Investigating *in vitro* anticancer properties of Malaysian Rainforest plants:

Acalypha wilkesiana Müll, Arg.

Archidendron ellipticum (Blume) Hassk.

Duabanga grandiflora Walp.

Pseuduvaria macrophylla (Oliv.) Merr

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Abstract

Malaysia is ranked the 12th richest country for its biological diversity of plant species by the Convention on Biological Diversity and this project aims to contribute to existing knowledge of Malaysian rainforest plants, *Acalypha wilkesiana, Duabanga grandiflora, Archidendron ellipticum* and *Pseuduvaria macrophylla,* for the treatment of cancer.

A. wilkesiana (Euphorbiaceae) whole plant EtOH and EtOAc extracts inhibited growth of breast cancer MDA-MB-468 cells (GI_{50}: 22.7 and 15.9 μg/ml) and revealed preference over non-transformed MRC5 fibroblasts (GI_{50}: 46.6 and 53.3 μg/ml, respectively). EtOH and HEX extracts were able to impair cell survival and colony-forming abilities in MDA-MB-468 cells after 24 h. Detection of increased MDA-MB-468 sub-G1 cell populations after 48 h treatment to EtOH and HEX extracts, suggest that cells may be undergoing apoptosis.

A. ellipticum (Leguminosae) crude polar bark and leaf extracts inhibited MDA-MB-468 cell growth (GI_{50} of bark EtOH, EtOAc extracts: 1.7 and 40.4 μg/ml, respectively and leaf EtOH and EtOAc extracts: 9.3 and 9.3 μg/ml, respectively). However, MDA-MB-468 cell growth was unaffected by HEX extracts (> 200 μg/ml). Separation of crude extracts revealed sub-fractions of greater activity, in particular a 50-fold enhanced potency in sub-fractions of HEX extract thus overcoming masking or antagonistic activity in the crude mixture. Following 24 h, bark and leaf extracts impaired MDA-MB-468 cells' proliferative and colony-forming abilities at 1X and 2X GI_{50} values suggesting significant damage was induced leading to observed cellular senescence and inhibition of cell proliferation. After 48 h exposure to EtOH and HEX extracts, MDA-MB-468 cells accumulated cellular damage, possibly affecting microtubule functions resulted in activation of apoptosis as shown by increased of sub-G1 and G2/M MDA-MB-468 cell populations and presence of phosphatidyl serine on the outer membrane of cells. Modest levels of flavonoid and phenolic compounds were found in bark and leaf extracts, which correlated to moderate level of free radical scavenging activity observed.

D. grandiflora (Lythraceae) bark and leaf extracts revealed growth inhibitory effects against colorectal cancer HCT116 cells (GI_{50} of bark Water, EtOH, EtOAc and HEX extracts: 42.4, 37.5, 21.69 and 28.9 μg/ml, respectively; leaf Water, EtOH, EtOAc and HEX extracts GI_{50}: 38.0, 40.9, 24.7 and > 200 μg/ml, respectively). Separation of crude bark extracts resulted in fractions of greater activity, whereas separation of leaf extracts revealed reduced activity suggesting synergistic activity in the mixture. Following 24 h, bark and leaf extracts impaired HCT116 cells' proliferative and colony-forming abilities at 1X and 2X GI_{50} values indicating significant damage incurred leading to observed cellular senescence and cell proliferation inhibition. After 48 h exposure to *D. grandiflora* extracts, increased sub-G1 HCT116 cell population and a G1/0 cell cycle block accompanied by decreased S and G2/M cell populations were measured. Detection of phosphatidyl serine on cells' outer membrane and activated apoptotic caspase 3 protein confirmed *D. grandiflora* extracts induced apoptosis. Highest levels of flavonoid and phenolic compounds were found in polar bark and leaf *D. grandiflora* extracts, which may correlate to the highest free radical scavenging activity measured.

P. macrophylla (Annonaceae) extracts collectively displayed greatest HCT116 cell growth inhibition (EtOH, EtOAc and HEX extracts GI_{50}: 5.2, 1.6 and 5.4 μg/ml) and separation of EtOH and EtOAc extracts revealed even greater activity in sub-fractions. After 24 h, polar extracts at 2X GI_{50} completely impaired HCT116 cells' proliferative and colony-forming abilities suggesting extract-induced significant damage led to inability of cells to proliferate. Analysis of cell cycle distribution revealed increased sub-G1 HCT116 cell population and high levels of early apoptotic HCT116 cells following 48 h exposure to *P. macrophylla* extracts coupled with detection of caspase 3 activation confirming execution of apoptosis. Modest levels of flavonoid and phenolic compounds were detected in EtOH, EtOAc and HEX extracts, which correlated to modest free radical scavenging activity.

The findings in this project should justify further separation and *in vitro* investigations.

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Abbreviations

ADME	Adsorption, distribution, metabolism and excretion
B	Biological drug
Calcein-AM	Calcein acetoxymethylester
CDK	Cyclin-dependent kinase
CAM	Complementary and alternative medicine
CML	Chronic myeloid leukemia
COX	Cyclooxygenases
CRC	Colorectal cancer
DAPI	4’,6-diamidino-2-phenylindole
DNP	Dictionary of Natural Products
DOS	Diversity Orientated Synthesis
DTP	Developmental Therapeutics Program
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallate
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
FADD	Fas-Associated protein with Death Domain
FC	Folin-Ciocalteau
FDA	US Food and Drug Administration
GA	Gallic acid
GAE	Gallic acid equivalent
GC	Gas chromatography
GI_{50}	50% Growth inhibition concentration
GR	Glucocorticoid receptors
G6PD	Glucose-6-phosphate dehydrogenase
HPLC	High performance liquid chromatography
HPV	Human papillomavirus
HSV	Herpes simplex virus
HTS	High-throughput screening
IF	Interferon
IL	Interleukin

IP	Intellectual property
KCM	Korean Chinese Medicine
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MIC	Minimum Inhibitory concentration
MMP	Metalloproteinase
MS	Mass Spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBI	National Biodiversity Index
NCDDG	National Cooperative Drug Discovery Group
NCE	New chemical entity
NCI	National Cancer Institute
ND	Natural product derivative
NF–κB	Nuclear factor–κB
NF-1	Neurofibromatosis type I
NHF	Normal human fibroblast
NM	Natural product mimic
NMR	Nuclear Magnetic Resonance
NP	Natural product
NPDD	Natural product drug discovery and development
NSCLC	Non-small cell lung cancer
PARP	Poly(ADP-ribose) polymerase
PI	Propidium iodide
P13K	Phosphatidylinositol-3-kinase
PR	Progesterone receptor
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
QE	Quercetin equivalent
Rb	Retinoblastoma
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RO5	Rule-of-five
S	Synthetic drug

S*	Synthetic drug with a natural product pharmacophore
SAR	Structure-activity relationship
SRB	Sulforhodamine B
TCM	Traditional Chinese Medicine
TM	Traditional Medicine
TNF-α	Tumour necrosis factor α
TPSA	Topological polar surface area
TSG	Tumour suppressor gene
TSP	Tumour suppressor protein
t_R	Retention time
T_0	Initial treatment time (0 hour)
UNMC	University of Nottingham, Malaysia Campus
UNMC 9	*Acalypha wilkesiana*
UNMC 20	*Pseuduvaria macrophylla*
UNMC 35	*Archidendron ellipticum*
UNMC 37	*Duabanga grandiflora*
UV	Ultraviolet
V	Vaccine
VEGF	Vascular endothelial growth factor
WCMC	World Conservation Monitoring Centre
WDI	Derwent World Drug Index
WHO	World Health Organisation
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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1

Introduction

1.1 The history of the application of medicinal plants in treatment of diseases

Prehistoric evidence has revealed mankind's application of natural products to treat various symptoms as well as benign and malignant diseases.[1-3] Ancient accounts, dated approximately 2,600 BC, originating from the Sumerians and Akkaidians describe uses for approximately 1,000 plants and their derived substances such as oil, resin and juice.[3, 4] Our ancestors chewed on particular herbs to relieve pain, used plants as tinctures or dressings to heal wounds and also used plants as poisons.[3-5] Several surviving ancient recipes of plant-based treatment of diseases are still in use, for example the Egyptian *Ebers Papyrus* dated around 1550 to 2900 B.C, which includes over 700 drugs and 800 formulae consisting of plants, animal organs and some minerals. The Chinese *Materia Medica* dated from circa 1100 B.C describes the use of over 600 plants in treating diseases.[6, 7] Natural products used traditionally for treatment could incorporate entire or parts of organisms such as plants (flower, stems or roots), animal products (glands or organs), microorganisms or inorganic salts without extensive processing.[5, 8] The selection and application of natural products for human well-being have prospered and evolved over the centuries through a process of trial and error.[9] Indigenous knowledge and practices (or systems) are passed down generations worldwide for example, Traditional Chinese Medicine (TCM; China), Japanese Chinese Medicine or Kampo (Japan), Korean Chinese Medicine (KCM; Korea), Jamu (Indonesia), Ayurvedic Medicine (India), and more recently Phytotherapy (Europe).[2] The World Health Organisation (WHO) defines traditional medicine (TM) as indigenous practice, which "*incorporates plant, animal, and/or mineral based*

medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness".[8] TM is also known as 'complementary and alternative' (CAM) or 'non-conventional' medicine in countries where TM is not a part of the country's national health care system.[10, 11] At present, an estimated 80% of the world's population, primarily in undeveloped countries, is reported to use TM as their primary healthcare.[8] Whereas the other 20% of the world's population, primarily in developed countries, is being prescribed drugs that are derived directly from natural products or designed from semi-synthetic natural product pharmacophores.[12] Since there is an extended record of using natural products for human consumption and treatment of diseases, it deems rational to investigate plant materials as a source for biologically active therapeutic compounds.[13]

Nevertheless, mankind's desire to pursue a better life-style has led to increased commercial pressure for habitable areas, food sources and employment (e.g. farming). As a result, there is an ever-growing concern over the effect of the deforestation and industrialisation of the planet on the natural habitat of wildlife and plant species. The decline and potential extinction of species could result in the loss of interesting novel therapeutic compounds as well as the gradual loss of indigenous traditions and ethnobotanical knowledge.[14, 15] Therefore the race to identify useful natural compounds could ease commercial pressures and preserve natural habitats if governments and authority bodies enforce stricter regulations on deforestation.

1.2 Advances in traditional medicine: Ethnopharmacology and Molecular Pharmacognosy

Herbal remedy recipes are rich sources of phytochemicals (the term 'phyto-' is derived from the Greek word for plant) and thus provide guidance for potential drug-leads.[16, 17] The observation of natural products' use by indigenous groups accompanied by the scientific investigation of herbal recipes to identify active phytochemicals is an area of drug research termed ethnopharmacology.[3] Ethnopharmacology is enhanced by multicultural exposure and global communication enabling methodological collection and validation of medicinal valuable ethnobotanical evidence.[18] In the 18th century when medicines were mainly limited to natural products, Johann Adam Schmidt (1759–1809) adopted the term 'pharmacognosy' to incorporate the area of drug knowledge and 'ethnobotanic medicine'.[3, 11, 14] Consequently, pharmacognosy has advanced to incorporating various disciplines such as ethnobotany, phytochemistry, biochemistry, organic chemistry, taxonomy and pharmacology. Each discipline contributes to the accurate identification of plant species (plant selection), extraction of plant materials, verification of biological activity and the isolation and structure elucidation of novel active compounds or of already known active compounds by validating on compound databases (de-replication); this is termed 'molecular pharmacognosy', which "*explores naturally occurring structure-activity relationships (SAR) with a drug potential*" to increase chances of finding new structures, biological activity and targets (Figure 1-1).[19]

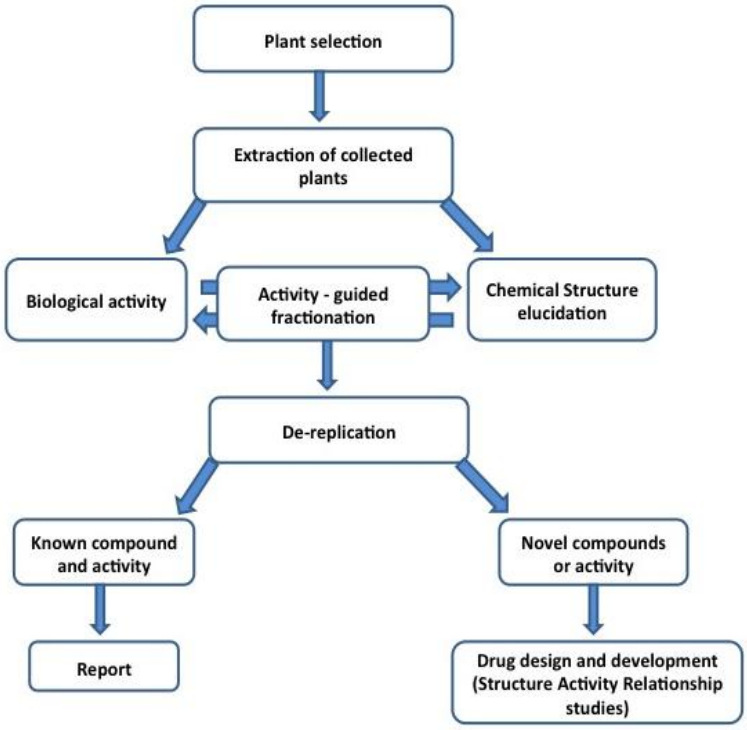
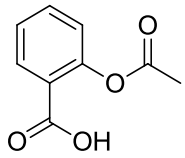


Figure 1-1: Explanatory model for molecular pharmacognosy**.**

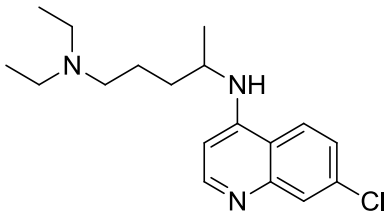
At the beginning of the 19th century, approximately 80% of all medicines were derived from different plant sections e.g. root, leaf and/or bark.[20] For instance, *Solanum dulcamara* bark extract is used in TCM for its analgesic properties to relieve pain.[21] By the late 19th century, aspirin (or acetylsalicylic acid) (1), from the bark of *Salix alba* was developed. Aspirin is the first synthetic pharmaceutical drug for the relief of minor pains, which highlights the significance of nature in drug development.[5, 22, 23] Upon tissue damage, algogenic chemicals such as prostaglandins are released to cause hyperalgesia, and aspirin acts to inhibit the biosynthesis of such molecules.[23-25] Later, morphine was isolated from opium poppy, *Papaver somniferum*, and the search and isolation for new drug leads continues today with approximately 75% of drugs for infectious and parasitic diseases consisting of natural products or natural product derivatives.[11, 20]



1

Figure 1-2: The chemical structure of acetylsalicylic acid (1).

Between 1981 and 2002, 52% of the 868 new chemical entities (NCEs) were natural products, natural product derivatives or compounds containing a pharmacophore from natural products.[26] In 2004, 70 natural product-related compounds were in clinical trials.[5] Recently, approved drugs based on natural products include artemether (isolated from *Artemisia annua* L. (Asteraceae) used in TCM), galantamine (isolated from *Galanthus woronowii* Losinsk (Amaryllidaceae)), calanolide A (isolated from *Calophyllum lanigerum* var. *austrocoriaceum* (Whitmore) (Clusiaceae)) and nitisinone (modified from mesotrione, from *Callistemon citrinus* Stapf. (Myrtaceae)), which are available in the United States (U.S.) for indications such as malaria, Alzheimer's, HIV/AIDS and tyrosinaemia, respectively.[11] Patients commonly acquire resistance to their treatment e.g. malaria patients develop resistance to chloroquine (2) over prolonged use, therefore drug development is a continual process to ensure new leads such as cryptolepine (3), an alkaloid from the African *Cryptolepis sanguiolenta*, with an alternative mechanism of action will be available as another treatment option.[27]



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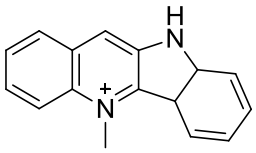


3

Figure 1-3: The chemical structures of chloroquine (2) and cryptolepine (3).

Fabricant and Farnsworth (2001) outlined several key objectives of investigating plants for therapeutic compounds, including the use of isolated bioactive compounds as direct drugs, structural templates for semisynthesis, pharmacologic tools and/or using the crude plant as a herbal remedy.[14, 28] The number of unexplored higher plant species, angiosperms and gymnosperms, in the world is estimated between 215,000 to 500,000.[2, 29] Of these, only 6% have been screened for their biological activity and < 20% have been subjected to laboratory investigation for potential therapeutic use.[13, 30] Sneader (2005) suggested that < 3,200 (1%) plant species are used in TM, highlighting that there are many unexplored areas in drug discovery from plant species.[2] Interestingly, Farnsworth *et al.* (1985) identified that of the 122 active compounds found in 94 different ethnomedicinal plant species, 80% of the active compounds have been used to treat identical or related indications to their ethnomedicinal use. Consequently, attesting extracts' traditional application could provide direction and selection of more specific assays for the plant species.[28]

1.2.1 Approaches to Natural Product Drug Discovery

Many different approaches are available to identify biologically active phytochemicals. Random collection and screening of phytochemicals is perhaps one of the most economical and popular approaches to isolate phytochemicals such as flavonoids, alkaloids, isothiocyanates, triterpenes etc.[1, 7, 12, 27] *In vivo* and *in vitro* bioassays could be employed to determine the activity of crude plant extracts prior to committing to extensive extraction, isolation and characterisation procedures for active fractions.[1, 31] Alternatively, guidance from archived literature documenting traditional medicinal systems could assist in identification of plants (extracts) with interesting bioactivity followed by isolation and characterisation of compounds present to enable investigation of their potential application in disease.[32] Collection and organisation of ethnomedicinal information should facilitate the identification of possible positive hits.[14]

Between the period of 1970 and 1990, the emergence of high-throughput screening (HTS) and generation of chemical compound libraries *via* combinatorial chemistry enabled identification of defined molecular targets and NCEs, respectively.[33-36] These technologies have the potential to produce and screen numerous drug candidates where typically a small team of researchers can screen over a million of samples annually. This is appealing compared to natural product drug discovery and development (NPDD), which often demand high expenditures, long discovery timelines, high possibility of re-discovery and sourcing of natural products as well as the fierce competition between big pharmaceutical companies to deliver first-in-class drugs with well-defined mechanism of actions.[35, 37] HTS enabled vast numbers of plant species to be validated, and companies with such facilities have the ability to screen thousands of plant extracts per week. Hence Lipinski's 'rule-of-five' (RO5) criteria were established to rationalise and respond to numerous chemical compound libraries and to assess the relationship between a compound's chemical structure and biological properties. Lipinski's RO5 predicts and selects for orally active compounds by fulfilling the criteria shown in Table 1-1.[38]

Table 1-1: Lipinski's Rule of Five (RO5) criteria for predicting orally active compounds.[38]

Lipinski's Rules	**Criterion**
Molecular weight (MWT)	≤ 500
Log *P*	≤ 5
Hydrogen bond donors	≤ 5
Hydrogen bond acceptors	≤ 10

In 2004, Lipinski suggested other factors for example the number of aromatic rings, number of rotational bonds and topological polar surface area (TPSA) should be included. Collectively, physicochemical features affecting drug absorption, distribution, metabolism, excretion (ADME) and interaction with target receptor will impact the quality of lead-like compounds used in screening libraries such as small molecule fragment and chemical libraries.[38, 39] However, applying the RO5 on a single combinational chemical core will

restrict the structural diversity of combinatorial libraries and satisfying the RO5 does not guarantee a drug-like compound.[38] Zhang *et al.* (2007) reported that just over 50% of the 1,204 US Food and Drug Administration (FDA)-approved orally administered small-molecule drugs obey the RO5, therefore the RO5 should be followed with caution and its compatibility in rational drug design and development should be re-evaluated. It has already been stated that natural products do not comply with Lipinski's RO5.[26] Kumar and Soni (2010) revealed statistical results from their data collection suggesting medicinally useful natural products tend to contain physicochemical features violating the Lipinski's RO5 criteria (Table 1-2).

Table 1-2: Physicochemical properties of medicinally useful natural products.[34]

Lipinski's Rules	**Criterion**
Molecular weight (MWT)	$201 \leq \text{MWT} \leq 600$
Log *P*	$-2 \leq \log P \leq 5$
Hydrogen bond donors (HBD)	$0 \leq \text{HBD} \leq 5$
Hydrogen bond acceptors (HBA)	$2 \leq \text{HBA} \leq 16$
Nitrogen atoms	$0 \leq$ nitrogen atoms ≤ 6
Oxygen atoms	$2 \leq$ oxygen atoms ≤ 7
Topological polar surface area (TPSA)	$26 \leq \text{TPSA} \leq 225$
Aromatic rings	$0 \leq$ aromatic rings ≤ 2
Rotatable bonds	$0 \leq$ rotatable bonds ≤ 12

Lee and Schneider (2000) have cross-analysed 1,748 and 807 different ring systems from natural products (in the BioscreenNP collection) and from trade drugs (in the Derwent World Drug Index (WDI)), respectively. Interestingly, results emphasised the diversity of natural product structures revealing that approximately 35% of trade drugs contained a ring system also present in the natural product collection whereas only 17% of natural products share the same ring system present in trade drugs.[13] Natural products have significantly higher numbers of chiral centres and greater steric complexity.[40] Therefore, information gained from the structure of natural products will introduce

diversity and increase opportunities for strong drug candidates not necessarily complying with the RO5, hence encouraging new drugs with novel mechanisms of action.[41, 42] This revived interest could be due to the low success rate of chemical libraries over the last two decades, with only one *de novo* compound approved by the FDA in 2005. Sorafenib (BAY43-9006; Nexavar®) is an oral multiple kinase inhibitor for the treatment of advanced renal cell carcinoma from Bayer Pharmaceuticals. Despite the short discovery timeline of 11 years, more systematic approaches should be employed to maintain the progression of drug discovery.[43] This could be achieved by changing from large libraries to using smaller focused libraries (termed Diversity Oriented Synthesis (DOS)) with approximately 100 to 3,000 compounds, and most of which are structurally related to natural products.[44] Additionally, natural product skeletons could act as templates in combinatorial chemistry to generate compound libraries of novel natural product scaffolds for screening.[13, 35] The extended history of phytochemicals used in medicine and evidence of their structural complexity could be deemed unsurprising since the co-evolution between plants and animals has resulted in compatibility between the building blocks present in both species (Figure 1-13).[33] Natural products have structural homogeneity across many plant species hence sharing comparable structural domains to biological targets.[5, 20] Therefore, phytochemicals may fit better into the 'chemical space' of target proteins e.g. interaction with targets in human signal transduction pathways.[16]

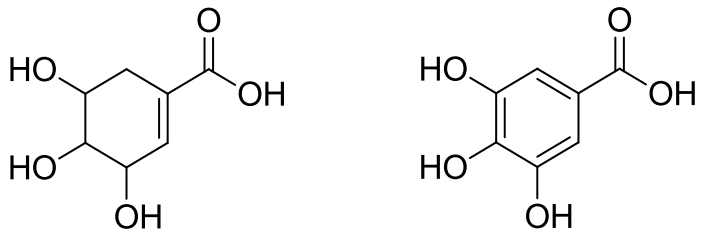
1.2.2 Purposes and classes of phytochemicals

Plants produce phytochemicals to serve different purposes including self-protection from destructive toxins and predators e.g. carcinogens or mutagens. Several tree species produce tannins as their defense mechanism in response to damaged leaves caused by predators or for communication with neighbouring plants to increase their survival chances.[2, 45, 46] As previously mentioned, exploring phytochemicals can offer advantages such as uncovering new drug leads or additional sources for currently available compounds.[11] Nonetheless, undesired side effects are also associated with phytochemicals because their purpose is to enhance survival and competitiveness of plants by defending

against possible threats and not for treating human diseases.[2, 20] Many natural products possess structures that are beyond the imaginative designs of scientists and often contain multiple stereocentres, '*chiral centres, aromatic rings, complex ring systems, degree of molecule saturation, and number and ratio of heteroatoms'*.[11] Phytochemicals can be grouped in different ways such as by their chemical structures, physiological effects, or by their biosynthesis mechanisms.[3]

1.2.2.1 Shikimic acid derivatives

Shikimic acid (4) is a common precursor of aromatic amino acids (phenylalanine, tryptophan and tyrosine) as well as other proteins and peptides, alkaloids, phenols, coumarins, chromones, xanthones, stilbenes, flavonoids, lignans and gallic acid.[47] Gallic acid is the precursor for many types of tannin, which is abundant in all plants, and forms insoluble compounds with proteins. Tannins can be obtained from galls produced on various plants and used to treat diarrhoea and bleeding gums.



4

Figure 1-4: The common chemical structures of Shikimic acid (4).

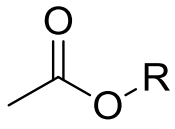
1.2.2.2 Carbohydrates

Carbohydrates, $C_n(H_2O)_n$, are the main constituents of living organisms and are present in abundance on cell surfaces with diverse fundamental functions including molecular recognition and signal transduction.[48] In plants, carbohydrates are one type of photosynthetic products, whose functions include providing structural support (e.g. cellulose), and energy storage (e.g. starch). Carbohydrates can be grouped into monosaccharides (3 to 9 carbons), disaccharides, oligosaccharides (2 to 10 monosaccharide molecules) and

polysaccharides (over 10 monosaccharide molecules). One example of medicinal use of mucilage (polysaccharide) from Linseed, (flax; *Linum usitatissimum* L. (*Linaeceae*)), is use as a laxative.[47]

1.2.2.3 Acetate derivatives

Acetate (5) is a common precursor for fatty acids (saturated and unsaturated triglycerides), fats and waxes, phospholipids, polyketides, monoterpenes, sesquiterpenes, diterpenes, triterpenes and steroids. Synthetic steroids are used for cancer treatments and to reduce adverse reactions to chemotherapy. Low doses of glucocorticoids can treat adverse reactions such as chemotherapy-induced emesis and dyspnoea in cancer patients *via* interaction with intracellular glucocorticoid (steroid) receptors (GR) to trigger signaling pathways and down-regulation of GR.[49]



5

Figure 1-5: The chemical structure of the acetate group (5).

1.2.2.4 Alkaloids

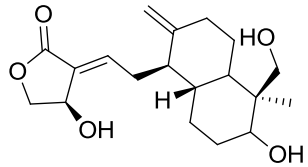
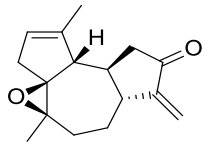
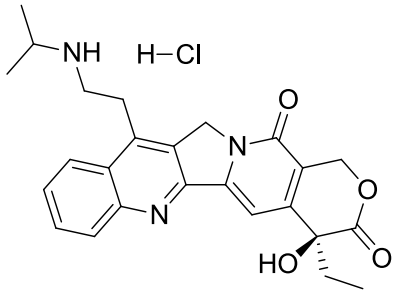
Alkaloids are derived from amino acids and there are over 3,000 known alkaloids such as cocaine, vincristine and vinblastine. Alkaloids are naturally occurring organic compounds that have nitrogen as part of a heterocyclic ring system. Only present in some plant families, the types of alkaloids range from pyridine and piperidine, tropane, isoquinoline, indole, quinolone and imidazole. Common alkaloids include caffeine and nicotine.

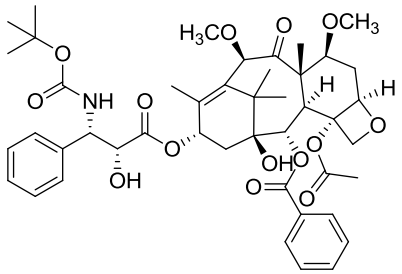
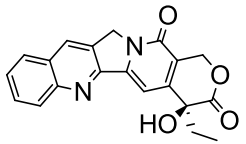
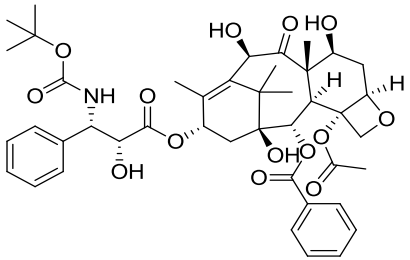
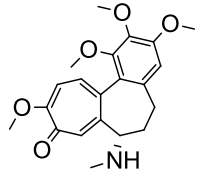
1.2.3 Common procedures in Natural Products Drug Discovery

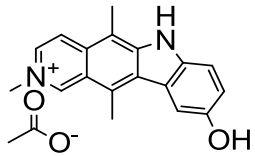
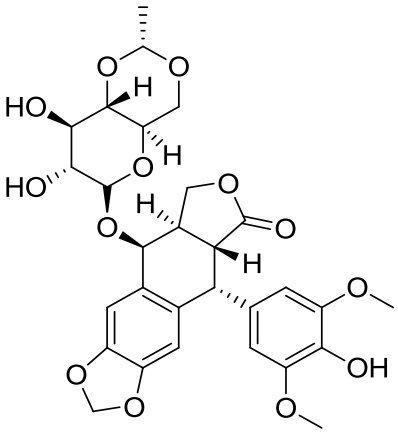
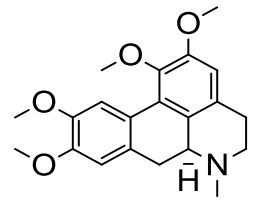
Application of modern technologies such as analytical, structural chemistry and various '-omics' techniques has enabled thorough studies to be conducted into compounds' SARs and their biological effects. Collecting and verifying such information has resulted in comprehensive databases such as the *Dictionary of Natural Products* (DNP), which has > 214,000 entries of natural compounds and their derivatives.[50] Rational design of chemical compounds could offer a secondary role for natural products in drug discovery and development by providing a template for combinatorial chemistry and molecular biology. Only by addressing the challenges would the enormous potential to discover novel compounds from natural sources be unlocked.[2] Bruhn and Bohlin (1997) mentioned four possible relationships of a bioactive compound: known activity and known structure, known activity and new structure, new activity and known structure or new activity and new structure; the last being of greatest interest.[19]

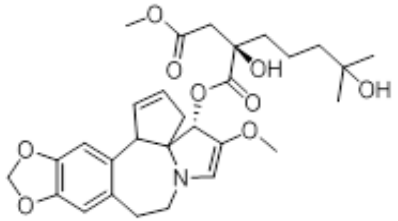
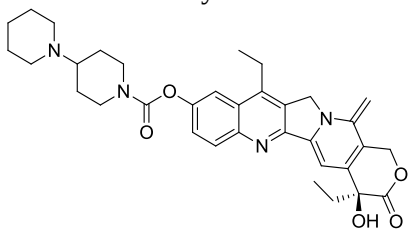
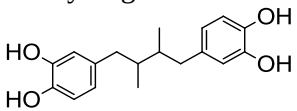
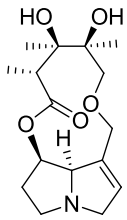
There are thousands of reports of antiviral, antibacterial and antifungal drugs derived from plants that are used to treat various indications. Newman and Cragg (2012) published a comprehensive list of NCEs with accompanying plants source and treatment indication(s).[51] Interestingly, several plants containing anticancer compounds were also used traditionally as anticancer agents. Diseases such as cancer where resistance is common will require continuous efforts to find novel compounds. Table 1-3 illustrates a compilation of the anticancer agents that are either directly from nature or natural products-derived in clinical use between the periods of 1981 to 2010.

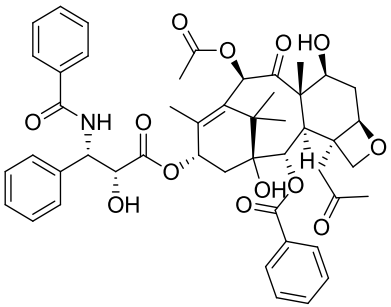
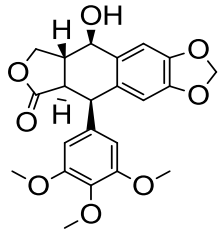
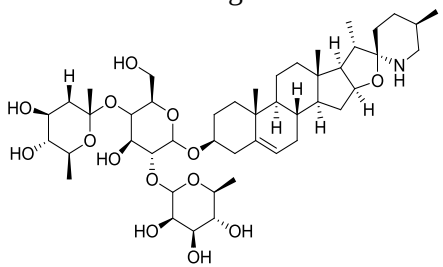
Table 1-3: A list of drugs derived from plants with their corresponding uses and source discovered between the years 1981 and 2010.

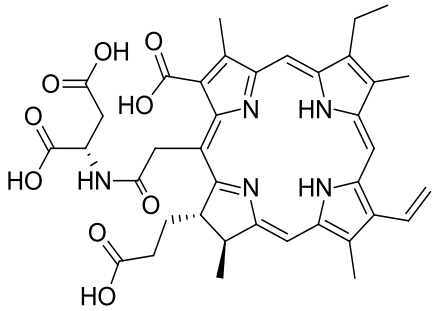
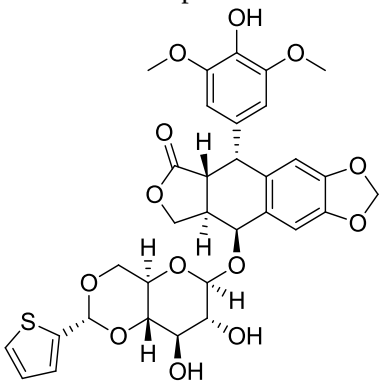
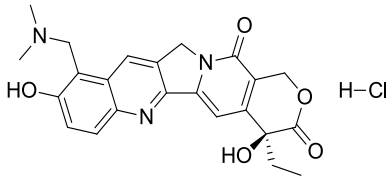
Drug	Plant Source of Natural Product-derivatives	Phytochemical compound class	Main Target(s)	Ethno-medical correlation/ Reference
Andrographolide 	*Andrographis paniculata*	Diterpenoid	Caspase-3 and -8, p16, p21, p27	Yes [52]
Arglabin 	*Artemisia glabella*	Sesquiterpene	Raf kinase cascade	No [53]
Belotecan hydrochloride 	*Camptotheca acuminate* Decne	Alkaloid	Topoisomerase I	No [54]

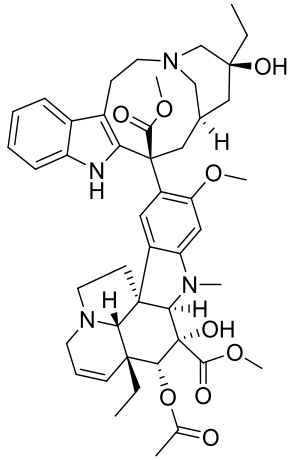
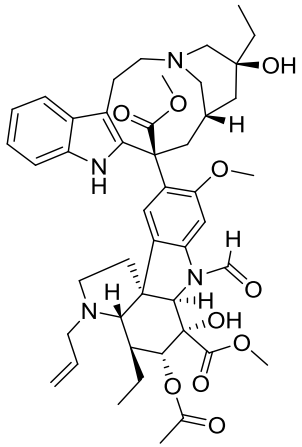
Cabazitaxel 	Taxol derivative	Diterpene	Tubulin α-1 and β-1 chains	No [55]
Camptothecin 	*Camptotheca acuminate* Decne	Alkaloid	DNA Topoisomerase I	No [56, 57]
Docetaxel 	Taxol derivative	Diterpene	Tubulin β-1 chain, VEGF	No [58]
Demecolcine 	*Colchicum autumnale* L.	Alkaloid	30S ribosomal protein S9 and 30S ribosomal protein S4	Yes [59]

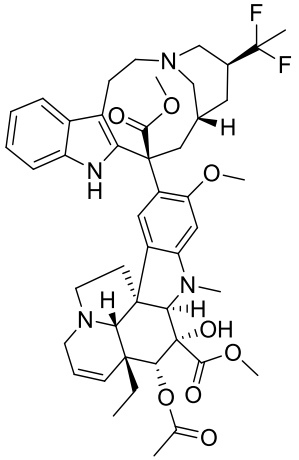
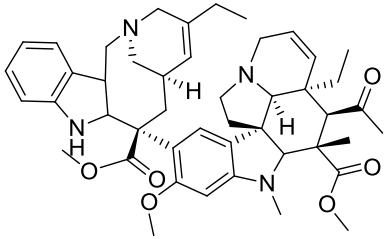
Elliptinium acetate 	*Bleekeria vitensis*	Alkaloid	DNA Topoisomerase II-α	No [60]
Etoposide Etoposide phosphate[c] 	Podophyllotoxin derivative	Alkaloid	DNA Topoisomerase II-α	Yes [61]
Glaucine 	*Glaucium flavum* Crantz	Alkaloid	DNA	No [62, 63]

Homoharringtonine Omacetaxine mepesuccinate 	*Cephalotaxus harringtonia* var. *drupacea*	Alkaloid	80S ribosome (eukaryotes)	No [64]
Irinotecan (prodrug) Irinotecan hydrochloride 	Camptothecin derivative	Alkaloid	DNA Topoisomerase I	No [65, 66]
Masoprocol Nordihydroguaiarectic acid 	*Larrea divaricata* Cav.	Phenolic	Arachidonate 5-lipoxygenase	No [67, 68]
Monocrotaline 	*Crotolaria sessiliflora* L	Alkaloid	Microtubules	Yes [69]

Paclitaxel Paclitaxel nanoparticles 	*Taxus brevifolia* Nutt Taxol derivative	Diterpene	Tubulin β-1 chain and Bcl-2	No [70, 71]
Podophyllotoxin 	*Podophyllum peltatum* L.	Alkaloid	Structure used for derivatives	Yes [72, 73]
Solamargines 	*Solanum incanum*	Alkaloid	TNFR and Bcl-2	No [74]

Talaporfin sodium 	Chlorophyll	Aspartyl chlorin	Tumour tissues	No [75, 76]
Teniposide 	Podophyllotoxin derivative	Alkaloid	DNA Topoisomerase II-α	Yes [77]
Topotecan hydrochloride 	Camptothecin derivative	Alkaloid	DNA Topoisomerase I	No [78]

Vinblastine 	*Cantharanthus roseus* (L.) G. Don	Alkaloid	Tubulin -α-3, -β, -δ, -ϒ and -ε chains and Transcription factor AP-1	No [79]
Vincristine 	*Cantharanthus roseus* (L.) G. Don	Alkaloid	Tubulin α-1 and β chains	No [80]

Vinflunine 	Vinca alkaloids derivative	Alkaloid	Tubulin	No [81]
Vinorelbine (di-)tartrate 	Vinca alkaloids derivative	Alkaloid	Tubulin-β chain	No [82]

The listed anticancer compounds are either isolated directly from plants or are a plant derivative from the period of 1981 to 2010. The ethnomedical correlation is noted for each plant and its active compound(s). Information regarding the mechanism of action of each drug was obtained from DrugBank, the NCI and/or the given references.[78, 83, 84]

1.2.4 Selection of plant materials

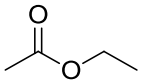
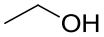
The selection of plants (or specific plant sections) for investigation could begin with searching random collections, literature and/or ethnopharmacology systems.[18, 85] The accuracy of documentation and authentication of the plant specimen should be verified by experts prior to standardised methodical processes. Specimen selection based on ethnopharmacological evidence should acknowledge the intellectual property (IP) of the traditional healers. A record of collection including harvest time (day or evening), season (month, weather and soil condition), age of the plant and location (specific area) should be kept because inconsistency will lead to differences in phytochemical compositions between samples. Furthermore, the endemic status of the plant and deforestation status are factors affecting the collector's judgment to investigating certain plants for their therapeutic properties.[85] The order of procedures from selection to identification of active plant extracts using bio-guided fractionation and subsequent structure elucidation of the active compound(s) has been extensively described.[85-87] The following procedures in this chapter were adapted from Houghton and Raman (1998), Samuelsson (1999) and Arnason *et al.* (1995).[18, 47, 87]

1.2.5 Preparation of crude extracts

In general, the starting plant material must be reduced in size through processes such as grinding and pulverisation prior to extraction. There are three types of extracts: dry i.e. prepared without solvent (*extracta sicca*), soft (*extracta spissa*) and fluid (*extracta fluida*) which are prepared with solvents of varying polarity (Table 1-4).[3] The choice of solvent determines which type of phytochemicals are eluted from the crude extract and the polarity provides indication of the electron distribution across the molecule such as π electrons (aromatic rings, carbon-carbon double bonds and carbonyl groups) and lone-pair electrons (electronegative atoms).[18] Other pressures on solvent selection include boiling temperature, reactivity, viscosity, vapour pressure, safety, cost and recovery. Plant extracts should be checked for contamination e.g. microorganisms, prior to preservation of samples. The samples could be dried

either by leaving in a warm, dry atmosphere or by freeze-drying then stored in the dark and at a sub-zero temperature.

Table 1-4: Polarity of solvent molecules used for UNMC extraction process.

Solvent	Structure	Polarity	Chemical class extracted
Hexane		Non-polar	waxes, fats and fixed oils (volatile oils)
Ethylacetate		Moderate polarity; electrons (π and lone-pair) on oxygen	alkaloids, aglycones, glycosides
Ethanol		Polar	glycosides
Water		Polar, no lipophilic non-polar areas	sugars, amino acids, glycosides

1.2.6 Extraction methods

The selection of a suitable extraction method is essential as this will impact on (active) phytochemicals present in the crude plant mixture for validation in bioassays. Many extraction methods are available to extract chemicals from plant extracts (see Table 1-5). Description of general extraction procedures are adapted from Houghton and Raman 1998, Gunnar and Samuelsson 1999 and Arnason *et al.*1995 and will be briefly described.[3, 18, 87]

Table 1-5: Common methods employed for extracting chemicals from crude plant extracts.

Extraction method	Procedure
Maceration	Coarsely powdered extract is soaked in a solvent contained in a sealed container at room temperature for at least three days. Material should be stirred until soluble matter has dissolved before straining; it is then pressed and filtered with liquid.
Infusion	Like maceration, plant materials undergo intermittent exposure to cold and boiling water and soluble matter should be contained in the solution.
Digestion	Like maceration, plant material is subjected to gentle heating during the whole extraction process.
Decoction	The material is boiled in a specified volume of menstruum for a set period at a fixed ratio. The macerated material is then cooled and strained.
Percolation	The material is placed in a sealed container with a specified volume of menstruum and incubated for 4 h; then packed into a percolator with added menstruum to macerate overnight. The liquid is allowed to drip slowly.
Soxhlet	The material is placed in folded filter paper then in apparatus where heat is applied to small volume of menstruum so the vapours condense and the condensed extractant drips into another chamber for collection.
Fermentation	The sample is soaked for a specified time to enable fermentation, hence alcohol is formed.
Counter-current	The material is processed into a slurry and fine sample prior to being placed with a solvent in a cylindrical extractor and moved in a unidirectional manner.
Sonication	The ultrasound frequency used ranges from 20 kHz to 2000 kHz to extract chemicals from material following disruption of permealising cell walls.
Supercritical Fluid	The use of CO_2 or argon as the extracting fluid means a low temperature can be used as well as recycling the solvents.
Phytonics Process	A technology using fluorocarbon (1,1,2,2-tetrafluoroethane) solvent for the extraction of plant materials.

Description of extraction processes are adapted from Handa 2008.[85] The term material refers to the crude plant material and menstrum refers to the extraction solvent. Some of the techniques are used in the Ayurveda system.

A series of other procedures will follow extraction such as filtering the extract obtained from the residues following extraction (marc) and spray drying to obtain a dry powder.[85] Extracts should be stored correctly at low temperatures (< -20°C) and away from light to minimise deterioration, decomposition and formation of artefacts to allow identification of constituent compound(s).

1.2.7 Activity-guided fractionation

Extract fractions should be tested in the appropriate *in vitro* systems so any extract fractions that are more active than the crude extract can be further separated and re-tested until the active substance(s) is isolated and identified. With respect to anticancer drug discovery, many cell-based assays intend to measure cancer cells' viability after exposure to an investigational compound since cancer cells are commonly associated with dysregulated cell proliferation and death; results could be indicative of an effective anticancer candidate.[88] Alternatively, mechanism-of-action-based bioassays can be used where the agent is directed to a molecular target of interest e.g. proteasome inhibition assay.[11, 86, 89]

It is of interest to determine the characteristics of any observed cytotoxic effects induced by the extracts such as those consistent with the hallmarks of cell death e.g. the loss of plasma membrane integrity, fragmentation of cells into apoptotic bodies or the fragments taken up by neighbouring cells. Several assays can indirectly test for induced cytotoxicity by measuring biochemical processes of viability markers since a reduction in metabolism is expected in dying cells.[88] A common approach to assessing *in vitro* cell viability is to measure its ability to inhibit vital dyes from crossing the intact plasma membrane. Vital dyes are used in a variety of techniques such as those that only label dead cells (e.g. trypan blue, propidium iodide and 4',6-diamidino-2-phenylindole (DAPI)) by entering the cell *via* a compromised plasma membrane. These dyes are widely adopted in flow cytometric techniques. In contrast, fluorogenic esterase substrates (e.g. calcein acetoxymethylester (calcein-AM)) are taken up by viable cells and hydrolysed by intracellular esterases to its active fluorescent form to be captured by microscopy.[90, 91] Alternatively, the loss of plasma membrane integrity resulting in the release of

intracellular protein such as glucose-6-phosphate dehydrogenase (G6PD) and lactate dehydrogenase (LDH) or intracellular ATP concentrations could be measured using conventional assay kits to assess cell viability. Furthermore, several colorimetric assays such as sulforhodamine B (SRB) and tetrazolium salts are dependent on the binding between the cationic aminoxanthine (pink dye) to basic amino acids in the cells or viable cells reducing tetrazolium salts to formazan compounds, respectively, and the absorbance intensity measured is proportionate to the cell numbers (indicating cell growth, viability or death).[92-94] Since the abovementioned cell death detection methods could be subjected to interference from other intracellular processes, it is important that the results are validated in a subsequent assay, which measure *bona fide* cell death markers such as phosphatidylserine or (cleaved) poly(ADP-ribose) polymerase (PARP) protein.[94]

Assays that are simple, effective and economical should be considered for initial screening. The choice of cytotoxicity assay is also of importance to avoid false negatives since active compounds may be present in trace amounts or their activity may be masked by other toxins in the crude extract.[2, 95] Since 1990, the NCI has been screening for potential active compounds using *in vitro* screening methods comprising a panel of 60 different human tumour cell-lines from nine organ sites, (including leukemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney) and a simple cell viability/cytotoxicity assay (e.g. SRB and tetrazolium salt assay).[96, 97] Since 1990, this programme has allowed the screening of > 80,000 compounds.[93, 98]

1.2.8 Isolation, structure elucidation and de-replication of active compounds

Analysis of active compounds can be assisted by techniques such as solid phase chromatography, nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC or LC) linked to ultraviolet (UV) absorption detector and mass spectroscopy (MS).[5] These well-established technologies can be 'linked' together, otherwise known as 'hyphenated' methods, for facilitating the separation, isolation and identification of phytochemicals in

mixtures. The combined, hence hyphenated, use of HPLC, NMR, gas chromatography (GC) and MS systems (e.g. LC-NMR, LC-MS, LC-NMR-MS) are becoming increasingly common in facilitating the drug discovery process. These systems provide information on the molecular mass, carbon skeleton and proton distribution and characteristics (e.g. polarity) of compounds, which enable accurate determination of compound structures.[37] Furthermore, hyphenated techniques minimise wasted efforts by supporting the process of de-replication (e.g. avoiding re-discovery) *via* reconciliation with established databases of already known compounds thus prioritising unknown or novel compounds for further testing.[2, 11, 35] A thorough investigation concluded that approximately 80% of several global database entries regarding plant information, genome, phytochemical composition, taxonomy, ethnomedicinal uses and authentication information are inaccurate.[99] For instance, approximately 75% of the GenBank plant records are incorrect.[99, 100] At present, lack of reliable genomic data for the metabolomes of medicinal plants proves unaccommodating to creating authenticated libraries of 'metabolite fingerprints' of plants.[100] Metabolomic analysis is a very useful tool to avoid de-replication, misidentification and SAR determination of metabolomes from medicinal plants, the latter can improve estimation of the efficacy and toxicity of investigational compounds before commercial development.[101] Employment opportunities are promising if medicinal plants are to be grown on a larger scale for pharmaceutical use and may persuade growers of 'illegal plants' to cultivate these profitable crops instead.[20] Nevertheless, the issue of inaccurate and insufficient plant records is acknowledged and considerable efforts are being invested into creating accessible international databanks of information such as The Plant List for validated entries of information.[102]

1.2.9 Synergism in phytomedicine

Crude plant extracts contain a mixture of secondary metabolites, which are biologically active. Whole plant extracts represent some of the most therapeutic drugs in the market and may rely on the synergistic effect of these bioactive compounds including their by-products.[16, 46, 103] Synergy is an overall greater effect caused by using a combination of drugs than the total effect of using a single compound.[104, 105] Williamson (2001) presented several examples whereby volunteers given ginseng and gingko together have improved cognitive function compared to those who were only given one of the extracts.[105, 106] There are two broad types of synergy described by Spinella (2002), which are pharmacodynamic or pharmacokinetic.[109]

Pharmacodynamic synergy occurs when two compounds are targeting a similar receptor target or physiological system. Pharmacokinetic refers to absorption, distribution, biotransformation, or elimination of the drug e.g. some polyphenols can facilitate absorption of agents.[16, 103, 107] Interaction between phytochemicals can also evoke antagonism, where the overall effect is less than that of a single compound or potentiation whereby an inactive compound can further increase the activity of an active compound. Another possible outcome is 'masking' where two compounds with opposing effects cancel each other out therefore, resulting in no net effect.[16] Berenbaum's isobol method is used to "*differentiate between real synergy and additional effects of a mixture of two plant constituents or extracts*".[16, 104] Recently, a multidrug approach is preferred over monodrug therapy in conventional medicine. This could be due to activation or inhibition of multiple targets in signaling pathways giving rise to synergistic effects.[103, 104, 108] Hence, perturbation of multiple modes of action could increase therapeutic efficacy allowing use of lower dosage.[109] Also using a lower dose of multiple agents acting *via* distinct mechanisms of action could reduce toxicities as well as minimise emergence of acquired drug resistance. However, this approach (common in standard cytotoxic chemotherapy use) raises concerns in natural product medicinal use as it is difficult to rationalise and standardise treatment without identifying the mechanisms that generate the synergistic effects.[103, 105]

1.3 Cancer

1.3.1 Cancer: a global problem

Cancer is a group of fatal diseases with more than 200 cancer types and each are quite distinct to each other (e.g. secretion, expression and or mutation of different proteins), of which the most common are breast, colorectal, lung, prostate and stomach cancers, however the highest incidence of mortality rates occurs within lung, breast, stomach, liver and colorectal cancers.[110-114] In 2011, WHO published a Global Status Report, which revealed cancer accounts for approximately 7.6 million (13%) world deaths in 2008.[115, 116] There are approximately 6 million new cancer incidences worldwide per year, those figures are predicted to increase to 27 million new cancer cases and 17.5 million cancer deaths by 2050.[117] Around 325,000 people are diagnosed with cancer annually in the United Kingdom (UK) and it is the second leading cause of death in the U.S.[118, 119]

1.3.2 Molecular biology of cancer and possible targets

The process of carcinogenesis is a multistep process requiring initiation, promotion and progression and metastasis. Cells undergoing unregulated growth within a specific tissue of any organ could, over time, usually decades, accumulate further mutations. As a result, acquiring the ability to invade surrounding areas and metastasise to distant sites by hijacking vessels formed by tumour angiogenesis.[120-122] This process presents various opportunities to intervene at the different stages.[112, 123] Cancers arising from epithelial cells (carcinoma) are the most common (85%) compared to those from mesoderm layer (sarcoma) or glandular tissue (adenocarcinomas).[112] During treatment tumour cells may progress into a more aggressive phenotype and become resistant to conventional anticancer drugs, therefore anticancer drugs with original modes of action e.g. that molecular targeted, are desired and so constant efforts to discover novel agents are needed. The biology of cancer is very complex and its surrounding microenvironment is equally as important in tumourigenesis. Different types of cancers acquire different genetic mutations

and phenotypes, however there are several essential capabilities shared by most, if not all, cancers enabling neoplastic cells to progress in multistep carcinogenesis.[124] The hallmarks of cancer described by Hanahan and Weinberg (2011 and 2000), unveil promising pathways as targets for therapeutic anti-cancer intervention.[122, 124] These hallmarks include self-sufficiency in growth signals, insensitivity to anti-growth signals, apoptosis evasion, sustained angiogenesis, tissue invasion and metastasis, limitless replicative potential, evasion of immune destruction and reprogramming of energy metabolism (Figure 1-6).[124]

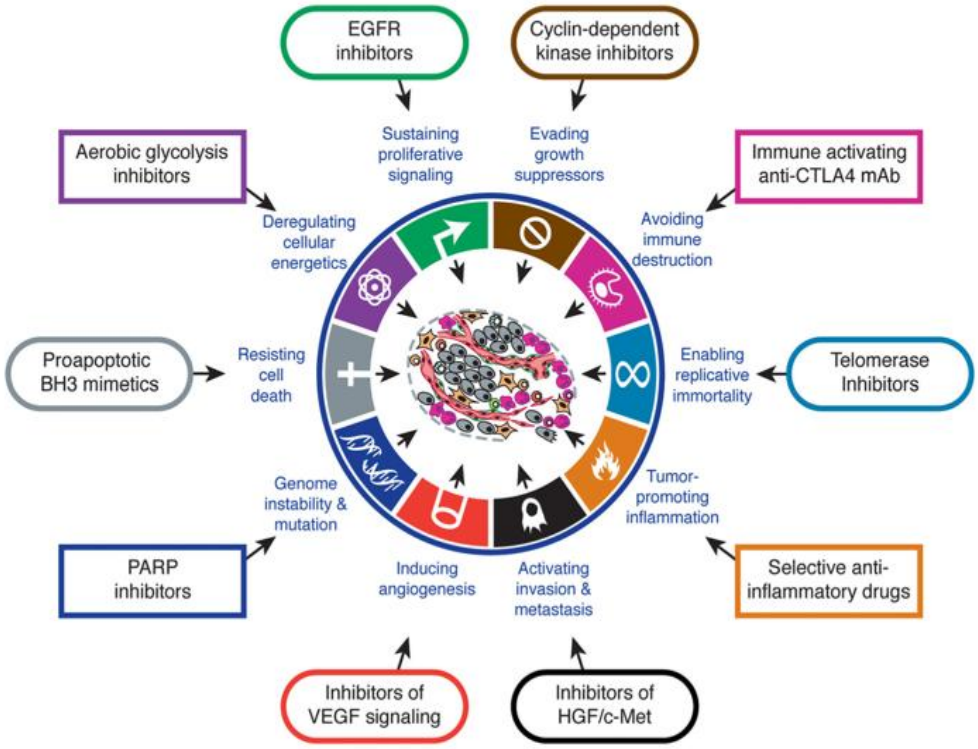


Figure 1-6: Depiction of Hanahan and Weinberg's hallmarks of cancer illustration. The diagram illustrates the cancer hallmarks as representative of drug targets and the examples of compounds that interfere with the targets' functions. Many drugs under development are found to be both intentionally and unintentionally directed to interfere with these hallmarks.[124]

Self-sufficiency in growth signals: Transformed cells, unlike normal cells, are self-sufficient in mitogenic growth signals, which sustain continuous cell proliferation. Cancer cells may produce growth factor ligands, which act autonomously on their own receptors or by increasing the level of protein

receptors on the cell membrane enabling capture of minute/normal levels of growth factors, translating into an exaggerated response. An example is the mutation of the ras gene, which is present in 30% of all human cancers. The Ras proteins are membrane-associated guanine nucleotide-binding proteins, and its activation requires the transient binding of an extrinsic ligand e.g. growth factors, resulting in cell proliferation. However, mutated Ras is permanently in its activated conformation and is independent of GTP binding resulting in constitutive activation of downstream signaling cascade (Ras-Raf-MEK-ERK) and cell proliferation.[125]

Insensitivity to anti-growth signals: Transformed cells are unresponsive to antigrowth signals e.g. soluble growth inhibitors and immobilised inhibitors, that are found on the surface of neighbouring cells hence disregarding such anti-growth signals will maintain the transformed cells in a permanently active state.[122] An example is the loss of the retinoblastoma protein (pRb) function where pRb is a mediator of anti-proliferative signals. In a hypophosphorylated state, pRB is bound to E2F (transcription factor) thus altering its function and inhibiting cell cycle progression from G_1 phase. Mutations in the pRB gene caused by the human papillomavirus (HPV) E7 oncoprotein liberate E2F allowing transcription hence cell cycle progression through the restriction 'R' point into S phase.[126]

Evading apoptosis: Homeostasis is maintained by a balanced level of cell proliferation and cell attrition (e.g. apoptosis). Cancer cells acquire various mechanisms, which overcome cell death such as through the loss of tumour suppressor proteins (TSPs) and overexpression of survival proteins. For instance, the wild-type p53 protein is a DNA damage sensor and a component of the apoptotic signaling pathway. Loss of p53 protein (a consequence of point mutation in p53 gene) is found to occur in over 50% of human cancers and 75-80% of colon carcinomas are without both p53 alleles.[127] One of the roles of p53 act as a regulator of cell cycle by making necessary cell cycle arrests at G_1/S phase and binding to any damaged DNA, repair can be initiated before commitment to the entire cycle upon passing the R point. If the damage is irreparable then p53 can initiate apoptosis.[128] Over-expressions of certain

anti-apoptotic Bcl-2 family members e.g. Bcl-2, Bcl-X_L and Mcl-1, are essential in inhibiting apoptosis, hence supporting deregulated oncogene expressions.

