast cells (Hs578 Bst). However, rhein significantly inhibited the viability of MCF-7 cells in a time and dose-dependent manner under normoxic and hypoxic conditions (Figure 5.5). For example, after 48 hours incubation with 50, 100, and 200 μM rhein there was an imperative inhibition on the viability of MCF-7 cells under normoxic (77%, 43%, and 32%, respectively) and hypoxic (74%, 36%, and 28%, respectively) conditions. Furthermore, even though the growth inhibition of MCF-7 was concentration-dependent under both conditions, it was more evident at shorter times under hypoxic conditions. The IC_{50} value (50% growth inhibition) of rhein for MCF-7 after 48 hours incubation under normoxic and hypoxic conditions was 81.3 and 71.3 μM, respectively. This specifically indicates that the treatment of the non-invasive cells with rhein under hypoxic conditions was relatively more effective than under normoxic conditions.

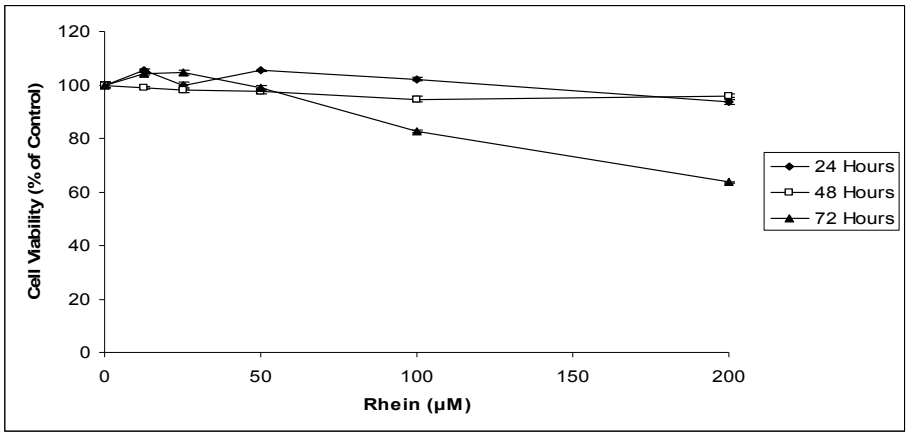


Figure 5.4 Effect of rhein on cell viability of Hs578 Bst normal breast cells under normoxic conditions. Cells were incubated with increasing concentrations (12.5 to 200 μM) of rhein for 24, 48, or 72 hours after which the cell viability was determined using the MTS assay. Measurements were performed in triplicate in two different cultures. Results are expressed as the mean % of MTS absorbance.

The viability of MDA-MB-435s cells under normoxic and hypoxic conditions was also significantly inhibited in a time and dose-dependent manner (Figure 5.6). However, under hypoxic conditions there was a slower but gradual decrease in the proliferation of the invasive

cells (Figure 5.6B). In contrast to MCF-7 cells, the growth inhibition of MDA-MB-435s cells was more evident under normoxic than hypoxic conditions. In addition, after 48 hours incubation of the invasive cells with 50, 100, and 200 µM rhein under normoxic conditions, the viability was significantly inhibited and reduced to 51%, 32%, and 16%, respectively. On the contrary, under hypoxic conditions after 48 hours incubation with 50, 100, and 200 µM rhein the viability was only reduced to 82%, 61%, and 22%, respectively. This is understandable, because under hypoxic conditions cancer cells are more resistant to most inhibitors. The IC_{50} value of rhein for MDA-MB-435s after 48 hours incubation under normoxic and hypoxic conditions was 52.1 and 127.3 µM, respectively. Based on the obtained results for both cell lines, we selected for further studies the 48 hours incubation with treatment of 50, 100, and 200 µM rhein.

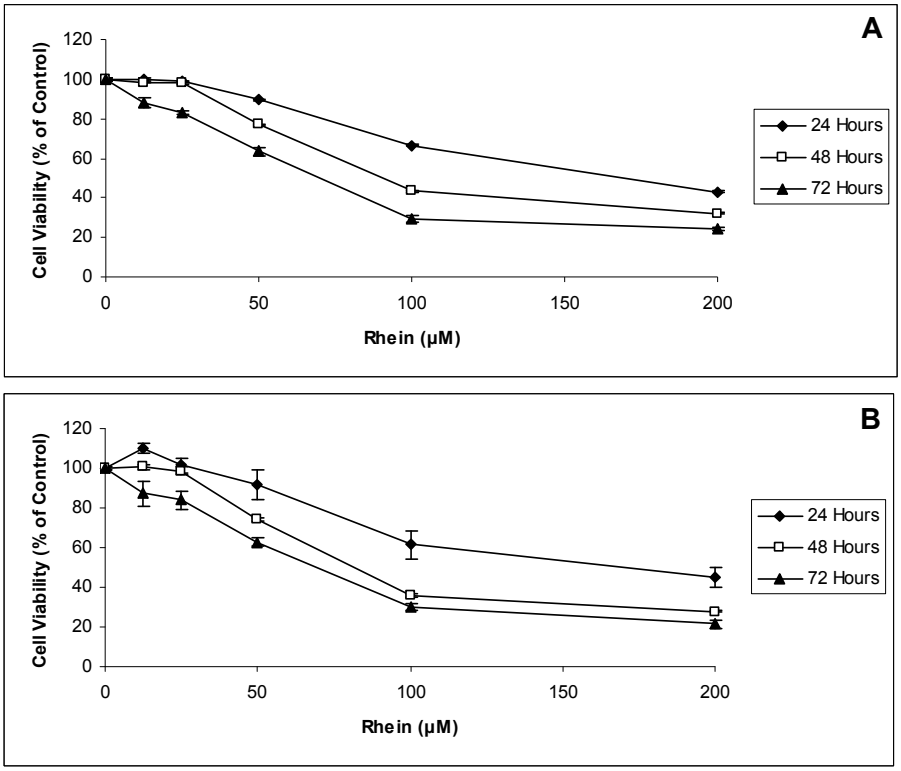


Figure 5.5 Rhein inhibits cell viability of MCF-7 cells under **(A)** normoxic conditions and **(B)** hypoxic conditions. Cells were incubated with increasing concentrations (12.5 – 200 µM) of rhein for 24, 48, or 72 hours after which the cell proliferation was determined using the MTS assay. Measurements were performed in triplicate in two different cultures. Results are expressed as the mean % of MTS absorbance.

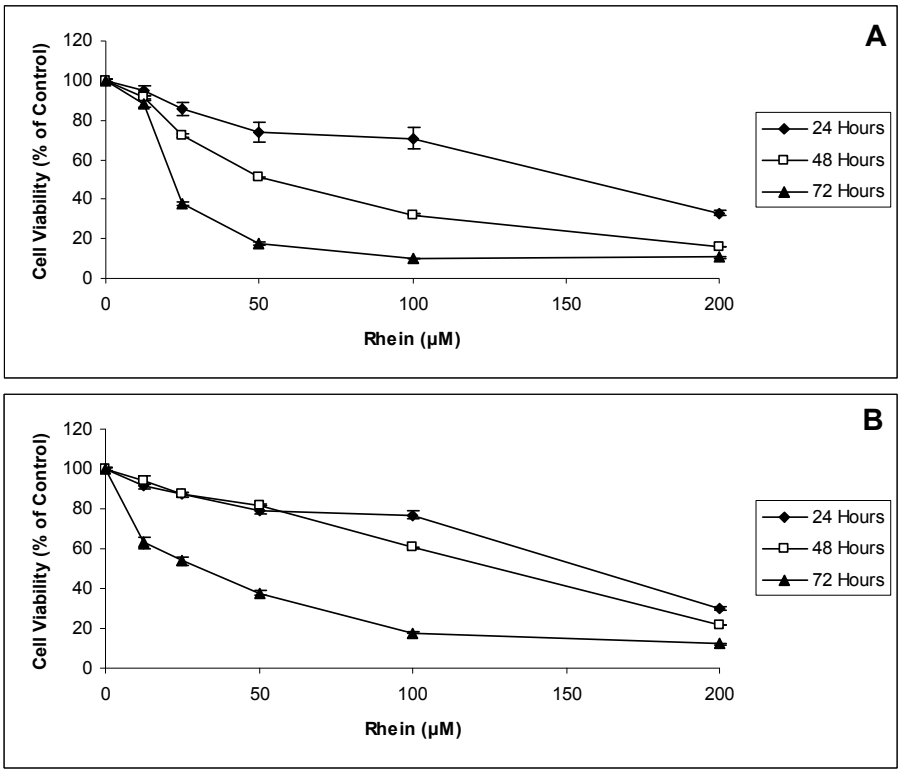


Figure 5.6 Rhein inhibits cell viability of MDA-MB-435s cells under **(A)** normoxic conditions and **(B)** hypoxic conditions. Cells were incubated with increasing concentrations (12.5 – 200 μM) of rhein for 24, 48, or 72 hours after which the cell proliferation was determined using the MTS assay. Measurements were performed in triplicate in two different cultures. Results are expressed as the mean % of MTS absorbance.