Sustained angiogenesis: Cancer cells can initiate angiogenesis and generate blood vessels from the existing capillary network; this 'angiogenic switch' is vital for tumour growth and metastasis. The tumour can modulate its own supply of angiogenic factors, classed into activators (e.g. vascular endothelial growth factor (VEGF) and angiogenin) and inhibitors (e.g. thrombospondin and angiostatin); production levels are increased and decreased by the tumour cells, respectively.[129] However, the tumour vasculature formed under the influence of VEGF is of poor quality and highly disorganised leading to hypoxia and additional VEGF production hence leading to a more aggressive phenotype.[130]

Tissue invasion and metastasis: Transformed epithelial cells can invade local tissues and metastasise via various processes such as the 'epithelial-mesenchymal transition' (EMT) process. Although less than 0.01% of metastatic cells are successful in colonisation at the distant site, metastases confer a highly unfavourable patient prognosis, and are responsible for >90% of cancer deaths.[131] A tumour cell can acquire the ability to remodel the host tissue extracellular matrix (ECM) and secrete proteases such as matrix metalloproteinases (MMPs). Increased MMPs levels e.g. MMP-2, MMP-9 and MMP-13, in cancer cells can facilitate the detachment of tumour cells from the primary site, cell migration towards the lymph and/blood vessels, degradation of the vascular basement membrane and penetration of the vascular lumen to invade a distant site.[132]

Limitless replicative potential (immortalisation): Normal cells are constricted to undergo a finite number of replications (Hayflick number) and enter senescence due to the degradation of the protective cap of DNA, telomeres (a repeating DNA sequence TTAGGG), found at the end of chromosome arms. Eventually, the shortened chromosome ends will be exposed resulting in chromosomal fusion and apoptosis.[133] Approximately 85-90% of cancerous cells become immortalised by upregulating telomerases

(DNA polymerase) to replenish telomeres on the chromosomes, hence cells become capable of unlimited cycles of replications.[122]

Evading immune destruction: The immune system offers several mechanisms to prevent or detect and eliminate tumour cells by infiltrating tumour masses e.g. eliminating virus-induced tumours, which account for 20% of tumours. Secondly, during inflammation many growth factors, survival factors, pro-angiogenic factors and enzymes facilitating EMT and ROS are released into the tumour microenvironment contributing to tumourigenesis and progression.[124] Also, immune immunoediting, which is based on the concept of detection and elimination of tumour cells expressing tumour-specific antigens or stress-induced antigens, consists of three phases: designated elimination, equilibrium and escape.[134] Firstly the immune system will eliminate the detected tumour cells, however elimination may only be partial therefore the remaining tumour cells may exist in an equilibrium state. However, the immune system will continue to eliminate any tumour clones that continue to grow and accumulate further alterations translating to antigen expression. Any tumour clones that escape elimination are more resistant and surpass immune surveillance.[134]

Reprogramming energy metabolism: The rate of cancer cell metabolism should support the rate of rapid neoplastic cell proliferation, resulting in further accumulation of genetic alterations and instability, which may translate to additional cancerous traits. A subpopulation of cancer cells can reprogramme 'switch' their metabolism to glycolysis even in the presence of oxygen, and it has been shown to be associated with activated oncogenes Ras, Myc, P13K (phosphatidylinositol-3-kinase) and mutated TSPs, p53, PTEN (Phosphatase and tensin homolog) and NF-1 (neurofibromatosis type I).[135, 136] Adoption of glycolysis may present several advantages for cancer cells such as providing macromolecules and organelles for new cells formation as a result of the upregulation of glucose transporters and enzymes of the glycolytic pathway as well as providing various glycolytic intermediates for various biosynthetic pathways.[124, 137]

1.3.3 The role of medicinal plants in treating cancer

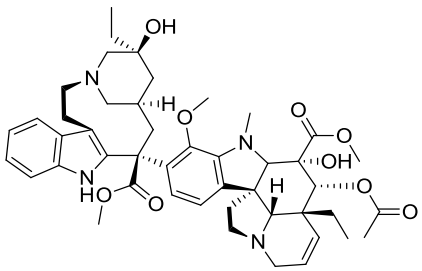
A series of publications by Dr J. Hartwell has described the traditional application of over 3,000 plant species in cancer treatment.[30, 32] The task of cancer diagnosis is not straightforward therefore evaluation of historical evidence of plants in cancer treatment should be validated using appropriate techniques. During the peak of NP drug discovery between 1960 and 1982, the NCI collected and tested over 114,000 plant-derived extracts for their cytotoxicity. Introduction of new screening technologies in the following years (1986 to 2004) led to the establishment of the Natural Products Branch of the Developmental Therapeutics Program (DTP) by the NCI. The DTP has collected approximately 60,000 higher plant samples worldwide for screening in a cell-line panel consisting of 60-tumour cell lines and further *in vivo* evaluation. More recently extensive reviews by Newman and Cragg (2007 and 2012) classified all the therapeutic compounds marketed between 1981 and 2010 into to their treatment category and origin.[44, 51] NCEs approved between 1940 to mid-2006, have been subdivided into the following categories biological (B), natural product (N), natural product derivative (ND), totally synthetic drug (S), synthetic drug with a natural product pharmacophore (S*), natural product mimic (NM) and vaccine (V). Of the 1,184 NCEs, 174 are anticancer agents related to natural products (52%; 10% S/NM, 4% S*, 10% S*/NM, 5% N, 23% ND), and the majority are inspired by natural products (48%; 4% V, 14% B, and 30% S).[26] There have been reviews highlighting that the compounds in the synthetic category are essentially isosteres of peptide substrates and therefore act as NMs.[44, 138] Chemotherapy for many common solid tumours has limited anti-solid tumour activity without significant effects on patients' survival rates; moreover, many tumours will acquire resistance to currently available antineoplastic treatments.[45] Therefore a continuous effort to search for novel active compounds from nature to create a library for high throughput screening is vital in order to impede carcinogenesis. Such a goal has inspired this project combined with a long history of successful anticancer drug candidates from nature.[5, 139]

1.3.4 Classes of anticancer compounds

Since the 1940's, > 60% of all the available anticancer drugs introduced into the clinic are related to natural products and > 70% of compounds in clinical trial in 2004 were related to natural products.[140-142] The sources of these active natural products is not limited just to plants but also include microorganisms and marine organisms as well since many marine compounds possess antibiotic and anti-inflammatory properties and as such could play a role in cancer treatment.[5] The current plant-based anticancer drugs used in the clinic can be categorised into four main classes of compounds: vinca (or Catharanthus) alkaloids, epipodophyllotoxins, taxanes, and camptothecins (shown in Table 1-3).[11, 86, 140] The success of natural compounds is reflected by their worth in the global anti-cancer marker of over $2.75 billion US dollars in 2002.[11]

1.3.4.1 Vinca alkaloids

Vinca alkaloids isolated from the Madagascan periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae), were the first natural product-based anticancer agents to be used clinically.[11] Vinca alkaloids such as vinblastine (7) and vincristine (8) were found to be active against murine lymphocytic leukaemia. This discovery led to development of semi-synthetic analogues of these active agents vinflunine, vinorelbine and vindesine, which are used in multidrug therapy to treat various cancers including leukaemias, lymphomas, advanced testicular cancer, breast and lung cancers and Kaposi's sarcoma.[143] Vinca alkaloids and their derivatives bind to tubulin leading to microtubule depolymerisation in metaphase therefore they act as anti-microtubule, anti-mitotic agents.[11, 26, 140]

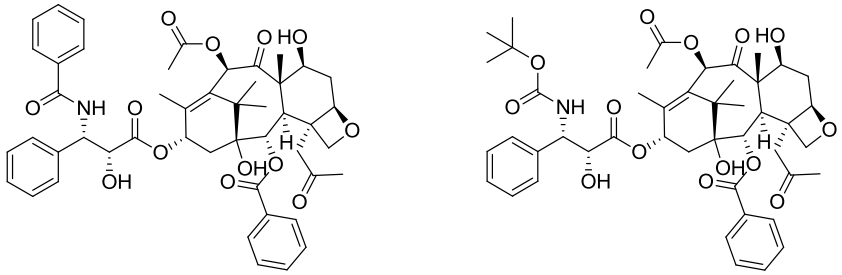


7; R= CH_3 8; R= CHO

Figure 1-7: The chemical structure of vinblastine (7) and vincristine (8).

1.3.4.2 Taxanes

Paclitaxel (taxol®) isolated from the bark of the Pacific Yew, *Taxus brevifolia* Nutt (Taxaceae), is used to treat several cancers of the breast, ovary and non-small cell lung cancer (NSCLC).[140] Paclitaxel (9) was discovered during the NCI's screening program and serves as a precursor for 23 analogues, which are currently in pre-clinical development for various cancers indications. The taxanes bind to and stabilise microtubules to halt cell division at the G2-M phase of cell cycle.[5]

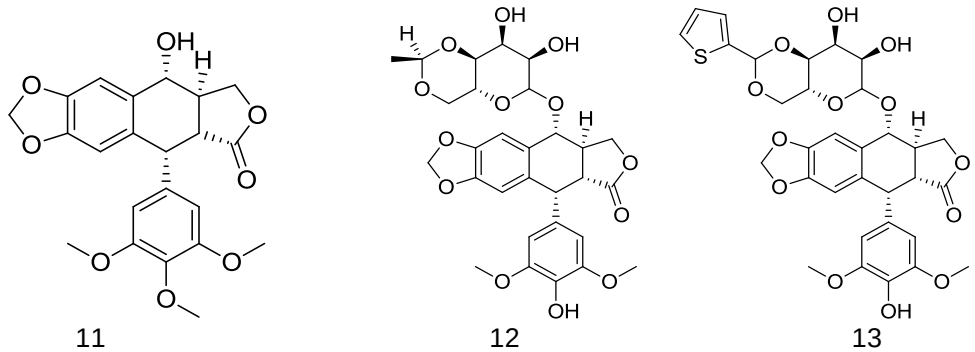


9 10

Figure 1-8: The chemical structure of taxol (9) and docetaxel (10).

1.3.4.3 Epipodophyllotoxins

Podophyllotoxin and other closely related lignans isolated from the *Podophyllum* species (Podophyllaceae) revealed antitumour activity accompanied by serious toxicity. This observation resulted in development of safer semi-synthetic analogues such as etoposide and teniposide. These analogues exert cytotoxicity against lymphomas, bronchial and testicular cancers via DNA strand breaks, induced by permanently binding to DNA topoisomerase II in the G2 phase of the cell cycle.[140]

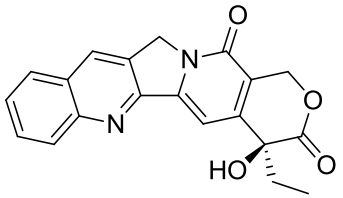


11 12 13

Figure 1-9: The chemical structure of podophyllotoxin (11), etoposide (12) and teniposide (13).

1.3.4.4 Camptothecins

Camptothecin is isolated from the Chinese *Camptotheca acuminata* Decne (Nyssaceae), which contains an α-hydroxylactone system and an unsaturated conjugated pyridine moiety.[144] Initial studies demonstrated severe bladder toxicity and therefore more water soluble and potent derivatives topotecan and irinotecan were developed for treating advanced ovarian and small cell lung cancers and metastatic colorectal cancers, respectively.[140] The mechanism of action involves selectively inhibiting topoisomerase I, which interferes with the cleavage and reassembly of DNA.



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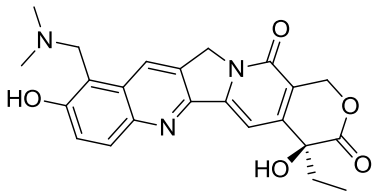

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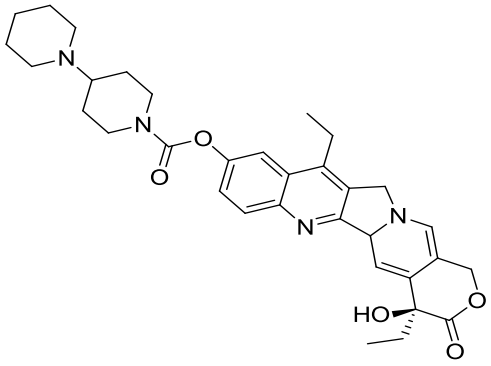


16

Figure 1-10: The chemical structure of camptothecin (14), topotecan (15) and irinotecan (16).

Many other compounds from plants with antitumour activity have already been used traditionally such as homoharringtonine (*Cephalotaxus harringtonia* var. *drupaecea*), an active alkaloid administered to chronic myeloid leukemia (CML) patients. Homoharringtonine and its analogues with reduced methyl groups can inhibit DNA topoisomerase II.[89] It also inhibits CDK9 and protein translation, downregulating the survival protein Mcl-1. It is thought that CDK9 inhibition contributes to anticancer activity. Salvia species roots and rhizome extracts have shown to inhibit proliferation of cancer cell lines of the liver, colon (colon-205), cervical (HeLa), nasopharynx (KB) and larynx (Hep-2).[89]

Extracts from several other plant species including *Brucea, Euphorbia, Rubia, Cocculus trilobus, Curcuma* (turmeric) and *Maytenus* are also used in therapy. Many analogues of existing anticancer drugs isolated from plants are in clinical trials and many are found to affect the cell cycle (Figure 1-11).

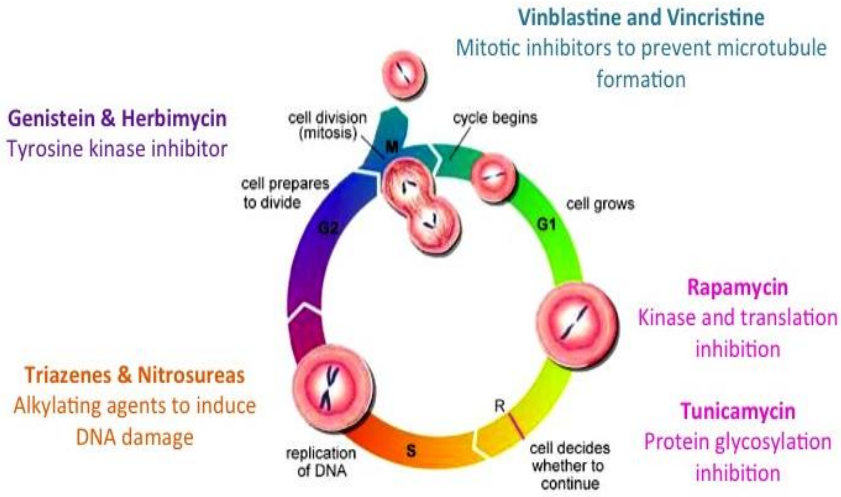
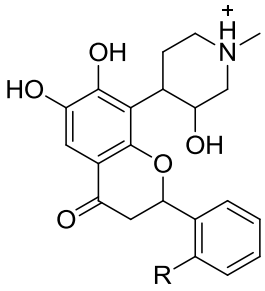


Figure 1-11: A simple representation of the cell cycle phrases, G_1, S, G_2, M, and the corresponding anticancer compounds that targets those specific phases.

A totally synthetic flavone, flavopiridol (17), was synthesised based on the structure of the natural product rohitukine, which was initially found to induce cell-cycle arrest and apoptosis in murine ovarian cancer cells, OCA-I.[140, 145] Flavopiridol is the first cyclin-dependent kinase inhibitor in clinical trial.[146-148] Flavopiridol is currently being investigated in eighteen Phase I and Phase II clinical trials either alone or in combination with other chemotherapy agents.[89]



17

Figure 1-12: The chemical structure of the cyclin-dependent kinase inhibitor, flavopiridol (17).[149]

1.3.5 Chemoprevention

Besides importance as drugs, natural products can be a vital source of compounds to "*suppress, arrest or reverse carcinogenesis, in its early stages*".[17, 110] A balance between oxidants and antioxidants (phytochemicals) obtained from diets rich in fruits and vegetables are essential for an optimal physiological state.[150, 151] Many epidemiologic studies support the proposed inverse correlation between increased consumption of fruits and vegetables in the diet and cancer risk.[110, 152, 153] Several phytochemicals possess chemopreventive properties that act on various pathways (apoptosis, angiogenesis and metastasis) and targets (oncogenes, tumour suppressor genes (TSG) etc.) to maintain cellular health and homeostasis. Certain phytochemicals act on normal cellular processes such as cell division, and hence inhibit proliferation of (cancer) cells. Further considerations to facilitate drug discovery from plants include improved sourcing and advances in combinatorial biosynthesis, microbial genomics and synthetic biology by addressing issues of complex structural synthesis.[41]

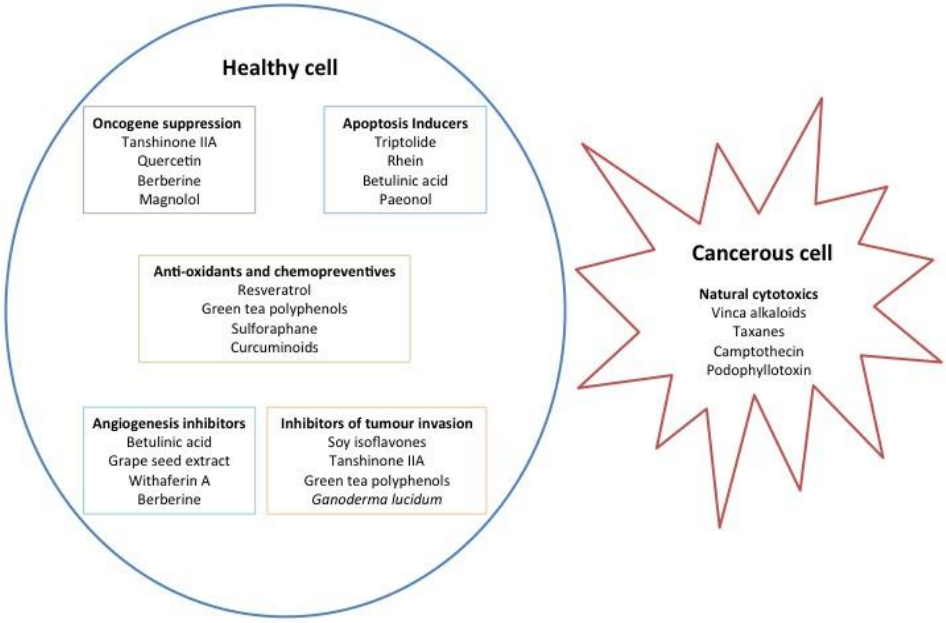


Figure 1-13: Diagram displaying some relevant targets for anti-cancer activity and examples of phytochemicals which interact with these targets, adapted from Williamson (2010).[16]

Phytochemicals have the ability to modulate certain molecules in key signal transduction pathways relating to cancer such as the nuclear factor–κB (NF–κB), receptor tyrosine kinases, mitogen-activated protein kinase (MAPK) and cyclooxygenases (COX).[46] Additionally, phytochemicals may modulate the immune system and possess antimicrobial, anti-inflammatory, anti-asthmatic and anti-anaphylactic activity.[154, 155] Exogenous antioxidants found only in plants can be classed as water-soluble or lipid-soluble e.g. vitamin C and β-carotene respectively.[156] Sufficient amounts of antioxidants are required to counteract endogenous free radicals otherwise the resulting oxidative stress could lead to oxidative damage to macromolecules, lipids, proteins and DNA.[16, 140, 141] Data from *in vitro* measurements and cell-free models suggest that compared to other cellular processes such as methylation, deamination and depurination, oxidation from normal metabolism causes the highest levels of DNA bases (around 26,000) loss daily subsequently leading to an increased risk of developing chronic diseases such as cancer.[157, 158] Many research projects such as that of the National Cooperative Drug Discovery Group (NCDDG) are characterising potential anticancer compounds from plants of the tropical rainforest to isolate compounds from edible plant species and dietary supplements.[11] Several clinical trials are being conducted by the NCI on potential cancer chemopreventive agents including curcumin (phenolic acid; Phase I colon), genistein (flavonoid; Phase I breast and endometrial), soy isoflavones (isoflavonoid; Phase II prostate), epigallocatechin gallate (flavonoids; Phase II breast, Phase I unspecified cancer, Phase II bladder recurrence) and resveratrol (alkaloid; Phase I unspecified cancer).[11]

Phytochemicals can be classified into the categories of terpenes, carotenoids, phenolics e.g. flavonoids, alkaloids, nitrogen-containing compounds and organosulfur compounds, which may be sub-divided into further categories (Figure 1.15).[17]

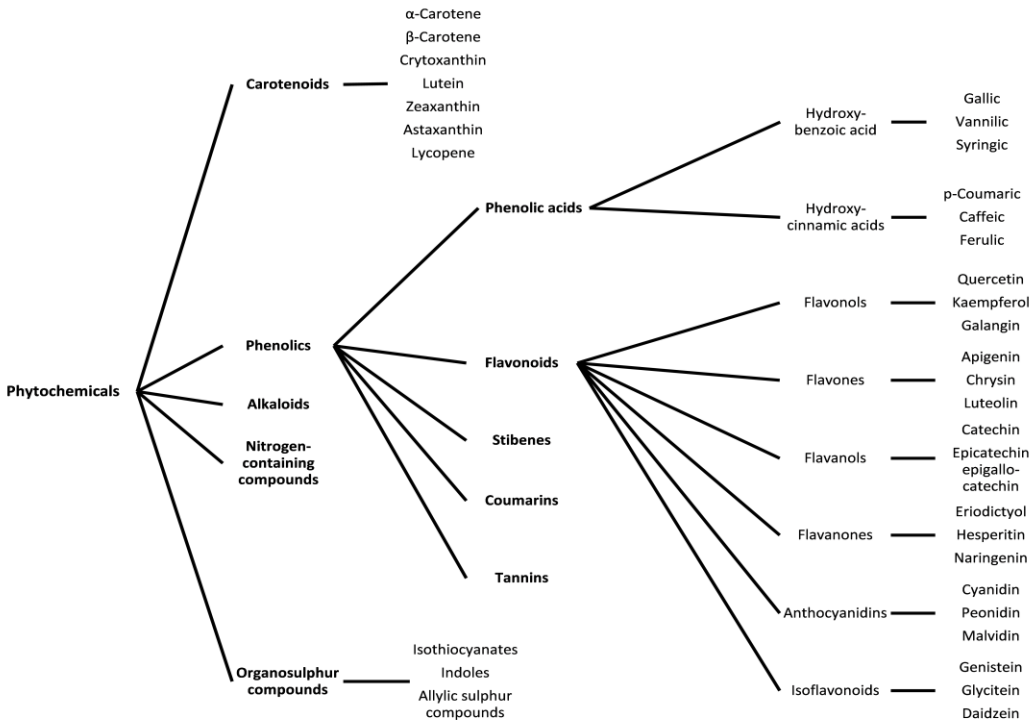


Figure 1-14: A flow chart listing some sub-families of phytochemicals: carotenoids, phenolics, alkaloids, nitrogen-containing compounds and organosulphur compounds.[17]

1.3.5.1 Terpenes

There are over 600 different caroteniods identified, of which there are two main classes: xanthophylls (oxygen containing) and carotenes (non-oxygen containing).[159] This predominant pigment possesses provitamin and antioxidant activities. The basic structure usually contains a 40-carbon skeleton structure of isoprene units and often occur in the all-*trans* form.[17] The number of double bonds determines the shape, chemical reactivity and light-absorbing properties of carotenoids.[159] Carotenoids have the ability to quench and inactivate reactive oxygen species (ROS) preventing genomic instability and carcinogenesis.[118, 160, 161] Diets rich in carotenoid pigments (vitamin A (retinol)) e.g. eggs and carrots can prevent the development and progression of epithelial cancers.[11, 110, 162] However, studies have suggested that in the long-term high levels of retinol are associated with an increased risk of osteoporotic hip fracture in women due to reducing bone mineral density.[163]

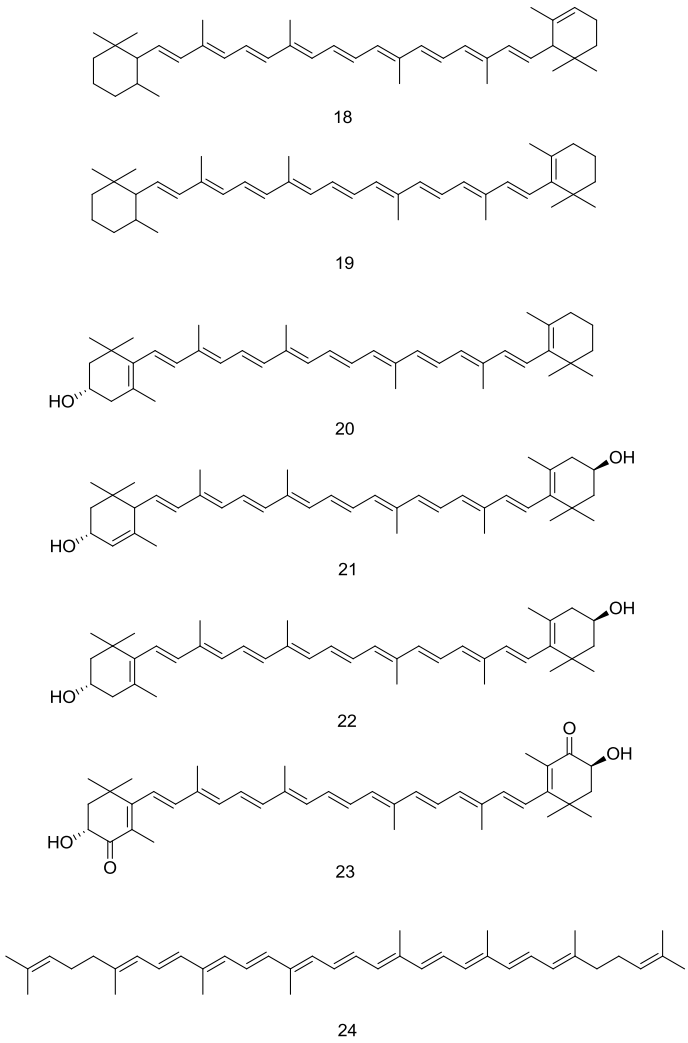


Figure 1-15: The chemical structures of carotenoids such as α-carotene (18), β-carotene (19), β -cryptoxanthin (20), lutein (21), zeaxanthin (22), astaxanthin (23) and lycopene (24).[17]

1.3.5.2 Phenolics

Phenolics are protective secondary metabolites produced by plants. Their structure consists of ≥ 1 aromatic ring(s) with ≥ 1 hydroxyl group.[17] The phenolic ring can exist in an oxidised or reduced states giving rise to a quinone or phenol, respectively.[156] Phenolics can be grouped into phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavanols, flavones, flavonols, flavonones, anthocyanidins and isoflavonoids), stibenes, coumarins and tannins, as shown in Figure 1-16: and Figure 1-17. It is estimated that 2/3 of the phenolics consumed in our diet (in foods such as nuts, fruits, vegetables, red wine, coffee and tea) consist of mainly flavonoids and

1/3 phenolic acids.[17] Flavonoids may also have a role in chemotherapy to induce or inhibit the metabolism of drugs by interfering with the function of cytochrome P450 (CYP) enzymes.[105, 164]

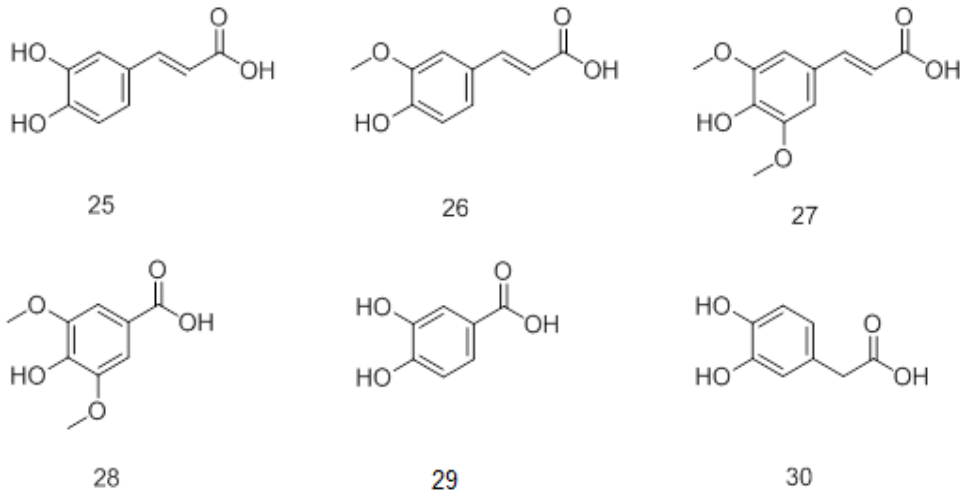


Figure 1-16: The chemical structures of phenolic acids such as caffeic acid (25), ferulic acid (26), sinapic acid (27), syringic acid (28), protocatechuic acid (29), 3,4-dihydroxyphenylacetic acid (30).[156]

However, tannins may act adversely on health by forming complexes with proteins, starch and digestive enzymes, thus lowering nutritional values. Tannins may also have an adverse effect on the absorption of amino acids and alkaloids.[105, 154, 165] The net effect may include "*a decrease in food intake, growth rate…net metabolisable energy, protein digestibility…damage of the mucosal lining of gastrointestinal tract and alteration of excretion of certain cations, and increased excretion of proteins and essential amino acids*." [165] Paradoxically, certain tannins also appear to possess chemopreventive properties and together with the above mentioned adverse activities make tannins a concern for use.

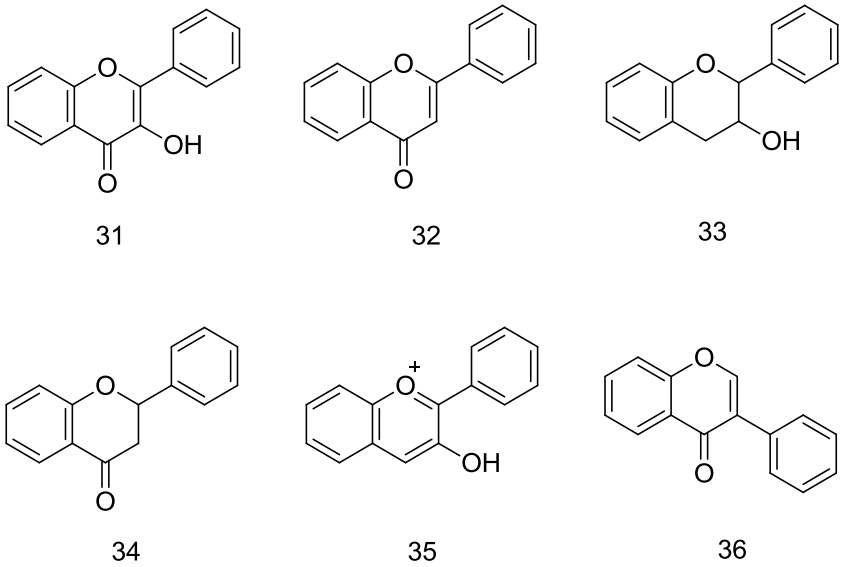


Figure 1-17: The chemical structures of flavonoids such as flavonols (31), flavones (32), flavanols (catechins) (33), flavanones (34), anthocyanidins (35) and isoflavonoids (36).[17]

1.3.5.3 Alkaloids

Alkaloids are nitrogen-containing compounds and thousands have been identified, and many have been used for their medicinal value for centuries.[154] Examples of alkaloids used medicinally include atropine, cocaine, hyoscine, morphine, emetine and ergometrine.[166] As discussed, vinca alkaloids are used in treatment of cancers such as leukaemias and lymphomas. Currently under Phase II/III clinical trial for chronic myeloid leukemia (CML) is the plant alkaloid homoharringtonine (6), which functions by inhibiting protein synthesis at the ribosome level.[30, 143]

1.4 A megadiverse country: Malaysia

Malaysia has been ranked the 12th richest country for its biological diversity of plant species according to the National Biodiversity Index (NBI), which is evaluated by the World Conservation Monitoring Centre (WCMC).[167, 168] In Malaysia, the estimated 40,000 plant species can be sub-divided into approximately 12,500 flowering plants, 15,000 higher plants and over 1,100 ferns and related species.[167, 169]

Malaysia's favourable climate offers a variety of habitats to suit diverse life forms. The majority of the total land area (57.5% - 59.9%) is forest area of which large proportions (57%) are tropical rainforests on the hills and mountains.[168] Areas in Pasoh, Peninsular Malaysia, Lambir, Sabah, and Sarawak contain particularly dense forests with hundreds of tree species.[170] The lowlands have been largely converted to agricultural land, mainly for growth of cash crops, oil palm and rubber. Apart from other crops such as coconut, rice and sugar cane, the agricultural lowlands support pineapple, cocoa, tapioca, maize and coffee plant growth. The main economic product of Malaysia's forest is timber and the most important timber producing families are the Dipterocarpaceae and Leguminosae.[169]

1.4.1 Plant profiles

Colleagues from the University of Nottingham Malaysia Campus (UNMC) collected various plants in Malaysia, and the following have been selected for investigation.

1.4.1.1 *Acalypha wilkesiana* Müll, Arg. (Euphorbiaceae)

Latin name*: Acalypha wilkesiana* Müll, Arg.

Synonymous name: *A. amentacea* f. *circinata* (Müll, Arg.) Fosberg, *A. Wilkesiana* (Müll, Arg.) Fosberg, *A. compacta* Guilf. ex C.T. White, *A. godseffiana* Mast, *A. godseffiana* var. *heterophylla* L.H. Bailey, *A. hamiltoniana* Briant, *A. macafeeana* Veitch, *A. musaica* auct, *A. tricolor* Seem, *A. triumphans.* Linden & Rodigas, *A. wilkesiana* f. *appendiculata* J.W. Moore, *A. wilkesiana* f. *circinata* (Müll, Arg.), *A. wilkesiana* f. *illustris* J.J. Sm, *A. wilkesiana* f. *macrophylla* J.J.Sm, *A. wilkesiana* var. *marginata* E. Morren, *A. wilkesiana* f. *monstrosa* J. J. Sm, *A. wilkesiana* f. *triumphans* (Linden & Rodigas) J. J. Sm, *Ricinocarpus wilkesianus* (Müll, Arg.) Kuntze.[171]

Vernacular name: Beef steak plant, copper plant (leaf), fire dragon and Jacob's coat [171]

Order: Malpighiales [171]

Family: Euphorbiaceae [171]

Genus: *Acalypha* [171]

Species: *Acalypha* [171]

Description: Tropical bush of up to 1.5 m high with red spiral leaves. Flowers are apetalous and without a nectary disc. The male inflorescences are spikes found at the top whilst the female are found at the base. Fruits are three-lobed capsules splitting into 3 valves.[172]

Distribution: Asia-Pacific [173]

Uses: In Malaysia, this plant is boiled in goat's milk and the milk is drank to lower blood pressure, treat fever and pimples and relieve coughs or it is boiled in sugar to treat thrombocytopaenic purpura and allergic purpura. In Nigeria, the leaves are boiled in water and given to those with gastrointestinal disorders or suffering from skin infections such as *Impetigo contagiosa*, *Candida intetrigo*, *Pityriasis versicolor*, *Tinea corporis*, *Tinea pedis* and *Tinea*

versicolor.[174] It is also recognized as an abortifacient herb in Nigeria besides its common use to treat headache, common cold and swelling.[175]

Constituents: Anthraquinone, corilagin, gallic acid, geraniin, kaempferol- and quercetin-3-0-rutinoside.[176]

Pharmaceutical Interest: It has shown antibacterial and antifungal properties in the aqueous and ethanolic extracts by inhibiting the growth of bacteria (*Staphylococcus aureus*) and fungus (*Candida albicans, Trichophyton rubrum, Trichophyton mentagrophytes* and *Aspergillus flavus*).[177] It has been shown to cause apoptosis in lymphocytes and stimulate the release of tumour necrosis factor α (TNF-α), interleukin (IL) -5 and -6 and interferon-γ.[175]
The collection took place in May 2007 at Broga forest, Malaysia. The whole plant was extracted in increasing polarity solvents: hexane (HEX), ethyl acetate (EtOAc) and ethanol (EtOH).

1.4.1.2 *Archidendron ellipticum* (Blume) Hassk. (Leguminosae)

Latin name: *Archidendron ellipticum* (Blanco)

Synonymous name: *Abarema elliptica* (Blanco) Kosterm, *Abarema elliptica* (Blume) Kosterm, *Albizia asciculate* (Benth.) Kurz, *Inga elliptica* Blume, *Pithecellobium ellipticum* (Blanco) Hassk, *Pithecellobium ellipticum* (Blume) Hassk, *Pithecellobium fasciculatum* Benth, *Pithecellobium waitzii* Kosterm.[102, 178, 179]

Vernacular name: Langir (antu), Borneo Indelebah, Jaring, Jering-jering.[180]

Order: Fabales [171]

Family: Fabaceae [173]

Genus: Archidendron [178, 179, 181]

Species: Ellipticum [181]

Description: A small flowering tree which can grow up to 20 m in height and 50 cm in diameter.[178] The leaves are flat and the rachis can be between 4 and 15 cm bearing yellow and yellowish-green flowers (0.3 cm in diameter). The fruits are orange-red (13.8 cm) containing black seeds.[178, 180]

Habitat: It is found in lowland primary and secondary rain forest, along rivers, on hillsides with sandy soils and in areas of limestone.[181, 182]

Distribution: Widespread in Malaysian states: Johor, Kedah, Kelantan, Melaka, Megeri Sembilan, Pahang, Perak, Penang, Perlis, Selangor, Singapore, Terengganu and Langkawi. Also found in Nicobar Islands, Peninsular Thailand, Sumatra, Java, Borneo and Philippines.[178]

Uses: The fruits of this plant can be consumed for food flavouring, the leaves are used for lice problems and the roots are use as piscicide.[178, 180, 183]

Constituents: Possibly djenkol acid, rotenoids, coumarins, lignans, terpenoids, polyacetylenes, alkaloids, saponins, toxalbumins, which are present in other piscicidal plants.[178, 184]

Pharmaceutical Interest: Traditional healers often employ many piscicidal plants in their practice. Previously, active antitumour compounds have been isolated from several piscicidal plants.[184] Kunitz proteinase inhibitors were identified in the seeds and are commonly used to inhibit the digestive proteinases of insects leading to possible starvation.[185] Whereas, trypsin inhibitors of *Glycine max* inhibited 50% of MCF-7 breast cancer cell proliferation at a low concentration of 4.3 μM.[186]

Plant material was collected on 21st April 2008 in Sg. Congkak forest, Malaysia. The bark and leaf specimens were extracted in solvents of increasing polarity: HEX, EtOAc and EtOH.

1.4.1.3 *Duabanga grandiflora* Walp. (Lythraceae)

Latin name: *Duabanga grandiflora* (DC.) Walp

Synonymous name: *Duabanga sonneratioides* Buch.-Ham, *Lagerstroemia grandiflora* Roxb. Ex DC and *Leptospartion grandiflorum* (Roxb. ex DC.) Griff.[181, 187]

Vernacular name: Khykan, Hokol, Lomatia, Magasawih, Bondraphulla

Order: Myrtales [181]

Family: Lythraceae [170, 187]

Genus: Duabanga [181]

Species: Grandiflora [181]

Description: A fast-growing flowering tree of 30 - 40 m in height with straight trunks, light brown branches with leaves of 25 cm in length and 10 cm broad. The flowers are 5-7cm in size and are white in colour with a strong unpleasant smell. The calyx is bell-shaped and separated into six segments with many stamens. The fruit contains many seeds in a leathery capsule and can be collected in April.[188-191]

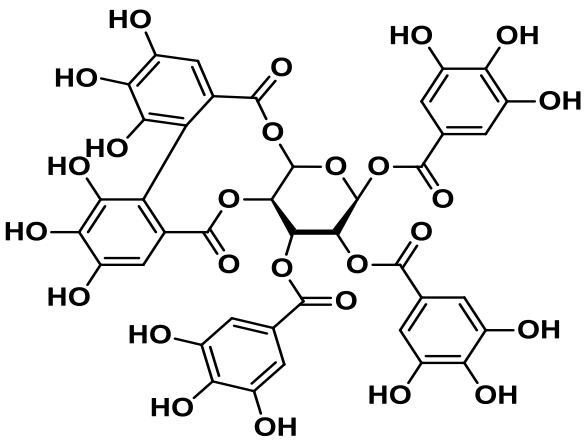
Habitat: They live in mixed evergreen and deciduous forests at an altitude range of 650 – 1450 M, also commonly found in stream valleys.[190, 192]

Distribution: Eastern Himalayas (riverbanks of sub-Himalayan valleys), southwards through Assam to Burma, Andaman Island, India, Malaysia and Bangladesh, the Philippines, Japan, Thailand, Nepal, Bhutan, Cambodia, Eastern India, Laos, Myanmar, Northern Thailand and Vietnam.[188, 189, 193]

Traditional value: In Nepal, the bark of *D. grandiflora* has been used as a piscicide.[194] The leaf extracts are used traditionally in Thai medicine for their

skin whitening, anti-aging and anti-inflammatary properties.[195] Additional dermatological properties include improving sagging, burns, liver spots and dullness. In Northern Thailand, the seeds of *D. grandiflora* are used traditionally for abdominal pain, food poisoning and peptic ulcer.[196] The timber is used for furniture.[191]

Constituents: Eugeniin (ellagitannin), ellagic acid, hentriacontane, hentriacontanol, hentriacontanone, b-sitosterol, a-amyrin, epioleanolic acid, epifriedelinol, lignoceryl ferulate, betulinic acid, acacetin, tetramethylellagic acid, quercetin 3-glucoside and 3-galactoside, genkwanin and gentianin 40-galactoside.[195-197] Furthermore,duabanganals, latifolinal, 3b-hydroxyursane-9(11),12-diene, 3b-hydroxy-12-oleanen-11-one, oleanolic acid, 3-O-(E)-coumaroyloleanolic acid, 3b-hydroxyolean-9(11),12-diene, hyptatic acid, betulenic acid 12, divergioic acid, (E)-5-(2-form-ylvinyl)-7-methoxy-2(3,4-methylenedioxyphenyl)benzofuran, 3,4-O-methylenedioxy-30,40,50-tri-O-methylellagic acid, vanillin, b-sitosterol, b-sitosterol glucoside, 3-hydroxy-4-methoxycinnamaldehyde and 5-formyl- furfurol.[193] Eugeniin (37) has also been isolated from *Geum japonicum* and *Syzygium aromaticum*.[198]



37

Figure 1-18: The chemical structure of eugeniin (37).

Pharmaceutical Interest: The extracts contain antioxidants and can inhibit collagenase activity, fat accumulation and melanin formation. Eugeniin has been shown to stimulate strong dose dependent type III collagen production.[196]

Eugeniin is also found in the bud of *Syzygium aromatica* and possesses virucidal effects against herpes simplex virus (HSV) (GI_{50}: 10 μg/ml) and type A influenza virus.[199] Antiviral activity is achieved by hindering RNA and DNA replication.[198, 200] Further investigations on the antiviral properties of Eugeniin have been published.[198-204] Antimicrobial activity was seen in a range of Gram-negative and Gram-positive bacteria e.g. *E. coli* and *S. aureus*, respectively.[205] Cytotoxicity in Walker Carcinosarcoma 256 has also reported.[197] Recently, crude methanolic extracts of *D. grandiflora* have been shown to act as a repellent for rice weevil, *Sitophilus oryzae*.[206]

The plant material was collected on 21st April 2008 at Semenyih Dam, Malaysia. HEX, EtOAc, EtOH and water bark and leaf extracts were prepared.