5.3.3 Rhein Inhibits the Migration and Invasion of MDA-MB-435s Cells

Cell migration is also an important process during angiogenesis. In this study, the invasive breast cancer cells (MDA-MB-435s) were utilized. The cells were treated with 50 μM rhein for 48 hours, since at this concentration the viability was reduced to approximately 50%. The angiogenesis-inducing growth factor VEGF causes endothelial cells to migrate (chemotaxis). Therefore, cell migration was assessed in the presence of VEGF as chemo-attractant. The migration capability of the cells after treatment with rhein was normalized to levels in untreated control cells to determine the relative percentage of inhibition. According to the results in Figure 5.7, there was a significant inhibition of migration of cancer cells at 50 μM rhein under both normoxic (60%) and hypoxic (42%) conditions. Thus, under hypoxic conditions fewer cells

migrated, which indicate that rhein (under these conditions) more effectively inhibited the migration of the invasive cells.

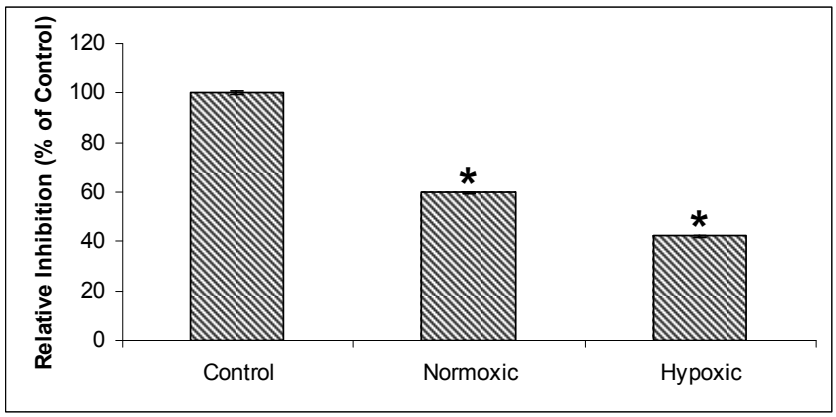


Figure 5.7 Cell migration of control and rhein treated MDA-MB-435s cells under normoxic and hypoxic conditions. Results are expressed as the mean ± S.E.M. *$p < 0.05$, compared with the control.

In addition, the invasive breast cancer cell line MDA-MB-435s was used for invasion studies. The effect of rhein on the invasion was examined by treating the cells with 50μM rhein. The invasion capacity of the cells after treatment with rhein was normalized to levels in untreated control cells to determine the relative percentage of inhibition. It is apparent in Figure 5.8 that rhein, compared to the control, significantly inhibited the cell invasion under normoxic and hypoxic conditions (both at 47%). These results suggest that rhein effectively inhibited the invasion of the cancer cells. It is instructive to note that the invasion of endothelial cells depends on the stability of extracellular matrix (ECM), which in turn can be imbalanced by proteases, including matrix metalloproteinases.[45, 46] It is therefore, possible that rhein could inhibit the protease activity as will be shown in the paragraph on MMP activities (section 5.3.10), thereby stabilizing the ECM, which helps in the decrease of breast cancer cell invasion.

5.3.4 Rhein Induces Cell death

Cell death detection ELISA was used to determine whether the anti-proliferative activity of rhein is due to induction of apoptosis. The amount of nucleosomes during nuclear DNA denaturation of apoptotic MCF-7 and MDA-MB-435s cells was measured after 48 hours

incubation with rhein (50, 100, and 200 μM). Cell death was normalized to levels of untreated cells (control) in order to determine the enrichment factor of the apoptotic cells. The enrichment factor is the ratio of the absorbance of the sample (dying/dead cells) and the absorbance of the control (viable cells). Whenever the MCF-7 cells were treated with 50 and 100 μM, there was no significant difference observed in the nucleosome production, which indicates that cell death did not occur by apoptosis at these concentrations. Therefore, the decreased cell viability correlated to cell death at these concentrations is most possibly due to inhibition of the cell cycle or activation another cell death mechanism.[47] However, the nucleosome production was significantly increased at 200 μM, suggesting that cell death occurred by apoptosis at 200 μM rhein, both under normoxic and hypoxic conditions (Figure 5.9).

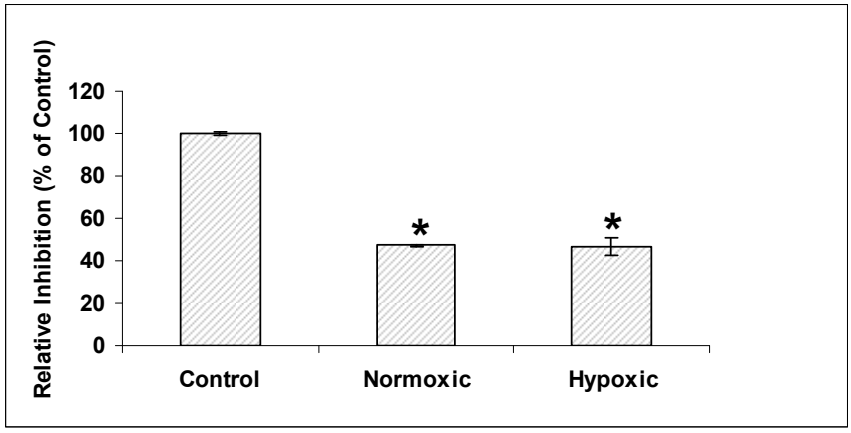


Figure 5.8 Cell invasion of control and rhein treated MDA-MB-435s cells under normoxic and hypoxic conditions. Results are expressed as the mean ± S.E.M. *$p < 0.05$, compared with the control.

In contrast to MCF-7, rhein did not induce apoptosis in MDA-MB-435s cells in a concentration-dependent manner. Instead, at low concentrations (50 μM of rhein) no significant increase was observed in the nucleosomal production of MDA-MB-435s cells, suggesting the absence of apoptosis. Subsequently, at 100 μM rhein the nucleosomal production was significantly increased, which indicates the occurrence of apoptotic cell death (Figure 5.10). Nevertheless, the nucleosomal production was remarkably decreased to an insignificant level

upon treatment with 200 μM rhein, suggesting the possible deactivation of apoptosis. The afore-mentioned results of rhein-induced cell death in MDA-MB-435s cells were both observed under normoxic and hypoxic conditions.

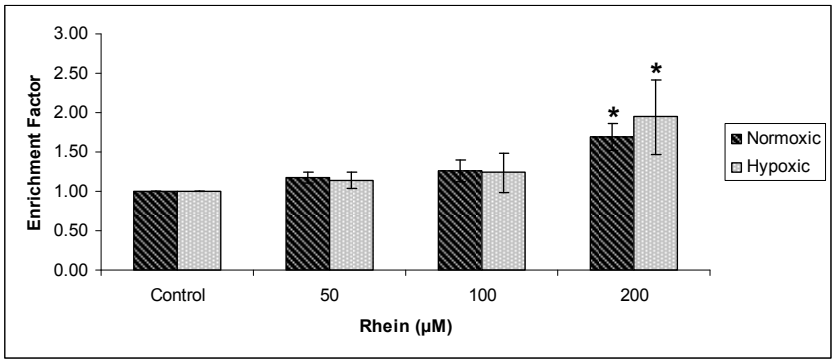


Figure 5.9 Effect of rhein on cell death of MCF-7. Cells were incubated with rhein in culture medium at 50, 100, 200 μM or culture medium alone (control) under normoxic and hypoxic conditions for 48 hours. The induced apoptosis was detected by use of cell death ELISA. Results are expressed as the enrichment factor (mean ± S.E.M.). All experiments were performed in triplicate with two replicates each. *Indicates significant difference at $p < 0.05$.

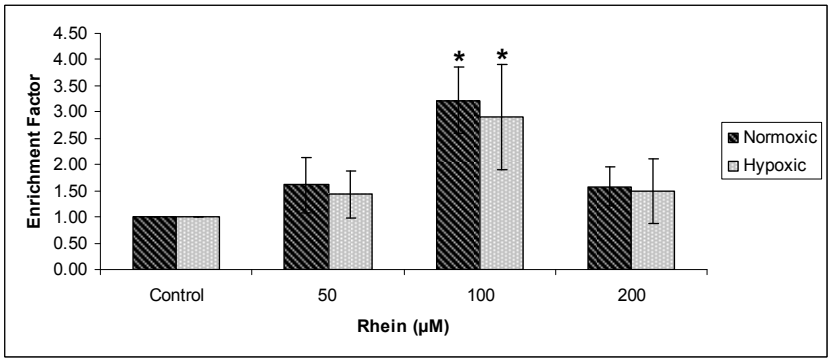


Figure 5.10 Effect of rhein on cell death of MDA-MB-435s. Cells were incubated with rhein in culture medium at 50, 100, 200 μM or culture medium alone (control) under normoxic and hypoxic conditions for 48 hours. The induced apoptosis was detected by use of cell death ELISA. Results are expressed as the enrichment factor (mean ± S.E.M.). All experiments were performed in triplicate with two replicates each. *Indicates significant difference at $p < 0.05$.

5.3.5 Western Blot Analysis

Cytoplasmic extracts of MCF-7 and MDA-MB-435s breast cancer cells were analyzed using Western Blot to evaluate the effect of rhein on Hsp 90α, Hsp 90β, COX-2, and HER-2. Analysis of these proteins, which are directly or indirectly transcribed by HIF-1, gave better

insight into the overall mechanism of rhein. In general, the treatment of MCF-7 cells with 100 and 200 μM rhein under normoxic and HIF-1 induced conditions decreased Hsp 90α, COX-2, and HER-2 levels (Figure 5.11A, C, and D), while it had no significant effect on the expression of Hsp 90β (Figure 5.11B). The relative densities compared with the control of these four factors in rhein-treated MCF-7 cells are reported in Figure 5.11E and F.

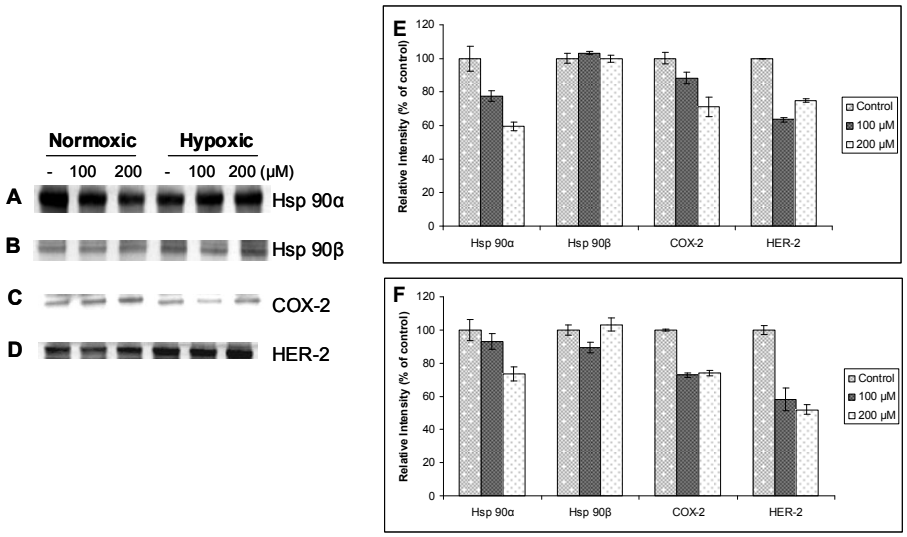


Figure 5.11 Effect of rhein on **(A)** Hsp 90α, **(B)** Hsp 90β, **(C)** COX-2, and **(D)** HER-2 expressions in MCF-7 cells. Cells were untreated (control) or treated with 100 and 200 μM rhein under normoxic and hypoxic conditions for 48 hours. Cell extracts were prepared and subjected to immunoblotting and Western Blot analysis to investigate Hsp 90α, Hsp 90β, COX-2, and HER-2 protein level. Blots are a representative of three. **(E, F)** Relative band intensity of the expression factors Hsp 90α, Hsp 90β, COX-2, and HER-2 determined by use of densitometry under normoxic and hypoxic conditions, respectively. Bars represent the mean ± S.D. from three independent experiments.

The treatment of hormone-dependent MDA-MB-435s cells with 100 and 200 μM rhein under normoxic conditions had various effects on the investigated factors. Levels of Hsp 90α were decreased only after treatment with 200 μM rhein. In addition, after treatment with 100 and 200 μM rhein, COX-2 expression was not significantly reduced, while the HER-2 levels were decreased in a concentration-dependent manner (Figure 5.12E). On the contrary, rhein-treated MDA-MB-435s cells contained, after treatment with 100 and 200 μM rhein under HIF-1 induced conditions, decreased levels of Hsp 90α, COX-2, and HER-2 as shown in Figure 5.12F. In

addition, both under normoxic and hypoxic conditions rhein (at 100 and 200 μM) had no significant inhibition effect on Hsp 90β levels. Treatment of MCF-7 and MDA-MB-435s cells with rhein did not effect the level of Hsp 90β, which is a good indication that rhein is not toxic to Hsp 90β.

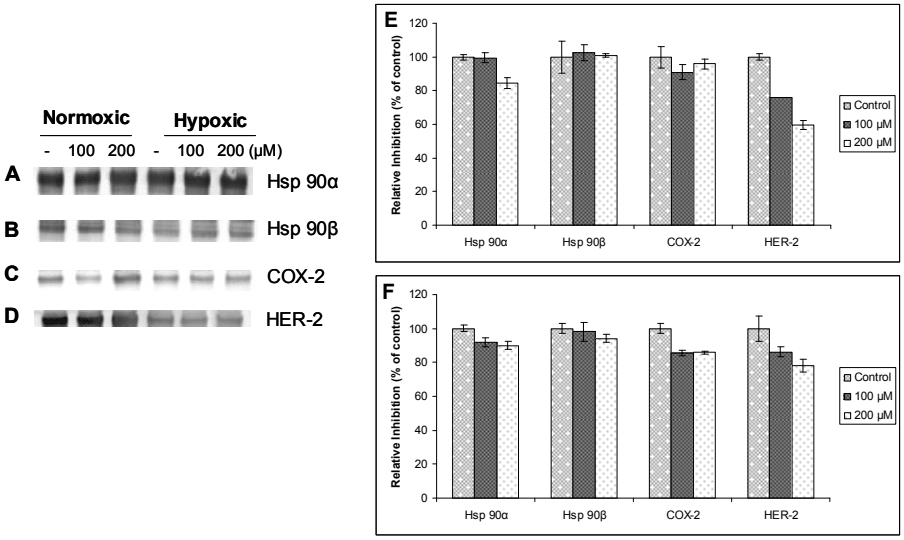


Figure 5.12 Effect of rhein on **(A)** Hsp 90α, **(B)** Hsp 90β, **(C)** COX-2, and **(D)** HER-2 expressions in MDA-MB-435s cells. Cells were untreated (control) or treated with 100 and 200 μM rhein under normoxic and hypoxic conditions for 48 hours. Cell extracts were prepared and subjected to immunoblotting and Western Blot analysis to investigate Hsp 90α, Hsp 90β, COX-2, and HER-2 protein level. Blots are a representative of three. **(E, F)** Relative band intensity of the expression factors Hsp 90α, Hsp 90β, COX-2, and HER-2 determined by use of densitometry under normoxic and hypoxic conditions, respectively. Bars represent the mean ± S.D. from three independent experiments.