1.4.1.4 *Pseuduvaria macrophylla* (Oliv.) Merr (Annonaceae)

Latin name: *Pseuduvaria macrophylla* (Oliv.) Merr [207, 208]

Synonymous name: *Mitrephora macrophylla* Oliv [207, 208]

Vernacular name:	Unknown
Order:	Magnoliales [170]
Family:	Annonaceae [200]
Genus:	Pseuduvaria [201]
Species:	Macrophylla [170, 207, 208]

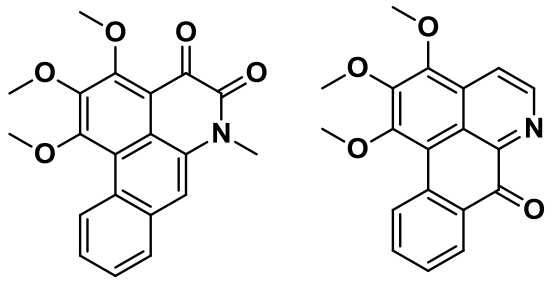
Description: A small tree with a wide spread growing up to 20-30 ft.[173] *Pseuduvaria* genus could be informally classed into: basal grade, long branch clade (47 genera and 1,500 species) and short branch clade (50 genera and 700 species) based on the differing rates of molecular divergence recorded. The flowers appearance resembles three sepals and two whorls of three petals,

usually unisexual. The inner petals are differentiated into a blade and basal claw and usually longer than the outer petals.[209]

Distribution: Malaysia hosts 12 species of the short branch clade, of which, eight are endemic. Found in the lowland and hill forest of Pulau Tioman, Peninsular Malaysia, Sungai Air Besar and southern Thailand.[173, 210, 211]

Traditional value: *P. macrophylla* has been reported to treat coughs, fever, nausea and vomiting.[211, 212]

Constituents: Dioxoaporphine alkaloid: 1,2,3-trimethoxy-4,5-dioxo-6*a*,7-dehydroaporphine and *O*-methylmoschatoline.[213] 1,2,3-trimethoxy-4,5-dioxo-6*a*,7-dehydroaporphine (yellow colour, melting point 199-201 °C) was also isolated from *Peperomia sui* and *Pseuduvaria setsoa*.[214]



38 39

Figure 1-19: The chemical structures of 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine (38) and O-methylmoschatoline (39).

Pharmaceutical Interest: Antimicrobial activity was seen in a range of Gram-negative and Gram-positive bacteria such as *Escherichia coli* and *Staphylococcus aureus,* respectively.[205] Due to the decreasing endemic status of this species, not many reports have been published on this species.[209] Other dioxoaporphines such as cepharadione and artabotrine have shown antimicrobial activies as well as anticancer activity against P-388 leukaemia cell lines, respectively.[215]

The collection took place on 8th August 2007 at Sg. Congkak forest in Malaysia. Extracts from whole plant were prepared in HEX, EtOAc and EtOH.

1.5 Research aims and objectives

Mankind has a long history of using natural products to prevent and treat diseases such as cancers. The biology and chemistry of natural products and their associated mechanisms of action are becoming better understood as research methods and technologies progress. Previously, limited reports described antitumour activities of *A. ellipticum* and *D. grandiflora* extracts whereas *P. macrophylla* has never before been examined for its biological activity therefore these plants' extracts are worthy of investigation to validate their use(s), to build on existing knowledge and explore the potential of these traditional medicinal plants for treatment of cancer and/or other diseases.

The aims of this project were to investigate selected Malaysian Rainforest plants for their potential antitumour properties and possible mechanism(s) of action. The rationale for selecting the aforementioned plants for investigation is based on the lack of available data on the biological activities of the species in general or the lack of scientific evidence for it as a potential anticancer activity specifically. Plant specimens were selected to coincide with studies being conducted by University of Nottingham, Malaysia Campus (UNMC) colleagues. A systematic approach was taken to study these plant extracts. It is the major goal of this project to determine whether UNMC plant extracts contain active phytochemicals, which may affect the survival of cancer cells. This project is in its infancy and will require interdisciplinary skills and techniques to evaluate the *in vitro* biological activities of UNMC plant extracts. This will support mechanistic studies of the UNMC plant extracts in order to justify the use of these plants to treat malignant disease.

The objectives of this thesis are:

1. To evaluate the *in vitro* growth inhibitory activities of crude extracts in a panel of cancer cell lines by measuring the cell viability and analysing cell cycle distribution. The assays employed to achieve the objectives are:
   - **i.** MTT assay
   - **ii.** Clonogenic assay
   - **iii.** Flow cytometric techniques using fluorescent staining of DNA with propidium iodide (PI)

2. To investigate whether the UNMC plant extracts induce apoptosis and to confirm any positive findings in a sensitive cell line.
   - **i.** Using flow cytometric analysis to measure dual Annexin V and PI florescent staining
   - **ii.** Confirmation of apoptosis by Western blotting techniques by employing specific antibodies to detect caspases and survival proteins.

3. To evaluate additional putative anticancer properties of extracts including the ability to
   - **i.** Inhibit cancer cell migration adopting the wound-healing (scratch) assay.
   - **ii.** Induce cellular senescence, an irreversible process leading to cell death. The assay employed to achieve this objective is intracellular detection the senescence marker β-galactosidase.

4. To evaluate potential chemopreventive properties of UNMC plant extracts by determining extract chemical contents as well as measuring the *in vitro* free radical scavenging activity. The following methods were employed:
   - **a.** Aluminium chloride method to determine the total flavonoid content

b. Folin Cioulteau methods to determine the total phenolic content

c. DPPH assay to measure the free radical scavenging activity.

5. To conduct initial activity-guided fractionation of crude extracts to enable identification of fraction(s) containing possible active components, if any. The following methods were employed:

i. Crude fractionation of UNMC extracts using extraction cartridge then analysing the collected material via HPLC techniques.

ii. To determine growth inhibitory properties of the crude extract fractions in selected cell lines using the MTT assay.

A literature search was performed on the medicinal uses and chemical constituents of *A. wilkesiana, P. macrophylla, A. ellipticum* and *D. grandiflora*. For instance *D. grandiflora* extracts have been used to treat stomach pain and for skin whitening through traditional practice in Thailand and its activity has been verified by identifying eugeniin, the active compound responsible.[196] There is a lack of evidence of *P. macrophylla*'s biological activity, however it was found to possess high levels of compounds possessing alkaloid functionality.[213, 214] A range of phytochemicals such as alkaloids found in plants possess therapeutic properties, thus suggesting the aforementioned plants are worthy of further investigation into their potential anticancer properties (refer to Plant profiles 1.4.1).[28, 32, 89, 216]

2

The influence of *Acalypha wilkesiana, Duabanga grandiflora, Archidendron ellipticum* and *Pseuduvaria macrophylla* crude extracts on cell viability

Approaches to select natural compounds as sources of potential anticancer drug leads include evaluating evidence of medicinal plants used in traditional systems or through random screening of natural compounds. Either approach would require validation *via* rapid, inexpensive and robust methods. Assays assessing cell viability are widely adopted in the preliminarily selection to identify those extracts with good activity. For instance the sulforhodamine B (SRB) assay is currently employed by the NCI *in vitro* anticancer-drug discovery program.[217] Other popular cell viability assays used in small-scale assessment of cancer cell viability after exposure to investigational compounds include tetrazolium salt-based assays such as 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thaizolyl blue; MTT).[93, 218] Identification of 'active' extracts in primary screening would facilitate selection of 'active' crude extracts to undergo further investigations.

2.1 Selection of UNMC plant extracts for investigation

Following the literature search, it was proposed that a primary screen investigating the anticancer activity of the plant extracts would enable preliminary selection of UNMC plant extracts. A range of bioassays has been considered and the robust and inexpensive, MTT and clonogenic assays were employed to examine *in vitro* growth inhibitory and cytotoxicity effects of the extracts in human-derived cancer cell lines. A cell panel was employed consisting of two breast adenocarcinoma cell lines, two ovarian carcinoma cell lines, two renal carcinoma cell lines, two colon carcinoma cell lines and a non-transformed lung fibroblast cell line. Non-transformed MRC-5 fibroblasts

were employed as a preliminary control to assess the putative selectivity of the *in vitro* growth inhibitory and cytotoxicity effects of UNMC plant extracts to cancer cells. A cervical cell line, SiHa, containing human papilloma virus (HPV) was included in the cell line panel to test *D. grandiflora* extracts due to previous reports of anti-viral activity by eugeniin.[202] Subsequently, sensitive cell lines were selected to conduct further *in vitro* examination to assess potential anticancer effects of UNMC plant extracts. The details of each type of cancer cell line and the specific cell lines utilised are shown in Table 2-1.

Table 2-1: Mammalian cell lines used for primary selection.

Cell type	Cell line	Characteristics	Reference
Breast adenocarcinoma	MCF-7	Er^+; $p53^+$; express WNT7B oncogene. Growth is inhibited TNF-α. Cellular products: insulin-like growth factor binding proteins BP-2, BP-4 and BP-5. Isolated from adult Caucasian female.	ATCC no': HTB-22; Brooks *et al.* 1973[219]
	MDA-MB-468	Er^- ; PR^- ; Her 2^-; expression of EGF and TNF-α. Isolated from African female.	ATCC no': HTB-132; Cailleau *et al.* 1978[220]
Ovarian adenocarcinoma	IGROV1	Isolated from adult female.	Bénard *et al.* 1985[221]
	SK-OV-3	Blood type B (Rh^+). Isolated from adult Caucasian female. Resistant to TNF-α and several cytotoxic drugs including diphtheria toxin, cis-platinum and adriamycin.	ATCC no': HTB-77; Frogh *et al.* 1975[222]
Renal cell carcinoma	Caki-1	Blood type O (Rh^-). Contains many microvilli, few filaments, many small mitochondria, well-developed Golgi and ER, lipid droplets and multilaminate bodies, secondary lysosomes. Isolated from adult Caucasian male.	ATCC no': HTB-46; Frogh *et al.* 1975[222]

	TK-10	Human spindle cell carcinoma isolated from adult male.	Bear *et al.* 1987[223]
Colorectal carcinoma	HCT116	TGFβ-1^{+}; P53 (wild-type) and mutation in codon 13 of ras protooncogene. Cellular products: keratin and carcinoembryonic antigen. Isolated from adult male.	ATCC no': CCL-247; Brattain *et al.* 1981[224]
	HCC2998	P53 (mutated) and K-ras (wild-type)	Goldwasser *et al.* 1995[225] and Kobayashi *et al.* 1999[226]
Cervical squamous cell carcinoma	SiHa	P53^{+}; pRB^{+}; HPV-16 (1 to 2 copies per cell). Grade II squamous cell carcinoma with an abundance of tonofilaments in the cytoplasm. Isolated from adult Asian female.	ATCC no': HTB-35; Durst *et al.* 1992[227]
Normal human foetal lung fibroblast	MRC-5	Isolated from 14 weeks gestation Caucasian male. Onset of senescence occurs after 42 to 46 population doublings.	ATCC no': CCL-171; Jacobs *et al.* 1970[228]

Information on the human cell lines used can be obtained from American Type Cell Culture (www.atcc.org) and National Cancer Institute (http://dtp.nci.nih.gov).

2.1.1 *In vitro* growth inhibitory and cytotoxicity effects of UNMC plant extracts in a cell line panel

An established method to observe the cell viability of a cell line after exposure to a compound of interest is the MTT assay.[229, 230] The method is mediated by substrates of mitochondrial dehydrogenases such as succinate, NADH and NADPH in viable cells to reduce soluble MTT dye into insoluble formazan with adsorption range between 510 and 570 nm.[231, 232] Therefore, the MTT assay can be used to investigate the effect of plant extracts on cell viability and proliferation in a panel of cancer cell lines. The mean absorbances were used to construct a dose-response curve, from which the estimated extract concentration inducing 50% growth inhibition (GI_{50} value) was calculated. The selection criteria for 'active' crude extracts are GI_{50} values of $\leq$ 20 µg/ml from *in vitro* assays.[95]

The cell lines were exposed to serial dilution of UNMC extracts for 72 h, followed by the addition of MTT. The cells were then incubated for 4 h at 37 °C before absorbance at 555 nm was read according to the MTT assay protocol outlined in Section 9.2.3. The absorbance of formazan for the cells at time of treatment (T_0) was also measured to determine basal level formazan produced by the viable cells. The T_0 value then allowed the calculation of the concentration of plant extract required to inhibit cell proliferation by 50% (GI_{50}). Furthermore, the anti-carcinogenic flavonoid, quercetin, was used as a positive control.[233] Quercetin was used to ensure that any activity seen for the extracts was not due to experimental error. Therefore, quercetin was tested against the cell line panel (n = 4) in at least three independent experiments.

2.1.1.1 *In vitro* growth inhibitory and cytotoxicity effects of *Acalypha wilkesiana* extracts in a cell line panel

A. wilkesiana (whole plant) EtOH and EtOAc extracts have demonstrated growth inhibitory effects across the cell line panel as shown in Table 2-2 (see appendix 1 for dose-response curves). Interestingly, both breast MCF-7 and MDA-MB-468, ovarian SK-OV-3 and CRC HCT116 cell lines appear to be

amongst the most sensitive to the growth inhibitory effects of the EtOH and EtOAc extracts.

Table 2-2: *In vitro* growth inhibitory effects of *A. wilkesiana* extracts in a cell line panel revealed by the MTT assay.

	Mean GI_{50} values (µg/ml)								
A. wilkesiana extracts	**MCF-7**	**MDA-MB-468**	**IGROV1**	**SK-OV-3**	**Caki-1**	**TK-10**	**HCT116**	**HCC2998**	**MRC-5**
EtOH	43.41 ± 9.85	*22.67 ± 3.66*	44.66 ± 1.47	41.80 ± 7.29	64.23 ± 4.51	77.99 ± 7.18	35.98 ± 1.53	88.74 ± 3.71	46.60 ± 8.03
EtOAc	35.96 ± 1.27	***15.88 ± 4.28***	60.32 ± 8.13	46.23 ± 8.52	120.99 ± 20.34	52.23 ± 5.96	39.14 ± 0.84	79.86 ± 3.42	53.26 ± 9.23
HEX	>200	>200	>200	>200	>200	>200	>200	>200	>200
Quercetin	5.33 ± 6.31	22.88 ± 8.08	9.66 ± 0.24	4.49 ± 1.37	5.03 ± 0.48	14.53 ± 1.24	21.47 ± 9.26	20.76 ± 12.61	18.56 ± 9.25

Extract concentration (µg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h exposure. Mean GI_{50} values (µg/ml) ± SEM were obtained from ≥ 3 (n = 4) individual experiments where active crude extracts (GI_{50} values ≤ 20 µg/ml) are highlighted in **bold**, extracts ± SEM values that could be considered active are highlighted in *italics*; the lowest GI_{50} values are underlined.

Fingerprint graphs (Figure 2-1) highlight MDA-MB-468 as the most sensitive cell line to EtOH and EtOAc extracts, with mean GI50 values of 22.67 µg/ml and 15.88 µg/ml, respectively. However EtOH and EtOAc extracts did not demonstrate selectivity between cancer cell lines and MRC-5 fibroblasts. EtOH and EtOAc extract-treated MDA-MB-468 cells produced GI_{50} values of 22.67 µg/ml and 15.88 µg/ml, respectively, which are near or lower than the selection criteria set (~ 20 µg/ml).

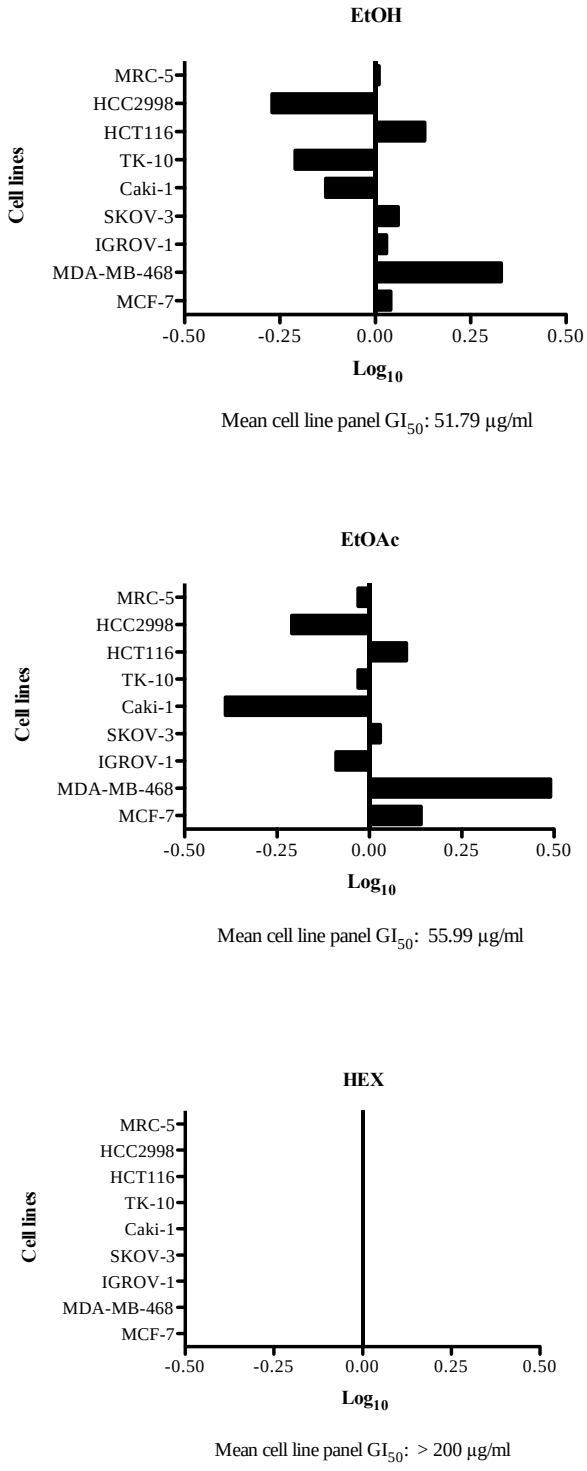


Figure 2-1: NCI-style mean fingerprint graphs for *A. wilkesiana* extracts. EtOH, EtOAc and HEX extracts were screened against a cell line panel and the bars represent the individual mean GI_{50} value of the cell line. The overall mean GI_{50} value of all the cell lines was set to the center (0.0); all values were to the function of $\log_{10}$. Bars to the left indicate cell lines that are less sensitive than the mean and bars to the right indicate cell lines that are more sensitive than the mean to the extract.

In addition, HCT116 cells revealed modest sensitivity towards EtOH and EtOAc extracts with GI_{50} values of 35.98 and 39.14 μg/ml, respectively. The difference between EtOH and EtOAc extract-treated mean cell line panel (GI_{50}) is 4.20 μg/ml, suggesting growth inhibitory and cytotoxic effects of both extracts are similar. There was no difference between the mean GI_{50} values for cell lines exposed to HEX extracts (> 200 μg/ml) since no growth inhibitory effects were demonstrated at the concentrations used.

2.1.1.2 *In vitro* growth inhibitory and cytotoxicity effects of *Archidendron ellipticum* extracts in a cell line panel

GI_{50} values for *A. ellipticum* extracts were determined using the MTT assay and are summarised in Table 2-3 (see Appendix 1 for dose-response curves). EtOH bark and leaf extracts inhibited the growth of cancer cell lines at low concentrations, MDA-MB-468 (1.73 and 9.32 μg/ml), Caki-1 (21.71 and 5.07 μg/ml), HCT116 (11.65 and 3.51 μg/ml) and HCC2998 (3.74 and 2.94 μg/ml) cells, respectively. To a lesser extent, growth of ovarian SK-OV-3 cells responded to EtOH bark extract (10.00 μg/ml), whereas renal TK-10 cells only responded to EtOH leaf extract (28.77 μg/ml).

In comparison, growth inhibitory effect of EtOH extracts appeared to be greater than EtOAc extracts as represented by mean cell line panel GI_{50} (EtOH bark: 45.28 and EtOH leaf: 45.73; EtOAc bark: 54.47 and EtOAc leaf: 138.82 μg/ml). Whereas, > 2-fold observed difference between the mean cell line panel GI_{50} values of EtOAc bark (54.47 μg/ml) and EtOAc leaf (135.82 μg/ml) extracts. Data from Table 2-3 revealed EtOH bark and leaf extracts demonstrated comparable activity (GI_{50} values) in MDA-MB-468, SK-OV-3, Caki-1, TK-10, HCT116, HCC2998 and MRC-5 cells (Figure 2-2).

Overall, EtOH extracts induced the greatest growth inhibitory effects across the cell line panel, particularly, in renal and CRC cell lines. It is interesting to note comparable mean GI_{50} values was observed across the cell line panel in bark and leaf extracts of the same solvents, e.g. EtOH bark = 45.73 μg/ml and leaf = 45.28 μg/ml; HEX bark = 189.81 μg/ml and leaf = > 200 μg/ml) extracts. In general, cell growth remained unaffected by HEX extracts even at high concentration of 200 μg/ml, which is 10X greater than the NCI's selection

criteria for crude extract activity. Furthermore, MRC-5 fibroblasts responded to EtOH and EtOAc bark and EtOH leaf extracts at low GI_{50} values: 3.82, 18.80 and 3.29 µg/ml, respectively.

Table 2-3: *In vitro* growth inhibitory effects of *A. ellipticum* extracts in a cell line panel revealed by the MTT assay.

		Mean GI_{50} values (µg/ml)								
A. ellipticum extracts		**MCF-7**	**MDA-MB-468**	**IGROV1**	**SK-OV-3**	**Caki-1**	**TK-10**	**HCT116**	**HCC2998**	**MRC-5**
BARK	EtOH	151.85 ± 5.82	***1.73 ± 1.52***	174.2 1± 19.37	***10.00 ± 4.01***	*21.71 ± 6.78*	28.77 ± 3.29	***11.65 ± 2.43***	***3.74 ± 0.26***	***3.82 ± 1.78***
	EtOAc	82.35 ± 4.07	40.43 ± 7.13	53.78 ± 12.80	62.26 ± 11.17	75.22 ± 7.38	32.45 ± 2.98	72.24 ± 2.21	52.68 ± 4.26	***18.80 ± 5.90***
	HEX	>200	>200	166.93 ± 10.46	>200	>200	>200	141.36 ± 10.07	>200	>200
LEAF	EtOH	97.64 ± 2.67	***9.32 ± 5.98***	242.05 ± 4.97	42.51 ± 4.49	***5.07 ± 0.59***	***5.24 ± 0.50***	***3.51 ± 0.16***	***2.94 ± 0.51***	***3.29 ± 0.42***
	EtOAc	>200	***9.28 ± 1.16***	198.50 ± 26.67	108.12 ± 7.10	>200	>200	122.22 ± 5.89	***19.97 ± 9.66***	69.73 ± 0.95
	HEX	193.42 ± 14.52	>200	>200	>200	>200	>200	>200	>200	>200
Quercetin		5.33 ± 6.31	22.88 ± 8.08	9.66 ± 0.24	4.49 ± 1.37	5.03 ± 0.48	14.53 ± 1.24	21.47 ± 9.26	20.76 ± 12.61	18.56 ± 9.25

Extract concentration (µg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h exposure. Mean GI_{50} values (µg/ml) ± SEM were obtained from ≥ 3 (n = 4) individual experiments where active crude extracts (GI_{50} values ≤ 20 µg/ml) are highlighted in **bold**, extracts ± SEM values that could be considered active are highlighted in *italics*; the lowest GI_{50} values are underlined.

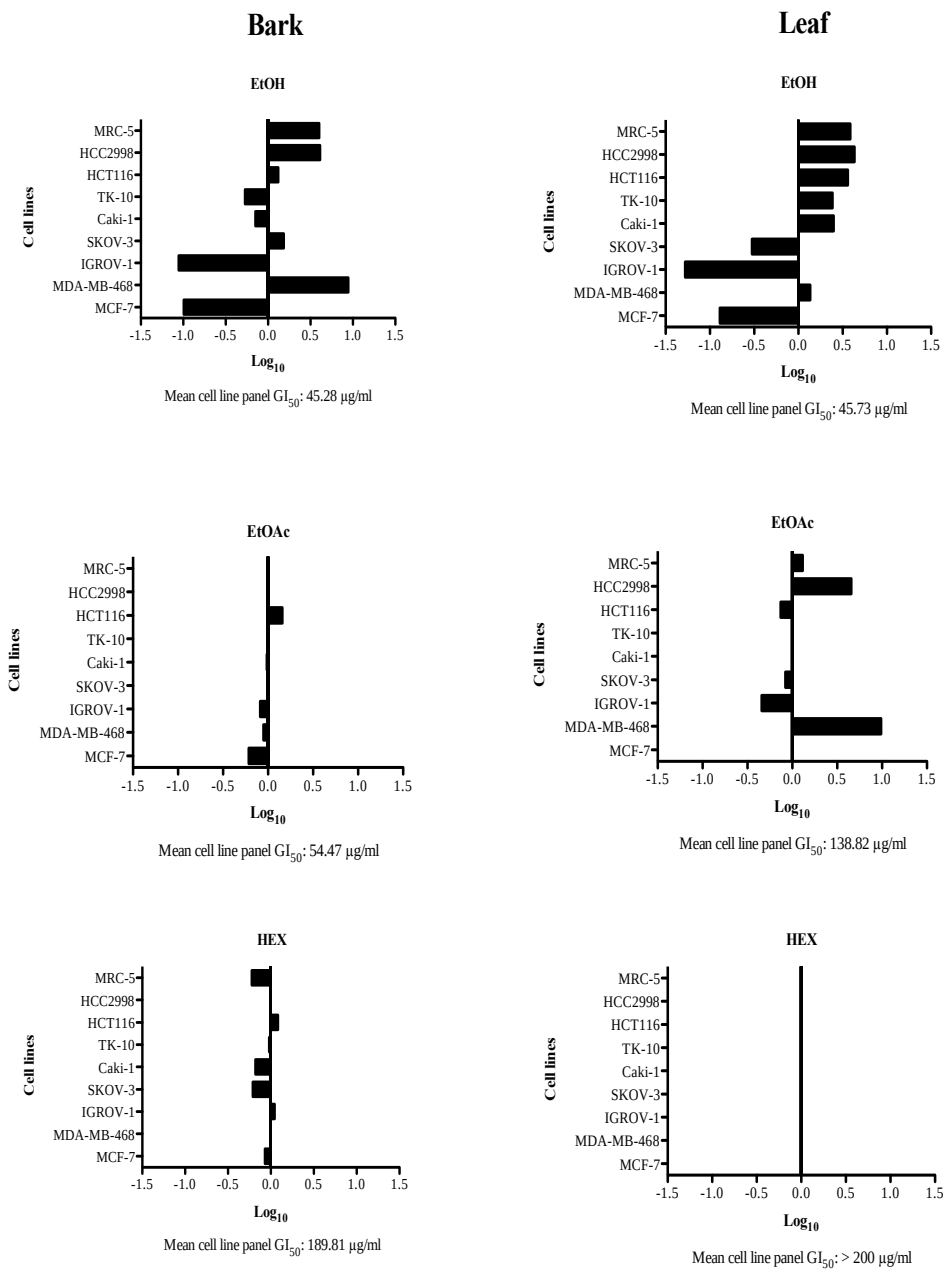


Figure 2-2: NCI-style mean fingerprint graphs for *A. ellipticum* extracts. EtOH, EtOAc and HEX extracts were screened against a cell line panel and the bars represent the individual mean GI_{50} value of the cell line. The overall mean GI_{50} value of all the cell lines was set to the center (0.0); all values were to the function of log_{10}. Bars to the left indicate cell lines that are less sensitive than the mean and bars to the right indicate cell lines that are more sensitive than the mean to the extract.

2.1.1.4 *In vitro* growth inhibitory and cytotoxicity effects of *Duabanga grandiflora* extracts in a cell line panel

The *in vitro* growth inhibitory and cytotoxicity activities of *D. grandiflora* bark and leaf extracts across the cell line panel are summarised in Table 2-4 (see Appendix 1 for dose-response curves). Interestingly, polar Water bark extracts induced growth inhibition only in breast cancer MCF-7 cells at concentrations within NCI's selection criteria (19.53 μg/ml). Growth inhibition of renal cancer Caki-1 and CRC HCT116 cells was observed at a lesser extent when exposed to EtOH bark extract (GI_{50} values: 34.40 and 37.54 μg/ml, respectively). EtOAc bark extract demonstrated greatest growth inhibitory effects across the cell panel, in particular, MCF-7 (19.87 μg/ml), HCT116 (21.69 μg/ml) and HCC2998 (4.65 μg/ml) cell lines. The cell lines most susceptible to growth inhibitory effects of HEX bark extracts were breast cancer MCF-7 (29.70 μg/ml) and CRC HCT116 (28.88 μg/ml) cells.

Overall, EtOAc and HEX extracts appeared to induce growth inhibitory effects in the greatest number of cell lines amongst the bark extracts. In comparison, EtOAc leaf extract emerged as effective in inhibiting the growth of CRC HCT116 (24.73 μg/ml) and breast cancer MDA-MB-468 (33.24 μg/ml) cells at similar concentrations to bark extracts. However, this trend was not seen in cells under the influence of Water leaf extract treated e.g. MDA-MB-468 (GI_{50} 37.40 μg/ml) and HCT116 (GI_{50} 38.02 μg/ml) cells and EtOH leaf extracts-treated breast cancer MCF-7 (34.47 μg/ml) and HCT116 (40.92 μg/ml) cells. A lack of cellular response to HEX leaf extract was observed at the highest concentration tested (> 200 μg/ml). In general, bark extracts presented the most number of active extracts against the cell line panel with mean cell line panel GI_{50} values of 65.04, 105.54, 31.58 and 36.36 and μg/ml for Water, EtOH, EtOAc and HEX extracts respectively, as compared to 79.80, 61.41, 43.78 and > 200 μg/ml for Water, EtOH, EtOAc and HEX leaf extracts, respectively. *D. grandiflora* bark extracts demonstrated broader growth inhibitory properties against the cell line panel compared to leaf extracts, suggesting different components are embedded in the extracts. MDA-MB-468, MCF-7 Caki-1, HCT116 and HCC2998 cells have shown broad sensitivity towards *D. grandiflora* extracts; contrarily IGROV1, SK-OV-3 and TK-10 cells were less sensitive to *D. grandiflora* extracts (Figure 2-3). Undesirably,

low GI_{50} (µg/ml) values of 7.37 and 20.88 in MRC-5 fibroblasts were obtained in both EtOAc and HEX bark extracts, respectively.

Table 2-4: *In vitro* growth inhibitory effects of *D. grandiflora* extracts in a cell line panel revealed by the MTT assay.

		Mean GI_{50} values (µg/ml)									
	D. grandiflora extracts	**MCF-7**	**MDA-MB-468**	**IGROV1**	**SK-OV-3**	**Caki-1**	**TK-10**	**HCT116**	**HCC2998**	**SiHa**	**MRC-5**
BARK	Water	***19.53 ± 3.97***	47.28 ± 7.34	69.98 ± 7.83	63.93 ± 6.12	46.69 ± 3.38	113.65 ± 11.03	42.44 ± 3.28	106.94 ± 11.07	-	74.91 ± 12.82
	EtOH	146.09 ± 9.35	40.04 ± 3.32	194.15 ± 15.77	135.69 ± 8.00	*34.40 ± 12.25*	153.36 ± 22.06	37.54 ± 5.68	141.46 ± 18.31	64.76 ± 9.41	67.12 ± 3.60
	EtOAc	***19.87 ± 1.43***	36.24 ± 4.10	29.49 ± 3.00	71.60 ± 8.00	31.53 ± 10.66	61.78 ± 0.56	21.69 ± 0.64	***4.65 ± 2.13***	69.01 ± 3.54	***7.37 ± 1.32***
	HEX	29.70 ± 0.53	34.95 ± 1.79	31.47 ± 2.57	56.59 ± 7.63	52.93 ± 4.97	36.65 ± 5.79	28.88 ± 1.55	35.22 ± 3.27	67.06 ± 3.28	***20.88 ± 1.34***
LEAF	Water	57.85 ± 6.40	**37.40 ± 5.45**	79.9 ± 3.78	66.58 ± 3.64	88.52 ± 2.71	150.17 ± 2.96	38.02 ± 3.25	111.34 ± 20.11	-	88.38 ± 15.79
	EtOH	**34.47 ± 2.78**	44.59 ± 7.84	71.01 ± 7.40	49.65 ± 9.12	40.79 ± 7.36	108.48 ± 12.99	40.92 ± 5.14	91.23 ± 8.45	49.21 ± 8.63	71.58 ± 2.96
	EtOAc	47.52 ± 2.77	33.24 ± 7.37	49.36 ± 3.94	36.13 ± 4.87	43.05 ± 8.86	62.85 ± 2.32	**24.73 ± 1.11**	66.33 ± 7.72	73.62 ± 3.05	30.77 ± 4.83
	HEX	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200

Extract concentration (µg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h exposure. Mean GI_{50} values (µg/ml) ± SEM were obtained from ≥ 3 (n = 4) individual experiments where active crude extracts (GI_{50} values ≤ 20 µg/ml) are highlighted in **bold**, extracts ± SEM values that could be considered active are highlighted in *italics*; the lowest GI_{50} values are underlined.

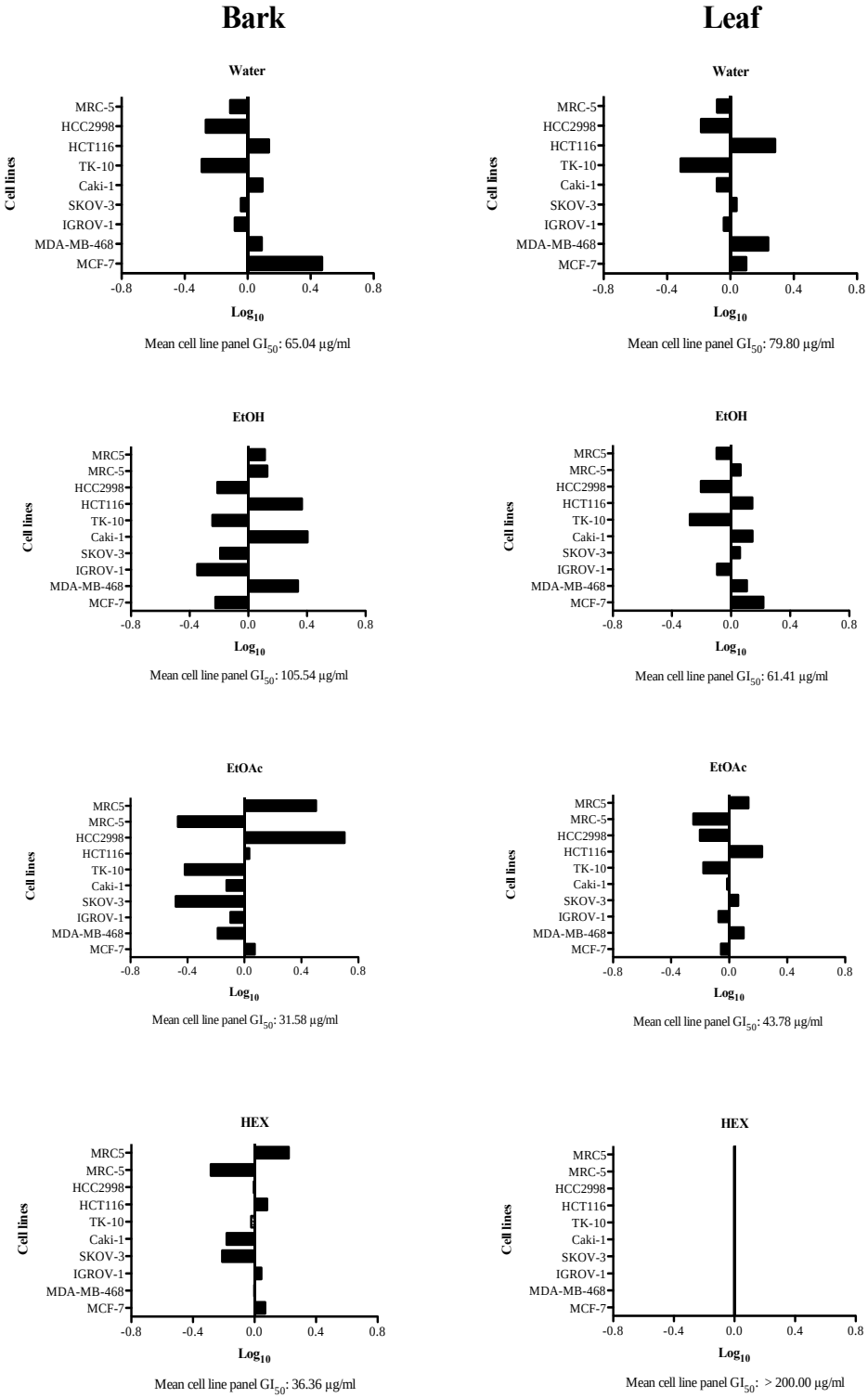


Figure 2-3: NCI-style mean fingerprint graphs for *D. grandiflora* extracts. Water, EtOH, EtOAc and HEX extracts were screened against a cell line panel and the bars represent the individual mean GI_{50} value of the cell line. The overall mean GI_{50} value of all the cell lines was set to the center (0.0); all values were to the function of log_{10}. Bars to the left indicate cell lines that are less sensitive than the mean and bars to the right indicate cell lines that are more sensitive than the mean to the extract.

2.1.1.5 *In vitro* growth inhibitory and cytotoxicity effects of *Pseuduvaria marcophylla* extracts in a cell line panel

The MTT assay demonstrated *P. macrophylla* extracts exerted *in vitro* growth inhibitory activity in all the cell line panel: potency EtOAc > EtOH > HEX (summarised in Table 2-5 and dose-response curves are shown in Appendix 1).

Table 2-5: *In vitro* growth inhibitory effects of *P. macrophylla* extracts in a cell line panel revealed by the MTT assay.

	Mean GI_{50} values (µg/ml)								
P. macrophylla extracts	**MCF-7**	**MDA-MB-468**	**IGROV1**	**SK-OV-3**	**Caki-1**	**TK-10**	**HCT116**	**HCC2998**	**MRC-5**
EtOH	***4.16 ± 1.84***	***5.36 ± 2.45***	***6.59 ± 1.07***	***3.73 ± 1.17***	***4.53 ± 1.49***	***6.98 ± 3.04***	***5.16 ± 1.07***	***3.96 ± 1.59***	***5.01 ± 1.13***
EtOAc	***1.23 ± 0.61***	***1.15 ± 0.37***	***5.38 ± 0.12***	***1.52 ± 0.66***	***1.47 ± 0.66***	***2.12 ± 0.48***	***1.61 ± 0.39***	***2.00 ± 1.08***	***1.71 ± 0.57***
HEX	***18.72 ± 4.00***	***6.49 ± 0.94***	***9.32 ± 2.06***	***5.88 ± 1.98***	***12.95 ± 6.92***	***13.10 ± 5.81***	***5.41 ± 1.58***	***9.82 ± 2.34***	26.23 ± 6.82
Quercetin	5.33 ± 6.31	22.88 ± 8.08	9.66 ± 0.24	4.49 ± 1.37	5.03 ± 0.48	14.53 ± 1.24	21.47 ± 9.26	20.76 ± 12.61	18.56 ± 9.25

Extract concentration (µg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h exposure. Mean GI_{50} values (µg/ml) ± SEM was obtained from ≥ 3 (n = 4) individual experiments where active crude extracts (GI_{50} values ≤ 20 µg/ml) are highlighted in **bold**, extracts ± SEM values that could be considered active are highlighted in *italics*; the lowest GI_{50} values are underlined.

EtOAc extract yielded GI_{50} values ≤ 2 µg/ml in MCF-7, MDA-MB-468, SK-OV-3, Caki-1, HCC2998 and HCT116 cancer cells as well as in MRC-5 fibroblasts. The activity between the EtOH and HEX extracts showed comparable growth inhibitory effects. HEX extracts revealed moderate selectivity (~ 5-fold) towards cancer cell lines MDA-MB-468, SK-OV-3 and HCT116 (GI_{50} values of 6.49, 5.88 and 5.41, µg/ml, respectively), over non-transformed MRC-5 fibroblasts (GI_{50} values: 26.23 µg/ml). However, EtOAc

and EtOH extracts did not demonstrate selectivity towards cancer cells compared with MRC-5 fibroblasts (Figure 2-4). Subtle differences in cell panel GI_{50} values were observed in cell line panel GI_{50} values following treatment with EtOH (GI_{50} value: 5.05 µg/ml) and EtOAc (GI_{50} value: 2.02 µg/ml) extracts whereas cancer cells' sensitivity towards HEX extract varied (GI_{50} values: 5.41 to 18.72 µg/ml).

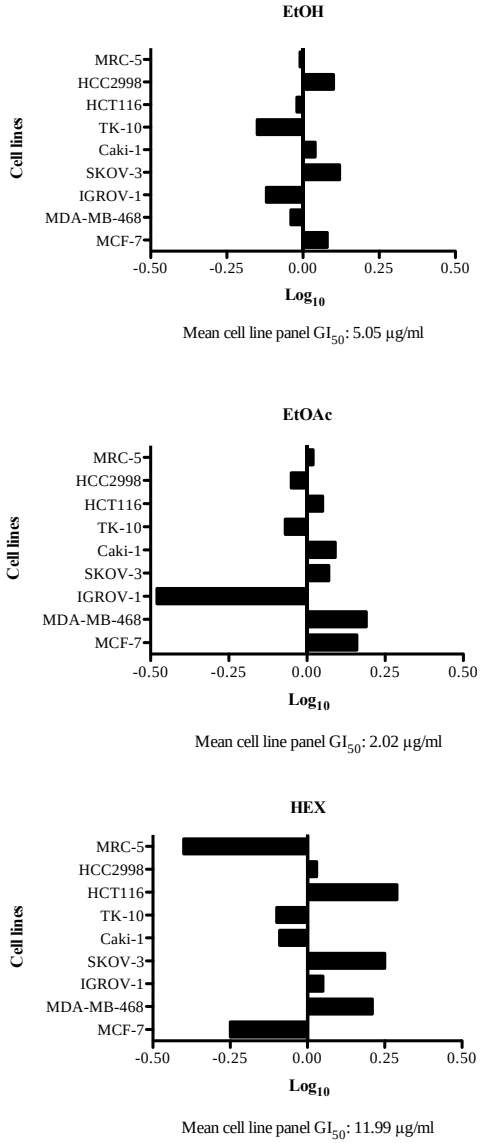


Figure 2-4: NCI-style mean fingerprint graphs for *P. macrophylla* extracts. EtOH, EtOAc and HEX extracts were screened against a cell line panel and the bars represent the individual mean GI_{50} value of the cell line. The overall mean GI_{50} value of all the cell lines was set to the center (0.0); all values were to the function of log_{10}. Bars to the left indicate cell lines that are less sensitive than the mean whereas bars to the right indicate cell lines that are more sensitive than the mean to the extract.

2.1.1.6 Summary of the *in vitro* growth inhibitory and cytotoxicity effects of UNMC extracts in a cell line panel

The MTT assay was used to examine *in vitro* growth inhibition and cytotoxicity of UNMC plant extracts' effects in human-derived cancer cells; extracts' selectivity against cancer cell line was compared in non-transformed fibroblasts.

***Acalypha wilkesiana**:* Extracts of *A. wilkesiana* primarily affected breast cancer cell line, MDA-MB-468 (GI_{50} values: EtOH: 22.67 and EtOAc: 15.88 μg/ml, respectively) and MCF-7 breast cancer (EtOH: 43.41 and EtOAc: 35.96 μg/ml, respectively). In support of this finding, Büssing *et al.* (1999) reported the verification of ethnopharmacological use of *A. wilkesiana* seeds to treat breast tumours and inflammation owing to their cytotoxic and immunomodulatory effects via generation of reactive oxygen intermediates (ROI), release of pro-inflammatory cytokines TNF-α and IL-6, T-cell-associated cytokines IL-5 and IL-γ.[175] This could be due to the presence of amphiphilic saponins in the extracts, which has ability to permeabilise cell membranes.

Another study has reported mean GI_{50} values of 28.03 and 89.63 μg/ml for U87MG (grade IV human gliobastoma) and A549 (human lung carcinoma) cells following 72 h exposure to *A. wilkesiana* EtOAc extracts. U87MG cells required almost two times greater concentration of EtOAc extracts to induce growth inhibitory effects when compared to MDA-MB-468 cells treated with EtOAc extracts (GI_{50} 15.88 μg/ml).[234] Both U87MG and A549 cells were insensitive to EtOH and HEX extracts (GI_{50} values between 166.30 to > 300 μg/ml), which corresponded to the trend observed for HEX extracts (see Table 2-2), suggesting unseparated components in crude HEX extract to be least effective in inducing growth inhibition.

There was minimal selectivity between MDA-MB-468 and MRC-5 cells. No evidence of growth inhibition was seen in HEX-treated cell lines. The absence of oestrogen (ER) and progesterone (PR) receptors on MDA-MB-468 cells may not to be a factor to cells' sensitivity towards these extracts, suggesting the active constituents may not be in the phytochemical class of isoflavonoids (phyto-oestrogens) or flavonoids (anti-oestrogens). Results from MTT assays

revealed MDA-MB-468 cells to be among the most sensitive to the growth inhibitory properties of *A. wilkesiana* extracts; therefore MDA-MB-468 cells were selected for further study with this plant.

***Archidendron ellipticum*:** EtOH extracts from *A. ellipticum* demonstrated good activity across the cell line panel. With the exception of ovarian carcinoma SK-OV-3 cells, the same cell lines responded to growth inhibitory effects of EtOH bark and leaf extract (MDA-MB-468 > HCC2998 > MRC-5 > HCT116 > TK-10 > Caki-1, in order of sensitivity), suggesting similar compounds are embedded. MDA-MB-468 cells were sensitive to EtOH and EtOAc extracts at low concentrations between 1.73 - 9.32 μg/ml. Only MCF-7 and IGROV1 cells revealed less sensitivity towards growth inhibitory effects of the extracts. Comparable GI_{50} values suggest that similar phytochemicals might be present in the EtOH bark and leaf extracts based on the similar sensitivity observed in MDA-MB-468, SK-OV-3, Caki-1, TK-10, HCT116, HCC2998 and MRC-5 cells.

Beutler *et al* (1997) stated that *in vitro* cytotoxicity of several crude legume extracts from the NCI natural product repository were comparable to that of *A. ellipticum*, such as *Albizia zygia* and *Albizia altissima*, sharing similar mean fingerprint patterns with saponins from *A. ellipticum*.[235] Many leguminous plant seeds contain biologically active compounds that could inhibit proteases and amylases. Kunitz (serine) proteinase inhibitor, which is present in germinative tissues of *A. ellipticum* and many *Mimosoideae* species possesses dual functions as storage protein and a part of the plant's defensive mechanism against seed predators.[236, 237] Inagaki *et al.* (2012) identified Kunitz-type protease inhibitors may affect ion channels (K^+, Na^+ and Ca^{2+}) and inhibit MMP2 activity hence may have a role in cancer metastasis inhibition.[238]

Results from MTT assays revealed MDA-MB-468 cells to be among the most sensitive to growth inhibitory properties of *A. ellipticum* extracts; therefore MDA-MB-468 was selected for further study with this plant.

Duabanga grandiflora: EtOAc bark extract of *D. grandiflora* exerted greatest activity across the cell line panel with growth inhibition in 70% of the cell line panel at near 20 μg/ml (NCI's selection criteria), in the order of sensitivity:

HCC2998 > MRC-5 > MCF-7 > HCT116 > IGROV-1 > Caki-1 > MDA-MB-468. Overall, HCT116 cells were most sensitive to *D. grandiflora* extracts EtOAc (bark) > EtOAc (leaf) > HEX (bark) > EtOH (bark) > Water (leaf) extracts with GI_{50} between 21.69 - 38.02 μg/ml. Interestingly, HEX extracts appeared to induce broad activity against MRC5 > HCT116 > MCF-7 > IGROV-1 > MDA-MB-468 > HCC2998 > TK-10 cells (in order of sensitivity) with GI_{50} values between 20.88 to 36.65 μg/ml. In contrast no growth inhibition was observed in cells exposed to HEX leaf extracts even at 200 μg/ml. The unresponsiveness of cells to HEX leaf extract (> 200 μg/ml) could either indicate a lack of potent compounds present in the extract or the masking of activity by other compounds present.

It is noted that the cervical cell line, SiHa, containing copies of HPV-16 (DNA) virus was included in the cell line panel due to reports identifying an anti-viral compound, eugeniin, present in *D. grandiflora* extracts. Eugeniin interferes with HSV DNA polymerase therefore it is also a reason to test if these conserved DNA reading proteins are also affected in HPV.[34] Traditional uses e.g. for anti-aging and anti-whitening, were supported by Tsukiyama *et al.* (2010) as the active hydrolysable tannin, eugeniin could stimulate type III collagen production.[196, 198] It was found that 2.74 μg/ml of eugeniin was effective at inducing cytotoxic effects against 50% of melanoma cells (RPMI-7951).[239] Another study revealed EtOH bark extract of *D. grandiflora* from India to be active in Walker carcinosarcoma 256 in rats.[197] Conversely, eugeniin extracted from leaf extracts using MeOH induced growth of human skin cells, normal human fibroblast (NHF) cells at concentration range between 31 – 125 μg/ml.[196] The constituents, oleanane triterpene (pentacyclic triterpenes) and lupine triterpene (betulinic acid) revealed cytotoxic activity against an acute lymphoblastic leukaemia cell line, MOLT-3, (GI_{50}: 9.5 and 14.1 μM) and a hepatocarcinoma, HepG2 (GI_{50}: 19.9 and 11.0 μM) cell line. These constituents were found to be inactive against (GI_{50} > 50 μM) HuCCA-1 (human chlolangiocarcinoma), A549 (human lung cancer), HeLa (human cervical carcinoma), and MDA-MB-231 (hormone independent breast cancer) cell lines.[193]

However, MTT results did not reveal any extract potency against or ability to promote proliferation of SiHa cells, which may be due to the 'masking' or antagonism of eugeniin's activity by other compounds in the mixture.
Results from MTT assays revealed HCT116 cells to be among the most sensitive to growth inhibitory properties of *D. grandiflora* extracts, therefore this cell line was selected for further study with this plant.