5.3.6 Rhein Inhibits HIF-1α

Hypoxia-inducible factor-1 (a dimer of HIF-1α and HIF-1β), the major transcriptional factor activated by hypoxia, was also examined in this study. This factor is responsible for increasing the invasion capacity, angiogenesis, and proliferation of breast cancer cells. The effect of rhein as potential inhibitor of HIF-1α activity in hypoxia-induced tumor angiogenesis breast cancer cells was evaluated. The breast cancer cells were treated with various concentrations of rhein (50 - 200 μM) for 48 hr under hypoxic conditions. Nuclear extracts of control and rhein-treated MCF-7 and MDA-MB-435s cells were analyzed for HIF-1α using ELISA.

The controls of hypoxia-treated breast cancer cells produced significantly high amounts of HIF-1α (Figure 5.13). Our results show that rhein at 50, 100, and 200 μM significantly inhibited $CoCl_2$-stabilized HIF-1α activities in both cell lines. These data indicate that rhein dose-dependently decreased HIF-1α levels in MCF-7 and MDA-MB-435s cells. At low concentration of rhein (50 μM) both cell lines exhibited different sensitivity to HIF-1α activity, whereas at high concentrations (100 and 200 μM) they showed about the same sensitivity. For example, rhein at 50 μM exhibited significant reduction in HIF-1α expression of MCF-7 and MDA-MB-435s cells to 22% and 40%, respectively. It is evident that at this concentration the level of HIF-1α activity was more effectively reduced in the MCF-7 cells than in the MDA-MB-435s cells. The obtained results for both cell lines are desirable because HIF-1α regulates the transcription of mitogenic growth factors associated with angiogenesis under hypoxic conditions.

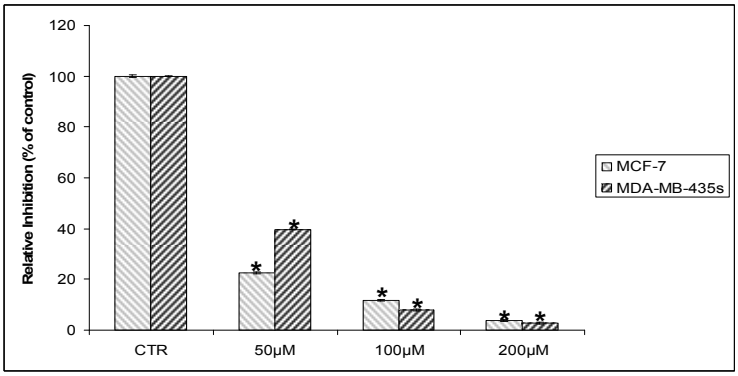


Figure 5.13 Rhein inhibits HIF-1α expression in MCF-7 and MDA-MB-435s cells. Breast cancer cells were untreated (control) or treated with 50, 100, and 200 μM rhein under hypoxic conditions for 48 hours before harvesting. Nuclear extracts were obtained as per manufacturer instructions, followed by protein determination. The normalized nuclear extracts were then subjected to HIF-1α analysis. Pretreatment of cells with different concentrations of rhein for 48 hours significantly diminished the translocation of HIF-1α. Results are expressed as the mean ± S.E.M. *$p < 0.05$, compared with the control.

5.3.7 Inhibition Effect of Rhein on I-κB and NF-κB p50, p65

Another factor that is stimulated by hypoxia is the nuclear factor-kappa B (NF-κB), which is implicated in the regulation of several genes involved in oncogenesis and angiogenesis.[48, 49] In addition, NF-κB stimulates cell proliferation and survival. This factor exists

in an inactive form in the cytoplasm, where it is composed of three major subunits: I-κB (inhibitory subunit), bound to the p50-p65 heterodimer. High levels of NF-κB are associated with cancer progression of several tumors, including breast cancer.[48, 50] Therefore, inactivation of NF-κB is crucial, since it leads to cancer cell death by autophagy or apoptosis.[51]

In this investigation, we first evaluated the effect of rhein on the phosphorylation of I-κB in MCF-7 and MDA-MB-435s breast cancer cells. The cells were treated with various concentrations of rhein (50 - 200 μM) for 48 hours under normoxic and hypoxic conditions. Whole cell extracts of control and rhein-treated breast cancer cells were analyzed using ELISA. The phosphorylation of I-κB in MCF-7 cells, treated with 50, 100, and 200 μM rhein, under normoxic conditions was reduced by 86%, 63%, and 27%, respectively, while under hypoxic conditions it was reduced by 80%, 46%, and 30% in comparison with their controls, respectively (Figure 5.14A). These data suggest not only that rhein at 100 and 200 μM significantly inhibited the phosphorylation of I-κB in MCF-7 cells under both conditions, but also that the dose-dependent rhein treatment under hypoxic conditions was more effective. In addition, rhein at 50 μM under hypoxic conditions significantly inhibited the phosphorylation of I-κB.

In Figure 5.14B are reported the results of the effect of rhein on I-κB phosphorylation in MDA-MB-435s cells. In contrast to MCF-7 cells, a significant inhibition of the I-κB phosphorylation in MDA-MB-435s cells by rhein in all treatments was observed. The invasive cells, when treated with 50, 100, and 200 μM rhein under normoxic conditions, showed a reduction in the I-κB phosphorylation by 57%, 46%, and 36%, respectively, whereas under hypoxic conditions a reduction by 67%, 33%, and 27% was observed, respectively. These results indicate that the phosphorylation of I-κB in MDA-MB-435s cells is effectively inhibited at a low concentration of rhein. Upon comparison of both cell lines, it can be stated that rhein more effectively inhibited the phosphorylation of I-κB in MDA-MB-435s than in MCF-7 cells. This is

important because MDA-MB-435s breast cancer is more life threatening than MCF-7. Altogether, this data suggest that rhein prevents degradation of I-κB in breast cancer cells; and hence, the translocation of the p50-p65 complex to the nucleus where it can bind to a κB motif.

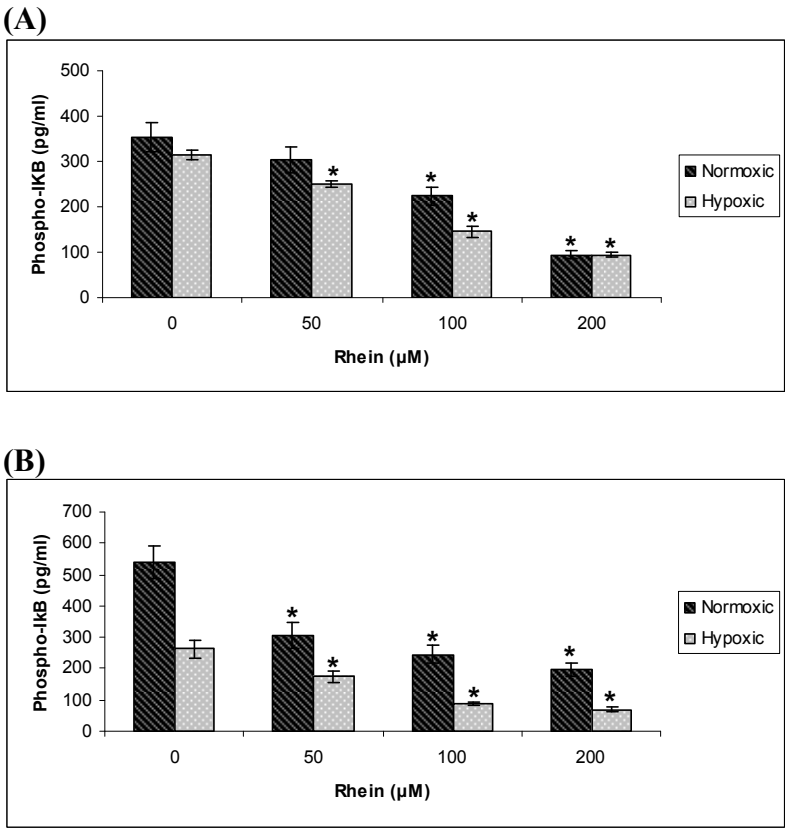


Figure 5.14 Effect of rhein on Iκ-B phosphorylation in **(A)** MCF-7 and **(B)** MDA-MB-435s cells. Breast cancer cells were incubated under normoxic or hypoxic conditions in the presence (50, 100, and 200 μM) or absence (control) of rhein for 48 hours. Whole cell extracts were acquired as indicated in section 5.2.11, followed by determination of the protein concentration. Normalized extracts of control and rhein-treated cells were assayed for Iκ-B levels using ELISA. Values are means ± S.E.M. *$p < 0.05$ compared with the control.