***Pseuduvaria macrophylla*:** The order of *P. macrophylla* extracts potency can be ranked as follow EtOAc > EtOH > HEX based on mean cell line panel GI_{50} values (5.05, 2.02 and 11.99 μg/ml, respectively). Remarkably, *P. macrophylla* extracts were able to inhibit growth across the cell line panel at concentrations lower than those observed for *A. wilkesiana, D. grandiflora* and *A. ellipticum* hence the most active plant in this collection. Good selectivity was observed between cancer cells and MRC5 fibroblasts treated with HEX extract. *P. macrophylla* plant extracts are the most active against cancer cell lines and exhibited GI_{50} effects < 10 μg/ml, which is lower (i.e. more potent) than selection requirement. There are no previous studies on the biological activity of *P. macrophylla*.[214] Aporphinoid (38) isolated from aerial parts of *Pseuduvaria setosa* has reveal cytotoxicity in small cell lung cancer cells, NCI-H187 at IC_{50} value of 3.0 μg/ml. Whereas reduced activity was observed in KB (epidermoid carcinoma) and BC (breast cancer) cells (IC_{50}: > 20 μg/ml).[214] CRC HCT116 cells were amongst the most sensitive to growth inhibitory properties of *P. macrophylla* extracts, in particular to HEX extract; therefore this cell line was selected for further study of *P. macrophylla in vitro* anticancer properties.

Further examination of *in vitro* activity of plant extracts were explored using other comparable cell-based assays. Growth inhibition was seen in most cancer lines treated with the above mentioned UNMC plant extracts; HCT116 and MDA-MB-468 cell lines were amongst the most sensitive cell lines hence chosen for further investigations into *in vitro* activity of UNMC extracts.

2.1.2 Influence of UNMC plant extracts on cell survival and clonogenicity

The clonogenic assay is another established method to examine the abilities of cancer cells to survive, proliferate and form colonies. Cytotoxic agents may exert their effects via various mechanisms including induction of cell cycle arrest or apoptosis leading to a loss of proliferative potential and protein degradation, respectively.[240, 241] This bioassay determines survival and subsequent proliferative and colony formation capacity of single cells following brief exposure (~ 24 h) to extracts of interest.[145, 242] The end-point is considered when the control populations contain ≥ 50 cells per colony. Subsequently, cell colony(ies) are fixed with methanol then stained with methylene blue dye then quantified by counting.[243, 244] Plumb *et al.* (1987) and Carmichael *et al.* (1987) have reported the positive correlation between the clonogenic and the MTT assay.[243, 245] Hence both assays were used in conjunction to compare the cytotoxic effects of the aforementioned plant extracts on the cell survival and proliferation of a panel of cancer cell lines. The cell lines and treatment concentrations (0.5X, 1X and 2X GI_{50} values) chosen to examine the sensitivity to the UNMC extracts were guided by results of MTT assays. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = $0.01 < p < 0.05$.

2.1.3 Influence of *Acalypha wilkesiana* extracts on MDA-MB-468 cells' survival and clonogenicity

Due to the unavailability of EtOAc extract only the clonogenicity of *A. wilkesiana* EtOH and HEX extracts were assessed. Results from clonogenic assay illustrated decreasing colony formation capacity of MDA-MB-468 cells with increasing extract concentration shown in Figure 2-5. Significant inhibition of colony formation was observed with EtOH extract at 36 and 72 μg/ml (1X and 2X GI_{50}) where < 40% of the cells formed colonies when at 1X GI_{50} EtOH and > 50% colony formation inhibition at 2X GI_{50}. Likewise, inhibition of colony formation following exposure to 1X and 2X GI_{50} of HEX

extracts was determined to be significant when compared to the control ($p < 0.05$). Although HEX extracts were able to inhibit colony formation, the treatment concentrations used (100, 200 and 400 μg/ml) were ≥ 5-fold greater than EtOH extract concentrations used.

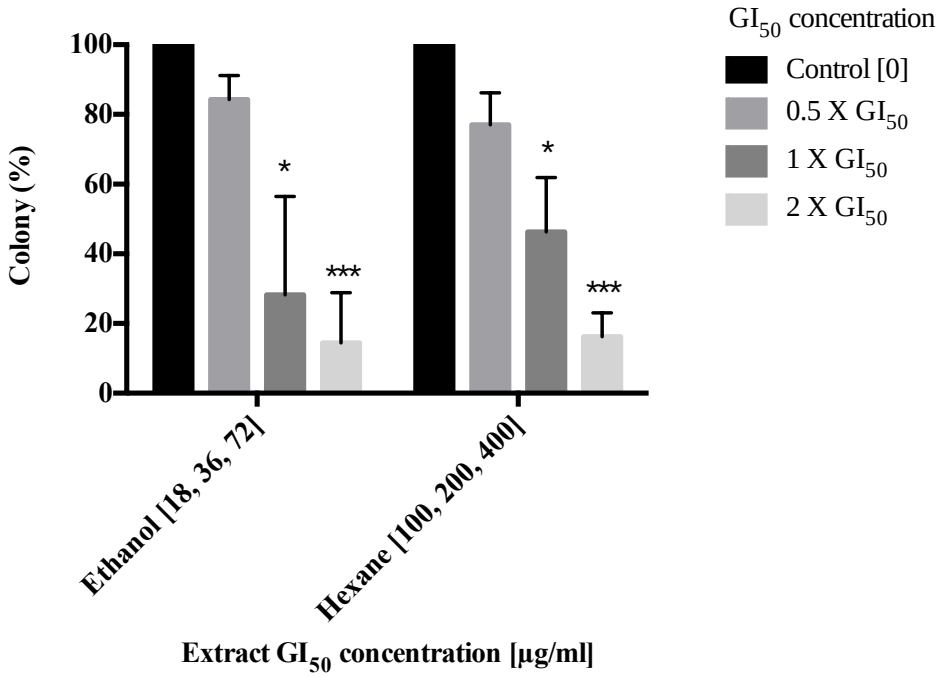


Figure 2-5: Effects of *A. wilkesiana* extracts to MDA-MB-468 cells' survival and clonogenicity. Bars (error bar = SEM) denote the mean % of HCT116 colonies formed after 24 h exposure to whole extracts from ≥ 3 (n = 4) individual experiments; plating efficiencies were ≥ 80% per experiment. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = $0.01 < p < 0.05$ and *** = $0.001 < p < 0.01$.

2.1.4 Influence of *Archichendron ellipticum* extracts to MDA-MB-468 cells' survival and clonogenicity

Dose-dependent inhibition of MDA-MB-468 colony formation was determined after 24 h exposure to *A. ellipticum* extracts (Figure 2-6). Constituents from bark EtOAc extractions appeared to be most active against breast cancer MDA-MB-468 cells with only 24% of treated cells retaining colony forming ability at 0.5X GI_{50}, which is significant compared to the control. Whereas EtOAc leaf extracts at ~ 5-fold less concentration than EtOAc bark extracts significantly inhibited colony formation. MDA-MB-468 cells' colony formation inhibition

ability was comparable at the same treatment range of EtOH and EtOAc leaf extracts (4.5, 9 and 18 µg/ml). Conversely, no significant difference in colony formation was observed when cells were exposed to EtOH and HEX bark extracts at 0.5X GI_{50} (77% and 88% control respectively; $p > 0.05$). Comparable numbers of MDA-MB-468 colonies (%) were counted after treatment with HEX extracts. Colony formation was affected by HEX leaf extract at high treatment concentrations (100, 200 and 400 µg/ml), which were up to 100-fold higher than other extracts used.

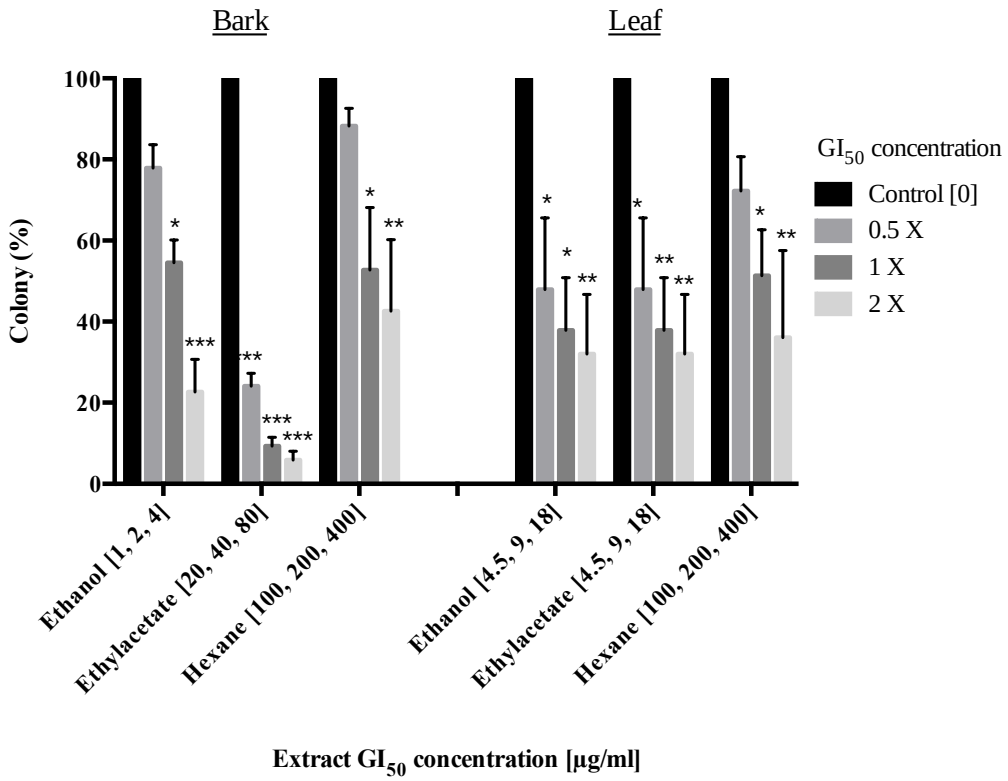


Figure 2-6: Effects of *A. ellipticum* extracts on MDA-MB-468 cells' survival and clonogenicity. Bars (error bar = SEM) denote the mean % of HCT116 colonies formed after 24 h exposure to bark and leaf extracts from ≥ 3 (n = 4) individual experiments; plating efficiencies were ≥ 80% per experiment. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = $0.01 < p < 0.05$, ** = $0.001 < p < 0.01$ and *** = $p < 0.001$.

Overall, bark and leaf EtOH and EtOAc extracts were most effective in inhibition of MDA-MB-468 colony formation with consideration of the low concentrations used (EtOH bark: 1, 2, and 4 µg/ml; EtOH leaf: 4.5, 9 and 18

μg/ml; EtOAc leaf: 4.5, 9 and 18 μg/ml), and verified to be statistically different from colony-forming ability of DMSO-treated control cells ($p < 0.05$). Again, a general dose-dependent trend of decreased colony formation with increasing extract concentration was observed. All treatments of *A. ellipticum* EtOAc extracts affected MDA-MB-468 cells' ability to proliferate ($p < 0.05$).

2.1.5 Influence of *Duabanga grandiflora* extracts on HCT116 cells' survival and clonogenicity

The effect of *D. grandiflora* extracts on HCT116 colony formation is shown in Figure 2-7. Polar Water and EtOH extractions appeared to be the most effective in inhibiting HCT116 cell survival and clonogenicity in comparison to EtOAc and HEX extracts.

Significant inhibition of colony formation was noted with 1X and 2X GI_{50} treatments of polar bark and leaf extracts, revealing > 90% colony formation inhibition. Overall, greatest cell response was noticed with *D. grandiflora* EtOH extracts at all concentrations tested and verified to be statistically different to the DMSO control cells ($p < 0.001$). HCT116 cells responded in similarly to both HEX extracts in regards to the number of colonies formed, however concentrations of HEX bark extract (14.5, 29 and 58 μg/ml) used were almost 7-fold lower than that of HEX leaf extract (100, 200 and 400 μg/ml). Therefore, polar extracts demonstrated greatest colony formation inhibitory effects on HCT116 cells.

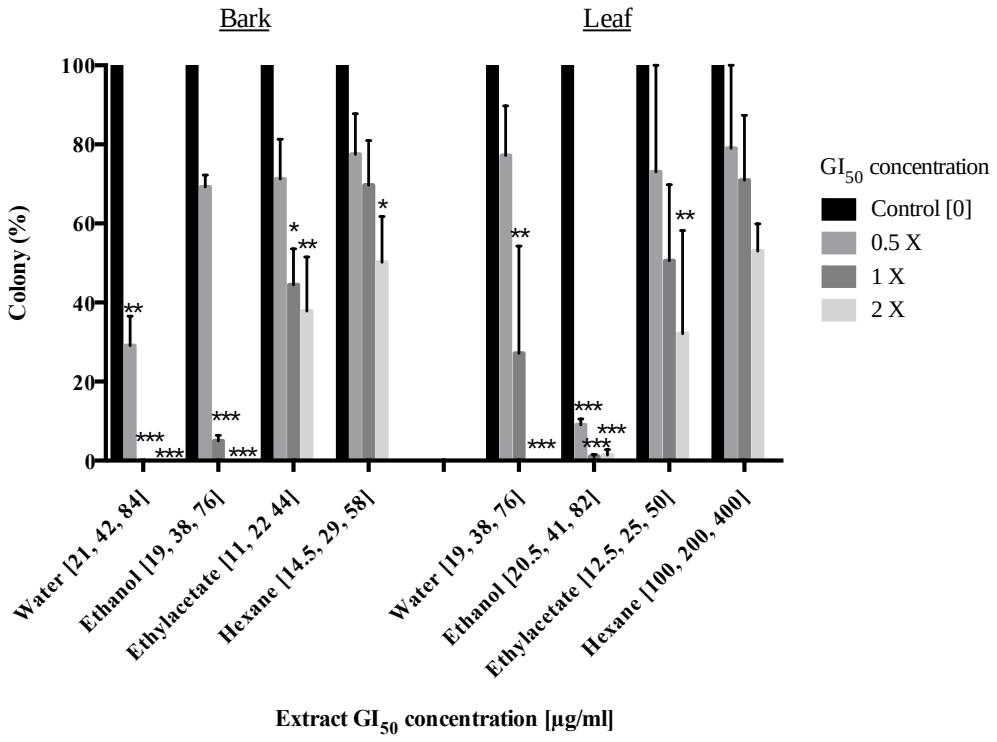


Figure 2-7: Effects of *D. grandiflora* extracts to HCT116 cells' survival and clonogenicity. Bars (error bar = SEM) denote the mean % of HCT116 colonies formed after 24 h exposure to bark and leaf extracts from ≥ 3 (n = 4) individual experiments; plating efficiencies were ≥ 80% per experiment. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = 0.01< p < 0.05, ** = 0.001 < p < 0.01 and *** = p < 0.001.

2.1.6 Influence of *Pseuduvaria macrophylla* extracts to HCT116 cells' survival and clonogenicity

The effects of *P. macrophylla* extracts on HCT116 colony formation after 24 h exposure are summarised in Figure 2-8. Both *P. macrophylla* EtOH and EtOAc extracts significantly inhibited HCT116 colony formation ($P < 0.001$) at 0.5X GI_{50} (47.35% and 42.87% colony counted, respectively), 1X GI_{50} (2.75% and 3.33% counted, respectively) and 2X GI_{50} (0.75% and 0.50% counted, respectively). Contrastingly, there was no evidence to suggest HEX extract induced significant inhibition of HCT116 colony formation at the concentrations tested at 2.5 µg/ml. Effects of HEX extract at 1X and 2X GI_{50} (5 and 10 µg/ml) revealed only a ~ 5% difference in the number of colonies formed. Whereas a greater ~ 40% difference in colony formation inhibition

was observed between EtOH and EtOAc extracts treated cells at 1X and 2X GI_{50}.

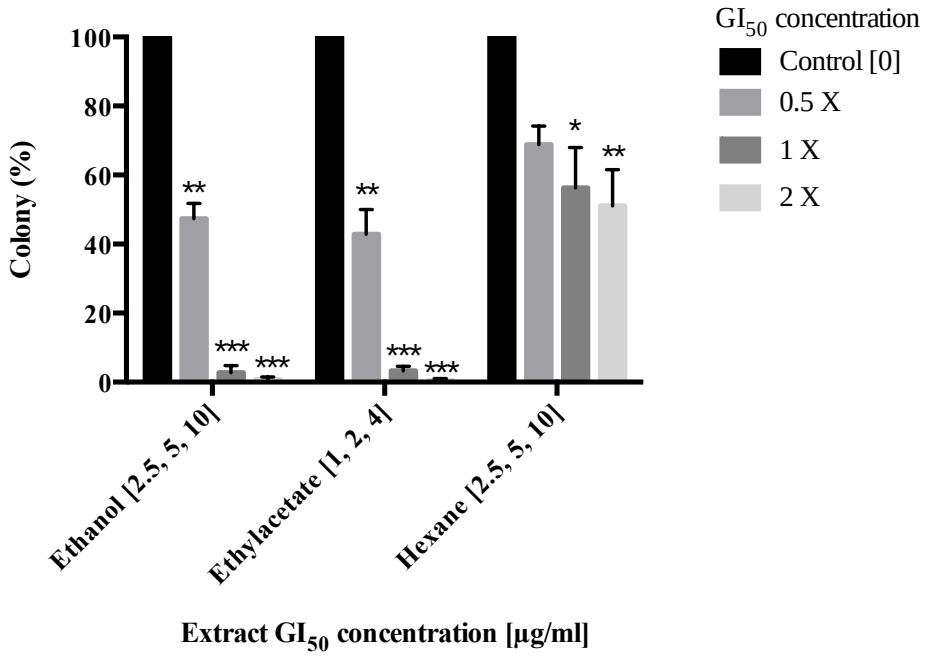


Figure 2-8: Effects of *P. macrophylla* extracts on HCT116 cells' survival and clonogenicity. Bars (error bar = SEM) denote the mean % of HCT116 colonies formed after 24 h exposure to whole extracts from ≥ 3 (n = 4) individual experiments; plating efficiencies were ≥ 80% per experiment. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = $0.01< p < 0.05$, ** = $0.001 < p < 0.01$ and *** = $p < 0.001$.

2.1.6.1 Summary of the effects of UNMC extracts on cancer cell colony formation

The clonogenic assay examined the *in vitro* anti-proliferative and clonogenicity of human-derived cancer cells post exposure to UNMC extracts, hence reveals the ability of individual cancer cells to survive UNMC extract challenge (24 h) and form colonies post-treatment, hence repopulate a tumour. HCT116 cells were exposed to *D. grandiflora* and *P. macrophylla* extracts, whereas MDA-MB-468 cells were treated with *A. wilkesiana* and *A. ellipticum* extracts for reasons stated earlier. Although significant inhibition of colony formation was observed in several extracts, further experiments are necessary to conclude whether the growth inhibition is a consequence of cytostasis or cytotoxicity.

Acalypha wilkesiana: EtOH and HEX extracts at 1X and 2X GI_{50} significantly inhibited > 50% MDA-MB-468 colonies, however, HEX extract required concentrations of > 5-fold higher than that of EtOH used to achieve similar colony formation inhibitory effects. Significant colony formation inhibitory effect following exposure of cells to EtOH extracts is consistent with irreversible cytotoxicity. EtOH extract concentration inhibited MDA-MB-468 colony formation by 50% (IC_{50}) at 35.51 µg/ml, therefore comparable to the GI_{50} value (35.98 µg/ml) obtained in the MTT assay.

***Archidendron ellipticum*:** Remarkably, EtOH extracts induced good inhibitory effects in MDA-MB-468 cells at 20-fold and 100-fold less concentration than EtOAc and HEX extracts, respectively. The presence of EtOAc extract resulted in fewest colonies formed (5.8 %) suggesting EtOAc possesses irreversible growth inhibitory (cytotoxic) effects (seen in MTT assay). Although, HEX extracts of *A. ellipticum* bark and leaf demonstrated modest anti-proliferative activity in MDA-MB-468 cells, the concentrations used were very high. At higher concentrations (1X and 2X GI_{50}), cells treated with bark and leaf polar extracts failed to recover suggesting irreversible cytotoxic effects; the cells may have died or failed to proliferate and remained as a single cell. The IC_{50} (µg/ml) values for EtOH, EtOAc and HEX bark extracts were 13.31, 18.93 and 69.78, respectively; EtOH, EtOAc and HEX leaf extracts IC_{50} values were 2.71, 61.22 and > 200, respectively. These values varied slightly from those obtained from the MTT assay. The difference in IC_{50} values suggests that HEX bark extract of *A. ellipticum* may not affect cell viability even at high concentrations yet cells' ability to proliferate and recover after treatment was affected, which could lead to senescence.

***Duabanga grandiflora*:** Inhibitory effects of *D. grandiflora* bark extracts in order of highest activity (lowest IC_{50}) on HCT116 colony formation was Water >EtOH >EtOAc >HEX. EtOH leaf extract showed the highest anti-proliferative activity even at a low concentration (20.5 µg/ml, 0.5X GI_{50}) and colony forming ability was almost completely inhibited by EtOH extracts at 1X and 2X GI_{50}. The presence of significantly fewer colonies with surviving cells

and maintained proliferative capacity after initial insult with polar extracts may suggest extracts to contain compounds, which exerts irreversible cytotoxic effects. Intriguingly, at 1X and 2X GI_{50} treatment of bark and leaf polar extracts, HCT116 cells failed to recover suggesting irreversible cytotoxic effects; cells may have died or remained as a single cell. The IC_{50} values for Water, EtOH, EtOAc, and HEX bark extracts were 15.02, 25.49, 16.47 and 18.09 μg/ml, respectively; these values are comparable to MTT GI_{50} values: 42.44, 37.54, 21.69 and 28.77 μg/ml, respectively. The IC_{50} values for Water, EtOH, EtOAc and HEX leaf extracts were 40.69, 16.95, 16.48 and > 200 μg/ml were also comparable to leaf GI_{50}: 38.02, 40.92, 24.73 and > 200 μg/ml.

***Pseuduvaria macrophylla*:** Both *in vitro* assays revealed the same order of potency of *P. macrophylla* extracts against HCT116 cells (EtOAc > EtOH > HEX) based on GI_{50} (1.61, 5.16 and 5.41 μg/ml) and IC_{50} values (0.33, 2.24 and 3.57 μg/ml, respectively). Less than 10% of cells were able to form colonies after exposure to EtOH and EtOAc extracts at 1X and 2X GI_{50} and modest anti-proliferative activity was observed in HEX extract. Results illustrated EtOH and EtOAc extracts induced significant inhibitory effects and the growth inhibitory effects of extracts observed in MTT assay were irreversible. Comparable EtOH and EtOAc extracts-treated HCT116 cell proliferation profiles suggest that similar phytochemicals may be embedded in these extracts. The anticancer activity of 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine (38) has been previously reported to be active against NCI-H187 (GI_{50}: 5.6 μg/ml). Interestingly, a similar dioxoaporphine with *N*-methyl substitution have shown increased activity against NCI-H187 cells (GI_{50}: 0.8 μg/ml).[214] Aristolochic acids and aristolactam share distinct similarity to the chemical skeleton of aporphinoids are known immunuostimulants and anticancer agents.[214]

In summary, greatest anti-proliferative effects observed with low extract concentration used resides with *D. grandiflora* bark and leaf EtOH and Water, *A. ellipticum* bark EtOAc and *P. macrophylla* whole EtOH and EtOAc extracts. Extracts active in both MTT and clonogenic assays suggest cells' viability is affected and therefore their ability to proliferate and recover after exposure.

Results illustrating modest activity in the MTT assay (~ 20 μg/ml to inhibit 50% of cell growth) yet high activity (almost complete colony formation inhibition) in the clonogenic assay could be due to the extracts' ability to induce irreversible anti-proliferative properties without affecting cells' viability e.g. *A. wilkesiana* EtOH, *A. ellipticum* bark EtOAc, *D. grandiflora* polar bark and leaf (Water and EtOH) extracts. Therefore, single viable cells with inhibited proliferative abilities could be present but fail to be detected, which may be the effects of more polar *D. grandiflora* bark EtOH and Water extracts. Therefore, cytotoxic and cytostatic effects of the extracts could be distinguished by comparing results from MTT and clonogenic assays; furthermore, if MTT results reveal a lower cell density than the seeding density it could imply a cytotoxic effect.

Overall, the IC_{50} values and GI_{50} values vary but are not too dissimilar and this may be due to low seeding densities, however the values still reveal anti-proliferative effects of UNMC extracts and a trend of decreased colony formation ability with increasing extract concentration.

Flow cytometric techniques were adopted to further investigate the observations of UNMC extracts on sensitive cell lines to determine whether embedded compounds halt cell cycle or exert cytotoxicity.

3

The influence of UNMC crude extracts on cell cycle and apoptosis induction

Flow cytometry is a widely used technique where single cell particles in sheath fluid are delivered rapidly to a focused light source (laser beam). The fluorescence or photons scattered by particles (e.g. cells sorted by their DNA content) are deflected at different angles, then pass through a series of optical filters, are captured by photomultiplier detectors, converted to digital signals, (that are proportional to the strength of emitted or deflected light) and sent to the computer.[91]

Agents that are effective modulators of cell cycle (checkpoints) can drive cells into apoptosis and potentially have a role on carcinogenesis.[246] It is now evident that anticancer agents can exert their activity by interfering with cell cycle mechanisms; camptothecin, etoposide and doxorubicin are S-phase blockers and inhibit cyclin-dependent kinases (CDKs).[247] One common application of flow cytometry is the measurement of DNA content to understand cell cycle distributions. Fluorescent stains such as propidium iodide (PI) bind stoichiometrically to nucleic acids and emit enhanced signals that can be detected by the instrument.[248] The detection of DNA-bound PI hence DNA content (hypodiploid, diploid, hyperdiploid and tetraploid compared to control cells) can reveal the cell cycle phase of the cell population i.e. sub-G1, G1/0, S and G2/M, respectively. Any disruptions in the DNA histograms (representative of the cell cycle) can be indicative of investigative agents having an (cytotoxic or growth arresting) effect on the cell cycle.[249]

3.1 UNMC plant extracts effects' on cell cycle distribution

The effect of UNMC plant extracts on cell cycle distribution was measured after 48 h exposure, using an Epics XL-MCL flow cytometer. Cells and treatment concentrations chosen to examine effects of UNMC extracts on cell cycle distribution were guided by results from the selection assays, and were 1X, 2X and 4X GI_{50} concentrations. The samples were kept overnight in a solution containing buffer, detergent and ribonuclease A, prior to analysis. The results were established from three or more individual experiments ($\geq$ 15,000 cells per sample, n = 2 per experiment). Data are represented as histograms and processed using EXPO32 software. Further analyses of cell cycle data were conducted using cell cycle analysis software, *Cylchred*. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = $0.01 < p < 0.05$. Regretfully, the investigation of *A. wilkesiana* EtOAc extract could not be examined due to a lack of supply.

3.1.1 *Acalypha wilkesiana* extracts' effects on MDA-MB-468 cell cycle distribution

MDA-MB-468 cell populations detected in sub-G1 phase increased with *A. wilkesiana* extracts concentrations, which is indicative of apoptotic cell death (Table 3-1). Following 48 h exposure to EtOH and HEX extracts at 4X GI_{50}, the cell population in sub-G1 population (2.7% and 2.3%, respectively) was at similar levels as control cells (2.4%) accompanied by a slight decrease in the G2/M population.

Table 3-1: Cell cycle distribution of MDA-MB-468 cells after exposure to *A. wilkesiana* extracts.

***A. wilkesiana* extract concentration GI_{50} value** [μg/ml]		**Cell cycle phase**			
		Sub-G1	G1/0	S	G2/M
DMSO control	[0]	2.4 ± 0.3	45.1 ± 5.3	15.1 ± 1.6	36.9 ± 3.4
EtOH	1X [36]	1.6 ± 0.0	49.6 ± 1.0	15.8 ± 1.0	32.7 ± 1.3
	2X [72]	3.8 ± 1.2	48.2 ± 8.8	17.7 ± 7.05	30.3 ± 0.6
	4X [144]	2.7 ± 12.3	47.1 ± 5.5	15.4 ± 1.2	34.4 ± 5.8
HEX	1X [200]	3.0 ± 1.0	45.4 ± 17.8	17.8 ± 1.50	33.2 ± 2.7
	2X [400]	4.9 ± 0.0	43.0 ± 0.0	18.4 ± 0.0	34.0 ± 0.0
	4X [800]	2.3 ± 1.3	42.1 ± 0.1	16.8 ± 0.8	39.4 ± 2.6

Mean MDA-MB-468 cell population (%) in each cell cycle phase (± SEM) obtained from DNA content histograms analysis generated by flow cytometry. Control (DMSO treated) samples were compared with extract-treated groups using Dunnett's multiple comparison (One-way ANOVA) analysis. Any statistically significant values ($p < 0.05$) are highlighted **bold**.

Figure 3-1 illustrates that at lower concentrations (1X and 2X $GI_{50:}$ 36 and 72 μg/ml, respectively) of EtOH extracts, the % of cell population in sub-G1, G1/0, S and G2/M were comparable to the control cells. The % of MDA-MB-468 cell population in each of the cell cycle phases of control cells were comparable to HEX extract-treated cells at very high concentrations (200, 400 and 800 μg/ml), as shown in Figure 3-2. There seem to no overall significant effects induced by extracts on MDA-MB-468 cells cycle.

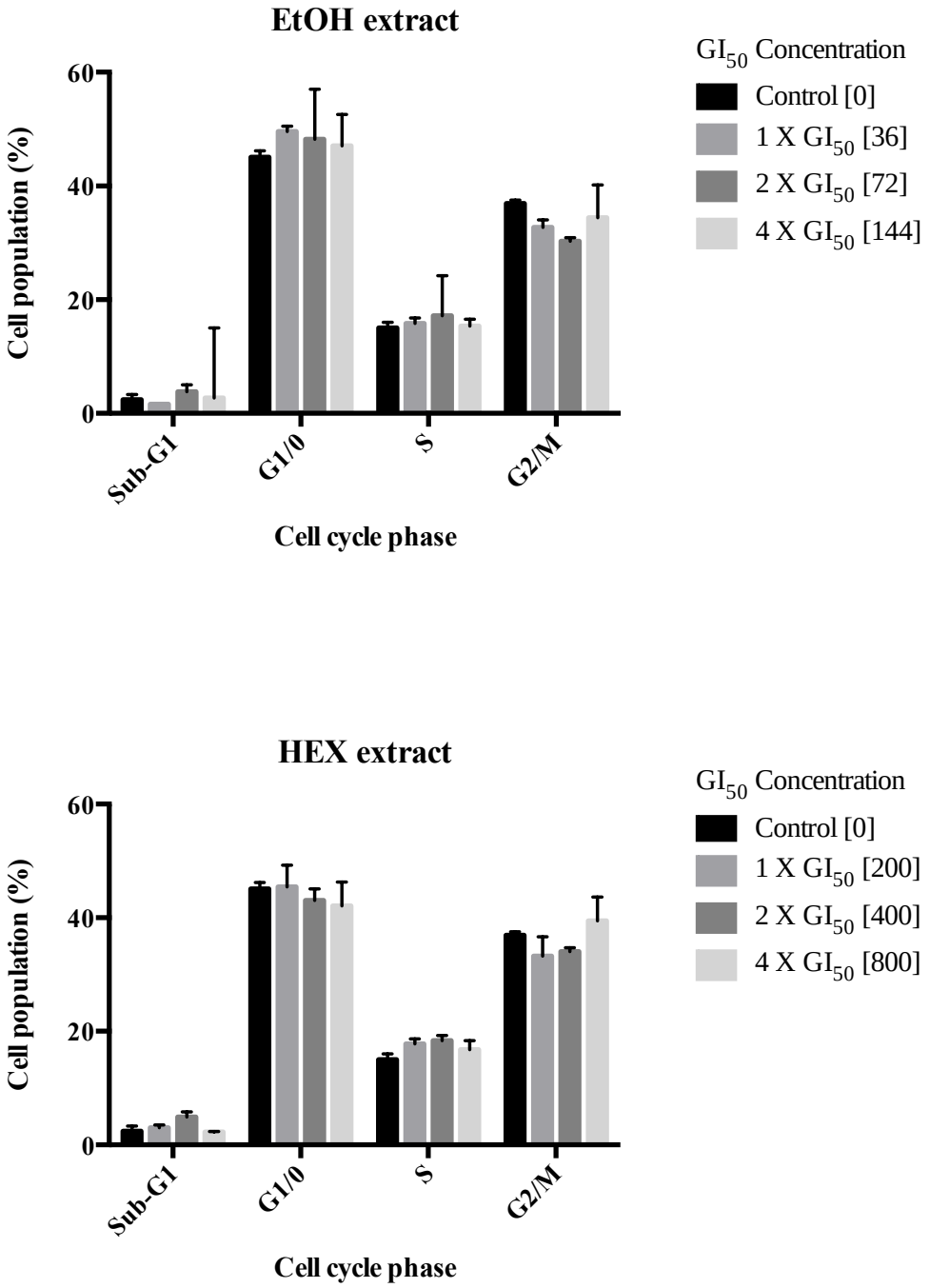


Figure 3-1: MDA-MB-468 cell cycle distributions after 48-hour exposure to *A. wilkesiana* extracts. Bars (error bar = SEM) denote the mean cell population (%) in each cell cycle phase after exposure to EtOH (A) and HEX (B) extracts from ≥ 4 (n = 2) individual experiments. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p < 0.05$).

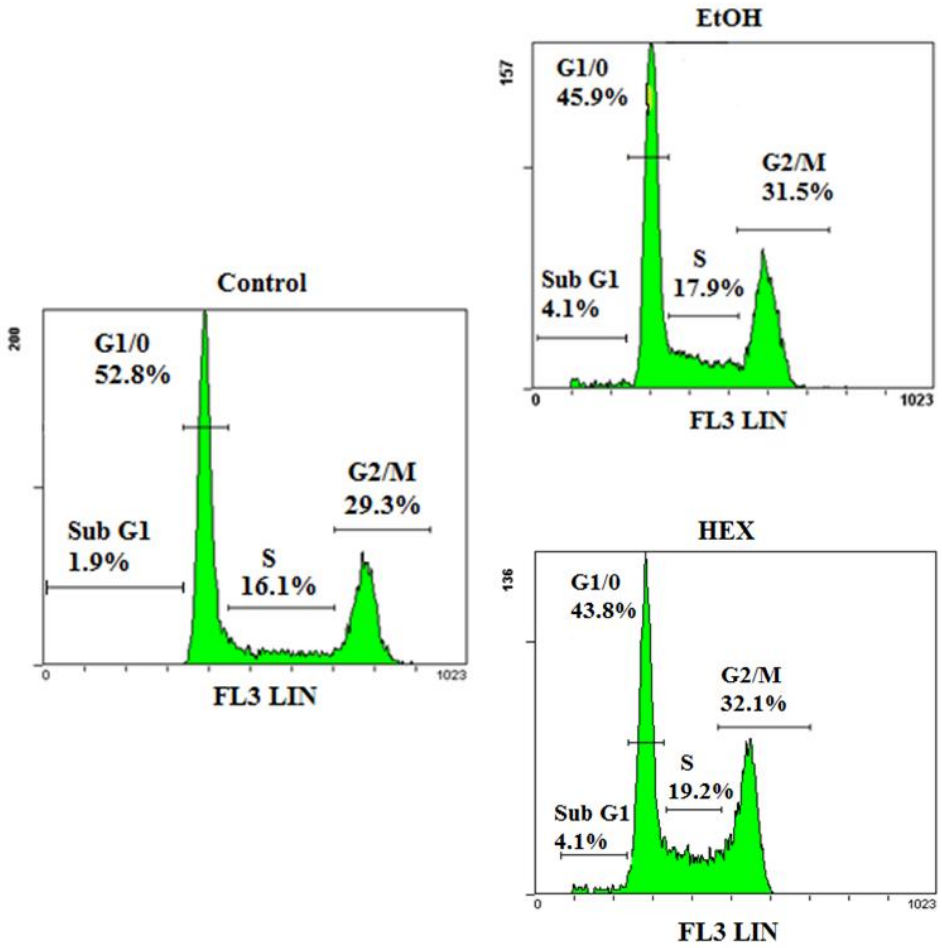


Figure 3-2: Detection of PI staining of MDA-MB-468 cellular DNA content after exposure to *A. wilkesiana* extracts at 4X GI_{50} concentration for 48 hour. Data is represented as histogram of cellular DNA content revealing % events in each cell cycle phase; images from a single representative experiment are shown. The samples were kept chilled overnight in a solution containing buffer, detergent and ribonuclease A prior to analysis. The results were established from three ≥ 15,000 cells per sample and processed using EXPO32 software. Image is representative of cell cycle distribution of one sample following treatment with UNMC extract as described above.

3.1.2 *Archidendron ellipticum* extracts' effects on MDA-MB-468 cell cycle distribution

MDA-MB-468 cell cycle distribution after exposure to *A. ellipticum* extracts varied between extracts and concentrations used, shown in Table 3-2. Flow cytometric analysis of cells exposed to EtOH bark extract revealed increased G2/M cell population (≥ 2-fold), which coincided with significant decrease in G1/0 cell populations ($p < 0.05$), while no comparable changes were noticed in sub-G1 and S phases ($p > 0.05$). Exposure of MDA-MB-468 cells to high concentrations of EtOAc bark extract (80 and 160 μg/ml) caused increased sub-G1 events by 3.8% and 8.6%, respectively, accompanied by decreased G1/0 cell populations compared to control cells (Figure 3-3). There is insufficient evidence to suggest changes in G1/0, S and G2/M cell cycle phases were influenced by EtOAc bark extract ($p > 0.05$). Concentrations of HEX bark and leaf extracts used were very high and appeared unable to cause perturbation to the distribution of the cell cycle.

Interestingly, EtOH bark and leaf extracts displayed similar trends where increased levels of sub-G1 and S phase cell populations were accompanied by a significant decrease in G1/0 and G2/M phase cell populations ($p < 0.05$). The slight changes in cell cycle distribution of EtOAc leaf extract were statistically insignificant when compared to the control cells. Again, at very high concentrations of HEX leaf extracts (193 to 772 μg/ml), no significant cell cycle distribution perturbation was observed with HEX leaf treated cells (Figure 3-4). Overall, comparable cell cycle distributions were observed following exposure to *A. ellipticum* extracts; therefore it is probable that similar extracts are present in the respective extracts.

Table 3-2: Cell cycle distribution of MDA-MB-468 cells after exposure to *A. ellipticum* extracts

	***A. ellipticum* extract concentration GI_{50} value [µg/ml]**		**Cell cycle phase**			
			Sub-G1	**G1/0**	**S**	**G2/M**
Bark	**DMSO control**	[0]	2.2 ± 0.3	50.0 ± 1.7	32.1 ± 1.0	15.7 ± 1.4
	EtOH	1X [2]	2.6 ± 0.4	**35.6 ± 2.3**	35.0 ± 2.4	**26.8 ± 0.8**
		2X [4]	2.6 ± 0.5	**33.1 ± 2.1**	36.9 ± 3.1	**27.4 ± 1.2**
		4X [8]	3.5 ± 1.1	**33.0 ± 3.0**	36.1 ± 2.7	**27.4 ± 2.4**
	EtOAc	1X [40]	3.9 ± 0.8	47.5 ± 2.2	32.3 ± 1.2	16.3 ± 0.5
		2X [80]	6.0 ± 1.1	44.2 ± 2.6	32.5 ± 2.7	17.3 ± 1.0
		4X [160]	**10.8 ± 1.2**	43.3 ± 3.1	25.8 ± 3.3	20.1 ± 3.7
	HEX	1X [200]	3.1 ± 1.1	46.4 ± 4.2	31.7 ± 2.4	18.8 ± 1.7
		2X [400]	2.5 ± 0.4	48.7 ± 3.5	31.4 ± 2.4	17.6 ± 1.7
		4X [800]	2.8 ± 1.2	44.1 ± 3.9	37.2 ± 3.0	15.9 ± 1.2
Leaf	**DMSO control**	[0]	2.2 ± 0.3	50.0 ± 1.7	32.1 ± 1.0	15.7 ± 1.4
	EtOH	1X [9]	3.2 ± 1.7	**32.6 ± 2.7**	34.9 ± 2.7	22.4 ± 3.6
		2X [18]	3.7 ± 1.8	**33.3 ± 1.4**	35.9 ± 2.7	21.0 ± 3.4
		4X [36]	5.3 ± 1.7	**29.4 ± 1.5**	**41.2 ± 0.8**	**24.1 ± 0.9**
	EtOAc	1X [9]	4.0 ± 1.3	45.9 ± 4.5	33.9 ± 2.6	16.1 ± 1.1
		2X [18]	3.2 ± 0.4	50.6 ± 2.1	32.0 ± 1.7	14.3 ± 1.1
		4X [36]	3.0 ± 0.4	51.9 ± 2.0	31.0 ± 1.7	14.2 ± 0.4
	HEX	1X [193]	3.5 ± 1.2	51.6 ± 2.2	29.8 ± 2.4	15.0 ± 1.3
		2X [386]	3.8 ±0.9	50.6 ± 2.6	30.6 ± 3.2	15.0 ± 0.6
		4X [772]	4.7 ± 1.4	52.8 ± 2.4	27.5 ± 2.5	15.0 ± 1.0

Mean MDA-MB-468 cell population (%) in each cell cycle phase (± SEM) obtained from DNA content histograms analysis generated by flow cytometry. Control (DMSO treated) samples were compared with extract-treated groups using Dunnett's multiple comparison (One-way ANOVA) analysis. Any statistically significant values ($p < 0.05$) are highlighted **bold**.

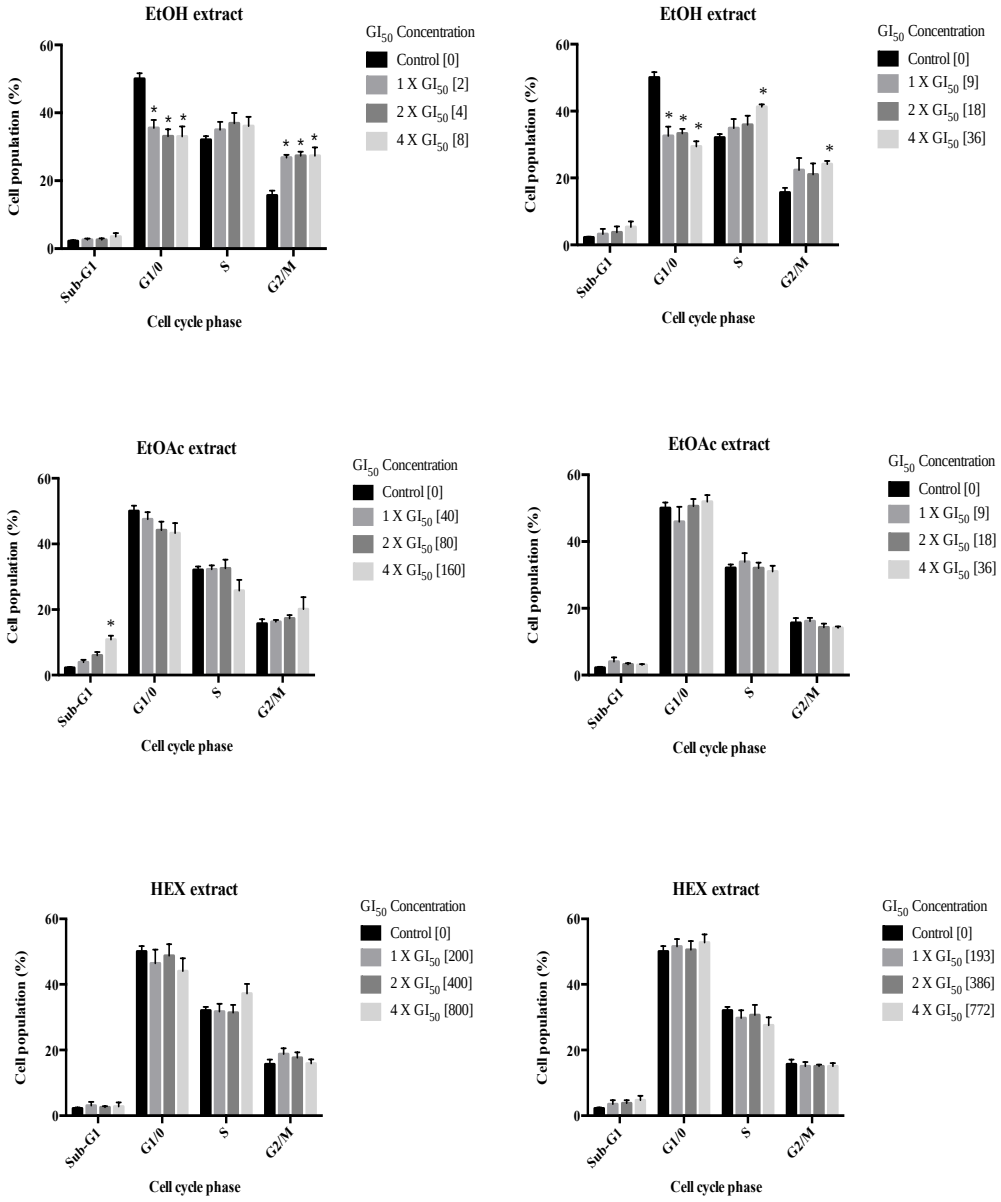


Figure 3-3: MDA-MB-468 cell cycle distributions after 48-hour exposure to *A. ellipticum* extracts. Bars (error bar = SEM) denote the mean cell population (%) in each cell cycle phase after exposure to EtOH, EtOAc and HEX extracts from ≥ 4 (n = 2) individual experiments. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p < 0.05$).

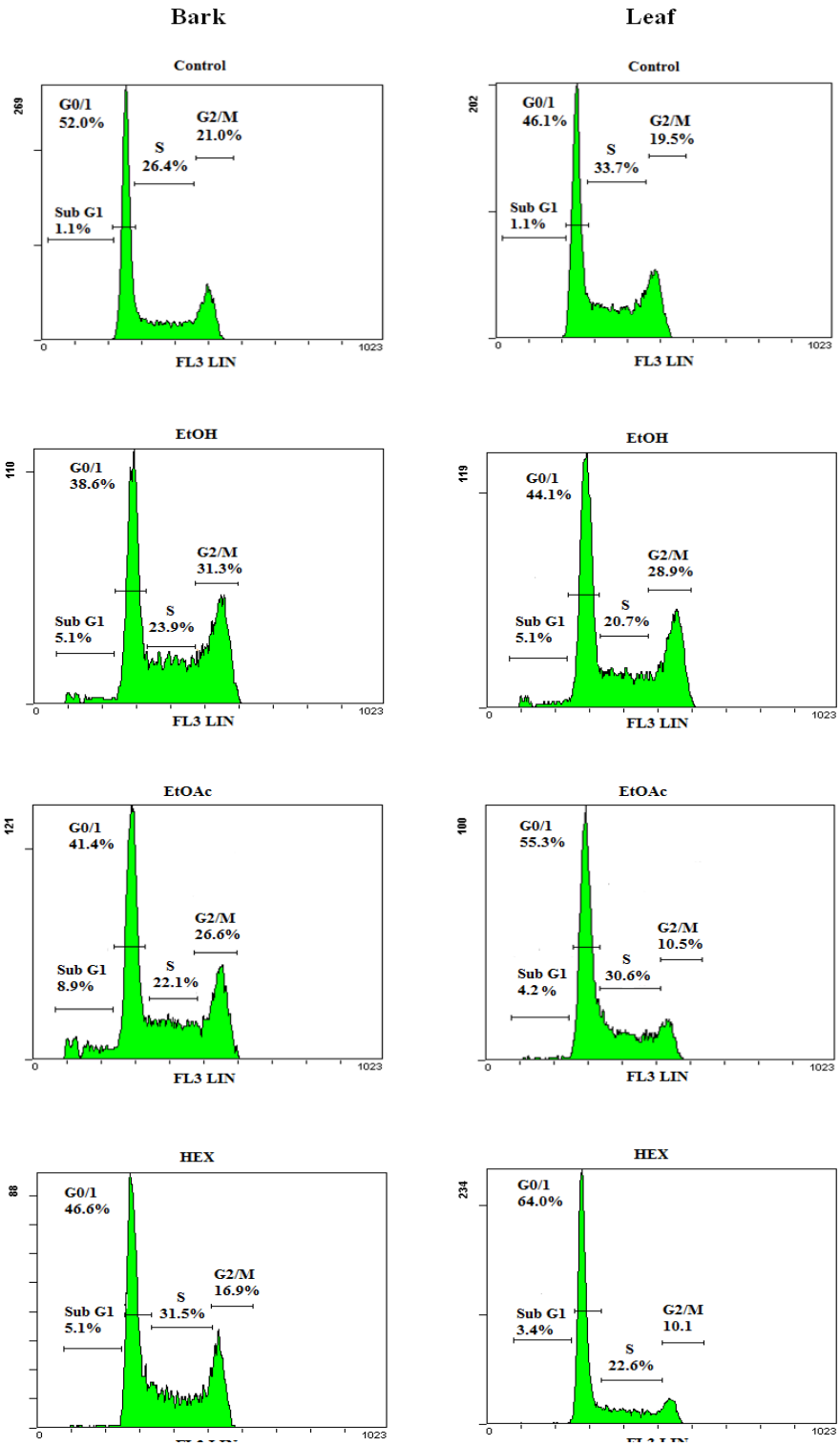


Figure 3-4: Detection of PI staining of MDA-MB-468 cellular DNA content after exposure to *A. ellipticum* extracts at 4X GI_{50} concentration for 48 hour. Data is represented as histogram of cellular DNA content revealing % events in each cell cycle phase; images from a single representative experiment are shown. The samples were kept chilled overnight in a solution containing buffer, detergent and ribonuclease A prior to analysis. Results were established from three ≥ 15,000 cells per sample and processed using EXPO32 software. Image is representative of cell cycle distribution of one sample following treatment with UNMC extract as described above.

3.1.3 *Duabanga grandiflora* extracts' effects on HCT116 cell cycle distribution

HCT116 cell cycle distribution after exposure to *D. grandiflora* extracts varied between extracts and concentrations used shown in Table 3-3. The % of cells measured in sub-G1 phase following exposure to bark extracts (Water, EtOH, EtOAc and HEX) was significantly higher than the control populations and accompanied by decreased cell populations in S-phase. HCT116 cells treated with HEX bark extracts displayed cell cycle distribution that is statistically different to the control population, with at least 20%, of cells in sub-G1 ($p < 0.05$) and S-phase populations accompanied by decreasing cell populations in G1/0 and G2/M phases (Figure 3-5). EtOAc bark extract induced similar cell cycle response to HEX extracts in HCT116 cells.

Overall, HCT116 cells exposed to *D. grandiflora* bark extracts displayed general trends, which includes emerging events in sub-G1 phase, accumulation of G1/0 events accompanied by decreased S and G2/M phase events. Whereas, high HEX leaf extract concentrations (200, 400 and 800 μg/ml) caused a decrease in G2/M cell populations (12.6, 12.6 and 8.8 %). Cell cycle perturbation effects of *D. grandiflora* bark extracts were distinctly more prominent than leaf extracts in HCT116 cells. There was insufficient evidence to suggest that cells treated with Water, EtOH and EtOAc leaf extracts induced any significant perturbations in cell cycle compared with the control population. Overall, *D. grandiflora* leaf extracts appeared to positively influence sub-G1 and G1/0 populations accompanied by decreasing HCT116 cells in S and G2/M phases.

Table 3-3: Cell cycle distribution of HCT116 cells after exposure to *D. grandiflora* extracts.

	***D. grandiflora* extract concentration GI_{50} value [µg/ml]**		**Cell cycle phase**			
			Sub-G1	**G1/0**	**S**	**G2/M**
Bark	**DMSO control**	[0]	0.9 ± 0.1	37.9 ± 3.4	41.8 ± 2.31	19.3 ± 1.9
	Water	1X [42]	1.9 ± 0.4	**51.4 ± 2.3**	**31.5 ± 2.1**	**15.2 ± 1.8**
		2X [82]	2.3 ± 0.5	**47.8 ± 3.5**	**33.0 ± 1.8**	16.9 ± 1.5
		4X [168]	2.9 ± 1.0	43.1 ± 6.3	36.4 ± 4.4	17.6 ± 2.0
	EtOH	1X [38]	2.1 ± 0.4	47.2 ± 3.2	**34.9 ± 1.8**	15.8 ± 1.9
		2X [76]	3.9 ± 1.3	40.7 ± 5.0	**36.9 ± 1.9**	18.5 ± 2.7
		4X [152]	3.1 ± 0.8	39.5 ± 6.7	41.6 ± 6.1	15.8 ± 0.9
	EtOAc	1X [22]	2.9 ± 0.7	46.1 ± 4.2	34.1 ± 3.0	16.9 ± 1.9
		2X [44]	4.8 ± 1.2	49.9 ± 4.5	31.9 ± 5.0	13.4 ± 1.1
		4X [88]	5.4 ± 1.9	**60.7 ± 2.3**	**22.3 ± 2.9**	**11.1 ± 2.1**
	HEX	1X [29]	**4.1 ± 0.9**	57.7 ± 3.5	27.3 ± 3.4	**10.9 ± 1.0**
		2X [58]	**9.0 ± 1.6**	57.8 ± 4.3	**23.4 ± 4.1**	**9.7 ± 0.6**
		4X [116]	**8.5 ± 1.9**	**60.1 ± 3.4**	**23.1 ± 3.0**	**8.2 ± 0.9**
Leaf	**DMSO control**	[0]	1.1 ± 0.3	43.7 ± 2.3	35.6 ± 2.6	19.6 ± 1.6
	Water	1X [38]	1.3 ± 0.2	**50.7 ± 1.5**	33.1 ± 2.5	**5.0 ± 2.1**
		2X [76]	1.6 ± 0.4	**49.1 ± 1.9**	33.1 ± 2.5	6.0 ± 2.1
		4X [152]	1.7 ± 0.3	47.6 ± 3.8	33.5 ± 2.6	7.2 ± 2.6
	EtOH	1X [41]	2.7 ± 0.9	48.0 ± 1.9	31.0 ± 1.0	8.4 ± 1.8
		2X [82]	3.6 ± 1.2	36.0 ± 4.5	42.6 ± 5.8	7.8 ± 2.3
		4X [164]	4.7 ± 1.3	33.5 ± 4.5	41.8 ± 4.7	0.0 ± 2.1
	EtOAc	1X [25]	1.3 ± 0.2	50.2 ± 2.1	33.5 ± 2.0	5.1 ± 1.5
		2X [50]	**2.6 ± 0.6**	**56.0 ± 3.2**	27.6 ± 1.9	3.9 ± 1.3
		4X [100]	**3.5 ± 0.3**	48.4 ± 3.9	30.7 ± 4.1	17.5 ± 1.4
	HEX	1X [200]	1.5 ± 0.5	51.7 ± 2.9	34.3 ± 2.0	2.6 ± 1.3
		2X [400]	1.2 ± 0.3	**58.0 ± 4.2**	28.1 ± 3.5	2.6 ± 3.0
		4X [800]	1.8 ± 0.4	**64.7 ± 4.9**	24.7 ± 3.5	**.8 ± 1.7**

Mean HCT116 cell population (%) in each cell cycle phase (± SEM) obtained from DNA content histograms analysis generated by flow cytometry. Control (DMSO treated) samples were compared with extract-treated groups using Dunnett's multiple comparison (One-way ANOVA) analysis. Any statistically significant values ($p < 0.05$) are highlighted **bold**.

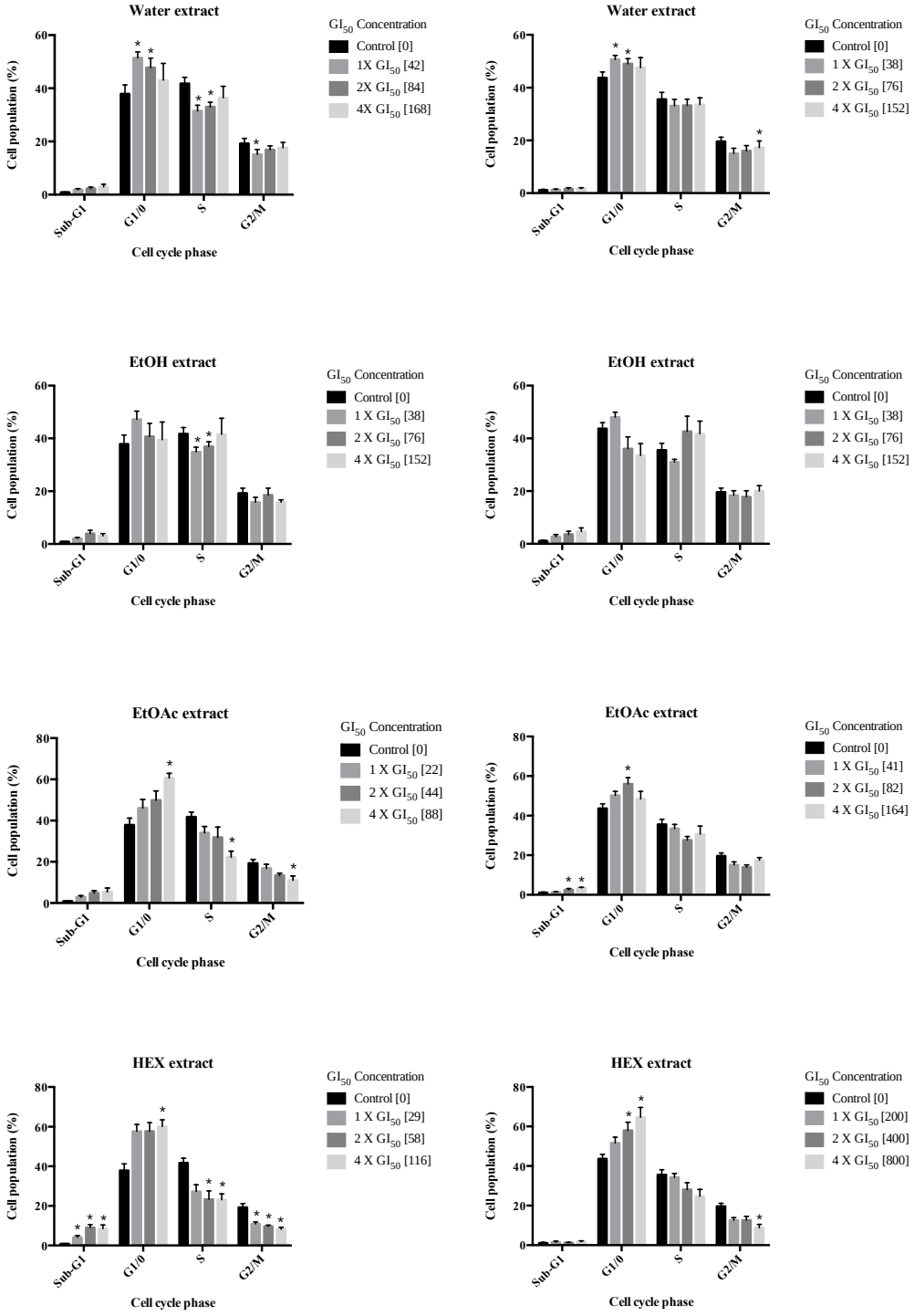


Figure 3-5: HCT116 cell cycle distributions after 48-hour exposure to *D. grandiflora* extracts. Bars (error bar = SEM) denote the mean cell population (%) in each cell cycle phase after exposure to Water, EtOH, EtOAc and HEX extracts from ≥ 4 (n = 2) individual experiments. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p < 0.05$).

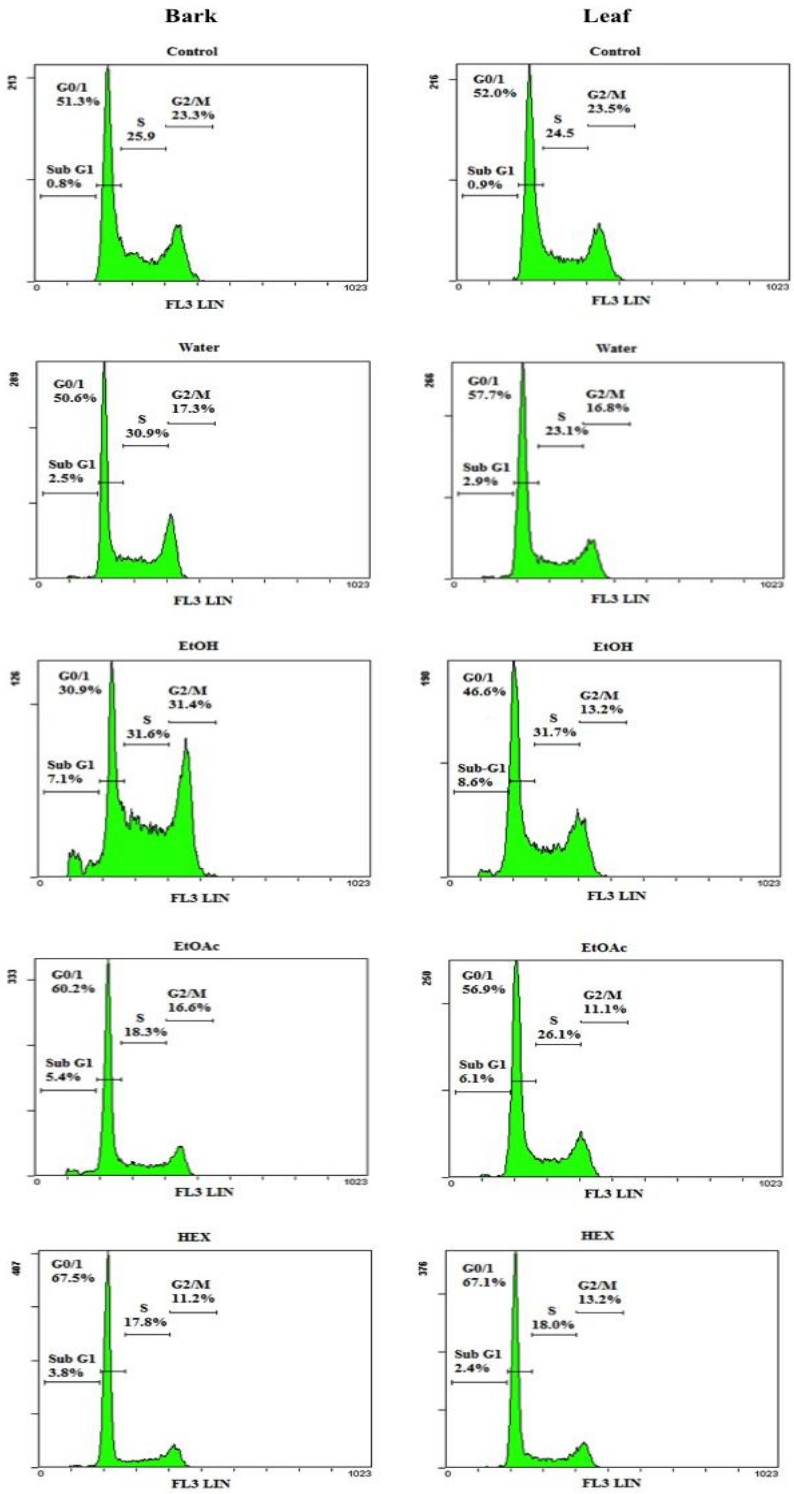


Figure 3-6: Detection of PI staining of HCT116 cellular DNA content after exposure to *D. grandiflora* extracts at 4X GI_{50} concentration for 48 hour. Data is represented as histogram of cellular DNA content revealing % events in each cell cycle phase; images from a single representative experiment are shown. The samples were kept chilled overnight in a solution containing buffer, detergent and ribonuclease A prior to analysis. The results were established from three ≥ 15,000 cells per sample and processed using EXPO32 software. Image is representative of cell cycle distribution of one sample following treatment with UNMC extract as described above.

3.1.4 *Pseuduvaria macrophylla* extracts' effects on HCT116 cell cycle distribution

The change in HCT116 cell distribution after exposure to *P. macrophylla* extracts is illustrated in Table 3-4. Flow cytometric analysis of HCT116 cells treated with *P. macrophylla* extracts revealed a significant rise in sub-G1 populations of ~ 1% - 8 % compared to control population (0.9 %), shown in Figure 3-7. In general, cells exposed to low concentrations of EtOH, EtOAc and HEX extracts displayed increased G2/M cell populations accompanied by a decreasing G1/0 cell population. Moreover, the level of sub-G1 events is significantly higher than the control when exposed to higher extract concentrations ($p < 0.05$). Overall, similar cell cycle distribution patterns were observed across *P. macrophylla* extract-treated HCT116 cells (Figure 3-8).

Table 3-4: Cell cycle distribution of HCT116 cells after exposure to *P. macrophylla* extracts

***P. macrophylla* extract concentration GI_{50} value [µg/ml]**		**HCT116 cell cycle phase**			
		Sub-G1	G1/0	S	G2/M
DMSO control	[0]	0.9 ± 0.1	37.6 ± 1.7	39.5 ± 0.7	22.1 ± 0.9
EtOH	1X [5]	**2.2 ± 0.3**	44.9 ± 3.7	35.0 ± 1.35	17.9 ± 2.1
	2X [10]	**9.0 ± 0.0**	29.2 ± 0.9	**33.0 ± 0.6**	28.8 ± 0.4
	4X [20]	**8.4 ± 0.2**	**25.7 ± 0.9**	42.2 ± 1.3	23.7 ± 0.1
DMSO control	[0]	2.1 ± 0.6	41.1 ± 2.5	38.4 ± 1.4	18.4 ± 2.2
EtOAc	1X [2]	3.4 ± 0.9	**25.3 ± 4.0**	44.7 ± 3.5	26.6 ± 2.7
	2X [4]	5.5 ± 1.0	**22.4 ± 2.1**	37.7 ± 3.8	34.4 ± 5.2
	4X [8]	**7.4 ± 0.9**	25.8 ± 3.0	41.1 ± 2.6	25.7 ± 3.0
DMSO control	[0]	2.1 ± 0.6	41.1 ± 2.5	38.4 ± 1.2	18.4 ± 2.2
HEX	1X [5]	3.2 ± 0.5	35.7 ± 3.0	36.2 ± 1.6	24.9 ± 2.3
	2X [10]	4.7 ± 0.9	34.9 ± 2.0	39.8 ± 2.1	20.6 ± 2.1
	4X [20]	**6.2 ± 1.0**	**33.4 ± 2.9**	38.1 ± 3.3	22.6 ± 2.5

Mean HCT116 cell population (%) in each cell cycle phase (± SEM) obtained from DNA content histograms analysis generated by flow cytometry. Control (DMSO treated) samples were compared with extract-treated groups using Dunnett's multiple comparison (One-way ANOVA) analysis. Any statistically significant values ($p < 0.05$) are highlighted **bold**.

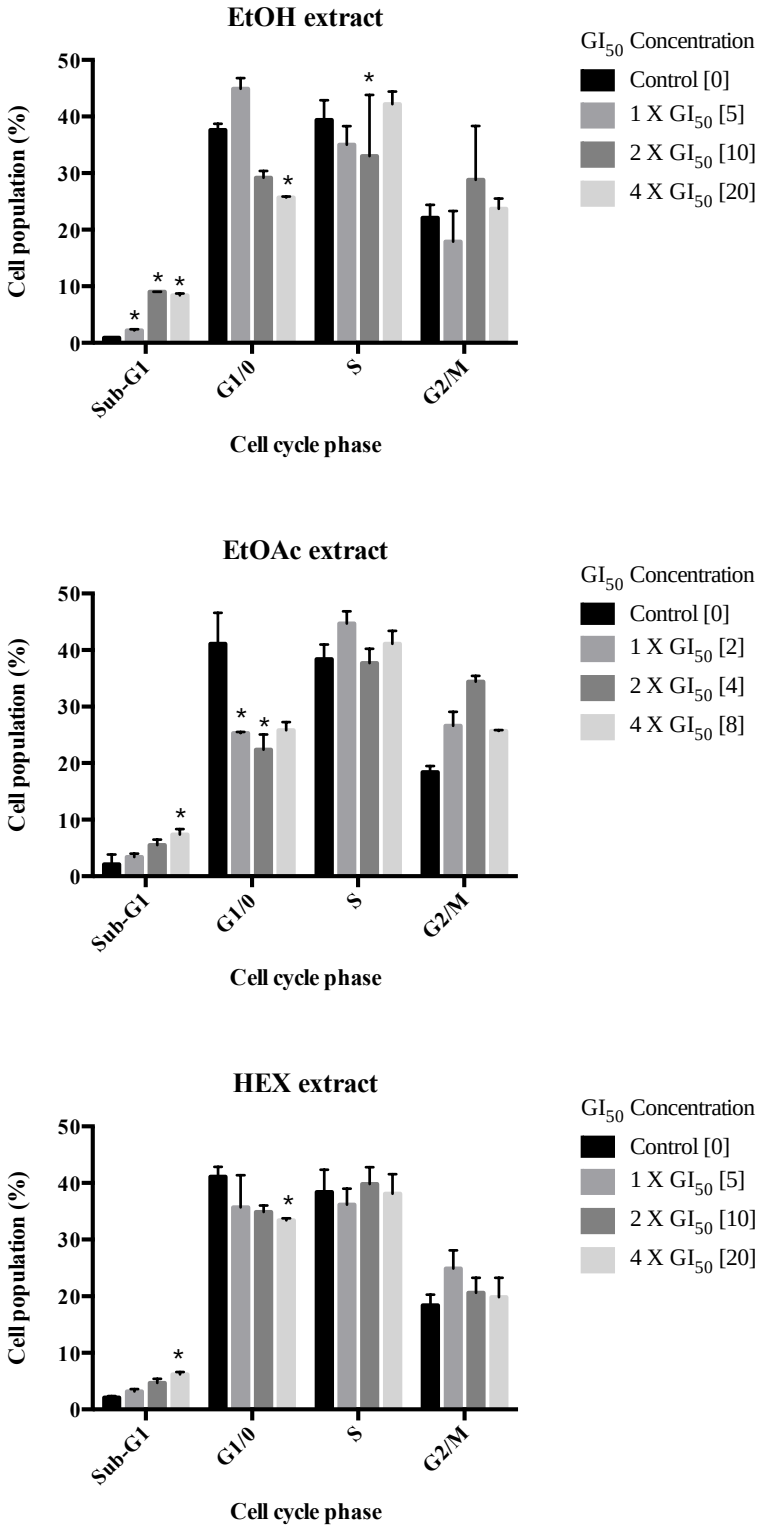


Figure 3-7: HCT116 cell cycle distributions after 48-hour exposure to *P. macrophylla* extracts. Bars (error bar = SEM) denote the mean cell population (%) in each cell cycle phase after exposure to EtOH, EtOAc and HEX extracts from ≥ 4 (n = 2) individual experiments. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p < 0.05$).

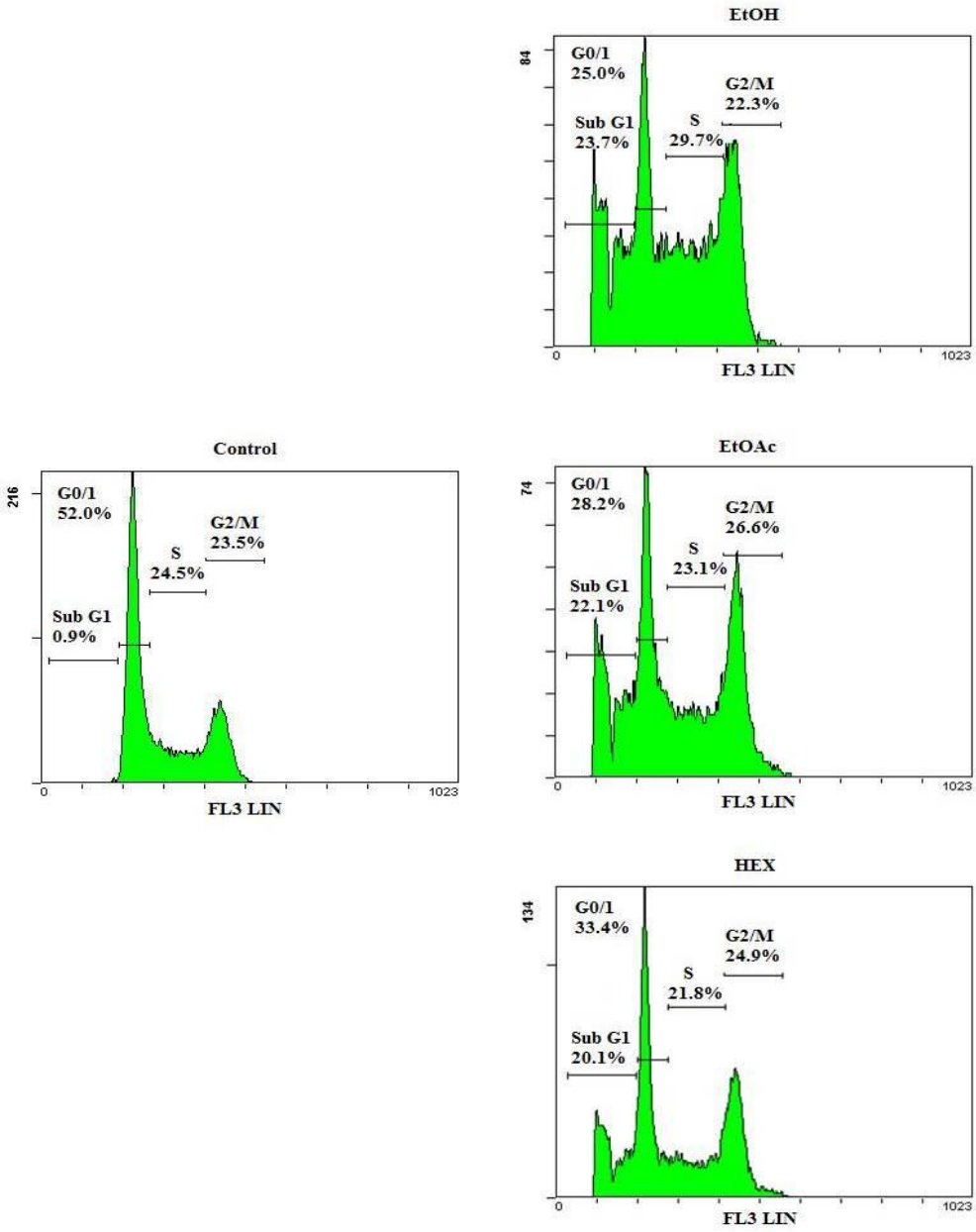


Figure 3-8: Detection of PI staining of HCT116 cellular DNA content after exposure to *P. macrophylla* extracts at 4X GI_{50} concentration for 48 hour. Data is represented as histogram of cellular DNA content representation of cell population % in each cell cycle phase; images from a single representative experiment are shown. The samples were kept chilled overnight in a solution containing buffer, detergent and ribonuclease A prior to analysis. Results were established from three experiments ≥ 15,000 cells per sample (n = 2) and processed using EXPO32 software. Image is representative of cell cycle distribution of one sample following treatment with UNMC extract as described above.

3.1.5 Summary of cell cycle analysis after exposure to UNMC extracts

The measurement of DNA content (hypodiploid, diploid and hyperdiploid) after exposure of cells to UNMC extracts revealed disturbances in specific cell cycle phases i.e. sub-G1, G1/0, S and G2/M.

Acalypha wilkesiana: Inhibition of HCT116 cell growth and clonogenicity was observed following exposure to EtOH extract, this could be associated to an increase in sub-G1 (apoptotic) cell population at higher treatment concentrations, hence inability to proliferate and form colonies. Overall, 2X and 4X GI_{50} EtOH and HEX treatment were more active in increasing cell numbers in sub-G1 and G1/0 phases and less active in causing G2/M arrest. This could be that compounds in EtOH extract are DNA damaging agents hence cell cycle halted at G1 phase to allow repair of DNA damaged cells before proceeding in the rest of the cell cycle. Since no significant differences were observed between DNA histograms of extract-treated cells and DMSO control cells, which can suggest DNA repair mechanisms are in place or that extracts might be eliciting a cytostatic effect. Neutral and alkaline comet assays have previously shown that U87MG and A549 cells following 72 h exposure to EtOAc and HEX extracts contain single and double DNA strand breaks resulting in apoptosis at concentrations as low as 0.1 μg/ml in a dose-dependent manner.[234] Bussing *et al.* (1999) have shown induction of apoptosis in Ficoll-isolated lymphocytes following treatment with *A. wilkesiana* seeds extract (1 - 100 μg/ml) as a possible consequent of granulocytes recruitment leading to excess ROI production hence cell death.[175]

***Archidendron ellipticum*:** MDA-MB-468 cells treated with EtOH extracts revealed greatest levels of G2/M cell population accompanied by a decreased G1/0 cell population, which suggests that cells in G1/0 phase could not proceed past mitosis, i.e. G2 block. Compounds which enhance the G2 block and pushing the cell to override G2/M block (e.g. disruption in mitosis) can enhance cytotoxicity of DNA damaging agents hence leading to the observed apoptotic sub-G1 cell population (treated cells in sub-G1 phase (%) is 4 - 8 %

greater than control). Interestingly, EtOH leaf and bark extracts displayed similar cell cycle effects, and is suggestive of similar phytochemicals are present in the extracts to arrest cells in the G2/M phase. There was not enough evidence to suggest EtOAc and HEX leaf extracts are affecting the cell cycle distribution.

***Duabanga grandiflora*:** Growth inhibitory effects of bark extracts complement effects seen in cell cycle profiles where a similar trend of potency was observed EtOAc > HEX > EtOH > Water. Cell cycle analysis revealed increased sub-G1 populations in all *D. grandiflora* extracts treated cells, however no significant differences was detected between cell cycle perturbation measured in *D. grandiflora* leaf extracts and control population. Increased sub-G1 and G1/0 populations in the presence of bark extracts is accompanied by decrease S and G2/M phases, which is indicative of cell death hence cells not proceeding pass G1/0 phase i.e. G1/0 block. Together with clonogenicity results revealing HCT116 cells were able to overcome the G1/0 block when extracts were removed from the cells; this may suggest DNA damage in cells are reversible and upon DNA repair, cells could proceed in the cell cycle.

***Pseuduvaria macrophylla*:** An emergence of HCT116 cells in the sub-G1 and G1/0 phase was observed in association with decreased S and G2/M cell populations after exposure to *P. macrophylla* extracts. Results imply cell death was induced by the extracts, and as sub-G1 cells increased, fewer cells were able to enter and progress past G1/0 phase. Generally extract-treated cells displayed increased G2/M cell populations accompanied by decreasing G1/0 cell populations, which is evidence of a G2/M block preceding cell death; as well as significantly higher levels of sub-G1 events (> 5-fold) when compared to than the control. The high potency and lack of clonogenicity suggests compounds may induce permanent cellular damage.

In summary, UNMC extracts contributed to increasing sub-G1 (hypodiploid) cell population, however further analysis was required to confirm if the sub-G1 populations were apoptotic cells induced by UNMC plant extracts since it is

unclear whether DNA fragmentation was a result of apoptotic enzyme cleavage or DNA damaging agents. Apoptosis serves several purposes throughout development in life e.g. when in response to pathological and physiological stimuli, cells respond by triggering signaling cascades ultimately resulting in caspase activation.[250] Characteristics of apoptosis include morphological changes (cell shrinkage) and biochemical features such as the degradation of DNA into oligomers (180 bp) by endonuclease; this could be measured and determined as hypodiploid (sub-G1) cells.[91]

3.2 Detection of apoptotic cells after exposure to UNMC plant extracts

Dysfunction of the apoptosis mechanism is often associated with cancers.[120] One of the hallmarks of early apoptosis involves the translocation of phosphatidylserine (PS) from the inner to the intact outer plasma membrane. The membrane integrity of an apoptotic cell diminishes when progressing into late apoptosis/necrosis. A calcium ion (Ca^{2+}) dependent phospholipid-binding protein, annexin V, possesses high PS binding affinity.[251] Dual staining with annexin V (conjugated to FITC, a green fluorescent dye) and PI was employed to detect and distinguish early apoptotic ($FITC^+/PI^-$) and late apoptotic/necrotic ($FITC^+/PI^+$) cell populations using flow cytometric techniques. Hence, detection of PS is one method of confirming apoptosis in cells treated with UNMC plant extracts.[94] The cell lines and treatment concentrations chosen to examine the ability of UNMC extracts to induce apoptosis were guided by results from the selection assays, 1X, 2X and 4X GI_{50} concentrations. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = $0.01 < p < 0.05$. Regrettably, due to the unavailability of *A. wilkesiana* extracts it could not be determined whether the significant increase in sub-G1 cell population caused by EtOH extracts was a consequence of cells undergoing apoptosis.

3.2.1 Time-dependent apoptosis induction in MDA-MB-468 cells after exposure to *Archidendron ellipticum* extracts

The increase in healthy cells ($FITC^-/PI^-$) entering apoptosis and progressing to late apoptosis/necrosis during exposure to *A. ellipticum* extracts is shown in Figure 3-9. At 24 h, the levels of apoptosis in EtOH, EtOAc and HEX bark extract-treated cells were greater than that seen in the control population by 36.1%, 26.5% and 35.6%, respectively ($p < 0.05$). Between 24 h to 48 h, EtOAc bark extract caused an overall 1.6% increase in early apoptotic MDA-MB-468 cells progressing to late apoptosis/necrosis. Conversely, a longer exposure period to EtOH and HEX bark extracts revealed that there were fewer

apoptotic cells measured than at 24 h (-2.4% and -5.0%, respectively; shown in Figure 3-10). Likewise a longer exposure period to EtOH and HEX leaf extracts displayed fewer apoptotic cells than at 24 h (-16.1% and -3.3%, respectively; shown in Figure 3-10). In addition, the decrease in healthy cell populations treated with EtOH, EtOAc and HEX leaf extracts was accompanied by increased apoptotic cell populations: 48.3%, 26.5% and 37.4%, respectively. Between 24 h and 48 h, HEX leaf extract caused an overall 3.3% increase of early apoptotic MDA-MB-468 cells progressing to late apoptosis/necrosis.

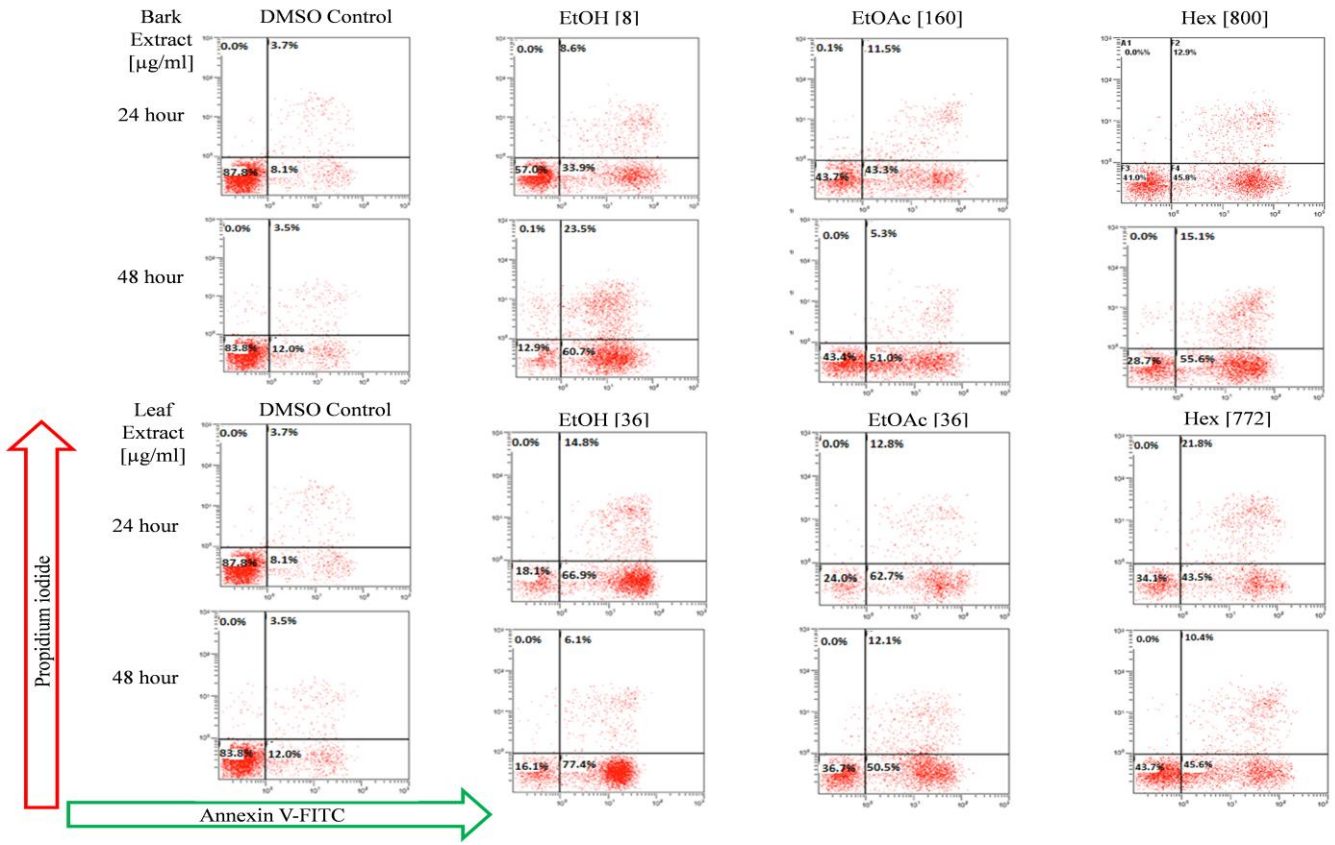


Figure 3-9: Phosphatidylserine detection in MDA-MB-468 cells after exposure to *A. ellipticum* bark (top) and leaf (bottom) extracts at 4X GI_{50} concentration after 24 and 48 hour exposure. Dot plots of annexin V-FITC (green dye) and PI (red dye) analysis from a single representative experiment is shown. Dual staining of FITC and PI were used to determine the number of cells (%) that are healthy ($FITC^-/PI^-$; lower left quadrant), undergoing early apoptosis, ($FITC^+/PI^-$; lower right quadrant) and late apoptosis/necrosis ($FITC^+/PI^+$ top right quadrant).

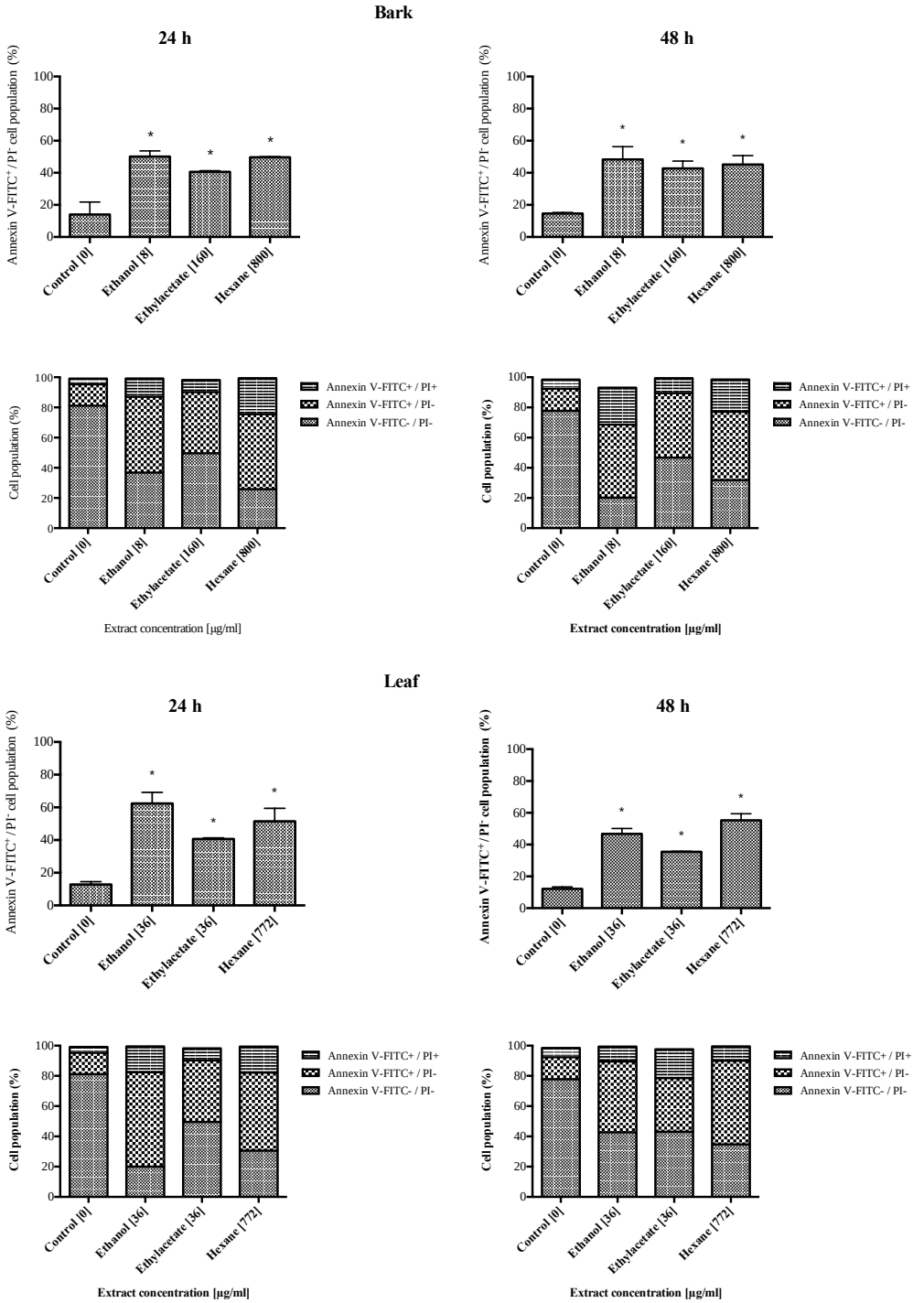


Figure 3-10 Apoptosis induced in HCT116 cells by *A. ellipticum* extracts after 24 and 48 hour exposure. Cells were exposed to 4X GI_{50} concentrations of bark and leaf extracts and stained with annexin V-FITC and PI. Bars denote the mean % cell population (± SEM) in early apoptosis (annexin V-$FITC^+$ /PI^-) (top). Bars denotes the mean % cell population (± SEM) in early apoptosis, late apoptosis/necrosis (annexin V-$FITC^+$ /PI^+) and healthy (annexin V-$FITC^-$ /PI^-) (bottom). ≥ 4 independent experiments (≥ 15,000 cells/sample; n ≥ 3) were performed. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

3.2.2 Time-dependent apoptosis induction in HCT116 cells after exposure to *Duabanga grandiflora* extracts

There was a gradual progression of early apoptotic cells (lower right quadrant) to late apoptotic/necrotic cells (top right quadrant) as exposure period to *D. grandiflora* extracts increased (Figure 3-11). Following 24 h exposure to bark extracts, approximately 33% to 49 % of HCT116 cells underwent early apoptosis ($FITC^+/PI^-$) as illustrated in Figure 3-12. Early apoptotic HCT116 cells were detected: 33.8%, 39.7%, 41.3%, and 48.5% of total population when exposed to Water, EtOH, EtOAc and HEX bark extracts, respectively; this is at least twice as many than that of early apoptotic control cell population (16.8%). Between 24 h and 48 h, in the presence of Water, EtOH and EtOAc and bark no considerable increase in levels of early apoptotic to late apoptotic/necrotic HCT116 cells (< 7%) was observed.

A gradual progression of early apoptotic cells to late apoptotic/necrotic cells as the cellular exposure period to *D. grandiflora* leaf extracts was observed (Figure 3-11). At 48 h, the percentage of early to late apoptotic cells increased significantly by 24.1%, and 38.1% in the presence of EtOH extract and Water leaf extracts, respectively, when compared to the control population ($p < 0.05$). The apoptotic/necrotic cells induced by Water leaf extracts at 48 h is significantly higher by 50.3% than the control population and comparable to the level of apoptotic cells following treatment with Water bark extract (49.9%). Overall, the concentrations used and cytotoxic effects of EtOH and Water extracts were comparable with only minor differences in apoptotic populations of 5.76% (24 h) and 5.23% (48 h), respectively. There was no considerable increase in the level of early apoptotic to late apoptotic/necrotic HCT116 cells treated with EtOAc leaf extract between 24 h and 48 h (6.7%); this is comparable to activity seen in EtOAc bark extract (Figure 3-12). The percentage of early to late apoptotic cells increased significantly by 31.9% in the presence of HEX leaf extracts ($p < 0.05$). HEX leaf extract at the highest concentration (800 μg/ml) appeared to be less active than lower concentrations of HEX bark extract (116 μg/ml).

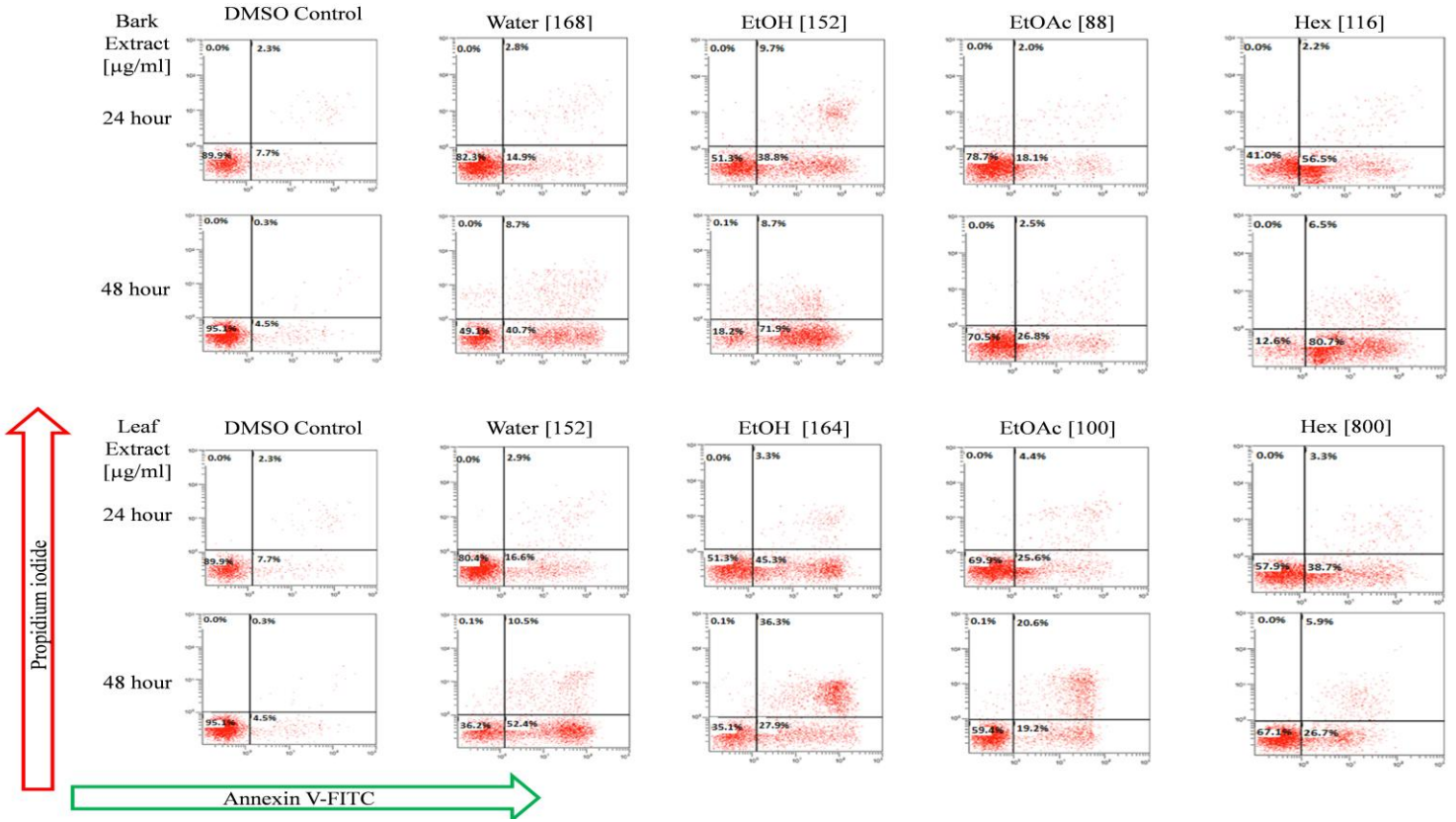


Figure 3-11: Detection of apoptotic populations of HCT116 cells after exposure to *D. grandiflora* bark (top) and leaf (bottom) extracts at 4X GI_{50} concentration after 24 and 48 hour exposure. Dot plots of annexin V-FITC (green dye) and PI (red dye) analysis from a single representative experiment is shown. Dual staining of FITC and PI were used to determine the number of cells (%) that are healthy ($FITC^-/PI^-$; lower left quadrant), undergoing early apoptosis, ($FITC^+/PI^-$; lower right quadrant) and late apoptosis/necrosis ($FITC^+/PI^+$ top right quadrant).

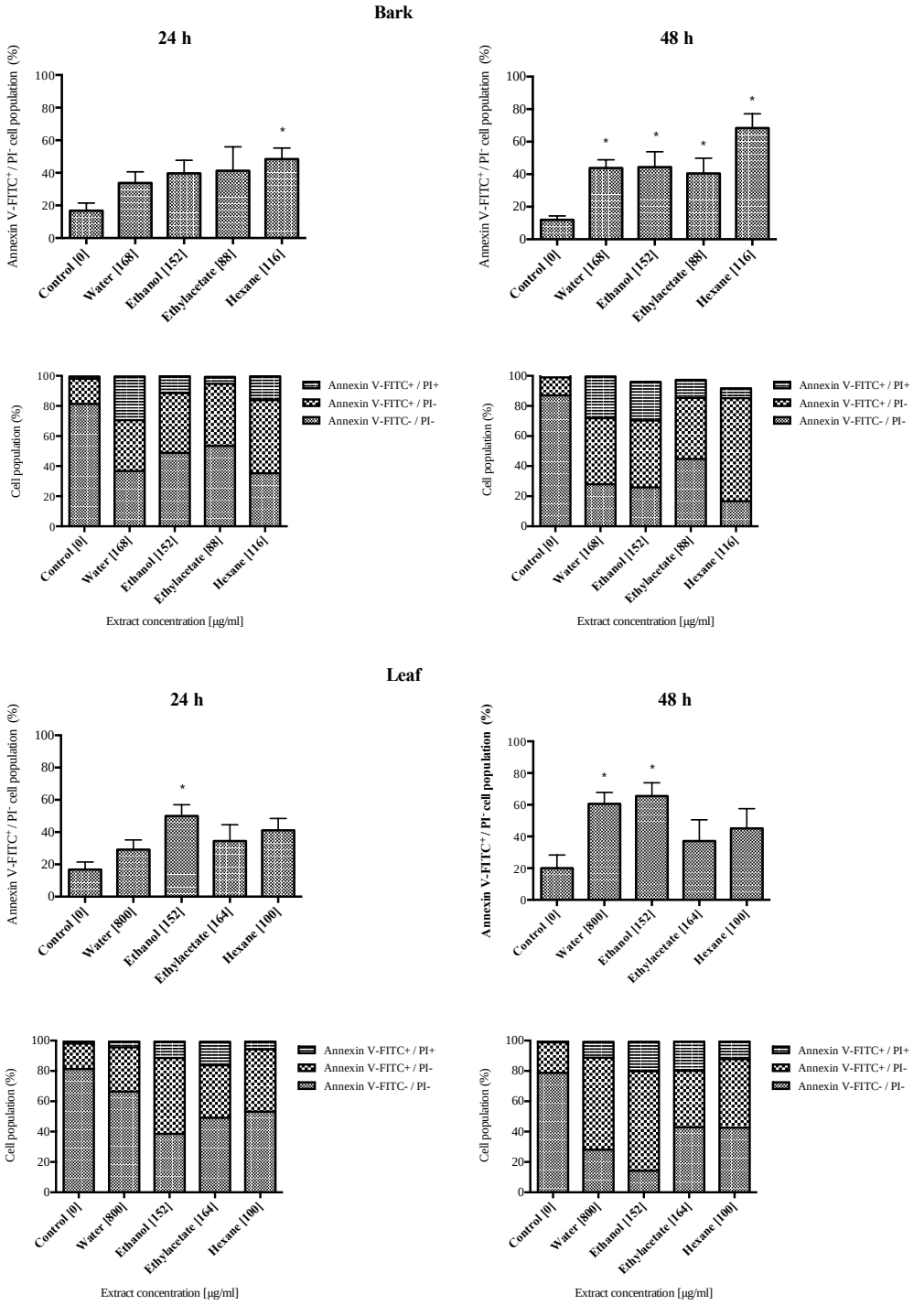


Figure 3-12: Apoptosis induced in HCT116 cells by *D. grandiflora* extracts after 24 and 48 hour exposure. Cells were exposed to 4X GI_{50} concentrations of bark and leaf extracts and stained with annexin V-FITC and PI. Bars denote the mean % cell population (± SEM) in early apoptosis (annexin V-$FITC^+$ /PI^-) (top). Bars denote the mean % cell population (± SEM) in early apoptosis, late apoptosis/necrosis (annexin V-$FITC^+$ /PI^+) and healthy (annexin V-$FITC^-$ /PI^-) (bottom). ≥ 4 independent experiments (≥ 15,000 cells/sample; n ≥ 3) were performed. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

3.2.3 Time-dependent apoptosis induction in HCT116 cells after exposure to *Pseuduvaria macrophylla* extract

The numbers of healthy cells ($FITC^-/PI^-$) entering apoptosis and progressing to late apoptosis/necrosis as the exposure period to *P. macrophylla* extracts increases is illustrated in Figure 3-13.