Secondly, the inhibition effect of rhein on the subunits NF-κB p50 and NF-κB p65 in MCF-7 and MDA-MB-435s breast cancer cells was evaluated. These cells were treated with various concentrations of rhein (50 - 200 μM) for 48 hours under normoxic and hypoxic conditions. Nuclear extracts of control and rhein-treated breast cancer cells were analyzed using ELISA. The relative activity of NF-κB p50 in MCF-7 cells treated with 50, 100, and 200 μM rhein was significantly decreased under normoxic and hypoxic conditions (Figure 5.15A). In

addition, the relative activity of NF-κB p50 in rhein-treated MDA-MB-435s cells was significantly inhibited under both conditions (Figure 5.15B). Furthermore, at high concentrations of rhein there was a more efficient inhibition under hypoxic than normoxic conditions. It is also apparent that there was a significant difference between normoxic and hypoxic conditions in the 200 μM rhein-treated MDA-MB-435s cells, which was not observed for the MCF-7 cells. In general, it was found that rhein more effectively inhibited the NF-κB p50 activity in MDA-MB-435s than in MCF-7 cells.

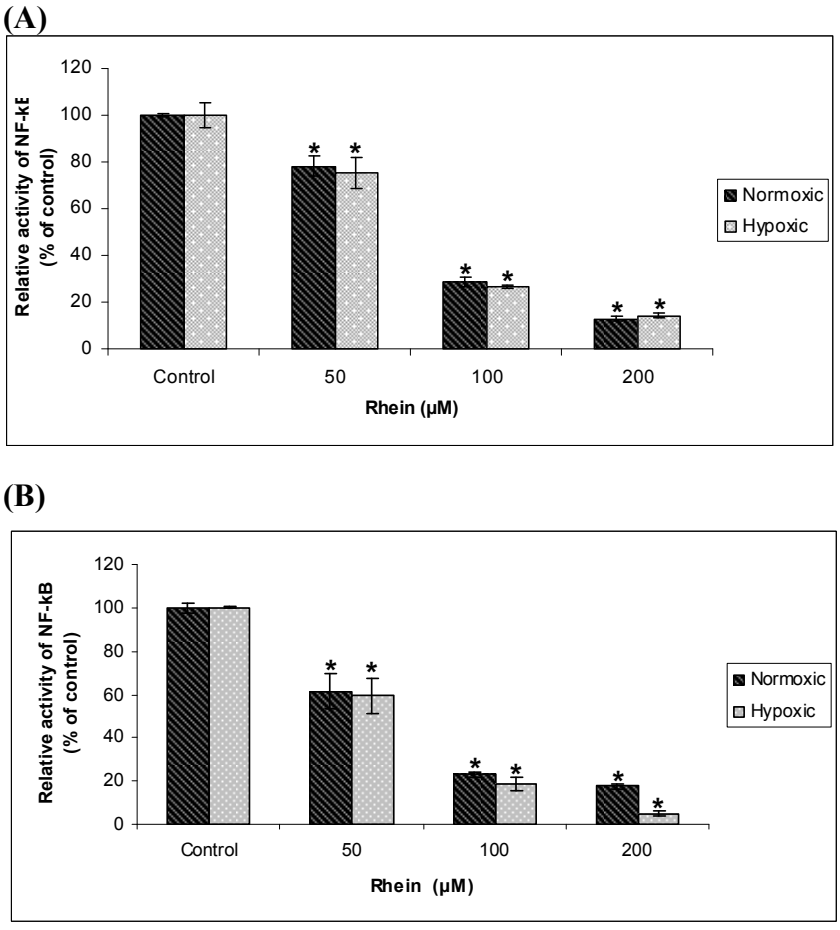


Figure 5.15 Rhein inhibits NF-κB p50 of MCF-7 and MDA-MB-435s cells under normoxic and hypoxic conditions. **(A)** NF-κB p50 activity of control and rhein treated MCF-7 cells. **(B)** NF-κB p50 activity of control and rhein treated MDA-MB-435s cells. After treatment of the cells with rhein (50 - 200 μM) for 48 hours, nuclear extracts were obtained as indicated in section 5.2.7, followed by determination of the protein concentration. Normalized nuclear extracts of control and rhein-treated breast cancer cells were assayed for NF-κB p50 activity levels using ELISA. Results are expressed as the mean ± S.E.M. *$p < 0.05$, compared with the control.

Subsequently, the inhibition effect of rhein (50 - 200 μM) on the activity of NF-κB p65 in MCF-7 cells was also evaluated under normoxic and hypoxic conditions (Figure 5.16A). Our data shows that rhein, in a dose-dependent way, significantly inhibited the activity of NF-κB p65 in non-invasive breast cancer cells under both conditions. Additionally, the treatment of 50 μM rhein was more effective under hypoxic than normoxic conditions, while the opposite trend was observed at 200 μM rhein. Finally, the effect of rhein on the activity of NF-κB p65 in rhein-treated MDA-MB-435s cells was investigated. Rhein at 50, 100, and 200 μM inactivated the activity of NF-κB p65 in invasive breast cancer cells under normoxic and hypoxic conditions (Figure 5.16B). Evidently, the inhibition effect of rhein on the activity of NF-κB p65 in MDA-MB-435s cells was dose-dependently more effective under hypoxic than normoxic conditions.

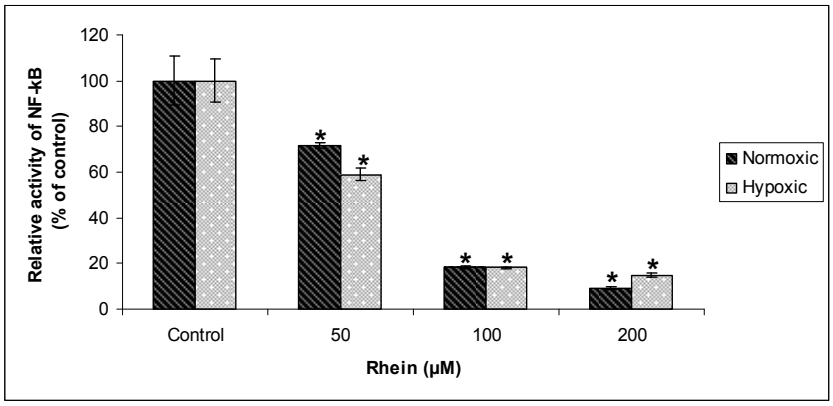
Overall, it can be stated that rhein, under normoxic conditions more effectively inhibited the activity of NF-κB p65 in MCF-7 than in MDA-MB-435s cells; while, on the contrary, under hypoxic conditions the inhibition effect was practically the same. In addition, rhein inactivated more efficiently the NF-κB subunit p65 than the p50, in both cell lines (Figure 5.15 and 5.16). Therefore, this data suggests that rhein is capable of inhibiting the process of oncogenesis, which in turn will inhibit angiogenesis, cell proliferation and survival, and induce cell death.