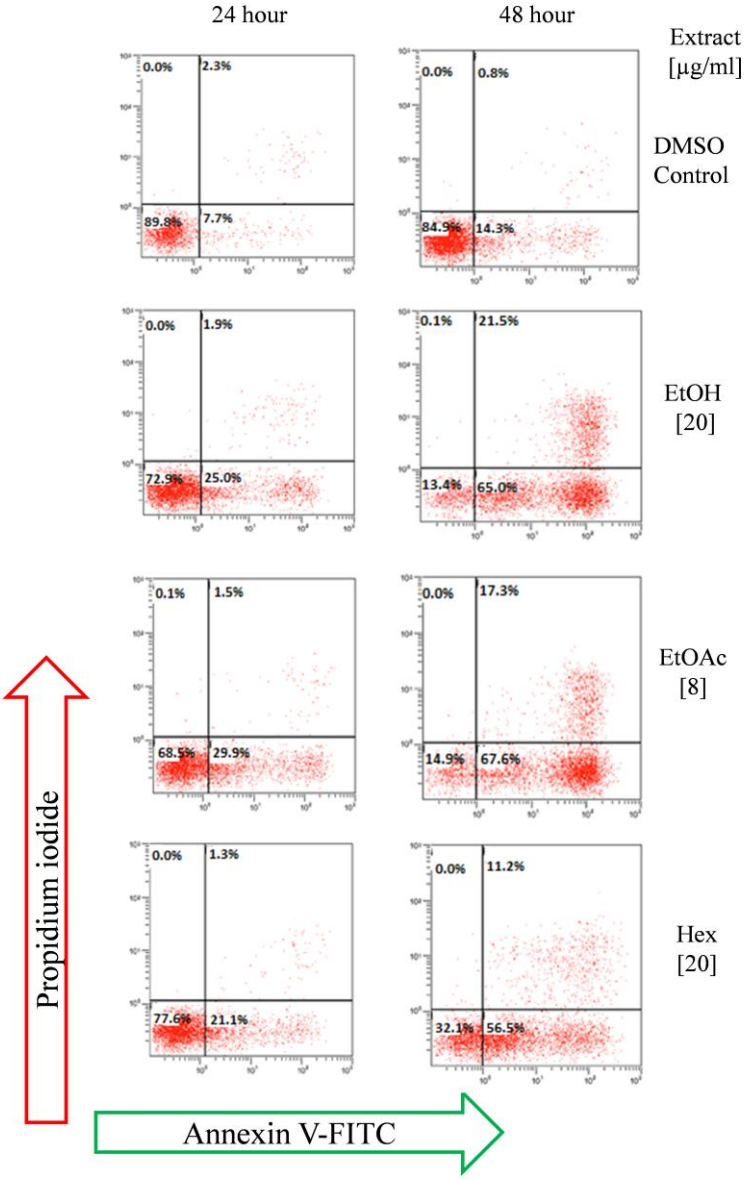


Figure 3-13: Phosphatidylserine detection in HCT116 cells after exposure to *P. macrophylla* extracts at 4X GI_{50} concentration after 24 and 48 hour exposure. Dot plots of annexin V-FITC (green dye) and PI (red dye) analysis from a single representative experiment is shown. Dual staining of FITC and PI were used to determine the number of cells (%) that are healthy ($FITC^-/PI^-$; lower left quadrant), undergoing early apoptosis, ($FITC^+/PI^-$; lower right quadrant) and late apoptosis/necrosis ($FITC^+/PI^+$ top right quadrant).

Following 48 h exposure, cell populations undergoing late apoptosis were evident, < 20% viable cells (FITC-/ PI-) were detected in cells exposed to EtOH extracts and < 40% viable cells following exposure to EtOAc and HEX extracts (Figure 3-14). During 24 h and 48 h, the decrease in healthy cell population was accompanied by an increase in apoptotic cell populations: 33.6%, 13.5% and 15.4% for EtOH, EtOAc and HEX extracts, respectively. At both time points, all *P. macrophylla* extracts caused significantly increased levels of early apoptotic HCT116 populations ($p < 0.05$).

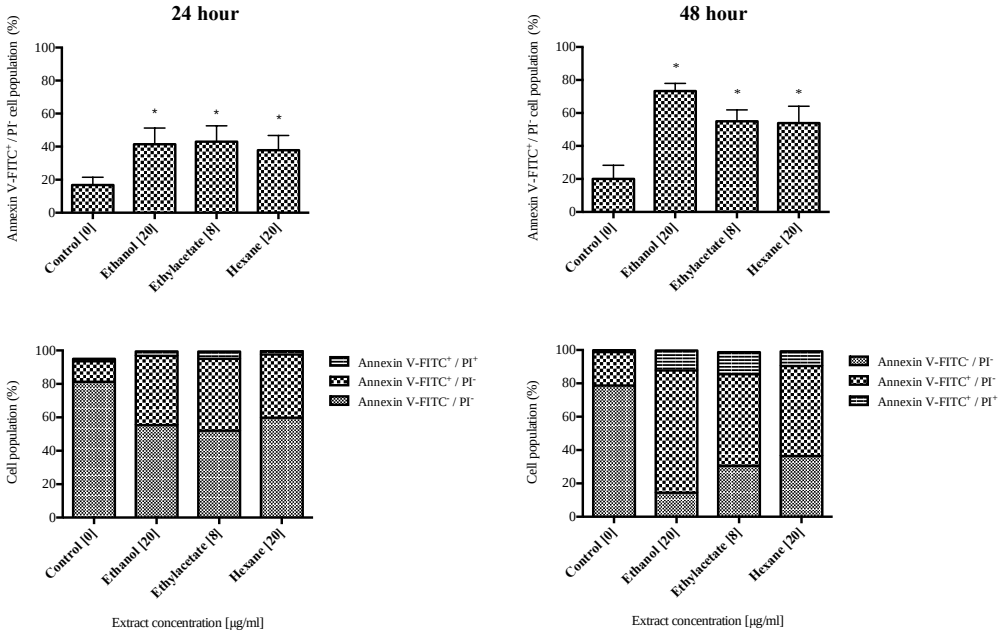


Figure 3-14: Apoptosis induced in HCT116 cells by *P. macrophylla* extracts after 24 and 48 hour exposure. Cells were exposed to 4X GI_{50} concentrations of extracts and stained with annexin V-FITC and PI. Bars denote the mean % cell population (± SEM) in early apoptosis (annexin V-$FITC^+$ /PI^-) (top). Bars denotes the mean % cell population (± SEM) in early apoptosis, late apoptosis/necrosis (annexin V-$FITC^+$ /PI^+) and healthy (annexin V-$FITC^-$ /PI^-) (bottom). ≥ 4 independent experiments (≥15,000 cells/sample; n ≥ 3) were performed. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

3.3 Identification of caspase 3 and Mcl-1 protein expressions

Detection of changes in protein expression in cells following their treatment with UNMC extracts could provide information on possible mechanism(s) of action. As mentioned in section 1.3.2, cancer cells gain the ability to evade apoptosis therefore disrupting homeostasis. Apoptosis is co-ordinated by initiator and executioner proteins involved in two main apoptotic pathways, known as the intrinsic (mitochondrial) and extrinsic (death receptor) pathways. Both pathways eventually converge on the same execution pathway, which is irreversibly initiated by executioner caspase 3. Caspase 3 is activated by cleavage commonly by multiple death signals and this activation is essential for apoptosis-associated DNA fragmentation.[260]

The overexpression of anti-apoptotic (pre-survival) proteins of the Bcl-2 family e.g. Bcl-2, Bcl-XL and Mcl-1 can inhibit apoptosis and enable deregulated oncogene expression. Apoptosis failure is a major contributor to tumourigenesis; therefore induction of cancer cell apoptosis by inhibition/downregulation of survival proteins is an important goal of experimental cancer therapies.

Western blot analysis was employed to detect presence of proteins within cellular extracts. Proteins could be extracted from samples, denatured and separated according to molecular weight by gel electrophoresis. Analysis of band densities as relative density to control was carried out using Image J software.

3.3.1 Effect of *Duabanga grandiflora* and *Pseuduvaria macrophylla* extracts on protein expression

The *in vitro* anticancer properties of *P. macrophylla* EtOH and EtOAc extracts and *D. grandiflora* EtOH and HEX extracts were examined. Water and EtOAc extracts induced anti-proliferative effects, which would be worthy of investigation, however stocks remaining were insufficient for repeating independent experiments. HCT116 cells were exposed to control treatments (DMSO and camptothecin) and available UNMC extracts. Whole cell lysates

were prepared 48 h later and SDS-PAGE/Western blot analysis was performed to examine Mcl-1 and caspase 3 expressions (Figure 3-16 and Figure 3-15).

Caspase 3 expression was examined in UNMC extracts treated HCT116 cell lysates after 48 h exposure. Distinct full length and cleaved forms of caspase 3 were expressed in *P. macrophylla* EtOAc and *D. grandiflora* EtOH and HEX extract-treated HCT116 cells (Figure 3-15). Caspase 3 expression was not detected in DMSO treated cells, however expression of full-length caspase 3 protein was detected in camptothecin-treated cells.

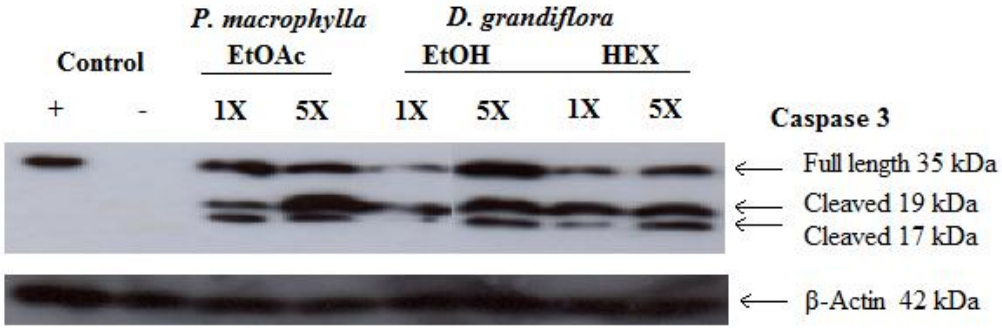


Figure 3-15 Caspase 3 protein expressions after 48-hour exposure to *D. grandiflora* and *P. macrophylla* extracts. HCT116 cells were treated with *P. macrophylla* EtOAc extract and *D.* grandiflora EtOH and HEX extracts for 48 h. Western blots of whole cell lysates representative of two independent experiments are presented. DMSO (-) and camptothecin (+) were used as control.

Noticeably, relative density of bands illustrating cleaved caspase 3 in cell lysates treated with 1X GI_{50} of *D. grandiflora* EtOH and HEX extracts increased as concentration increased to 5X GI_{50} and was accompanied by decreased relative density of full-length caspase 3 protein expression, therefore suggesting dose-dependent cleavage and expression of caspase 3. A similar trend was observed with *P. macrophylla* EtOAc extracts, higher relative densities of cleaved caspase 3 bands were noticed accompanied by lower relative density of full-length caspase 3 band (relative band density of 1X GI_{50}: 1.08 and 5X GI_{50}: 0.96). Expression of Mcl-1 in negative control cells was illustrated as a band that is ~6-fold higher in relative density than positive control cells. *D. grandiflora* EtOH and HEX (1X GI_{50}) treated cell lysates

illustrated Mcl-1 expression similar to relative band density of negative control (1.0). Whereas at HEX 5X GI_{50} treatment resulted in Mcl-1 expression that was less than half of the relative band density (0.3) than that detected in lysates of negative control.

In lysates of *P. macrophylla* EtOH extract-treated cells, Mcl-1 expression was comparable to that in control lysates (relative band density1.1 and 1.0, respectively), whereas lysates prepared from cells exposed to EtOAc extracts revealed bands of lower relative density (0.7).

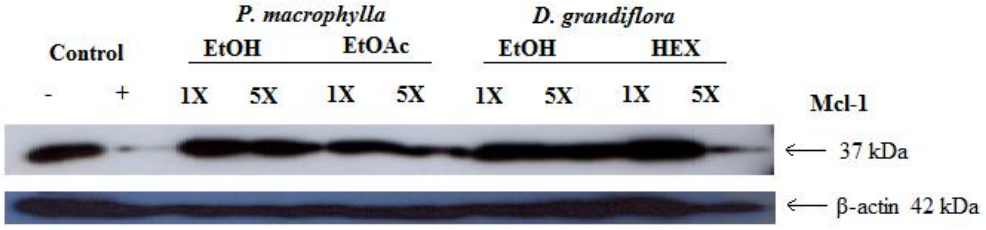


Figure 3-16: Mcl-1 protein expressions after 48-hour exposure to *D. grandiflora* and *P. macrophylla* extracts. HCT116 cells were treated with *P. macrophylla* EtOH and EtOAc extracts and *D.* grandiflora EtOH and HEX extracts for 48 h. Western blots of whole cell lysates representative of two independent experiments are presented. DMSO (-) and camptothecin (+) were used as control.

3.4 Summary of studies to investigate UNMC extract-induced apoptosis

Morphological changes in cancer cells were apparent under a phase-contrast microscope after 24 h and 48 h exposure to UNMC extracts. A proportion of cells appeared smaller, circular and 'blebbling' of the cell membrane was evident. Morphological observations accompanied by dual staining of cells with annexin V-FITC and PI confirmed the induction of apoptosis. The apoptosis-inducing capacity of UNMC extracts was highlighted by increased numbers of cells displaying $FITC^+/PI^-$ fluorescence compared with the DMSO-treated control cells after 24 h and 48 h exposures (Figure 3-14). Furthermore, Caspase-3 is susceptible to activation by any of the initiator caspases (caspase -8, -9 or -10). Detection of caspase 3 fragments suggests the execution pathway

has been initiated, however it is not certain whether initiation of caspase 3 was a consequence of the extrinsic or the intrinsic pathway activation. Activation of caspase 3 would lead to DNA fragmentation and these results are agreement with the sub-G1 cell population (lower DNA content) detected in the cell cycle distribution with apparent morphological changes. Quantifying expression of caspase 3 initiators e.g. caspase 8 or caspase 9 could reveal information on possible pathways employed by compounds in the extracts. Whereas, detection of anti-apoptotic Mcl-1 protein in *D. grandiflora* or *P. macrophylla* treated cells may suggest that Mcl-1 is not an influential factor in induction of apoptosis and that compounds embedded do not inhibit cdk-9.
It is noted that Mcl-1 and caspase 3 proteins expression were detected in *D. grandiflora*- and *P. macrophylla*-treated HCT116 cell lysates.

Archidendron ellipticum: Time-dependent induction of apoptosis by *A. ellipticum* extracts in MDA-MB-468 cells (increased sub-G1 cell populations) was confirmed by Annexin-V FITC/PI staining. At 48 h, significantly higher levels of early and late apoptotic HCT116 cells were detected than in control cells. Previously observed enhanced levels of EtOH extract-treated G2/M cell populations at 48 h suggested inhibition of or interference with mitosis; however, cellular/DNA repair mechanisms may be operating to overcome damage leading to the observation of slightly fewer apoptotic cells or cells may remain viable but unable to proceed to G2/M phase. Familiar examples of marketed anticancer drugs, which demonstrated similar effects on cells include taxol, an inhibitor of β-subunit of tubulin, which binds to the inner surfaces of microtubule leading to inhibition of microtubule depolymerisation and disrupts the normal dynamic reorganisation of the microtubule network causing G2/M cell cycle arrest and consequently interference with mitotic spindles resulting in cell death.[141] Additionally, effects of docetaxel are prominently observed in G2/M phase of the cell cycle and have minimal toxicity against cells in G1 phase. Contrastingly, vinca alkaloids can affect cells by inhibiting mitotic spindle assembly and reducing tension at the kinetochores of the chromosomes, also resulting in G2/M block. It has been reported that taxanes can cause cell arrest in G2/M phase (by inhibiting of depolymerisation) and phosphorylate Bcl-2 proteins and this provides additional basis to investigate possible pro-

apoptotic activity induced by UNMC extracts as cell cycle data of *A. ellipticum* revealed G2/M block and increased sub-G1 population. Kuntiz inhibitor found in *A. ellipticum* is able to activate caspase 3 and 8, which is suggestive of extrinsic pathway activation. *Acacia* genus of the Fabaceae family, which is also rich in saponins, has demonstrated apoptosis-inducing abilities in Jurkat cells *via* permeablisation of mitochondrial membrane (time-dependent cytochrome C release) and caspase 3 activation.

Duabanga grandiflora: The presence of sub-G1 HCT116 cell populations were observed following exposure of cells to *D. grandiflora* extracts and the Annexin-V FITC assay confirmed the sub-G1 cell population to be apoptotic cells. The observed G1/0 block is indicative of possible DNA damage resulting in inability to progress past G1/0 and apoptosis. Significant levels of early apoptotic HCT116 cells were measured in all *D. grandiflora* treated cells, in particular, following polar Water and EtOH extracts treatments. The level of apoptotic/necrotic cells emerging following exposure of cells to Water and HEX bark, Water and EtOH leaf extracts were time-dependent and significant compared to untreated control populations ($p < 0.05$). This is supported by the detection of cleaved caspase 3 proteins.

***Pseuduvaria* macrophylla**: The ability of *P. macrophylla* extracts to induce apoptosis in HCT116 cells was evident in this series of *in vitro* assays (potency: EtOH > EtOAc > HEX). Initially, early apoptosis was detected in all *P. macrophylla* extracts treated cells at significant levels (39 % to 48 %). As exposure time increased, a distinct progression of early apoptotic cells into late apoptosis was noticed hence appearance of apoptotic/necrotic cells was time-dependent (62% to 86 %; 24 h – 48 h). An aporphinoid, liriodenine (8H-benzo[g]-1,3-benzodioxolo[6,5,4-de]-quinolin-8-one), similar to that isolated from *P. macrophylla* revealed cytotoxicity against A549 lung cancer cells between 2 and 20 μM of liriodenine causing G2/M cell cycle arrest, together with 67% and 80% early apoptosis (Annexin-V FITC+/PI- cells), respectively.[252] As previously mentioned, liriodenine, similar to **38** isolated from *P. macrophylla* has revealed the ability to cleave PARP in A459 cells, which is suggestive of caspase 3 activation.[252] Both aporphinoids could be

exerting their cytotoxicity *via* similar mechanisms since similar trends of cell cycle perturbations were also observed.

Cell cycle perturbations and appearance of apoptotic cells have been detected, which suggest involvement of proteins such as cysteine-dependent aspartate-directed proteases caspases (executors of apoptosis). It has been reported that Fas, caspase -3 and -8 are involved in PS externalisation facilitating non-inflammatory phagocytic recognition hence uptake of apoptotic cells.[250]
Overall, detection of cleaved (activated) caspase 3 by Western blot and Annexin V positive, PI negative cells by flow cytometry provide robust evidence of cells undergoing early apoptosis. Both sets of data together with evidence of morphological changes can confirm caspase-dependent apoptosis; these preliminary results illustrating execution of apoptosis provide support for further investigation of constituents in UNMC plants as potential anti-tumour agents.

4

Effects of UNMC plant extracts on cell migration

The ability of malignant cells to migrate and invade surrounding tissue, vascular beds, ECM is crucial to cancer metastasis. Invasion and metastasis represent one of the hallmarks of cancer. It is interesting that the cancer cells' acquired resistance mechanism to VEGF inhibitors leads to enhanced migratory capacity.[124]

4.1 Effects of UNMC plant extracts on cell migration

A simple *in vitro* assay was used to mimic *in vivo* cell migration during wound healing. A 'wound' is created in a cell monolayer and observations (images captured) at timed intervals will enable analysis of the migration rate of the cells.[253] Wound images (n = 2) of the marked section were analysed using *ImageJ* and the wound area was measured as area covered (%). At 0 h, the wound is considered as 100% and over time, the area of the wound can decrease (as cells migrate), increase (cell death; floating cells) and/or remain the same (migration inhibition); if necessary, medium was replenished to remove floating cells for clearer images of the wound. The end point was considered when wound size of the control cells is 0% regardless of the wound size of treated cells. Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = $0.01 < p < 0.05$. Certain extracts were selected for anti-migration activities based on the acquired growth inhibitory/cytotoxicity data and extract availability.

4.1.1 Effect of *Archidendron ellipticum* extracts on HCT116 cell migration

The investigation of *in vitro* anticancer properties of HEX bark extract was terminated due to the lack of supply.

At 24 h treatment period, significant differences in wound area (%) were observed between bark extract-treated and control cells (Figure 4-1). A comparable migration rate in EtOAc bark and EtOH leaf extract-treated cells was observed after 24 h treatment, however EtOH bark and EtOAc leaf extract treatment led to emergence of a larger wound area observed as a consequence of detached cells ($p < 0.05$) . This result supports the observation seen in the MTT assay that EtOH bark and EtOAc leaf extracts were the most active against MDA-MB-468 cells. At 56 h, remaining wound area of MDA-MB-468 cells at 62.2% and 87.9% after exposure to low concentrations EtOH bark extracts (2 and 8 μg/ml) revealed strong anti-migration properties.

The remaining wound sizes of 42.1% and 43.5% after treatment of cells with 1X and 4X GI_{50} EtOAc bark extract, respectively, were significant compared to control cells; statistical analyses have shown significant differences in wound size ($p = 0.005$ and 0.020, respectively). To a lesser extent, a steady cell migration rate was observed following EtOH leaf extract treatment than compared to control cells until 64 h where a significant wound area was observed, ~ 30%, with both concentrations of EtOH extract. Whereas following 24 h treatment, similar effects of EtOAc leaf extract at 1X GI_{50} illustrate a steady migration rate comparable to the control cells. Only at 4X GI_{50} treatment, were cells unable to migrate completely due to cytotoxicity of EtOAc extracts; the cell monolayer was lost due to cell death. Anti-migration effects of HEX leaf extracts were only observed at very high concentrations (772 μg/ml) in MDA-MB-468 cells. It is interesting to note that between 0 h and 48 h, the healing (migration) rate of MDA-MB-468 cells supersedes the control cells under the influence of HEX extract ($p < 0.05$).

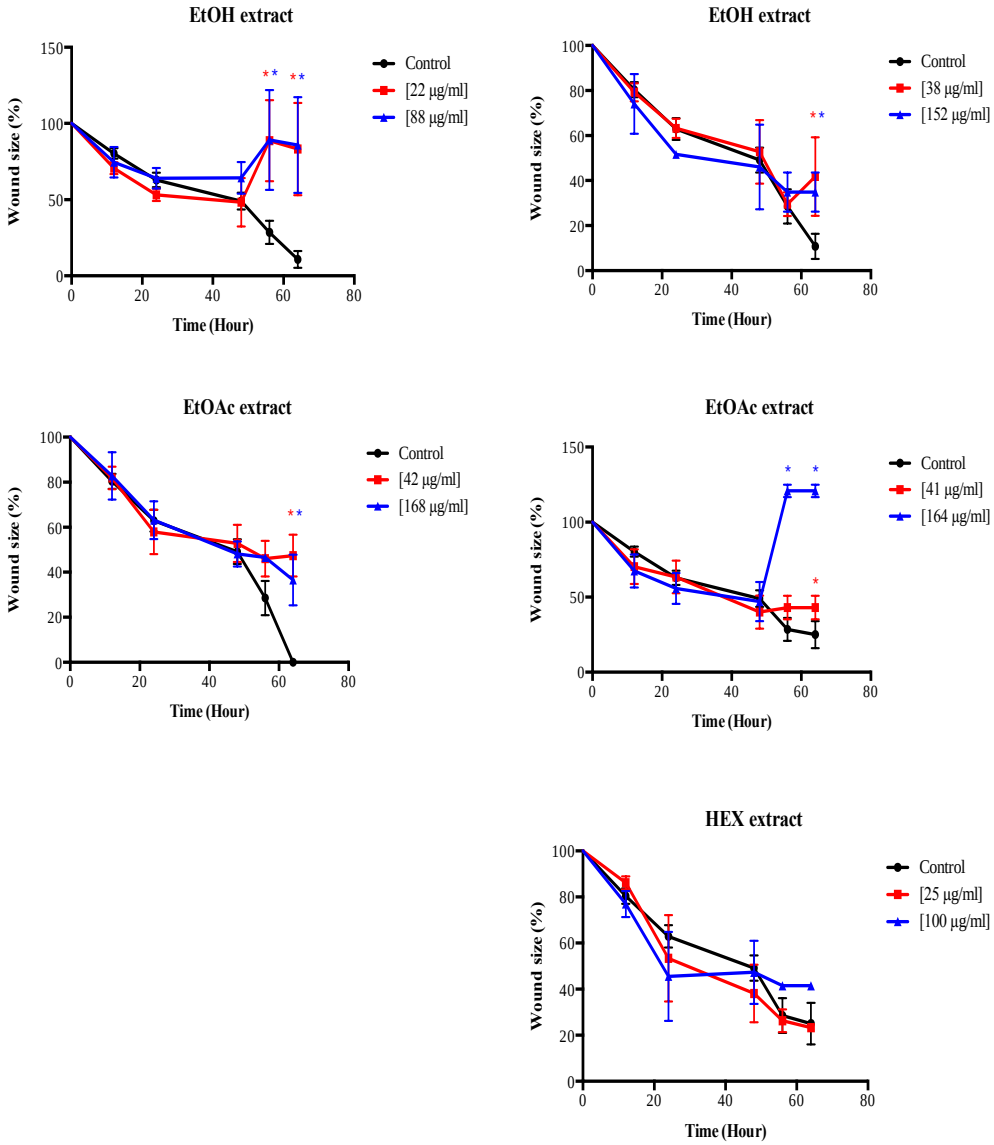


Figure 4-1: Inhibitory effect of *A. ellipticum* bark and leaf extracts on MDA-MB-468 wound healing (%) over a 64 h period. Cells were exposed to 1X and 4X GI_{50} concentrations of EtOH (A), EtOAc (B) and HEX (C) extracts measured at specific sections every 24 h (monitored closely when wound is ~ 10%). Mean measurements are from 3 independent experiments (n = 2). Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

4.1.2 Effect of *Duabanga grandiflora* extracts on cell migration

The investigation of *in vitro* anticancer properties of EtOH and HEX bark extracts was carried out. Water and EtOAc extracts induced anti-proliferative effects, which would be worthy of investigation, however supply was insufficient to allow for repeated independent experiments.

A positive correlation was observed between extracts' concentration and potency against HCT116 cell migration inhibition as prominent effects were observed (Figure 4-2). At 56 h, EtOH extracts at 38 and 152 μg/ml were able to significantly inhibit HCT116 cell migration as wound area remained at 38.6% and 53.6%, respectively ($p = 0.005$). A steady inhibition of HCT116 cell migration under the influence of EtOH bark extract was revealed; wound areas of > 50% remained without the loss of cell monolayer, suggesting that cells are viable but reluctant to migrate. Inhibitory effects on cell migration were observed when HCT116 cells were treated with 1X GI_{50} HEX extract, as wound sizes remained constant at 81.4% to 75.0%. While, detached dead cells were noticed with HEX extract treatment at 4X GI_{50}, resulting in a significantly greater wound area (> 100%); HEX extracts appeared to be cytotoxic to HCT116 cells as the cell monolayer was lost during longer treatment times (> 48 h; represented by a wound area of > 100%.

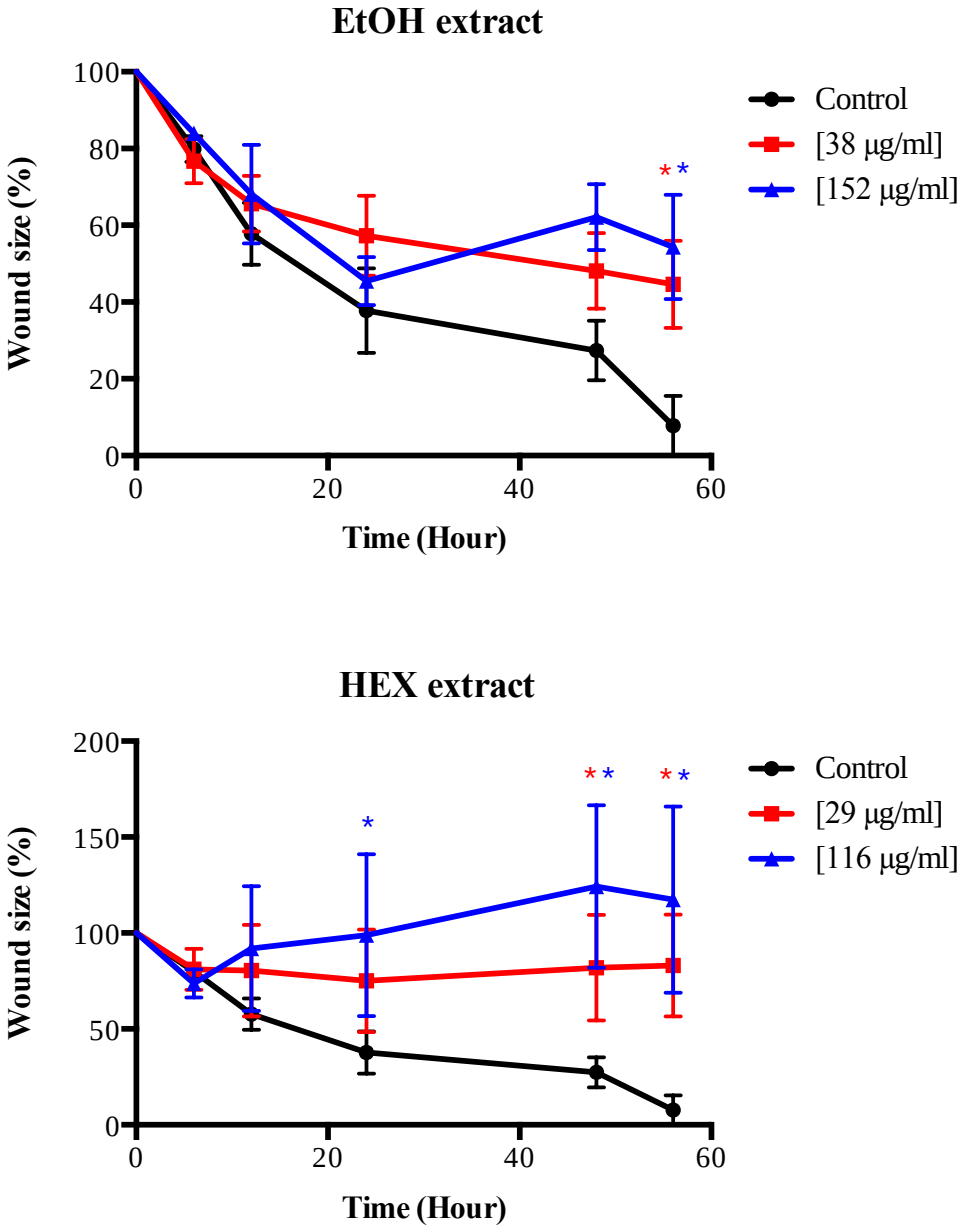


Figure 4-2: Inhibitory effect of *D. grandiflora* bark extracts on HCT116 wound healing (%) over a 56 h period. Cells were exposed to 1X and 4X GI_{50} concentrations of EtOH (A) and HEX (B) extracts and measured at specific sections every 24 h (monitored closely when wound is ~ 10%). Mean measurements are from 3 independent experiments (n = 2). Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

4.1.3 Effect of *Pseuduvaria macrophylla* extracts on HCT116 cell migration

The rate of wound healing decreased when HCT116 cells were treated with EtOH and EtOAc extracts, when compared to control cells at 56 h; hence results suggest the anti-migration effects were induced by *P. macrophylla* extracts. There was no significant difference in wound area between EtOH extract-treated and control cells ($p < 0.05$) between 0 h and 48 h (Figure 4-3).

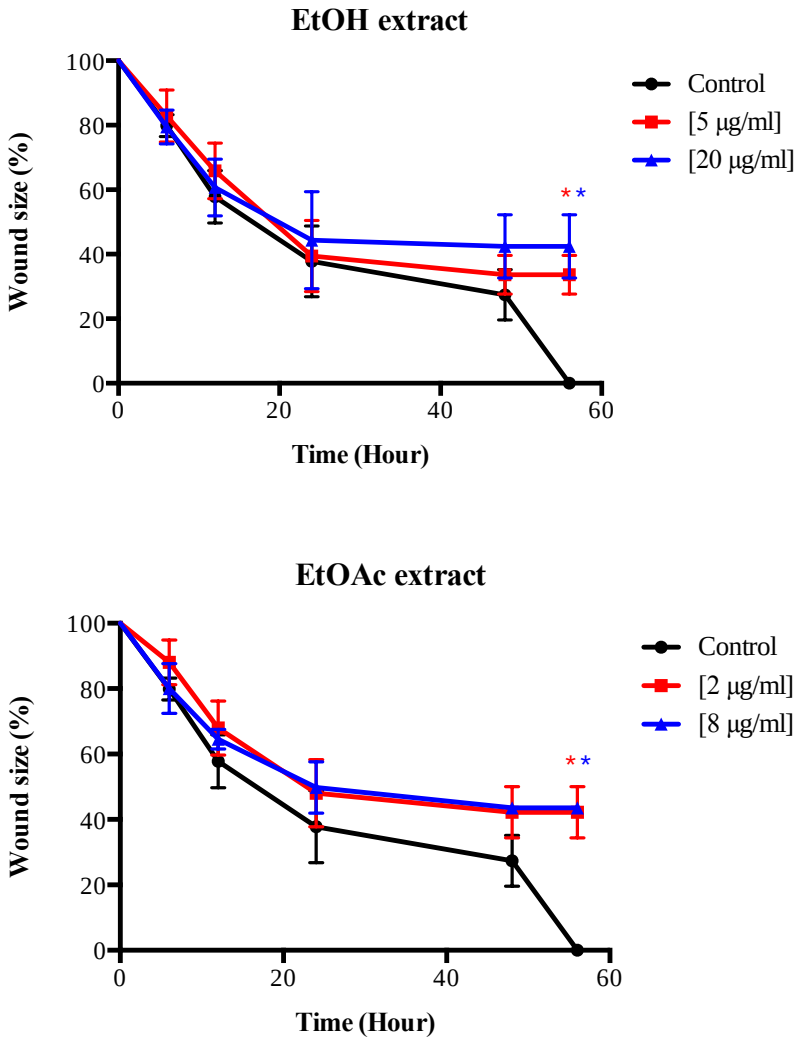


Figure 4-3: Inhibitory effect of *P. macrophylla* extracts on HCT116 wound healing (%) over a 56 hour period. Cells were exposed to 1X and 4X GI_{50} concentrations of EtOH (A) and EtOAc (B) extracts and measured at specific sections every 24 h (monitored closely when wound is ~ 10%). Mean measurements are from 3 independent experiments (n = 2). Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

At 56 h, the remaining wound area in cells exposed to 5 and 20 μg/ml EtOH were 33.7% and 42.5%, respectively and cell migration was significantly inhibited by 2 and 8 μg/ml EtOAc extract with remaining wound size of 42.1% and 43.5%, respectively compared to control cells. Statistical analyses have shown significant differences in wound size after 40 h of exposure ($p = 0.005$ and 0.020, respectively).

4.1.4 Summary of migration inhibition properties of UNMC extracts

A wound-healing assay was performed to assess the UNMC extracts' ability to inhibit cell migration as this could have potential therapeutic uses specifically for cancer metastasis inhibition and also to overcome acquired resistance to VEGF inhibitors.

Archidendron ellipticum: Comparable migration rates to the control cells were observed in MDA-MB-468 cells following 0 h to 48 h exposure to EtOAc bark and EtOH leaf. To a greater extent, this trend was also observed with EtOAc leaf extracts with remaining wound size of 43.0%. However, cell migration could no longer be observed due to loss of cell monolayer (> 100%, hence cell death) at higher concentrations of EtOAc leaf extract. EtOH bark induced greatest significant anti-migration effects at lowest concentrations used compared with wound area of control cells; the wound area of cells following EtOH bark treatment (40 and 160 μg/m) remained at 46.1% and 46.5%, respectively. It is interesting to note the enhanced migration rate of MDA-MB-468 cells under the influence of HEX extract, suggesting embedded compounds could encourage cell migration prior to inhibitory effects at longer exposure time. It is anticipated that HEX bark extract would have comparable low anti-migration effects as to HEX leaf extract based on previous *in vitro* assays results of HEX extracts on HCT116 cells e.g. MTT, cell cycle and annexin-V FITC.

Duabanga grandiflora: Inhibition of HCT116 cell migration was observed following treatment with EtOH and HEX bark extracts. A steady decrease in wound area was observed with EtOH-treated HCT116 cells. From 12 h onwards, inhibition of HCT116 cell migration following HEX extract treatment was evident as the remaining wound areas were preserved at 75% to 82%. Following 24 h, detachment of cells was clearly observed following replenishment of medium containing extracts, suggesting cytotoxicity with high concentrations of HEX extract where wound area (%) was measured at > 100%, which indicated cell death. It may be expected that EtOAc bark and leaf extracts would induce greater inhibitory effects on HCT116 migration, based on previous *in vitro* assays results of EtOAc extracts on HCT116 cells from e.g. MTT, cell cycle and annexin-V FITC assays.

***Pseuduvaria macrophylla*:** Extracts exhibited a gradual inhibition of cell migration with slightly greater effects seen with EtOAc extract than EtOH extract: larger wound areas were observed at lower concentrations of EtOAc applied. It is expected that HEX extract may induce similar inhibitory effects on HCT116 migration, based on previous *in vitro* assay results of HEX extracts on HCT116 cells (e.g. MTT, cell cycle and annexin-V FITC) which yielded comparable results to EtOH and EtOAc extracts.

Overall, inhibition of cell migration was observed in several of the UNMC extracts. It is speculated that compounds present in the extracts could be influencing the TNF-α pathway resulting in lack of survival and inflammation signals *via* inactivation of NF-κB signaling and induction of apoptosis by caspase 3 activation.[254] TNF-α can induce cytokines, angiogenic factors and metalloproteinases (MMPs). Polyphenols such as (-)-epigallocatechin-3-gallate (EGCG) can modulate MMP-2 and MMP-9, which affect the basement membrane turnover and tissue remodeling that are associated in tumour invasion and metastasis, which requires changing the dynamics of extra cellular matrix (ECM) to gain motility.[255, 256]

4.2 Effect of UNMC plant extracts on cellular senescence

Cellular senescence describes the irreversible growth arrest, which is the underlying mechanistic cause of aging in normal somatic cells upon reaching their Hayflick replicative limit; hence senescence is also a tumour suppression mechanism. Many tumours possessing unlimited and/or extended replicative potential, hence escaping mortality, can progress to malignant transformation; however several chemotherapeutic agents can induce senescence in tumours.[257] The biomarker, β-galactosidase, present in the lysosomes of senescent cells has been measured to determine the effects of UNMC plant extracts on senescence and, indirectly, cancer cell immortality. Increased detection of blue-green-stained β-galactosidase within the cytosol of cells undergoing senescence following incubation of cells with X-gal has been used to determine senescent cells. The level of β-galactosidase in cells treated with UNMC plant extracts at 1X and 2X GI_{50} was determined by counting the number of blue/green cells under the microscope as a result of the binding of X-gal to β-galactosidase in senescent cells. DMSO and RHPS4 were used as negative and positive control treatments for the detection of senescence in HCT116 cells (Figure 4-4). Pentacyclic acridine RHPS4 is a telomere-targeted agent that stabilises G-quadruplex structures within telomeric DNA, hence preventing telomere-telomere interaction.[258] Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = $0.01 < p < 0.05$.

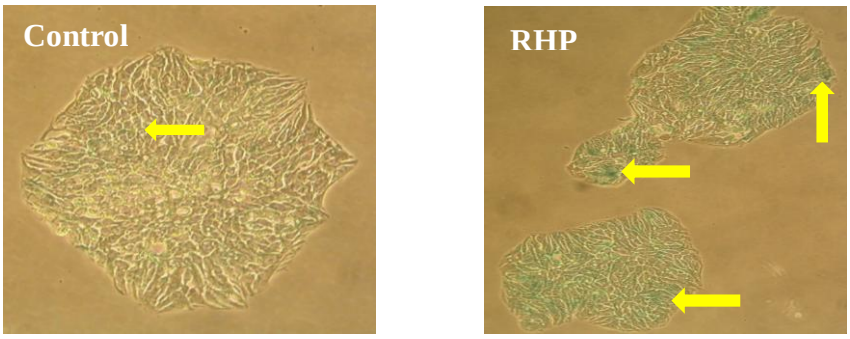


Figure 4-4: Images of β-galactosidase positive (senescent) HCT116 cells. Senescence of HCT116 cells treated with DMSO as negative control (left) and RHPS4 as positive control (right) were assessed using X-gal staining. Cells were exposed to DMSO and RHPS4 (1 μM) for 8 days; β-galactosidase^{+} cells appearing blue/green in colour. Images shown were captured by Nikon Coolpix camera (100X magnification).

4.2.1 Effect of *Archidendron ellipticum* extracts on cellular senescence

EtOH bark and all leaf extracts were investigated for senescence-inducing activity. The level of senescence in MDA-MB-468 cells induced by *A. ellipticum* extracts is illustrated in Figure 4-5 and Figure 4-6. All extract-treated cells have significantly higher numbers of β-galactosidase positive cells than the DMSO control population (1.9%) by 10- to 23-fold. The percentage of senescent cells was significantly higher than control cells by 21.7% and 25.3% when exposed to 1X and 2X GI_{50} of EtOH bark extract (2 and 4 µg/ml).

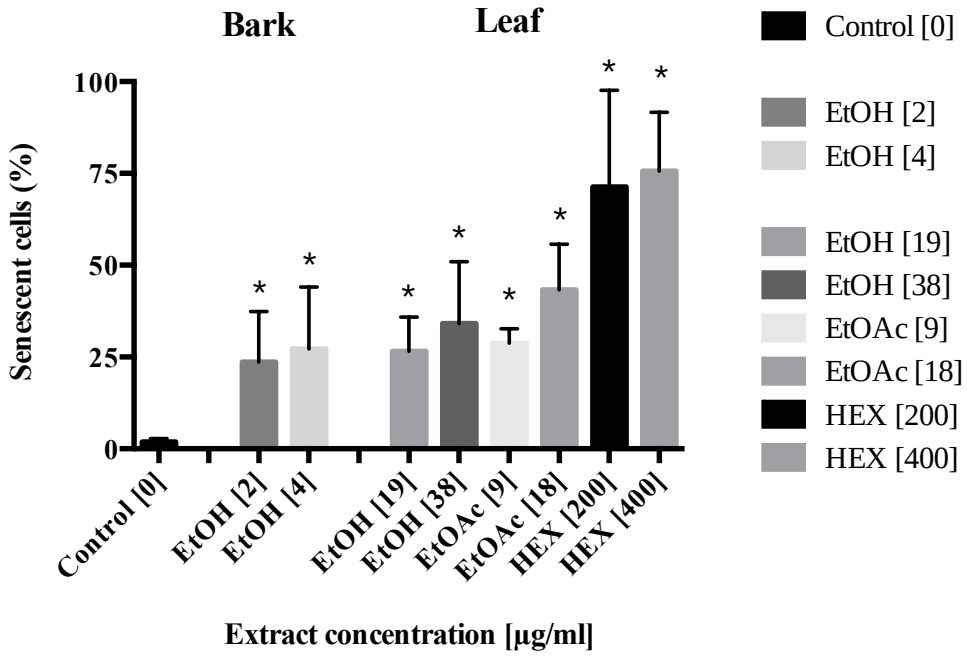


Figure 4-5: MDA-MB-468 cells undergoing senescence after exposure to *A. ellipticum* extracts. Cells were exposed to 1X and 2X GI_{50} concentrations of bark and leaf extracts for 7 days then stained with X-gal followed by 24 h incubation at 37 °C in the dark. Blue/green β-galactosidase^{+} cells were counted under a light microscope. Bars represent mean measurements are from 3 independent experiments (n = 2). Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

Levels of senescent cells were more significant following treatment with extract at high concentrations of EtOH and EtOAc leaf extracts ($p \leq 0.05$). The difference in the levels of senescent cells after exposure to 1X GI_{50} (26.5%)

and 2X GI_{50} (34.1%) EtOH leaf extracts was 7.7%. A larger 14.5% difference in the level of senescence cells between 1X GI_{50} (28.8%) and 2X GI_{50} (43.3%) between EtOAc leaf extracts was observed. Significant numbers of senescent cells were induced at very high concentrations of HEX leaf extracts ($p < 0.05$).

Images of MDA-MB-468 cells were captured to illustrate the appearance of senescent cells (Figure 4-6); cells exposed to *A. ellipticum* EtOH bark extracts appeared to have swelled up and are more circular whereas smaller circular shapes were seen with the EtOAc-treated MDA-MB-468 cells, which is suggestive of apoptotic bodies. Following statistical analysis, significant differences in the level of senescence observed between extract treated cells and control was measured.

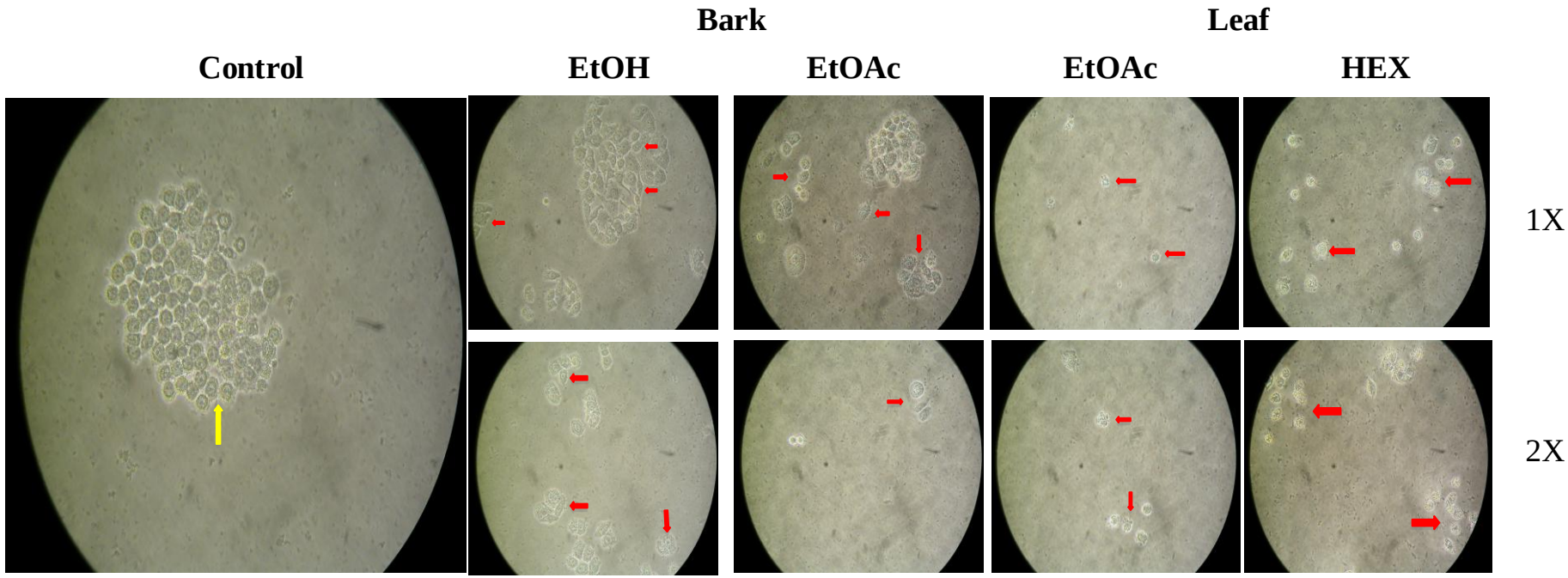


Figure 4-6: Images of β-galactosidase positive (senescent) MDA-MB-468 cells. Senescence was assessed using X-gal staining. Cells were exposed to 1X and 2X GI_{50} *A. ellipticum* extracts concentrations and β-galactosidase^{+} cells appearing blue/green in colour (highlighted by arrows) were counted under a light microscope. Images shown were captured by Nikon Coolpix camera (100X magnification) and are from a single representative experiment (n = 2).

4.2.2 Effect of *Duabanga grandiflora* extracts on cellular senescence

The investigation of *in vitro* anticancer properties of Water and EtOAc bark and leaf extracts was terminated due to the lack of supply. EtOH and HEX bark extracts were investigated for senescence-inducing activity.

The level of senescence in HCT116 cells induced by *D. grandiflora* extracts is illustrated in Figure 4-7. All extract-treated cells appeared to have a higher level of senescence than those measured in the DMSO control population (1.5%) by 2- to 5- fold. Levels of senescent HCT116 cells following exposure to EtOH bark extracts (38 and 76 μg/ml) were measured at 5.8% and 6.0%, respectively. The % of senescent HCT116 cells treated with 1X GI_{50} HEX bark extracts (29 μg/ml) was 2.2% higher than the control population and greater effects were seen at 2X GI_{50} of HEX extract (58 μg/ml) treatment yielding a significant senescent population of 5.6% compared with control cells. Images of HCT116 cells were captured to illustrate the appearance of senescent cells (Figure 4-8). Observation of single cells individually enabled careful examination of each cell to determine not only β-galactosidase status but also morphological changes. HCT116 cells became longer and fibroblast-like with long spindle-like protrusions. Following statistical analysis, significant differences were noticed between control and HEX bark extract-treated cells, however there was no significant difference in the level of senescence observed between the EtOH and low concentrations of HEX extract treated cells ($p > 0.5$).