5.3.8 Rhein Decreases the Levels of VEGF

The effect of rhein on the inhibition of the angiogenic stimulator VEGF was evaluated for both MCF-7 and MDA-MB-435s cells. The concentration of rhein used in the VEGF assessment ranged between 50 and 200 μM. The levels of VEGF in the rhein-treated MCF-7 and MDA-MB-435s cells were lower than the controls, both under normoxic and hypoxic conditions (Figure 5.17). In the MCF-7 breast cancer cells (Figure 5.17A) the levels of VEGF in the control under normoxic and hypoxic conditions were 668 and 657 pg/mL, respectively. After treatment with 100 μM rhein, under normoxic and hypoxic conditions, there was a 39% and 36% reduction in

the VEGF levels of MCF-7 cells, respectively. Under the same conditions, the VEGF levels in the 200 μM rhein-treated MCF-7 cells were reduced to 25% and 28%, respectively. This data indicates that rhein at 50, 100 and 200 μM under normoxic and hypoxic conditions significantly reduced VEGF levels in MCF-7 cell cultures (Figure 5.17A).

(A)



(B)

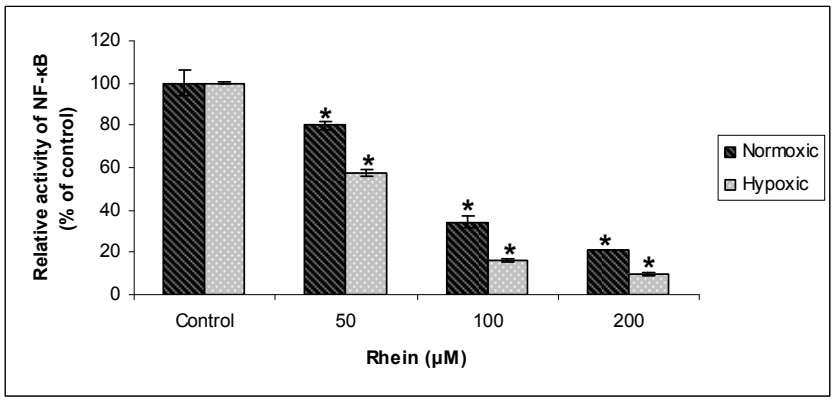


Figure 5.16 Rhein inhibits NF-κB p65 of MCF-7 and MDA-MB-435s cells under normoxic and hypoxic conditions. **(A)** NF-κB p65 activity of control and rhein-treated MCF-7 cells. **(B)** NF-κB p65 activity of control and rhein treated MDA-MB-435s cells. After treatment of the cells with rhein (50 - 200 μM) for 48 hours, nuclear extracts were obtained as indicated in section 5.2.7, followed by determination of the protein concentration. Normalized nuclear extracts of control and rhein-treated breast cancer cells were assayed for NF-κB p65 activity levels using ELISA. Results are expressed as the mean ± S.E.M. *$p < 0.05$, compared with the control.

The VEGF levels in controls of MDA-MB-435s breast cancer cells were remarkably higher under normoxic (816 pg/mL) and hypoxic (926 pg/mL) conditions than the MCF-7 cells, which

possibly explains the extreme resistance of the invasive cells. Over-expression of VEGF is one of the major factors involved in cancer metastasis. In addition, high levels of VEGF are a biomarker for cancer progression since this factor is a very important angiogenic stimulator in the migration and proliferation of endothelial cells. The levels of VEGF in 100 μM rhein-treated MDA-MB-435s cells were under normoxic and hypoxic conditions only reduced to 70% and 64%, respectively. Furthermore, under similar conditions the VEGF levels in the 200 μM rhein-treated invasive cells were diminished to 34 and 31%, respectively. The results reported in Figure 5.17B show that rhein dose-dependently decreased the levels of VEGF in MDA-MB-435s cells under hypoxic conditions. However, under normoxic conditions the VEGF levels in these cells were only significantly reduced when treated with 100 and 200 μM rhein.

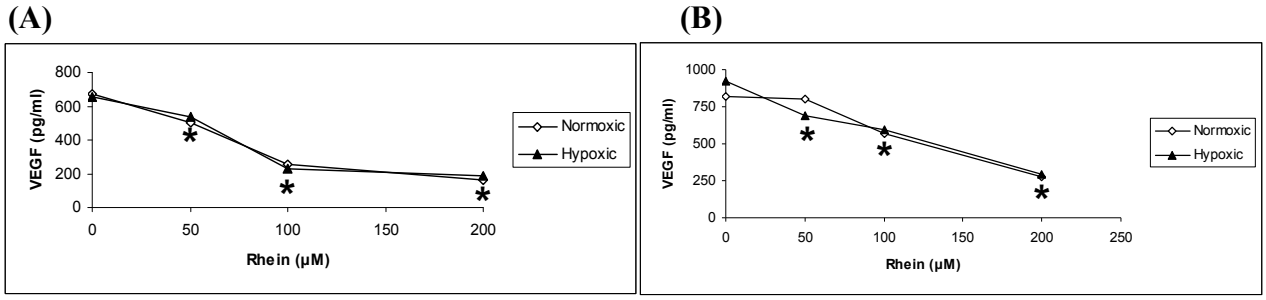


Figure 5.17 Effect of rhein on VEGF levels in **(A)** MCF-7 and **(B)** MDA-MB-435s cells. Rhein significantly decreased the VEGF protein in both cell lines. After treatment with the indicated concentrations of rhein, supernatants were collected and assayed for VEGF as indicated in section 5.2.12. Experiments were performed in triplicate. Bars represent the mean ± S.E.M. *, $p < 0.05$ versus control.

Altogether, the results indicate that rhein decreased the levels of VEGF in both MCF-7 and MDA-MB-435s breast cancer cells under normoxic and hypoxic conditions. In addition, it was found that the VEGF levels in invasive cells were less reduced than the non-invasive, suggesting greater resistance to the rhein treatment.

5.3.9 Rhein Reduces EGF Levels

The endothelial growth factor (EGF) is a gene protein responsible for cell proliferation and survival. Furthermore, it triggers angiogenesis, tumor growth, invasion, and metastasis. In this

study, the determination of EGF levels in rhein-treated MCF-7 and MDA-MB-435s breast cancer cells, under normoxic and hypoxic conditions, was performed using ELISA. The cells were treated in the concentration range of 50 to 200 μM rhein and incubated for 48 hours. Figure 5.18, illustrates the results of the effect of rhein on EGF levels in both cell lines. The concentration of EGF in controls of MCF-7 cells under normoxic and hypoxic conditions was 2.5 pg/mL, respectively. The EGF levels in 200 μM rhein-treated MCF-7 cells were reduced to 62% under both conditions. These results indicate that rhein dose-dependently decreased EGF levels in MCF-7 cells under normoxic and hypoxic conditions (Figure 5.18A).

(A) **(B)**

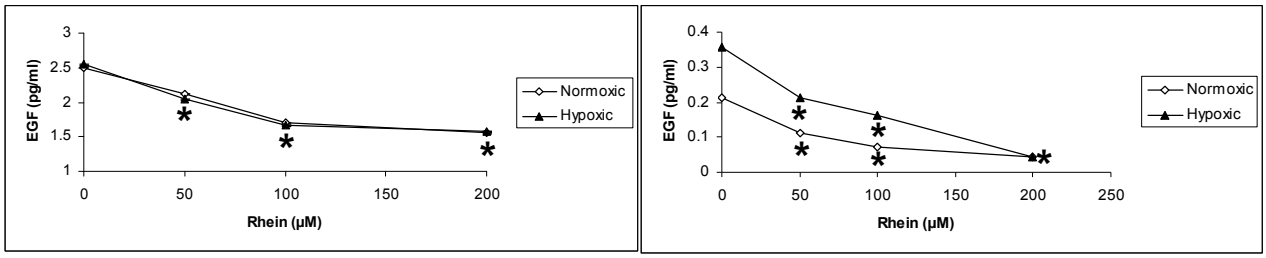


Figure 5.18 Effect of rhein on EGF levels in **(A)** MCF-7 and **(B)** MDA-MB-435s cells. Rhein significantly decreased the EGF protein in both cell lines. After treatment with the indicated concentrations of rhein, supernatants were collected and assayed for EGF as indicated in section 5.2.12. Experiments were performed in triplicate. Bars represent the mean ± S.E.M. *, $p < 0.05$ versus control.