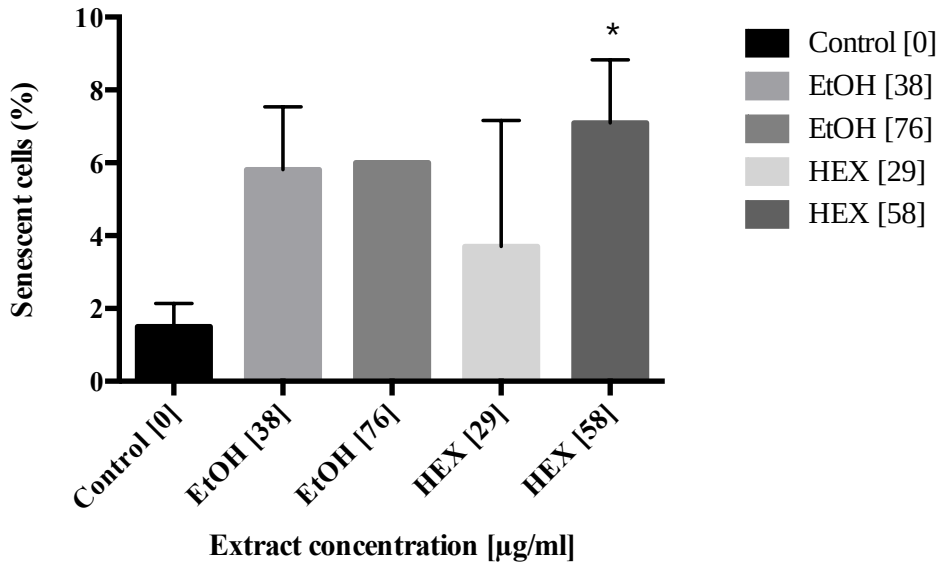


Figure 4-7: HCT116 cells (%) undergoing senescence after exposure to *D. grandiflora* extracts. Cells were exposed to 1X and 4X GI_{50} concentrations of extracts for 7 days then stained with X-gal followed by 24 h incubation at 37 °C in the dark. Blue/green β-galactosidase^{+} cells were counted under a light microscope. Bars represent mean measurements are from 3 independent experiments (n = 2). Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

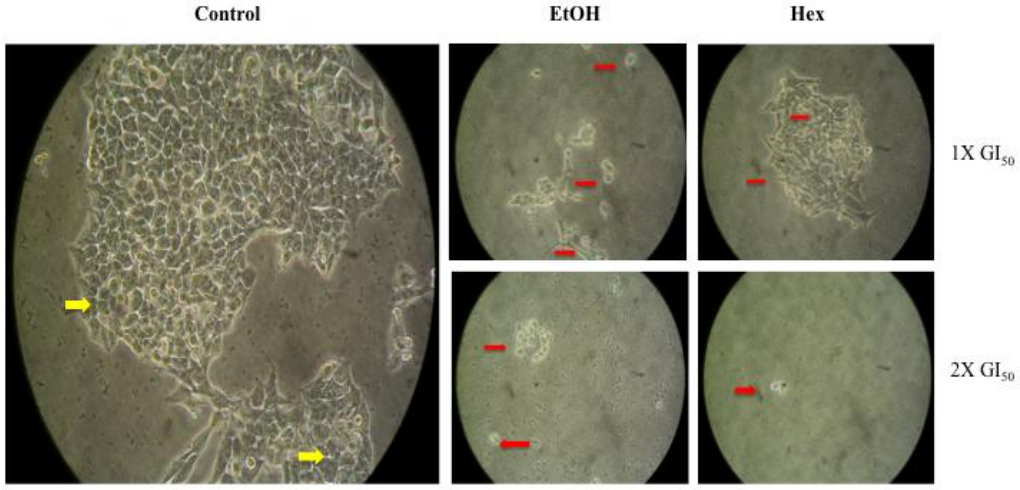


Figure 4-8: Images of β-galactosidase positive (senescent) HCT116 cells. Senescence was assessed using X-gal staining. Cells were exposed to 1X and 2X GI_{50} concentrations of *D. grandiflora* bark extracts and β-galactosidase^{+} cells appearing blue/green in colour (highlighted by arrows) were counted under a light microscope. Images shown were captured by Nikon Coolpix camera (100X magnification) and are from a single representative experiment (n = 2).

4.2.3 Effect of *Pseuduvaria macrophylla* extracts on cellular senescence

EtOH and EtOAc extracts were investigated for senescence-inducing activity. The level of senescence induced in HCT116 cells by *P. macrophylla* extracts is illustrated in Figure 4-9. All extract-treated cells had greater levels of X-gal (β-galactosidase) staining than the DMSO control population (1.5%) by 2- to 5- fold. The percentage of senescent cells was greater by 2.0% and 5.9% when exposed to 1X and 2X GI_{50} of EtOH extract (5 and 20 μg/ml) than control population. Similarly, EtOAc extracts induced greater levels of senescent cells compared to control cells by 3.2% and 7.0% at 2 and 4 μg/ml, respectively.

The difference in the level of senescent cells after exposure to 1X GI_{50} (3.5%) and 2X GI_{50} (7.4%) EtOH extracts was 3.9%. A larger difference in the level of senescence cells between 1X GI_{50} (4.7%) and 2X GI_{50} (8.5%) EtOH leaf extracts was 3.8%. There was not enough evidence to conclude *P. macrophylla* extracts induced significant levels of senescence in sensitive HCT116 cells (Figure 4-9).

Images of HCT116 cells were captured to illustrate the appearance of senescent cells (Figure 4-6). Increased number of 'blue/green' cells covering a wider area can be observed in extract-treated HCT116 cells (Figure 4-10) and extract-treated cells appeared small and circular which could be indicative of apoptotic bodies. A low level of naturally occurring senescent cells was detected in the control population, which may be due to high cell density in certain sections. Statistical analysis revealed significant difference in the level of senescence observed between extract treated cells at 2X GI_{50} and the control ($p < 0.5$).

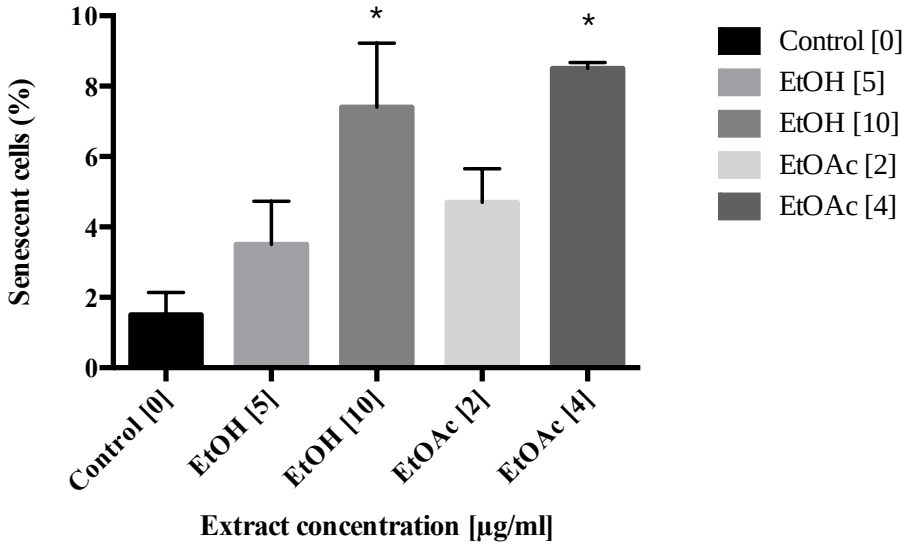


Figure 4-9: HCT116 cells undergoing senescence after exposure to *P. macrophylla* extracts. Cells were exposed to 1X and 2X GI_{50} extract concentrations for 7 days then stained with X-gal followed by 24 h incubation at 37 °C in the dark. β-galactosidase^{+} cells appear blue/green in colour and were counted under a light microscope. Blue/green β-galactosidase^{+} cells were counted under a light microscope. Bars represent mean measurements are from 3 independent experiments (n = 2). Dunnett's multiple comparison (One-way ANOVA) analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = statistically significant ($p \leq 0.05$).

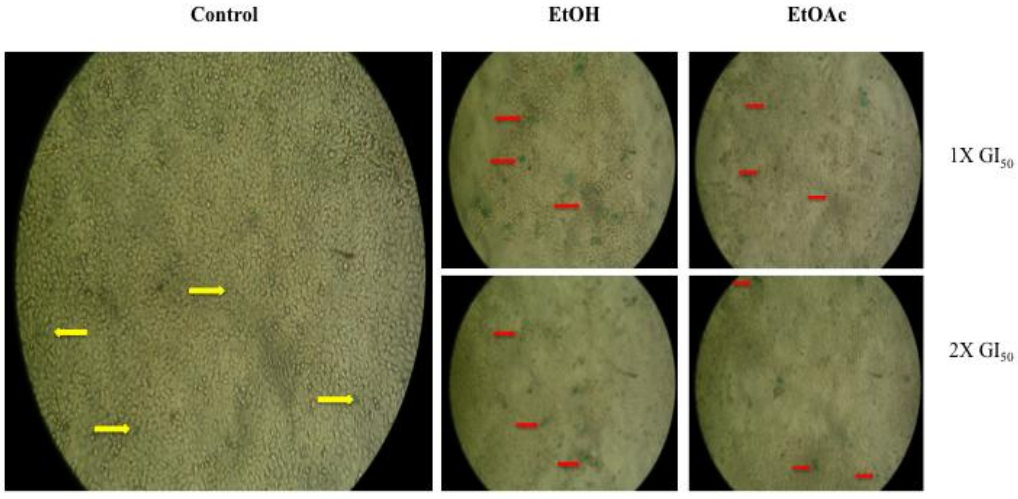


Figure 4-10: Images of β-galactosidase positive (senescent) HCT116 cells. Senescence was assessed using X-gal staining. Cells were exposed to 1X and 2X GI_{50} concentrations of *P. macrophylla* extracts and β-galactosidase^{+} cells appearing blue/green in colour (highlighted by arrows) were counted under a light microscope. Images shown were captured by Nikon Coolpix camera (100X magnification) and are from a single representative experiment (n = 2).

4.2.4 Summary of migration inhibition properties of UNMC extracts

The detection of β-galactosidase in senescence cell populations after exposure to UNMC plant extracts at 1X and 4X GI_{50} was determined by counting the numbers of blue/green (senescent) cells. Low levels of naturally occurring senescent cells seen in the control population could due to high cell density in certain areas.

***Archidendron ellipticum*:** All extracts examined were capable of inducing senescence in the cells at a significantly higher level than control cells. Considering the concentrations of *A. ellipticum* extracts and corresponding senescence, EtOH bark extract (2 and 4 μg/ml) appeared to be the most potent since higher concentrations (of ~ 1.5 to > 22 –fold) of EtOH (9 and 18 μg/ml), EtOAc (9 and 18 μg/ml) and HEX (200 and 400 μg/ml) leaf extracts induced only slightly greater levels of cellular senescence.

***Duabanga grandiflora*:** The ability of extracts to induce senescence in HCT116 cells was observed and in particular HEX bark extract (58 μg/ml; 7.1%) causing significant senescence compared to the DMSO control sample (1.5%). Slight increase in senescence was observed with higher concentrations of EtOH bark extract (76 μg/ml).

***Pseuduvaria macrophylla*:** EtOAc extract was slightly more effective at inducing cellular senescence than EtOH extract even at lower concentrations. The level of senescent cells measured after exposure to *P. macrophylla* extracts was similar to cells exposed to *D. grandiflora* extracts, however *A. ellipticum* extracts were most active at inducing cellular senescence in cells.

Certain compounds present in UNMC extract cells may induce low levels of DNA damage in cells which activate DNA repair mechanisms resulting in a slower transition through the S and G2/M phase, maintaining G1 phase senescence equilibrium, as shown by some sesquiterpene lactone dehydroleucodine.[259] Previous results also suggest accumulated damage will

stimulate the activation of apoptosis and impair cell proliferation. A telomerase inhibitor, telomestatin, isolated from *Streptomyces anultus* can bind to the terminal G-quatert of a DNA quadruplex thus affecting the binding of protective capping proteins with telomerase; this may induce a DNA damage response leading to cell growth arrest, senescence or death by inhibiting telomerase activities (e.g. elongation of telomeres).[260, 261]

5

Chemical content and fractionation of *Pseuduvaria macrophylla*, *Archidendron ellipticum* and *Duabanga grandiflora* crude extracts

5.1 Chemical content and free radical scavenging ability of UNMC crude extracts

Plants live in a conspecific and heterospecific environment. This can depend on complex phytochemical interactions to increase survival chances for themselves and surrounding plants. Plants are able to produce and emit phytochemicals to fend off harmful or unfavourable conditions. Recent reports explain that some plants are also able to emit semi-volatile compounds to 'communicate' to neighbouring plants in order to reduce harm caused by herbivores.[262] For many years, these phytochemicals have been exploited by mankind to treat many adverse biological conditions e.g. use as chemical structures templates to synthesise and develop therapeutic compounds. Plant extracts were subjected to analysis for their flavonoid and phenolic contents using the aluminium trichloride and Folin-Ciocalteau (FC) colorimetric methods. Subsequently, their corresponding antioxidant activity was measured using the DPPH assay.

The aluminium trichloride ($AlCl_3$) colorimetric method was adapted from Meda *et al.* (2005)[263] and Chang *et al.* (2002).[264] $AlCl_3$ can form acid-stable complexes with reactive groups of flavones and flavonols and form acid-labile complexes with the flavonoids. Quercetin is a widely distributed flavonoid found in nature hence was employed as a standard to measure against the UNMC extracts for total flavonoid content determination. Secondly, an FC colorimetric method was adapted from Singleton *et al.* (1999) and Meda *et al.* (2005).[263] FC reagent contains phosphomolybdate and phosphotungstate, which reacts with reducing substances such as polyphenolic compounds. Gallic acid (GA) is a widely distributed phenolic compound found in nature

and is the chosen standard used to measure against the UNMC extracts for total phenolic content determination. Pearson's correlation analysis was used to calculate the coefficient (*r*) to determine the strength of the association of the quercetin and gallic acid standard curves; a strong association is considered if *r* = (-) 1.

Phenolic compounds also act as antioxidants and scavenge free radicals, hence the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) assay was employed to determine antioxidant capacity of UNMC extracts. Antioxidant activity is correlated to phenolic content. In brief, $DPPH^{\cdot}$ is a stable free radical, which can be used to indirectly assess the radical scavenging activity of plant extracts. On reaction with an antioxidant such as polyphenol compounds, $DPPH^{-}$ is reduced to the molecular form (DPPHH) resulting in a colour change from yellow to purple. This change in absorbance can be used to measure the radical scavenging power of the plant extracts. The following method was adapted from the method described by Amic *et al*. (2003)[265] and Nara *et al.* (2006).[266] Antioxidants play a crucial role in the prevention of chronic diseases such as heart disease, cancer and Alzheimer's disease by reducing oxidative stress.[267]

Extracts of UNMC plants *A. ellipticum*, *D. grandiflora* and *P. macrophylla* were tested for their chemical contents and free radical scavenging activity. There was insufficient *A. wilkesiana* extracts for testing and the latest batch of *A. wilkesiana* specimen have been collected from a different location and was not subjected to analytical tests to determine its chemical profile and the variability between batches.

5.1.1 Flavonoid and Phenolic content and free radical scavenging activity of *Archidendron ellipticum* extracts

Flavonoid and phenolic content was determined using 1 mg of *A. ellipticum* extracts. There were comparable quantities of flavonoids detected in the leaf and bark extracts, shown in Table 5-1. The total flavonoids in bark extracts (EtOH, EtOAc and HEX) were estimated to be 2.65, 2.87 and 2.44 mg quercetin equivalent/g, respectively, which is slightly lower than the total flavonoids detected in leaf extracts: 3.57, 3.56 and 2.65 mg quercetin equivalent/g, respectively. Pearson's correlation analysis suggests a strong association of the quercetin standard curve, r = 0.84. Ranked in the order of highest flavonoids present in bark extracts: EtOAc > EtOH > HEX and in leaf extracts: EtOH > EtOAc > HEX.

Table 5-1: Flavonoid content in *Archidendron ellipticum* extracts

***A. ellipticum* extract**		**Total flavonoids as quercetin equivalent mg/ 1g extract (QE)**	**Total phenolics as gallic acid equivalent mg / 1 g of extract (GAE)**
Bark	EtOH	2.65 ± 0.00	1.03 ± 0.01
	EtOAc	2.87 ± 0.00	4.23 ± 0.02
	HEX	2.44 ± 0.00	3.09 ± 0.04
Leaf	EtOH	3.57 ± 0.00	2.44 ± 0.02
	EtOAc	3.56 ± 0.00	0.69 ± 0.00
	HEX	2.65 ± 0.00	0.69 ± 0.01

Flavonoid and Phenolic contents from 1 mg of *A. ellipticum* extracts are presented as quercetin equivalents (QE), mg/ gram of extract and gallic acid equivalents (GAE), mg, /gram of extract (n = 3). Pearson product-moment correlation coefficients were 0.84 and 0.83 for flavonoids and phenolic standard curves, respectively.

Phenolic compounds were detected in the leaf and bark extracts, shown in Table 5-1. The levels of phenolic compounds varied between bark and leaf extracts. The total phenolic compounds in *A. ellipticum* bark extracts (EtOH, EtOAc and HEX) were estimated to be 1.03, 4.23 and 3.09 mg GAE/g, respectively; this is considerably less than the total phenolic compounds

detected in leaf extracts: 2.44, 0.69 and 0.69 mg GAE/g. Pearson's correlation analysis suggests a strong association of the gallic acid standard curve, $r = 0.83$. Ranked in the order of highest phenolic content in bark extracts: EtOAc > HEX > EtOH and in leaf extracts: EtOH > EtOAc = HEX. Previously, Beutler *et al.* (1997) reported detection of a series of new saponins in *A. ellipticum* Ch_2Cl_2:MeOH extracts; plants of this genus have been found to contain sterols, triterpenes and coumarins, which may contribute to phenolic content.[235]

A. ellipticum extracts displayed modest direct antioxidant behavior (Table 5-2); EtOH extracts emerged as the most active and HEX bark extracts were least active compared with the other extracts. Ranked in the order of highest free radical scavenging ability (%) in bark and leaf extracts, respectively: EtOH (43.8, 66.7) > EtOAc (24.1, 41.8) > HEX (3.9, 24.1). Overall, a corresponding order of extract potency and % of radical scavenging ability was observed in leaf extracts. No particular trend was noted between antioxidant activity and the amount of phenolics present in the bark extracts and the amount of flavonoids present in the bark and leaf extracts.

Table 5-2: Free radical scavenging ability of *Archidendron ellipticum* extracts.

	***A. ellipticum* extract**	**Free radical scavenging (%)**
Bark	EtOH	43.8 ± 0.5
	EtOAc	24.1 ± 0.6
	HEX	3.9 ± 2.6
Leaf	EtOH	66.7 ± 0.5
	EtOAc	41.8 ± 0.6
	HEX	24.1 ± 2.6

Average % free radical scavenging ability of extracts ± SEM are shown; the antioxidant content was determined using a standard curve of quercetin (0 – 1.2 mg/ml). Pearson product-moment correlation coefficient was used to measure correlations of free radical scavenging (%) between extracts; three independent experiments were performed (n = 4).

5.1.2 Flavonoid and Phenolic content and free radical scavenging activity of *Duabanga grandiflora* extracts

Flavonoid and phenolic content were determined using 1 mg of *D. grandiflora* extracts. There were considerably greater amounts of flavonoids detected in the leaf extracts compared to bark extracts, shown in (Table 5-3). The total flavonoids in *D. grandiflora* bark extracts (Water, EtOH, EtOAc and HEX) were estimated to be 3.01, 0.08, 0.97 and 0.08 mg quercetin equivalent/g, respectively. This was considerably less than the total flavonoids detected in leaf extracts: 2.39, 3.17, 3.20 and 2.35 mg quercetin equivalent/g, respectively. Ranked in the order of highest flavonoids present in bark extracts: Water > EtOAc > EtOH = HEX and in leaf extracts: EtOAc > EtOH > Water > HEX. Phenolic agents were detected in the leaf and bark extracts. The total phenolic compounds in *D. grandiflora* bark extracts (Water, EtOH, EtOAc and HEX) were estimated to be 12.73, 34.87, 9.57 and 2.20 mg gallic acid equivalent (GAE)/g, respectively. This is less than the total phenolic compounds detected in leaf extracts: 12.60, 27.81, 17.92 and 0.37 mg GAE/g, respectively. Ranked in the order of highest phenolics present in bark extracts: EtOH > Water > EtOAc > HEX and in leaf extracts: EtOH > EtOAc > Water > HEX. The levels of phenolic compounds varied; however, the ranking order of phenolics present was comparable between corresponding extracts in bark and leaf. Overall, HEX extracts contained the least flavonoid and phenolic content. Othman *et al* (2011) found appreciable amounts of phenolic agents in EtOH bark and leaf extracts whilst moderate amounts were detected in EtOAc bark and leaf extracts. There were appreciable amounts of flavonoids in EtOAc bark and EtOH leaf extracts whereas trace amounts were noticed in EtOH bark extract.[205] Additionally, Othman *et al* (2011) investigated the presence of alkaloids, tannins, saponins, steroids and triterpenes in *D. grandiflora extracts;* the aforementioned chemicals were detected in *D. grandiflora* EtOH bark and leaf extracts excluding alkaloids and triterpenes. Whereas, trace amounts of saponins and triterpenes and steroids and triterpenes were found in EtOAc bark and leaf extracts, respectively.[205]

Table 5-3: Flavonoid and Phenolic contents in *Duabanga grandiflora* extracts

***D. grandiflora* extract**		**Total flavonoids as quercetin equivalent mg/ 1g extract (QE)**	**Total phenolics as gallic acid equivalent mg / 1 g of extract (GAE)**
Bark	Water	3.01 ± 0.00	12.73 ± 0.23
	EtOH	0.08 ± 0.00	34.87 ± 0.03
	EtOAc	0.97 ± 0.00	9.57 ± 0.01
	HEX	0.08 ± 0.00	2.20 ± 0.01
Leaf	Water	2.39 ± 0.00	12.60 ± 0.05
	EtOH	3.17 ± 0.00	27.81 ± 0.01
	EtOAc	3.20 ± 0.00	17.92 ± 0.01
	HEX	2.35 ± 0.00	0.37 ± 0.00

Flavonoid and Phenolic contents from 1 mg of *D. grandiflora* extracts are presented as quercetin equivalents (QE), mg/ gram of extract and gallic acid equivalents (GAE), mg, /gram of extract (n= 3). Pearson product-moment correlation coefficients were 0.84 and 0.83 for flavonoids and phenolic standard curves, respectively.

Table 5-4: Free radical scavenging ability of *Duabanga grandiflora* extracts

	***D. grandiflora* extract**	**Free radical scavenging (%)**
Bark	Water	94.4 ± 0.4
	EtOH	38.3 ± 0.5
	EtOAc	86.3 ± 0.6
	HEX	28.7 ± 2.6
Leaf	Water	95.3 ± 0.1
	EtOH	96.3 ± 0.1
	EtOAc	95.7 ± 1.1
	HEX	9.4 ± 1.0

Average % of free radical scavenging abilities of extracts ± SEM are shown; the antioxidant content was determined using a standard curve of quercetin (0 – 1.2 mg/ml). Pearson product-moment correlation coefficient was used to measure correlations of free radical scavenging (%) between extracts; three independent experiments were performed (n = 4).

D. grandiflora extracts displayed direct antioxidant behavior in the DPPH radical scavenging assay (Table 5-4), with Water and EtOAc extracts emerging as the most active (> 85%) and leaf extracts with greater free radical scavenging ability in general. Ranked in the order of highest free radicals scavenging ability (%) in bark extracts: Water (94.4)> EtOAc (86.3)> EtOH (38.3)> HEX (28.7) and in leaf extracts: EtOH (96.3)> EtOAc (95.7)> Water (95.3)> HEX (9.4).

The results suggest that flavonoid levels in bark extracts correlated to their ability in free radical scavenging ability, which was also observed with Water, EtOH and EtOAc extracts possessing high levels of flavonoids. However it was interesting to observe that HEX leaf extract contained similar levels of flavonoids to Water leaf extract, and did not yield comparable antioxidant activity as expected; this maybe due to the difference in different flavonoids, hence difference in activity. There was a general positive correlation between antioxidant activity and phenolic compounds in the extracts; the amount of phenolics detected in leaf extracts corresponded to level of free radical scavenging activity. Whereas no obvious correlation was observed between phenolics level in bark extract and the level of free radical scavenging activity. In general, the extracts (Water, EtOH and EtOAc) with higher quantities of phenolic agents displayed greater levels of DPPH scavenging ability.

5.1.3 Chemical content and free radical scavenging ability of *Pseuduvaria macrophylla* extracts

Flavonoid and phenolic contents were determined using 1 mg of *P. macrophylla* extracts. Flavonoid contents in the leaf and bark extracts is shown in Table 5-5. The total flavonoids in whole extract (EtOH, EtOAc and HEX) were estimated to be 2.39, 2.39 and 1.71 mg QE/g, respectively. Pearson's correlation analysis suggests a strong association of the quercetin standard curve, $r = 0.84$. Ranked in the order of highest flavonoids present in whole extract: EtOAc = EtOH > HEX.

Table 5-5: Flavonoid content in *Pseuduvaria macrophylla* extracts

***P. macrophylla* extract**	**Total flavonoids as quercetin equivalent mg/ 1g extract (QE)**	**Total phenolics as gallic acid equivalent mg / 1 g of extract (GAE)**
EtOH	2.39 ± 0.00	7.09 ± 0.07
EtOAc	2.39 ± 0.01	5.72 ± 0.02
HEX	1.71 ± 0.00	4.06 ± 0.02

Flavonoid and Phenolic contents from 1 mg of *P. macrophylla* extracts are presented as quercetin equivalents (QE), mg/ gram of extract and gallic acid equivalents (GAE), mg, /gram of extract (n= 3). Pearson product-moment correlation coefficients were 0.84 and 0.83 for flavonoids and phenolic standard curves, respectively.

Phenolic compounds were detected in whole extract, shown in Table 5-5. The total phenolic compounds in *P. macrophylla* extracts (EtOH, EtOAc and HEX) were estimated to be 7.09, 5.72 and 4.06 mg GAE/g, respectively. Pearson's correlation analysis suggests a strong association of the gallic acid standard curve, r = 0.83. Ranked in the order of highest phenolics present in extracts: EtOH > EtOAc > HEX. Othman *et al.* (2011) reported phytochemical tests for alkaloids, tannins, phenolics, saponins, flavonoids, steroids and triterpenes were performed revealing moderate amount of alkaloids and phenolics, trace amount of saponins and appreciable amount of steroids within EtOH extracts. In addition, moderate quantities of alkaloids, tannins and steroid, trace amount of triterpenes were present in EtOAc extracts.[205] Several of these phytochemicals were found in bark and leaf *P. macrophylla* EtOH and EtOAc extracts.

P. macrophylla extracts varied in their direct antioxidant ability summarised in Table 5-2, with EtOH extracts emerging as the most active. Highest DPPH radical scavenging ability was observed in the EtOH extract (58.9%), whereas the EtOAc and HEX extracts shared similar antioxidant activity (9.8% and 8.9% respectively). A positive correlation was noted between antioxidant activity and phenolic content.

Table 5-6: Free radical scavenging ability of *Pseuduvaria macrophylla* extracts

***P. macrophylla* extract**	**Free radical scavenging (%)**
EtOH	58.9 ± 15.9
EtOAc	9.8 ± 2.9
HEX	8.9 ± 3.0

Average % of free radical scavenging ability of extracts ± SEM are shown; the antioxidant content was determined using a standard curve of quercetin (0 – 1.2 mg/ml). Pearson product-moment correlation coefficient was used to measure correlations of free radical scavenging (%) between extracts; three independent experiments were performed (n = 4).

5.1.4 Summary of chemical contents and free radical scavenging activity in UNMC extracts

In general, UNMC extracts do possess moderate amounts of phenolic and flavonoid compounds and displayed potent antioxidant activity. As previously mentioned, many of the already known plant-derived drugs are naturally occurring phytochemicals e.g. flavonoids and phenolics and it is known that compounds with antioxidant activity can be used in chemoprevention.[255]

Archidendron ellipticum: Similar quantities of flavonoids were detected across the bark (~ 2.5 mg QE) and leaf (~ 3.5 mg GAE) extracts. The level of phenolic compounds varied across the extracts and bark extracts contained greater amounts than leaf extracts. Leaf EtOH extract contained good levels of flavonoids and phenolics and demonstrated greatest DDPH scavenging activity (66.7%). EtOH and EtOAc bark and EtOAc and HEX leaf extracts demonstrated modest antioxidant activity (24.1% to 43.8%). HEX bark extract was the least active in free radical scavenging (3.9%). It was interesting to observe that HEX leaf extract demonstrated greater antioxidant activity (24.1%) to HEX bark extract that are of similar levels of the aforementioned extracts.

Duabanga grandiflora: In general, Water bark extract and Water, EtOH and EtOAc leaf extracts contained ~ ≥ 2.35 mg of flavonoids (as QE) and ~ ≥ 12.60 mg of phenolics (as GAE) and yielded ≥ 94 % free radical scavenging activity. Interestingly, EtOH bark extract contained the greatest amount of phenolics and the least amount of flavonoids, however the antioxidant activity was only 38.3%. Whereas, EtOAc bark extract contained modest amounts of flavonoids (0.97 mg) and phenolics (9.57 mg) yet the antioxidant activity was 86.3% as compared 94.4% Water bark extract (flavonoids: 3.01 mg and phenolics: 12.73). Both HEX extracts contained the least amount of flavonoid and phenolic compounds accompanied by lowest % of DPPH scavenging.

Pseuduvaria macrophylla: EtOH and EtOAc extracts contained the same flavonoid content (2.39 mg, GE) and in comparison, HEX extract contained ~

2-fold less flavonoid compounds; and modest levels of phenolic compounds were present in all extracts. The highest antioxidant activity was seen in EtOH (58.9%) whereas EtOAc (9.8%) and HEX (8.9%) displayed similar levels of antioxidant activity. Previously, Mahmood *et al.* (1986) isolated an aporphinoid from *P. macrophylla* extracts.

Collectively the ranking of highest level of flavonoid compounds present in extracts is: *A. ellipticum* extracts leaf > *D. grandiflora* leaf > *A. ellipticum* extracts bark > *P. macrophylla* whole > *D. grandiflora* bark extracts. The ranking of highest level of phenolic compounds present in extracts is: *D. grandiflora* bark > *D. grandiflora* leaf > *P. macrophylla* whole > *A. ellipticum* extracts bark > *A. ellipticum* extracts leaf extracts. The ranking of highest level of free radical scavenging activity seen in extracts is: *D. grandiflora* leaf > *D. grandiflora* bark > *A. ellipticum* extracts leaf > *A. ellipticum* extracts bark > *P. macrophylla* whole extracts.

5.2 Chromotography of UNMC crude extracts and effects of fractions on cell growth

Compound mixtures can be separated using a mobile and a stationary phase in chromatography. Reverse column chromatography solid-phase extraction (SPE) can separate crude extracts using different eluents (of varying polarity) and a non-polar stationary phase into phytochemicals groups with similar physicochemical characteristics such as solubility, size, shape and electrical charge.[18, 193] Subsequent analysis using hyphenated techniques such as HPLC and MS can produce a unique spectrum for each extract. Each compound has a unique profile, which can be matched against in a database. UNMC crude extracts were separated using a C-18 extraction cartridge and solvents of varying polarity; subsequently the fractions were analysed using LC-MS (see Appendix 2 for the fraction yields (%)). Fractions were examined for their bioactivity by MTT assay to determine whether concentrating the active component(s) and/or reducing putative antagonistic or synergistic interactions with other phytochemicals in the complex mixture could achieve greater or the same activity (active fraction) or lower activity (inactive fraction). The results could provide fraction leads for further bio-guided sub-fractionation and lead to possible study into synergistic or antagonistic activities and isolation of active component(s).

5.2.1 Chromotography of *Archidendron ellipticum* crude extracts and *in vitro* growth inhibitory properties of fractions in MDA-MB-468 cells

HPLC spectra of *A. ellipticum* bark and leaf extracts measured at 220 and 254 nm, are shown in Figure 5-1 and the main dominant peaks are represented in Table 5-7. Due to insufficient HEX bark extracts, fractionation could not be performed on this extract. A solvent peak is illustrated in the spectra at t_R ~0.32 min. There was no dominant peak present in EtOH crude bark extracts, which suggest glycoside compounds are unlikely to be present. The most abundant peak detected in the EtOAc bark spectrum was at t_R 3.10 min amongst several peaks between t_R 2.10 to 3.00 min. This implies that alkaloids,

aglycones and glycosides could be present in these crude extracts. Similarly, the spectrum of crude HEX bark extract revealed various minor peaks detected and a major peak at t_R 3.09 min at 220 nm (Figure 5-1; C); the compounds embedded in HEX crude extract are likely to be waxes, fats and oils. Both spectra of EtOAc and HEX bark extracts reveal a peak detected at t_R ~3.10 min. The appearance of a major peak t_R ~2.10 min in EtOH and EtOAc could be indicative of similar components present in leaf extracts and not found in bark extracts. Whereas, the appearance of t_R ~ 3.11 min in HEX leaf crude extract suggests that some of the components are also present in the HEX bark crude extract; a dominate peak at t_R ~ 4.97 min was also observed in HEX leaf crude extract.

Table 5-7: Retention time and colour of *Archidendron ellipticum* extracts dominant peaks using reversed phase chromatography.

***A. ellipticum* extracts**		**Retention time (t_R, min) an colour of *A. ellipticum* extracts**						
		Crude	F1	F2	F3	F4	F5	F6
Bark	EtOH	2.08 *Golden brown*	2.41 *Clear*	4.24 *Pale yellow*	2.15 *Golden*	**2.34** *Yellow*	3.75 *Green tint*	4.24 *Clear*
	EtOAc	3.10 *Dark green/ brown tint*	- *Yellow tint*	- *Clear*	2.21 *Yellow tint*	**3.12** *Pale yellow*	**3.11** *Pale yellow*	4.15 *Pale yellow*
	HEX	4.09 Light green	-	-	-	-	-	-
Leaf	EtOH	2.10 Dark green	2.63 *Yellow tint*	4.21 *Clear*	2.27 *Yellow /brown hint*	**2.47** *Yellow*	**2.70** *Yellow /green tint*	4.14 *Pale yellow*
	EtOAc	2.12 *Very dark green*	4.27 *Clear*	2.64 *Clear*	2.11 *Clear*	**3.19** *Yellow tint*	**4.05** *Dark green*	**3.65** *Green*
	HEX	4.97 *Dark green*	2.91 *Clear*	4.23 *Clear*	5.09 *Clear*	**2.89** *Yellow tint*	**3.12** *Yellow*	**3.16** *Pale yellow*

Extracts (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm. Data of active fractions are highlighted in **bold**, data of inactive fractions are displayed in grey and '-' denotes not available data.

Following chromatography of *A. ellipticum* crude extracts of varying polarity into six fractions; *in vitro* growth inhibitory properties of these fractions in MDA-MB-468 cells were examined (Table 5-8). HPLC spectra of the active fractions are shown Figure 5-3 to Figure 5-6 and spectra of inactive fractions are shown in Appendix 3, Figure 8-15 to 8-21. Interestingly, fractionation of crude EtOH bark extract revealed that F4 was the only active fraction with a very low GI_{50} value (1.75 µg/ml), almost identical to that of whole extract, hence it is very likely that the active component is present in this fraction and is worthy of sub-fractionation. There appears to be a mixture of components in EtOAc bark F4 and F5 as represented by various peaks in the HPLC spectra; F4 and F5 exhibited greater *in vitro* growth inhibition in MDA-MB-468 cells compared to crude EtOAc extract.

Table 5-8: *In vitro* growth inhibition effect of *Archidendron ellipticum* extracts fractions in MDA-MB-468 cells.

***A. ellipticum* crude extract GI_{50} value** [µg/ml]		**GI_{50} values of *A. ellipticum* fractions (µg/ml)**					
		F1	F2	F3	F4	F5	F6
Bark	**EtOH** [1.73]	> 50	> 50	> 50	1.75	> 50	> 50
	EtOAc [40.44]	> 50	> 50	> 50	**26.35**	**32.13**	> 50
	HEX [>200]	-	-	-	-	-	-
Leaf	**EtOH** [40.92]	> 50	> 50	> 50	**2.00**	**7.66**	> 50
	EtOAc [24.73]	> 50	> 50	> 50	**14.26**	**5.63**	**7.61**
	HEX [>200]	> 50	> 50	> 50	**4.10**	**6.02**	**12.64**

Extract concentration (µg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h. The mean GI_{50} values (µg/ml) ± SEM were obtained from ≥ 3 (n = 4) individual experiments. Fractions exhibiting GI_{50} values less than GI_{50} parent crude extract are considered active and highlighted in **bold,** fractions with GI_{50} values > 50 µg/ml are considered as inactive and '-' denotes unavailable extracts.

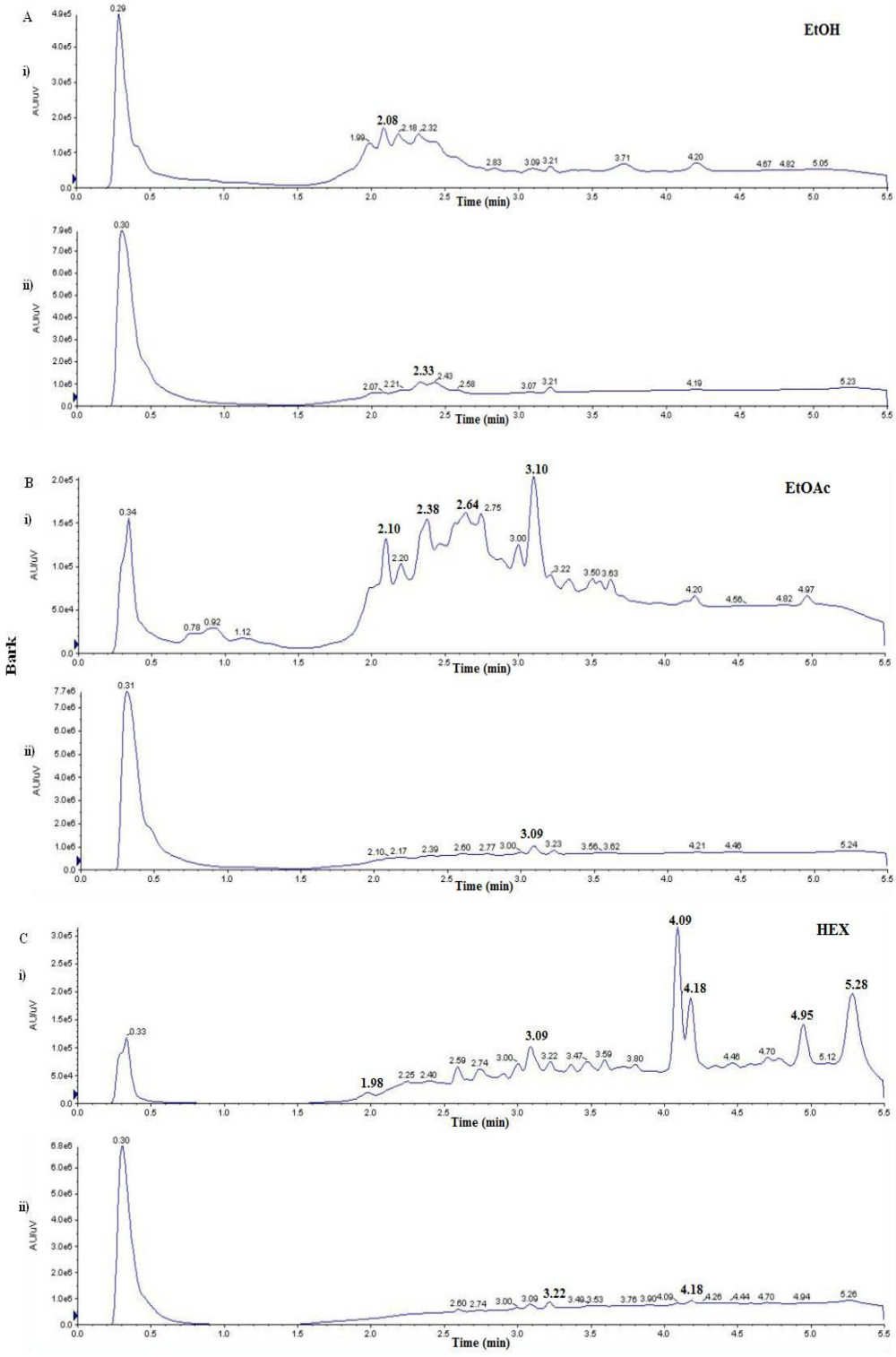


Figure 5-1: HPLC traces of *A. ellipticum* crude bark extracts using Reversed Phase chromatography. EtOH (A), EtOAc (B) and HEX (C) extract eluents (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

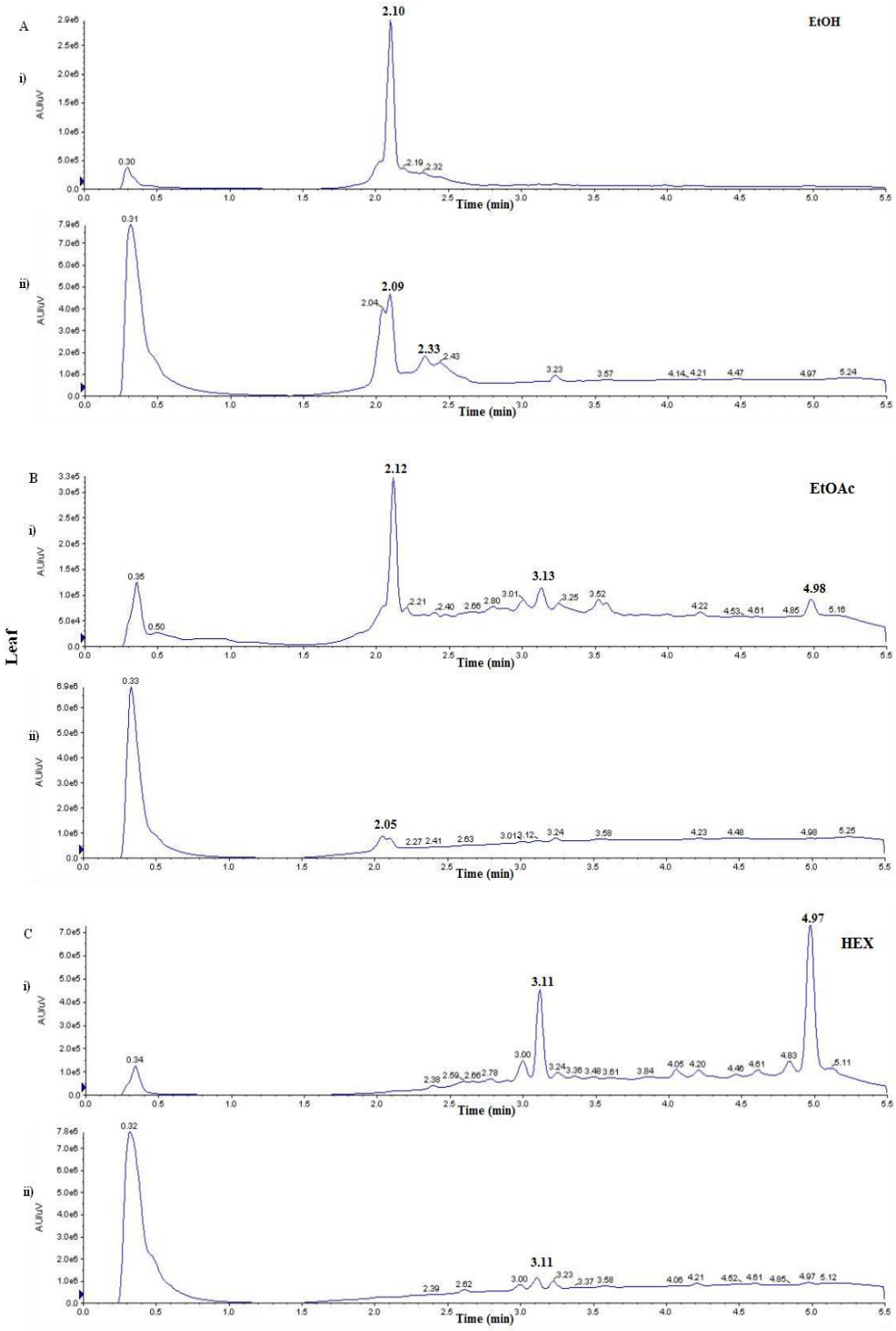


Figure 5-2: HPLC traces of *A. ellipticum* crude leaf extracts using Reversed Phase chromatography. EtOH (A), EtOAc (B) and HEX (C) extract eluents (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

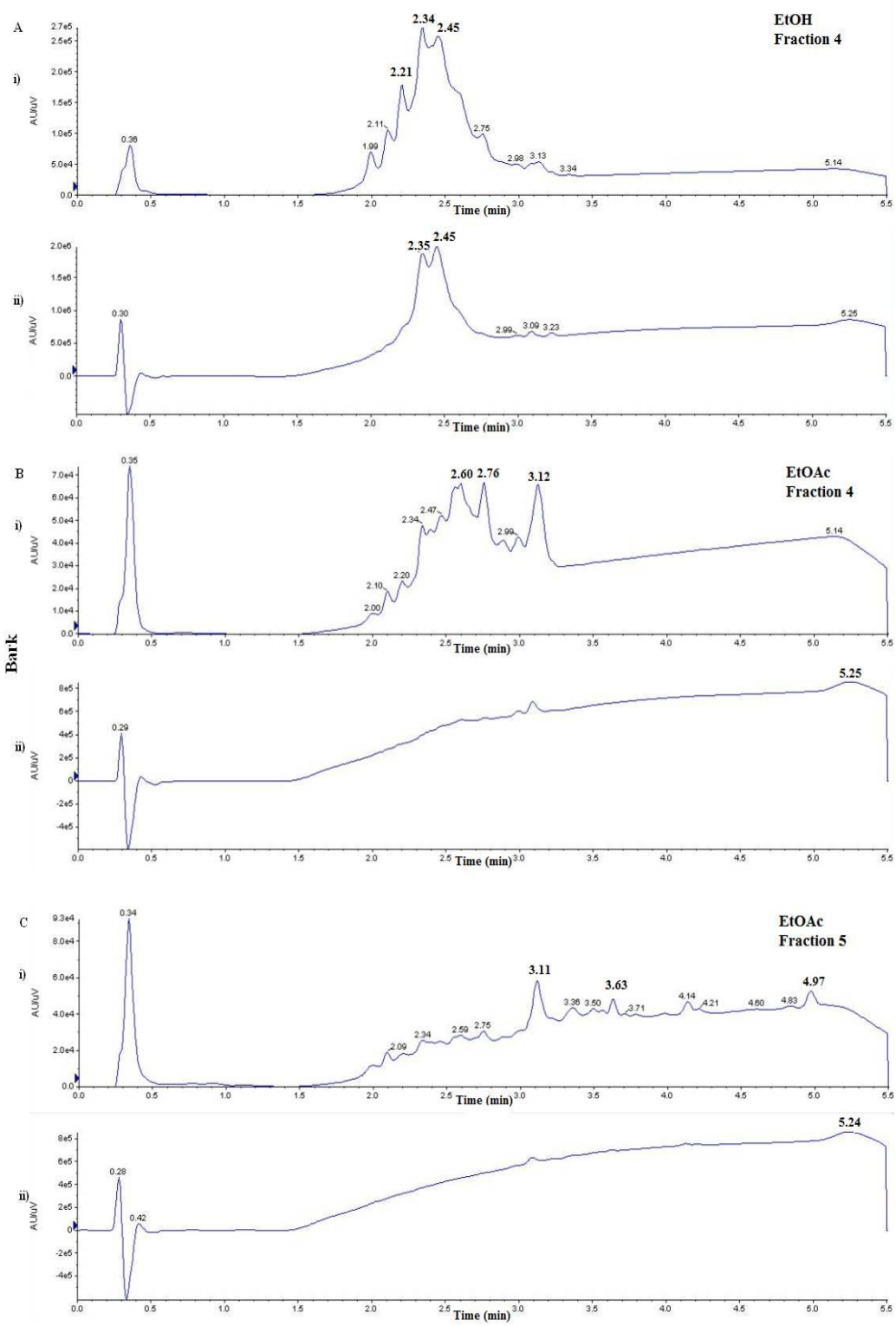


Figure 5-3: HPLC traces of *A. ellipticum* bark extract 'active' fractions using Reversed Phase chromatography. EtOH: F4 (A) and EtOAc: F4 (B) and F5 (C) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

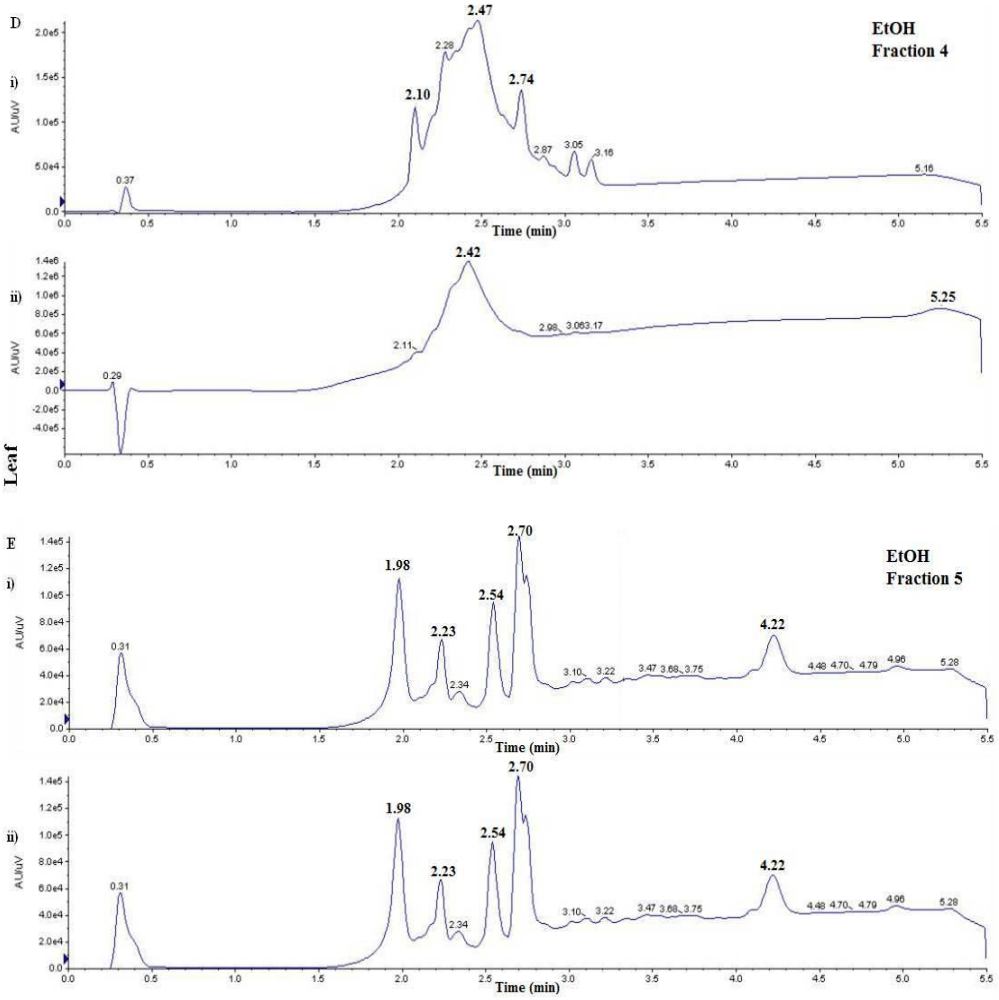


Figure 5-4: HPLC traces of *A. ellipticum* leaf extract 'active' fractions using Reversed Phase chromatography. EtOH: F4 (D) and F5 (E) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