The EGF levels in controls of the invasive breast cancer cells under normoxic and hypoxic conditions were 0.21 and 0.36 pg/mL, respectively (Figure 5.18B). These EGF levels were reduced by 33% and 44% when the MDA-MB-435s cells were treated with 100 μM rhein under normoxic and hypoxic conditions, respectively. Additionally, under the same conditions the EGF levels in the 200 μM rhein-treated invasive cells were decreased to 19 and 11%, respectively. Rhein significantly reduced levels of EGF in MDA-MB-435s cells (under normoxic and hypoxic conditions) in a concentration-dependent manner. The results of this study demonstrate that rhein is an inhibitor of EGF in MCF-7 and MDA-MB-435s breast cancer cells; and therefore, could

regulate angiogenesis in breast cancer cells. Taken together, it can be stated that rhein more effectively inhibited levels of EGF in MDA-MB-435s than in MCF-7.

5.3.10 Rhein Reduces the Enzyme Activity of MMP-2 and MMP-9

Both MMP-2 and MMP-9 have an important role in the invasion and metastasis of cancers cells.[45, 52] They can be used as biomarkers for the monitoring of disease onset having an ultimate effect on tumor growth and proliferation. Therefore, in this study we also examined the levels of enzymatic activity of MMP-2 and MMP-9. The results of the effect of rhein on MMP-2, and MMP-9 levels in MCF-7 and MDA-MB-435s breast cancer cells are displayed in Figures 5.19 and 5.20, respectively. The data shows a slightly decreased enzymatic activity under normoxic (P1-P4 for MCF-7 and P9-P12 for MDA-MB-435s) and hypoxic (P5-P8 for MCF-7 and P13-P16 for MDA-MB-435s) conditions, in which the brightness of the bands decreases as the dose concentration of rhein increased. MMP-9 is the first standard seen down the gel as it is higher in molecular weight than MMP-2. The effects of rhein can be seen as one follows from the controls across to the 200 μM concentrations of rhein, from the left to the middle (section A) and from the middle to the right (section B). It can be concluded that rhein effectively decreased the enzymatic activity of MMP-2 and MMP-9.

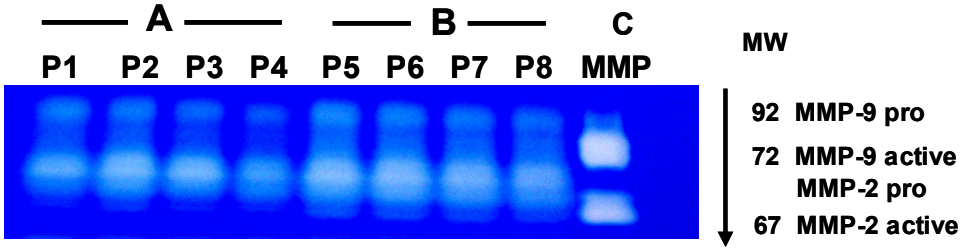


Figure 5.19 Zymogram of supernatant from rhein-treated MCF-7 cells. Rhein decreased the enzymatic activity of MMP-2 and MMP-9. **(A)** Normoxic conditions. Lane P1, control. Lanes P2, P3, and P4 represent the samples treated with rhein at 50, 100, and 200 μM, respectively. **(B)** Hypoxic conditions. Lane P5, control. Lanes P6, P7, P8 represent the samples treated with rhein at 50, 100, and 200 μM, respectively. **(C)** Zymogen standard mixture of pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa).

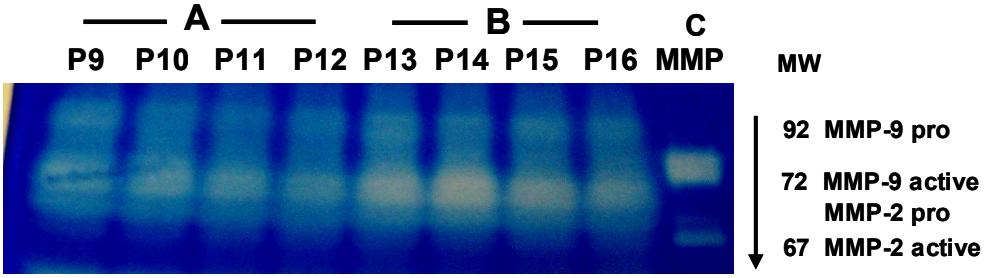


Figure 5.20 Zymogram of supernatant from rhein-treated MDA-MB-435s cells. Rhein decreased the enzymatic activity of MMP-2,-9. **(A)** Normoxic conditions. Lane P9, control. Lanes P10, P11, and P12 represent the samples treated with rhein at 50, 100, and 200 μM, respectively. **(B)** Hypoxic conditions. Lane P13, control. Lanes P14, P15, P16 represent the samples treated with rhein at 50, 100, and 200 μM, respectively. **(C)** Zymogen standard mixture of pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa).

5.4 Conclusions

Thus far, our data shows that rhein is a promising anti-angiogenic quinone for inhibiting the growth of non-invasive (MCF-7) and invasive (MDA-MB-435s) breast cancer cells under normoxic and HIF-induced conditions. This confirms the findings of Bolton et al. who concluded that quinone based-bioreductive drugs can have a versatile use for the inhibition of hypoxic tumor cell growth.[25] The migration of MDA-MB-435s cells under HIF-induced conditions was inhibited very effectively. This suggests that rhein may also be used as a preventative drug in the treatment of hypoxia-induced tumor angiogenesis of invasive cells.

Furthermore, our results suggest that the inhibition of VEGF production by rhein is through inhibition of HIF-1α. In addition, rhein impaired the phosphorylation of I-κB and inactivated the NF-κB subunits p50 and p65 in both breast cancer cell lines. Thus, HIF-1α and NF-κB inhibition is associated with decreased nuclear translocation of these factors and VEGF levels in cell supernatants. These results indicate that targeting HIF-1α and NF- κB with rhein has the potential to inhibit VEGF expression and limit MCF-7 and MDA-MB-435s tumor growth. In summary, our findings revealed novel *in-vitro* anti-angiogenic and antitumor activities of rhein, the primary anthraquinone in the roots of *C. alata*, in non-invasive and

invasive breast cancer. Hence, rhein is a potential therapeutic and preventive agent of breast cancer capable of inhibiting hypoxia-induced tumor angiogenesis.

5.5 References

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CHAPTER 6

CONCLUSIONS AND FUTURE STUDIES

The research presented in this dissertation primarily focused on the novel phytochemical analysis of root extracts of *Cassia alata* L. and the anti-angiogenic evaluation of rhein as a therapeutic agent against breast cancer. In order to conduct these studies, a combination of analytical and bio-analytical techniques was utilized. This chapter recapitulates the studies performed in this dissertation and provides recommendations for future research.

The first and second chapters contain an extensive review of the fundamental theory of many subjects discussed throughout this dissertation. This review was presented to provide background on the topics that were the major focus of this study, including the phytochemical analysis of root extracts from *Cassia alata* L. and the assessment of rhein as an anti-angiogenic agent in the treatment of breast cancer. In order to conduct these studies several analytical techniques were used including, high performance liquid chromatography, thin layer chromatography, and flash (column) chromatography. In addition, mass spectrometry, ultraviolet-visible, infrared, and nuclear magnetic resonance spectroscopy were employed together to characterize rhein. Furthermore, *in vitro* angiogenesis assays, enzyme-linked immuno-adsorbent assay, and Western Blot were used to investigate the effect of rhein on hypoxia-induced tumor angiogenesis.

In chapter 3, a simple and reliable LC-UV-APCI-MS method was developed for the simultaneous analysis and identification of six phenolic compounds in root extracts of *C. alata*. The extracts were purified by solid phase extraction and separated on a C_{18}-HPLC column using isocratic elution. The investigated phytochemicals were identified by their UV and MS spectra. These secondary metabolites were ionized using an APCI interface, and their molecular masses as well as their fragmentation patterns were determined. Finally, HPLC-UV was used to

determine the concentration of the six phenolic compounds in the root extract, and rhein was found to be the primary anthraquinone in the roots.

In chapter 4, rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) was fractionated and isolated from an ethanolic root extract using flash chromatography on a silica column with gradient elution. Additionally, rhein was purified using preparative thin layer chromatography, and finally characterized by use of mass spectrometry, nuclear magnetic resonance, ultraviolet, and infrared spectroscopy. The poor extraction yield (34 - 40%) and relative low purity (~ 70%) did not permit the use of the isolated rhein in bioassay analyses.

In chapter 5, a commercially available rhein was evaluated, *in vitro*, as a potential inhibitor of hypoxia-induced tumor angiogenesis in breast cancer cells. The anti-angiogenic activity of rhein was assessed using HUVECs, non-invasive (MCF-7) and invasive (MDA-MB-435s) breast cancer cells in combination with *in vitro* angiogenesis assays, enzyme-linked immunosorbent assays, and Western Blot analysis. It was found that rhein inhibited endothelial cell tube formation, MCF-7 and MDA-MB-435s cell viability, as well as MDA-MB-435s cell migration and invasion under normoxic and HIF-induced conditions. In addition, rhein significantly reduced VEGF and EGF levels in supernatant fractions and decreased HIF-1 activities in nuclear extracts of both cell lines. Furthermore, we examined cytoplasmic and nuclear extracts of both rhein-treated cell lines in relation to several other angiogenesis factors including NF-κB, HER-2, COX-2, and Hsp 90α and β. Our results suggest that the inhibition of VEGF and EGF production is through inhibition of HIF-1α. The inhibition of HIF-1α and NF-κB was associated with decreased nuclear translocation of these factors and VEGF and EGF levels in cell supernatants. The promising results indicate that targeting HIF-1 with rhein as a therapeutic agent has the potential to inhibit VEGF and EGF expression, and hence, suppress MCF-7 and MDA-MB-435s tumor growth. Therefore, these novel findings of rhein as an anti-

angiogenic agent could have a vital impact on the treatment of both non-invasive and invasive breast cancer.

Future studies in this arena should focus on making improvements in the extraction yield and increasing the purity of the isolated rhein by using more polar extraction solvents and supercritical fluid chromatography. In addition, possibly the most promising facet in this dissertation, is the demonstrated use of rhein as an anti-angiogenic agent against breast cancer cells. The ultimate aim of this project is to propose rhein as an oncologic drug, not only to treat breast cancer but also to treat other cancers, which are primarily dependent on angiogenesis for progression, invasion, and metastasis. Therefore, to gain better understanding of the mechanism of action of rhein it will be necessary to perform some additional *in vitro* studies (such as cell cycle analysis by flow cytometry) followed by *in vivo* and pre-clinical studies. Another potential area of research is to investigate the binding interactions of rhein with the various angiogenic proteins (such as HIF-1, VEGF, and Hsp 90) by use of anisotropy fluorescence spectroscopy, taking into account that rhein is fluorescent. This study could aid in providing a better insight into the mechanism of action of rhein.

APPENDIX

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VITA

Vivian Esther Fernand was born in Paramaribo, Suriname, to Rudi Fernand and Elize Fernand-Buyne. She is the last born child in a family of four children, with two elder sisters and an elder brother. She attended a private Moravian elementary and middle school, Maria Hartman School and Ritfeld School, respectively. She attended a public high school, Openbaar Atheneum. After obtaining her high school diploma, she was admitted to the Advanced Teachers Training Institute and to the University of Suriname for her undergraduate studies. In August 1996, she graduated from the Advanced Teachers Training Institute, division Chemistry, and in May 1998 she received her Bachelor of Science degree in agronomy with clear pass beta plus honor from the University of Suriname. During the course of her undergraduate studies, she did a research internship with the Department of Agriculture Chemistry at Louisiana State University, where she performed pesticide residues analyses in vegetables and verified formulations of pesticides using gas chromatography. After graduation, she taught chemistry in high school for two years and worked as a research associate for one year at the Center for Agricultural Research in Suriname, division tissue culture.

In the fall of 2000, Vivian was admitted to Louisiana State University for her graduate studies. In the spring of 2003 she received her Master of Science degree in the Department of Renewable Natural Resources, while she was also admitted to pursue her degree of Doctor of Philosophy in the Department of Chemistry under the guidance of Professor Isiah Warner. She was recipient of the Colgate Palmolive Teaching Award for her excellence in teaching. In addition, she received the Howard Hughes Medical Institute (HHMI) Graduate Mentor Award for her excellence in mentoring undergraduates.

The following is a list of excerpts from her dissertation research and collaborative work that have been published during her graduate studies:

- **Fernand, V. E.**; Losso, J. N.; Traux, R.E.; Villar, E.E.; Bwambok, D.K.; Fakayode, S.O.; Lowry, M.; Warner, I. M. "Rhein as Inhibitor of Hypoxia-Induced Tumor Angiogenesis in Non-Invasive Breast Cancer Cells." In preparation for publication.

- **Fernand, V. E.**; Losso, J. N.; Traux, R.E.; Villar, E.E.; Ishola, A.O.; Fakayode, S.O.; Lowry, M.; Warner, I. M. "Rhein Inhibits *In Vitro* Hypoxia-Induced Tumor Angiogenesis in MDA-MB-435s Breast Cancer Cells." In preparation for publication.

- **Fernand, V. E.**; Dinh, D. T.; Washington, S. J.; Fakayode, S. O.; Losso, J. N.; van Ravenswaay, R. O.; Warner, I. M. "Determination of Pharmacologically Active Compounds in Root Extracts of *Cassia alata* L. by use of High Performance Liquid Chromatography." *Talanta* **2008**, 74, 896-902.

- Bwambok, D. K.; Marwani, H. M.; **Fernand, V. E.**; Fakayode, S. O.; Lowry, M.; Negulescu, I.; Strongin, R. M.; Warner, I. M. "Synthesis and Characterization of Novel Chiral Ionic Liquids and Investigation of their Enantiomeric Recognition Properties." *Chirality* **2008**, 20, 151-158.

- Valle, B. C.; Morris, K. F.; Fletcher, K. A.; **Fernand, V. E.**; Sword, D. M; Eldridge, S; Larive, C. K.; Warner, I. M. "Understanding Chiral Molecular Micellar Separations Using Steady-State Fluorescence Anistropy, Capillary Electrophoresis, and NMR" *Langmuir* **2007**, 23, 425-435.

During her graduate studies, Vivian presented her work at a number of national scientific conferences including:

- **Fernand, V.E.**; Losso, J.N.; Bwambok, D. K.; Fakayode, S.O.; Warner, I. M. "Effect of Rhein on MDA-MB-435s Breast Cancer Cells under Hypoxic Conditions." American Chemical Society (ACS), New Orleans, LA, USA, April 2008.

- **Fernand, V.E.**; Losso, J.N.; Bwambok, D. K.; Fakayode, S.O.; Warner, I. M. "Evaluation of Rhein as Inhibitor of Hypoxia-Induced Tumor Angiogenesis in Breast Cancer Cells Using Several Bio-analytical Methods." Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (PITTCON), New Orleans, LA, USA, March 2008.

- **Fernand**, **V. E.**; Ishola, A. O.; Bwambok, D. K.; Fakayode, S. O.; Losso, J. N.; Warner, I. M. "Isolation, Characterization, and Cytotoxic Evaluation of Rhein from the Roots of *Cassia alata* L." Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (PITTCON), Chicago, IL, USA, February 2007.

- **Fernand, V. E.**; Dinh, D. T.; Fakayode, S. O.; Losso, J. N.; van Ravenswaay, R. O.; Warner, I. M. "Separation, Quantification, and Evaluation of Pharmacologically

Active Anthraquinones in *Cassia alata* L. Roots." Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (PITTCON), Orlando, FL, USA, March 2006.

- **Fernand, V. E.**; Dinh, D. T.; Huang, X.; Losso, J. N.; Warner, I. M. "Separation, Detection, and Evaluation of Quinones in Medicinal Plants." Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (PITTCON), Orlando, FL, USA, February 2005.

- **Fernand, V. E.**; Warner, I. M.; de Hoop, C.; Liu, Z. "Screening for Anti-tumor Activity of Extracts from Seven Medicinal Plants Related to Suriname." Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (PITTCON), New Orleans, LA, USA, March 2002.

Vivian will receive the degree of Doctor of Philosophy at the May 2009 Commencement.