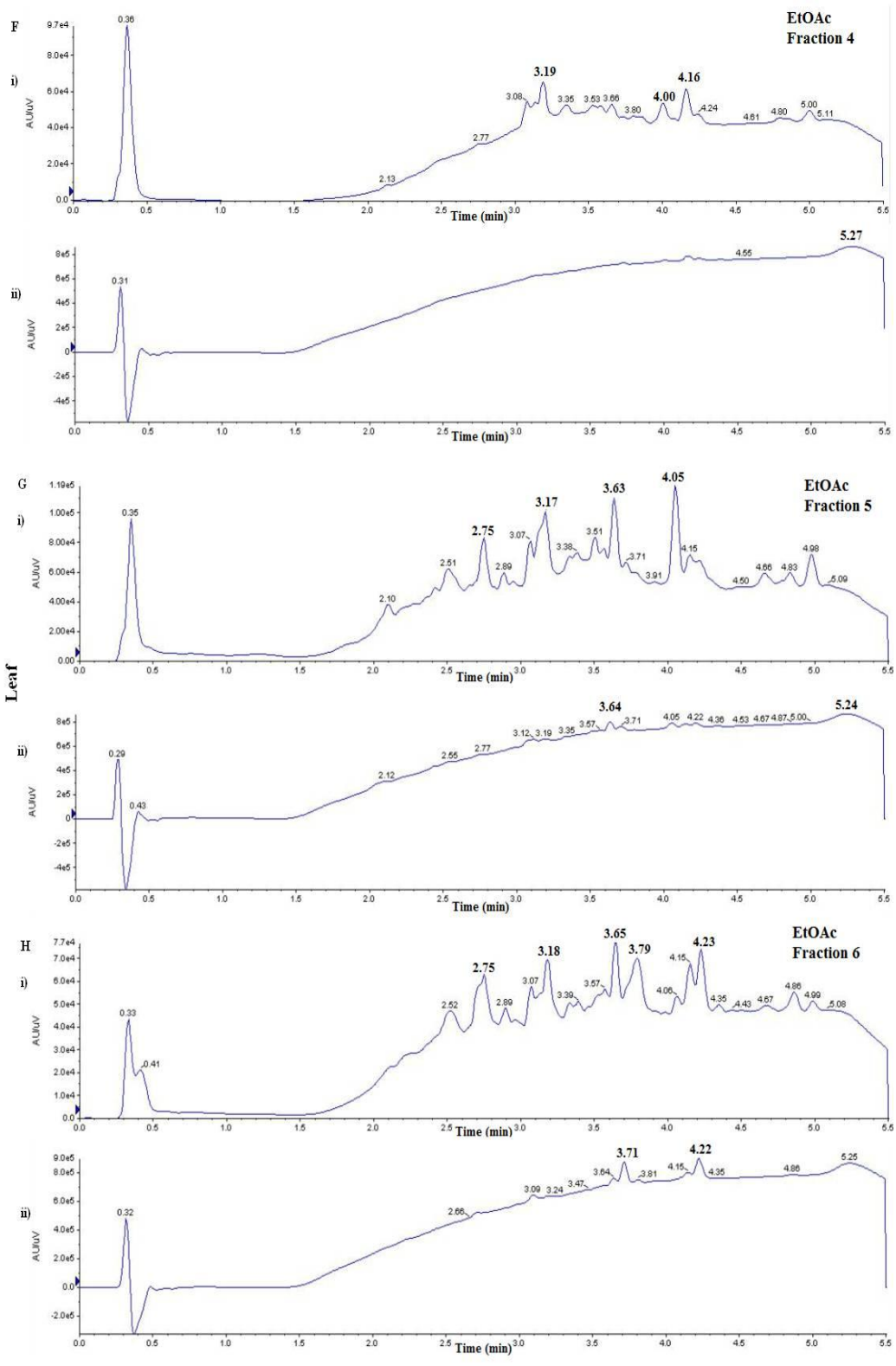


Figure 5-5: HPLC traces of *A. ellipticum* leaf extract 'active' fractions using Reversed Phase chromatography. EtOAc: F4 (F), F5 (G) and F6 (H) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

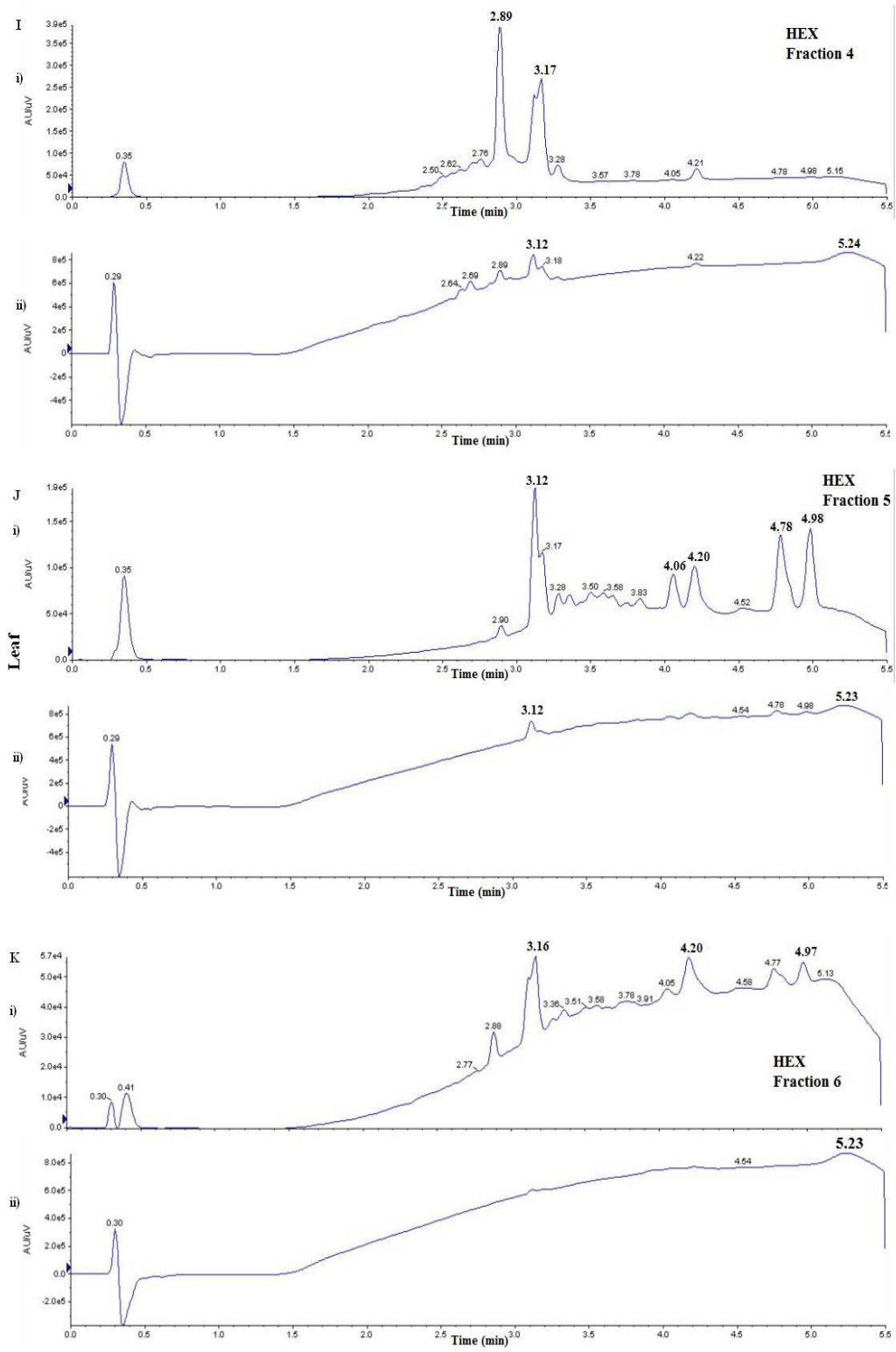
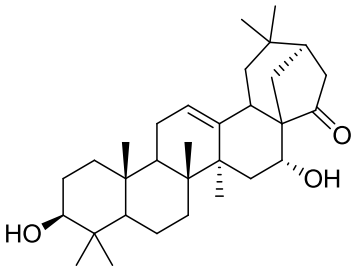


Figure 5-6: HPLC traces of *A. ellipticum* leaf extract 'active' fractions using Reversed Phase chromatography. HEX: F4 (I), F5 (J) and F6 (K) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

EtOH leaf F4 and F5 displayed by 20.5- and 5.3- fold greater activity, respectively against MDA-MB-468 cells compared to the crude extract; whereas all the other fractions induced no growth inhibition at ≤ 50 μg/ml; hence F4 and F5 should be further fractionated and apoptotic and anticancer properties of sub-fractions examined. The spectra of EtOH leaf F4 revealed an abundance of peaks between t_R ~ 2.10 to 3.16 min at 220 nm. Major peaks at t_R 1.98, 2.23, 2.54 and 2.70 were detected in EtOH leaf F5 at both wavelengths (220 and 254 nm), which could be indicative of amino acids with aromatic groups. Likewise, EtOAc leaf F4, F5 and F6 exerted *in vitro* cytotoxic effects greater than previously observed in the crude extract by 1.7-, 4.4 and 3.2- fold, respectively; the spectra of F4, F5 and F6 reveal that several components eluted in these fractions share similar retention time. Astoundingly, fractions from "inactive" crude HEX leaf extracts (GI_{50} > 200 μg/ml) displayed very potent *in vitro* activity revealing GI_{50} value < 13 μg/ml where previously no activity was seen in MDA-MB-468 cells at 200 μg/ml HEX crude leaf extract (Table 5-8). Two dominant peaks at t_R ~ 2.89 and 3.14 min were detected in HEX leaf F4as well as in F5 and F6 at a lower concentration. The peaks which appeared in all three spectra were measured at t_R ~2.89, 4.05, 4.20, 4.79 and 4.98 min. Overall, fractionation of *A. ellipticum* extract yielded fractions with greater *in vitro* activity than the crude extract. Interestingly, EtOH bark F4 was the only active fraction with an almost identical activity to that seen in the crude extract; HEX leaf extract fractions F4, F5 and F6 displayed intriguing activity suggesting that in the whole extract components interact antagonistically or the activity of the active component(s) was 'masked' by all the other components and needs to be concentrated for its activity to be detected.

Previous NCI 60 screening in the 60 cell line panel conducted by Beutler *et al.* (1997) illustrated *in vitro* cytotoxicity of an elliptoside isolated from *A. ellipticum* DCM:MeOH (1:1 v/v) leaf extract with mean panel GI_{50} of 1 μg/ml. This biologically active elliptoside possesses a novel structure yet shares similarities to saponins reported from other leguminosae.[235] The NCI-60 mean fingerprint graph for saponin of *Acer negundo* and 8 unrelated cytotoxic

saponins was reported to have a similar cytotoxicity profile to that of the elliptoside (40) (Figure 5-8).[235]



40

Figure 5-7: The chemical structure of elliptoside (40) isolated from *Archidendron ellipticum*.

However, *in vivo* screening results with a human melanoma (LOX) xenograft model in athymic mice and elliptoside (40) administered by intravenous and intraperitoneal methods only hinted at modest activity, which was expected as the fingerprint graph reveals that most melanoma cell lines including LOX1MV1, are insensitive to compound 40.[235] The fingerprint graphs of elliptoside (40) and *A. ellipticum* bark extracts reveal similar sensitivity patterns of the cell lines hence compound 40 could reside in bark sections of *A. ellipticum* plant.

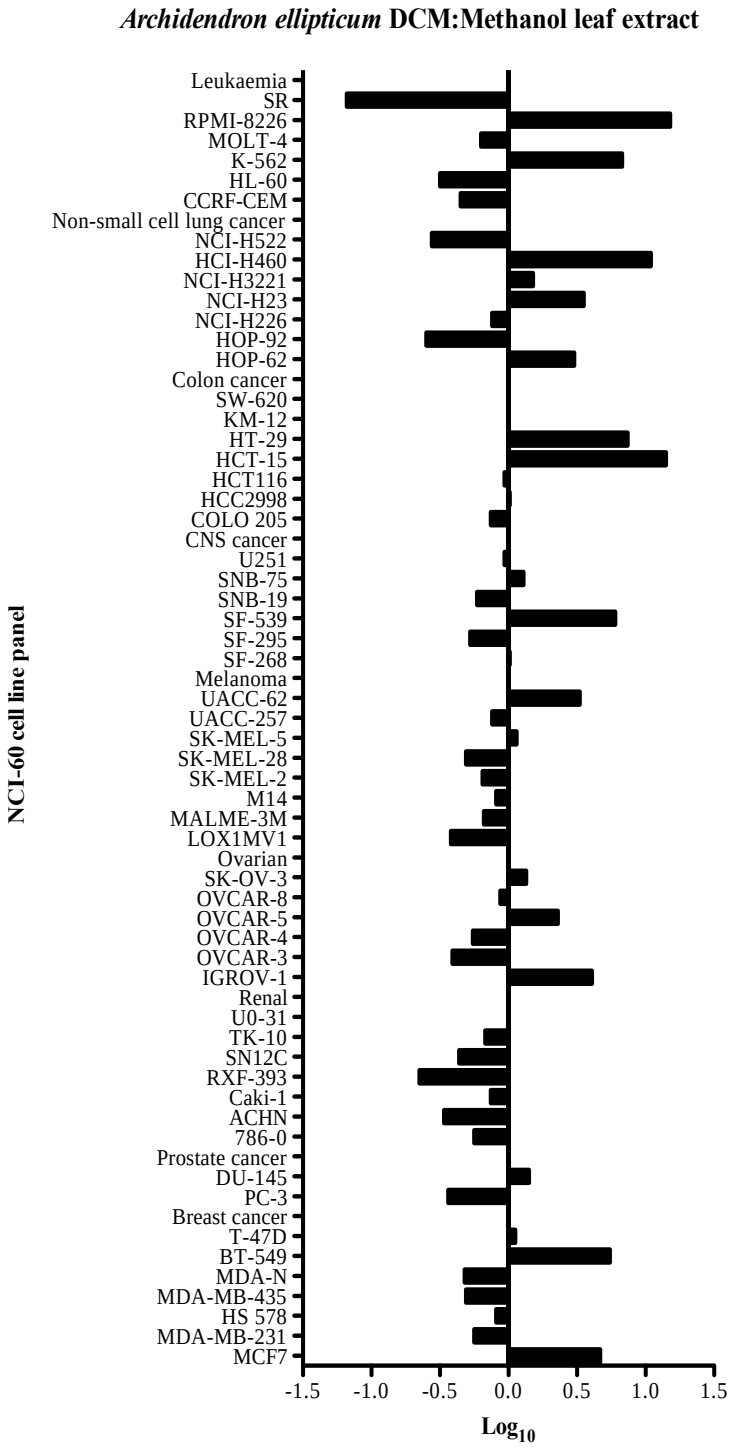


Figure 5-8: NCI-60 mean fingerprint graphs for an elliptoside extracted from *A. ellipticum* (synonym *Archidendron ellipticum*) leaf extracts adapted from GI_{50} values (log_{10}) published by Beutler *et al.* (1997).[235] Bars represent the individual mean GI_{50} value to the function of log10 of the cell line. The overall mean GI_{50} value of all the cell lines was set to the center (0.0). Bars to the left indicate cell lines that are less sensitive than the mean whereas bars to the right indicate cell lines that are more sensitive than the mean to the extract.

5.2.2 Chromotography of *Duabanga grandiflora* crude extracts and *in vitro* growth inhibitory properties of fractions in HCT116 cells

There was insufficient Water extract stock to perform analysis. HPLC spectra of *D. grandiflora* bark and leaf extracts were measured at 220 and 254 nm (Figure 5-9 and Figure 5-10, respectively) and dominant peaks recorded (Figure 5-2). A solvent peak is illustrated in all spectra at retention time (t_R) ~0.32 min. The major peaks in bark extracts were revealed at t_R 1.97 min (Water) and 1.96 min (EtOH) and minor peaks at t_R 2.10 min (Water) and 2.11 min (EtOH); similarly, the major and minor peaks in leaf extract were revealed at t_R 1.96 (Water) min and 1.98 min (EtOH). This suggests compounds (represented by the peaks) present in the crude extracts eluted are likely to be of the same class such as sugars, amino acids and glycosides. A major peak at t_R 1.99 min and a minor peak at t_R 2.10 from EtOAc bark and leaf analyses also implies that sugars, amino acids and glycosides could be present in these crude extracts. Nevertheless, the spectra of crude HEX bark extract revealed various minor peaks detected at 220 nm and several major peaks at t_R 4.09, 4.18, 4.95 and 5.28 min; significant peaks were not detected in HEX leaf extract (Figure 5-11; D) at 220 nm. Due to the non-polar nature of HEX, the compounds embedded in the crude extract are likely to be waxes, fats and oils.

Chromatography of *D. grandiflora* crude extracts into six fractions, the sub-fractions obtained were examined for their *in vitro* growth inhibitory properties using the MTT assay. HPLC spectra of the active fractions are shown in Figure 5-11 to Figure 5-13. Fractions from leaf extracts did not demonstrate any *in vitro* growth inhibition at concentrations ≤ 50 μg/ml (HPLC spectra of inactive fractions are presented in Appendix III-I to M). Several fractions from bark extracts displayed growth inhibitory effects at < 50 μg/ml in HCT116 cells (Figure 5-11 to Figure 5-13). A major peak at t_R ~1.97 min and minor peaks were revealed in F3, F4 and F5 from EtOH active fractions, which suggest this peak area contains some of the active components. Although EtOH bark F2 contained the same major peak, the concentrations detected were much less (2-fold). The presence of t_R ~1.97 major peak in inactive F2 and F3 EtOAc was found at a level similar to F1, F4, F5 and F6 of EtOAc

extracts in which activity was observed, however the spectra identifies several minor peaks suggesting minor components could be responsible for the activity or other components in F2 and F3 could cause antagonistic effects.

Table 5-9: Retention time and the colour of *Duabanga grandiflora* extracts dominant peaks using reversed phase chromatography.

	***D. grandiflora* extract**	**Retention time (t_R, min) and colour of *D. grandiflora* extracts**						
		Crude	F1	F2	F3	F4	F5	F6
Bark	Water	1.97 *Dark brown*	-	-	-	-	-	-
	EtOH	1.96 *Tan brown*	4.25 *Tan brown*	2.24 *Darker tan brown*	**1.98** *Light brown*	**1.97** *Clear*	**1.98** *Clear*	2.90 *Clear*
	EtOAc	1.99 *Light 'pine' brown*	**1.99** *Light brown*	2.51 *Pale brown*	2.74 *Golden*	**2.00** *Golden*	**1.97** *Golden*	**2.70** *Yellow hint*
	HEX	4.09 *Dark yellow/ green hint*	4.22 *Clear*	4.22 *Clear*	2.64 *Clear*	3.09 *Clear*	**4.12** *Pale yellow*	**4.11** *Pale yellow*
Leaf	Water	1.96 *Dark brown*	-	-	-	-	-	-
	EtOH	1.98 *Medium brown*	2.12 *Golden brown*	2.24 *Golden brown*	4.22 *Pale yellow/ brown hint*	2.83 *Clear*	4.13 *Yellow hint*	4.22 *Clear*
	EtOAc	1.99 *Dark green/ brown hint*	2.08 *Pale yellow*	2.06 *Pale yellow*	2.54 *Pale yellow*	2.91 *Pale yellow*	3.72 *Yellow*	3.64 *Yellow*
	HEX	4.96 *Green/ brown hint*	4.21 *Clear*	4.21 *Clear*	4.22 *Clear*	4.22 *Clear*	3.74 *Clear*	4.21 *Clear*

Extracts (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm. Data of active fractions are highlighted in **bold**, inactive fractions are displayed in grey and '-' denotes not available data.

Interestingly, active F5 and F6 of HEX bark fractions revealed a different set of minor and major peaks (t_R = 4.12 min; 2.75, 3.00 and 3.11 min, respectively); these peaks were absent from corresponding leaf fractions. A major peak at t_R ~1.98 min was detected in HEX bark fractions at low concentrations in F2, F3 and F4 amongst several minor peaks at 220 and 254

nm. Noticeably, *D. grandiflora* EtOAc bark F1 displayed a conjoined major peak at t_R ~1.99 min and a single major peak at t_R ~1.99 in all the other fractions. This major peak was the only peak detected in HEX F3 at 220 nm. Comparable spectra of EtOAc fractions containing a conjoined major peak in EtOAc leaf F1, which is similar to that seen in EtOH; the major peak at t_R ~1.99 was detected at 220 nm in all fractions. There were few significant peaks detected in HEX bark fractions at 220 and 254 nm suggesting that there is a lack of aromatic groups, amide bonds (proteins or amino acids) present.

Table 5-10: *In vitro* growth inhibitory effects of *Duabanga grandiflora* extract fractions in a HCT116 cells revealed by the MTT assay.

***D. grandiflora* crude extract GI_{50} value** [µg/ml]		**GI_{50} values of *D. grandiflora* fractions (µg/ml)**					
		F1	F2	F3	F4	F5	F6
Bark	**EtOH** [37.54]	> 50	> 50	30.01	**27.41**	**15.90**	> 50
	EtOAc [21.69]	38.76	> 50	> 50	39.30	28.99	27.09
	HEX [28.88]	> 50	> 50	> 50	> 50	29.62	41.60
Leaf	**EtOH** [40.92]	> 50	> 50	> 50	> 50	> 50	> 50
	EtOAc [24.73]	> 50	> 50	> 50	> 50	> 50	> 50
	HEX [>200]	> 50	> 50	> 50	> 50	> 50	> 50

Extract concentrations (µg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h treatment. Mean GI_{50} values (µg/ml) ± SEM were obtained from ≥ 3 (n = 4) individual experiments. Fractions exhibiting GI_{50} values less than GI_{50} parent crude extract are considered active and highlighted in **bold,** fractions with GI_{50} values > 10 µg/ml are considered as inactive and '-' denotes unavailable extracts.

Overall, only F4 and F5 of EtOH bark fractions revealed greater growth inhibitory activity than that of the crude extract by 1.4- and 2.4- fold, respectively, therefore these two fractions should be subjected to further bio-guided fractionation in order to isolate and identify the active components. The decrease in activity of EtOAc bark F2 suggests that the embedded phytochemicals interact synergistically. Furthermore, HEX bark F5 displayed a similar GI_{50} value to that of the crude suggesting that the active component(s) is embedded in this fraction and is worthy of further investigation. Since eugeniin has been isolated from *D. grandiflora*, the presence of aromatic groups should be expected. Cytotoxic effects of compounds extracted from *D. grandiflora* on HuCCA-1, A549, HeLa, HepG2 and MDA-MB-231 have been demonstrated by Kaweetripob (2012) *et al.*[193]

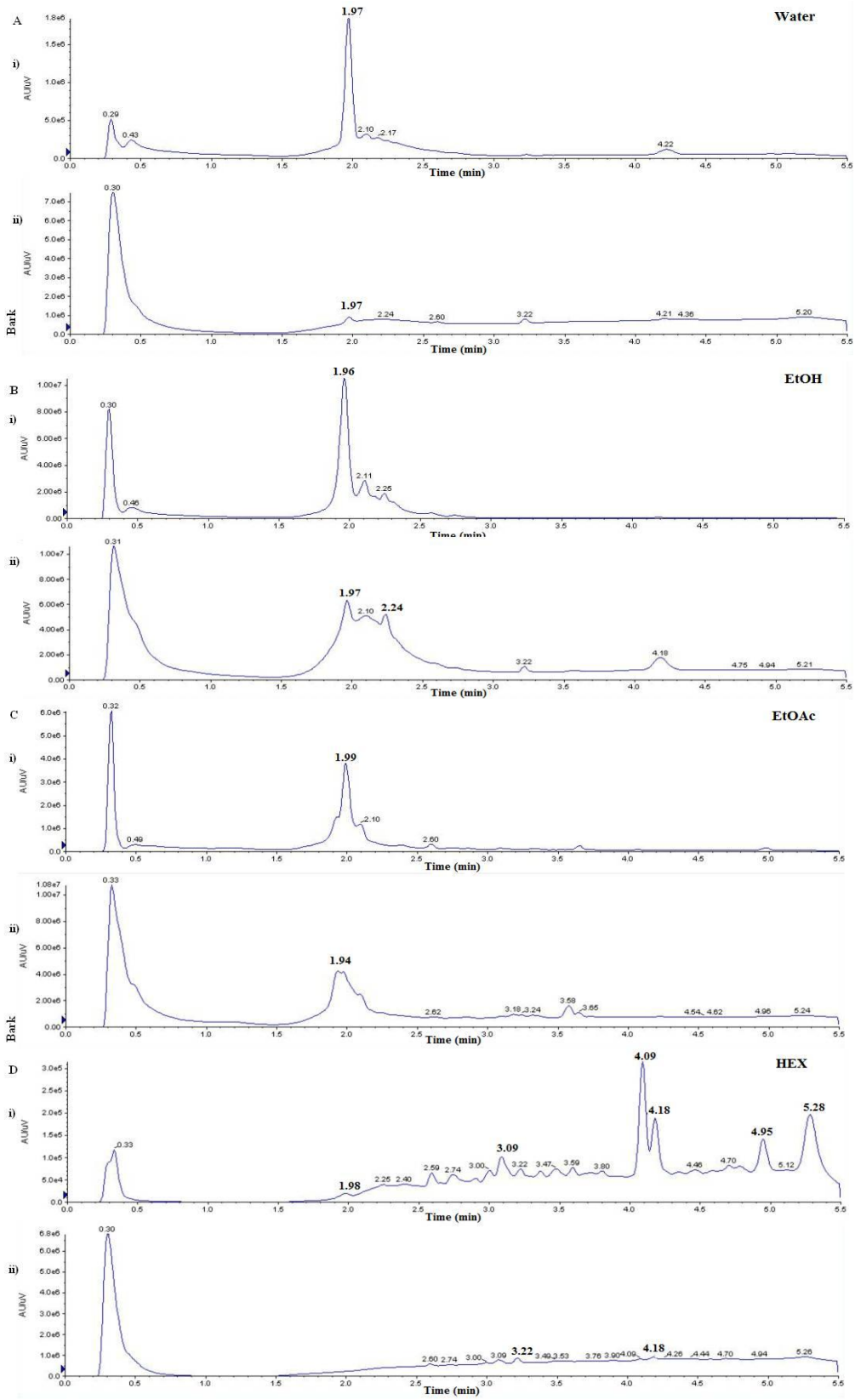


Figure 5-9: HPLC traces of *D. grandiflora* crude bark extracts using Reversed Phase chromatography. Water (A), EtOH (B), EtOAc (C) and HEX (D) extract eluents (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected with a UV light at λ = 220 (i) and 254 (ii) nm.

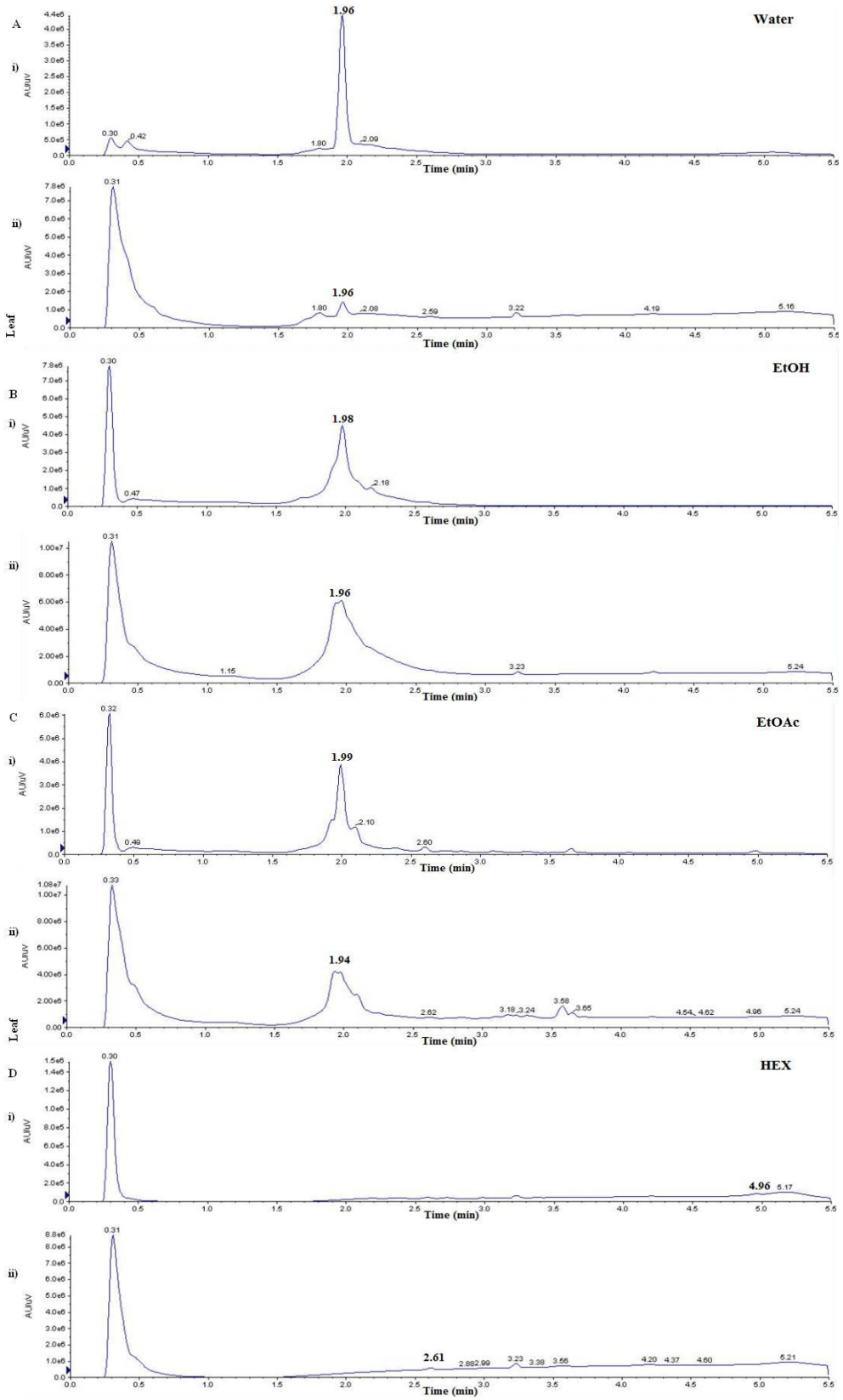


Figure 5-10: HPLC traces of *D. grandiflora* crude leaf extracts using Reversed Phase chromatography. Water (A), EtOH (B), EtOAc (C) and HEX (D) extract eluents (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50 x 2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

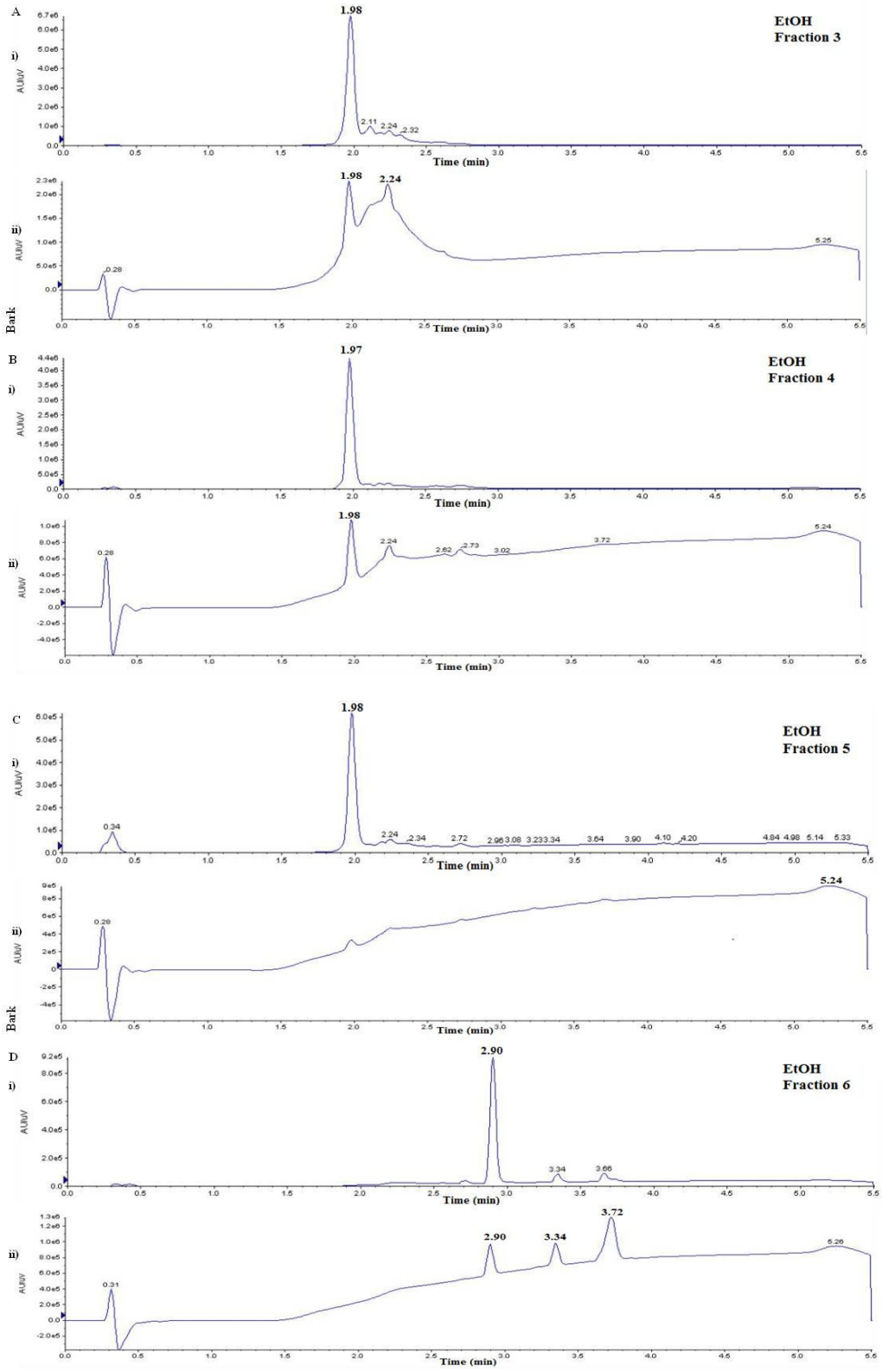


Figure 5-11: HPLC traces of *D. grandiflora* bark extract 'active' fractions using Reversed Phase chromatography. EtOH: F3 (A), F4 (B), F5 (C) and F6 (D) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

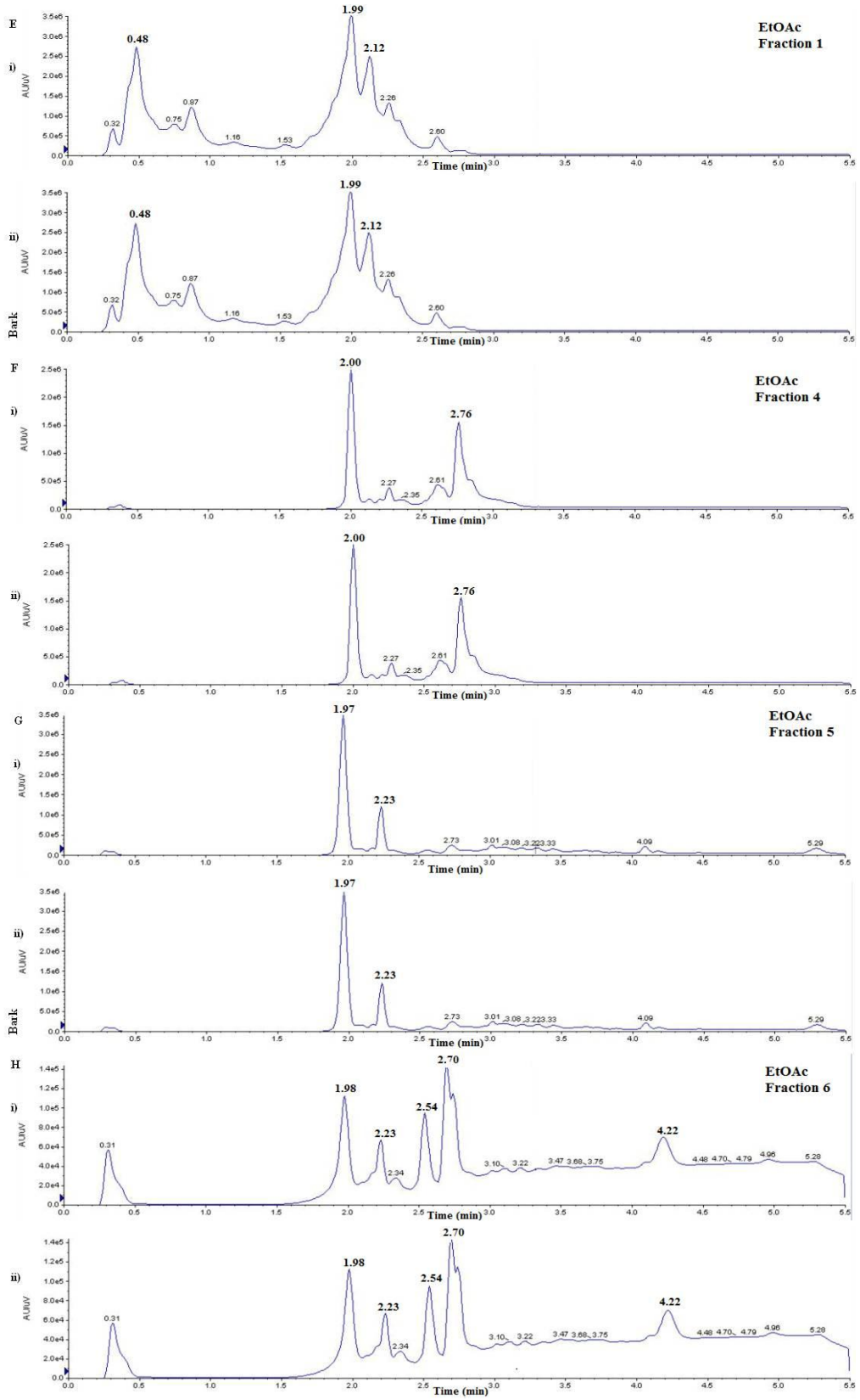


Figure 5-12: HPLC traces of *D. grandiflora* bark extract 'active' fractions using Reversed Phase chromatography. EtOAc: F1 (E), F4 (F), F5 (G) and F6 (H) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

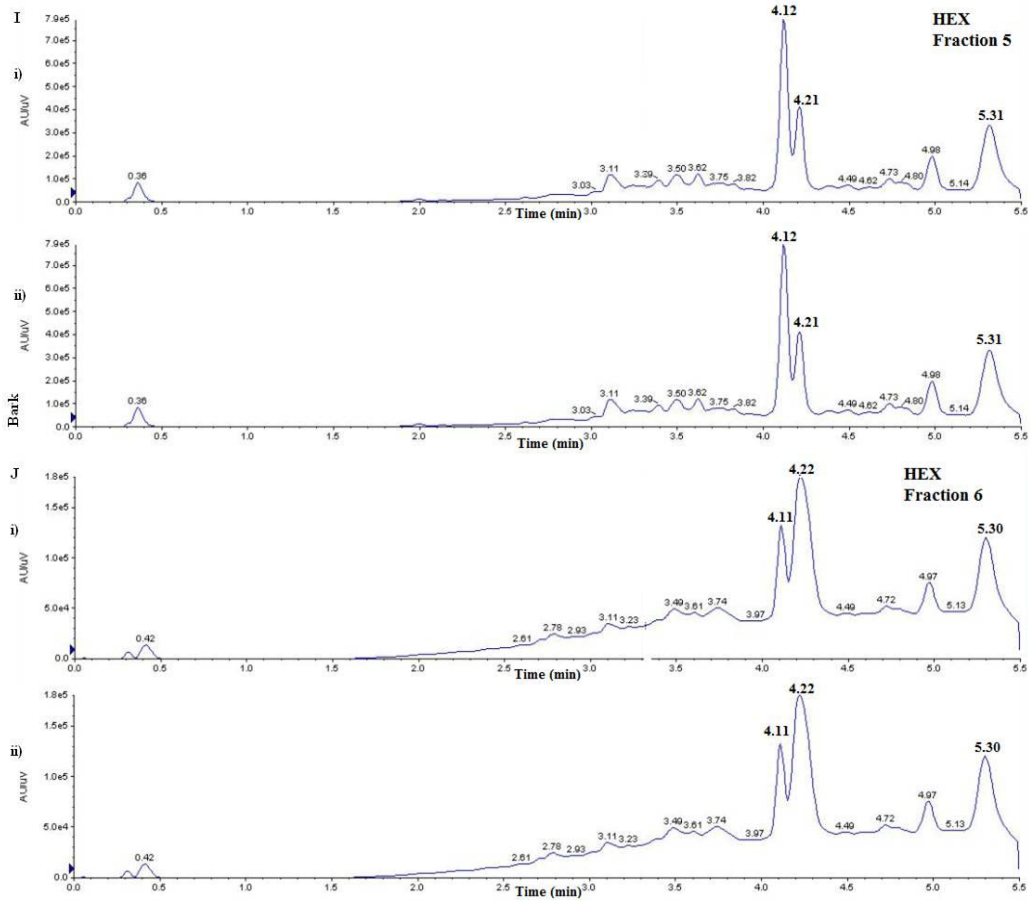


Figure 5-13: HPLC traces of *D. grandiflora* bark extract 'active' fractions using Reversed Phase chromatography. HEX: F5 (I) and F6 (J) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

5.2.3 Chromotography of *Pseuduvaria macrophylla* crude extracts and *in vitro* growth inhibitory properties of fractions in HCT116 cells

HPLC spectra of available *P. macrophylla* crude extracts measured at 220 and 254 nm are shown in Figure 5-14 and a summary of the main dominant peak is represented in Table 5-11. A solvent peak is illustrated in all spectra at t_R ~0.31 min. The major peak present in EtOH, EtOAc and HEX crude extracts was detected at t_R ~2.18 min. Compounds present in EtOH and EtOAc could be in the class of alkaloid, aglycone and glycoside compounds. Figure 5-14 displayed spectra, which revealed other peaks in both extracts were at t_R ~2.40 min and 2.74 min amongst several minor peaks.

Table 5-11: Retention time of *Pseuduvaria macrophylla* extracts dominant peaks using reversed phase chromatography.

***P. macrophylla* extracts**	**Retention time (t_R, min) and colour of *P. macrophylla* extracts**						
	Crude	F1	F2	F3	F4	F5	F6
EtOH	2.40 *Dark brown/ green hint*	4.24 *Yellow tint*	1.11 *Yellow tint*	**2.18 *Light brown***	**2.76 *Tan brown***	3.58 *Light brown*	**2.71 *Light brown***
EtOAc	2.16 *Dark brown/ green hint*	2.43 *Yellow tint*	1.11 *Yellow tint*	**2.18 *Yellow tint***	**2.73 *Yellow/ green hint***	3.58 *Yellow*	4.25 *Yellow tint*
HEX	- *Green*	-	-	-	-	-	-

Extracts (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm. Data of active fractions are highlighted in **bold**, data of inactive fractions are displayed in grey and '-' denotes not available data.

Comparable peaks were observed in HPLC spectra of EtOH crude extract analysed at 220 and 254 nm suggesting aromatic compounds and amide bonds are present in the fraction; the same observation was noticed for EtOAc crude extract. Following chromatography of *P. macrophylla* crude extracts of varying polarity were separated into six fractions. Following freeze-drying and reconstitution (100 mg/ml DMSO) growth inhibitory properties of fractions

were examined in HCT116 cells using the MTT assay. HPLC spectra of the active fractions are shown in Figure 5-16 to Figure 5-17 and spectra of inactive fractions are shown in Appendix 3, Figure 8-22 and 8-23. *P. macrophylla* EtOH and EtOAc F3 displayed potent growth inhibitory effects in HCT116 cells yielding GI_{50} concentrations lower than the crude extracts by 2.1-fold (Table 5-12). Results from the MTT assay revealed that the most active components in EtOH and EtOAc is embedded in F3; spectra of F3 illustrated a major peak at t_R ~2.18 min (254 nm) as well as several minor peaks. There were many minor peaks detected in EtOH F4 and F6, which suggest the presence of aromatic groups; however this fraction was less active than the crude extract. The detection of EtOAc F4 at both wavelengths suggests that components with aromatic rings and amide bonds were present in the fraction; however F4 is less active than the crude extract. Mahmood *et al* (1986) elucidated aporphinoid (1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine) in *P. macrophylla* and Wirasathien *et al.* (2006) reported the same alkaloid in *Pseuduvaria setosa*, described as a yellow amorphous powder, which may possibly be present in EtOH F1 and F2 and/or EtOAc F2 to F6 in varying quantities.[213]

Table 5-12: *In vitro* growth inhibition effects of *Pseuduvaria macrophylla* extract fractions in HCT116 cells.

P. macrophylla **crude extract** **GI_{50} value** [μg/ml]	GI_{50} values of *P. macrophylla* fractions (μg/ml)					
	F1	**F2**	**F3**	**F4**	**F5**	**F6**
EtOH [5.16]	> 10	> 10	**2.50 ± 0.74**	6.48 ± 1.27	> 10	8.06 ± 1.17
EtOAc [1.61]	> 10	> 10	**0.75 ± 0.13**	3.33 ± 0.61	> 10	> 10
HEX [5.41]	-	-	-	-	-	-

Extract concentration (μg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h. The mean GI_{50} values (μg/ml) ± SEM were obtained from ≥ 3 (n = 4) individual experiments. Fractions exhibiting GI_{50} values less than GI_{50} parent crude extract are considered active and highlighted in **bold,** fractions with GI_{50} values > 10 μg/ml are considered as inactive and '-' denotes unavailable extracts.

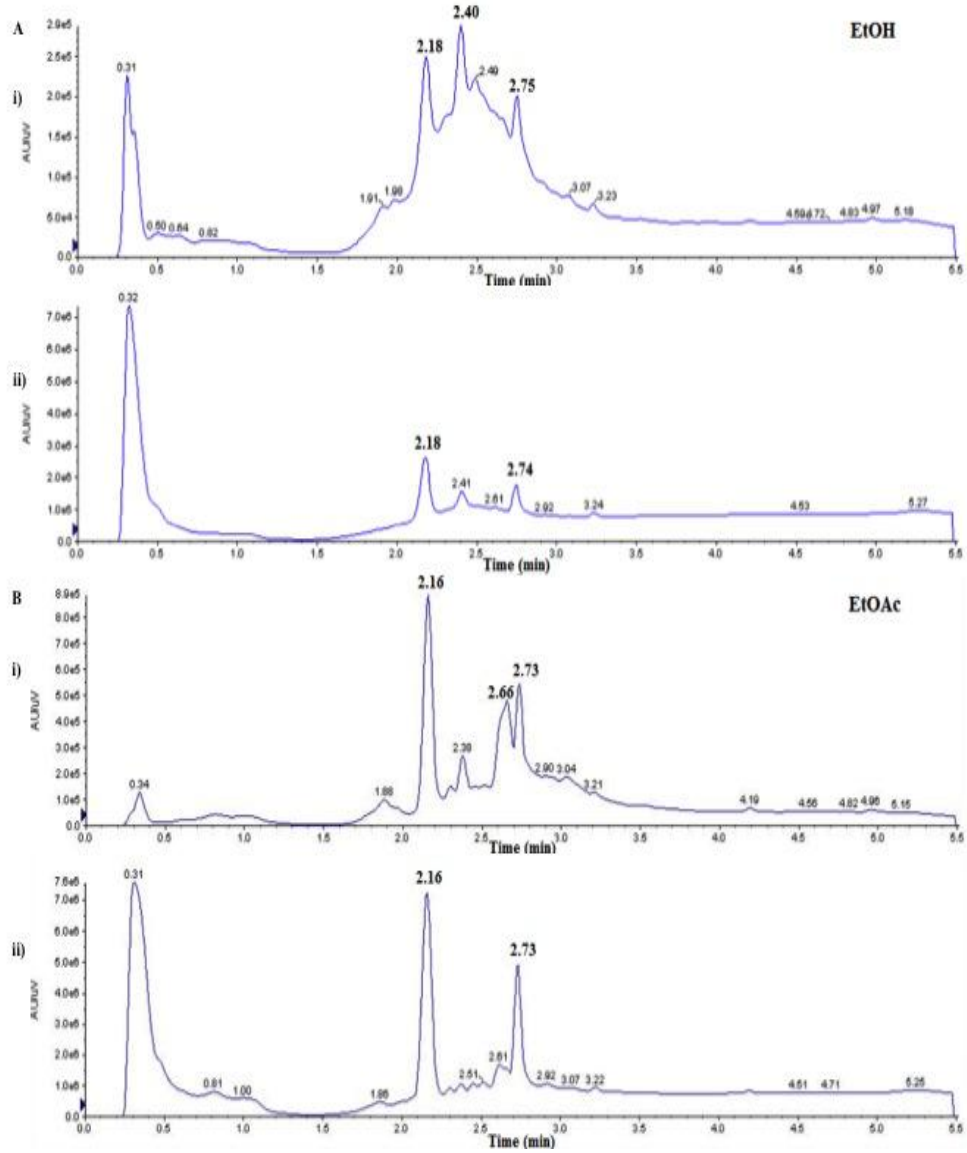


Figure 5-14: HPLC traces of *P. macrophylla* extracts using Reversed Phase chromatography. EtOH (A) and EtOAc (B) extract eluents (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

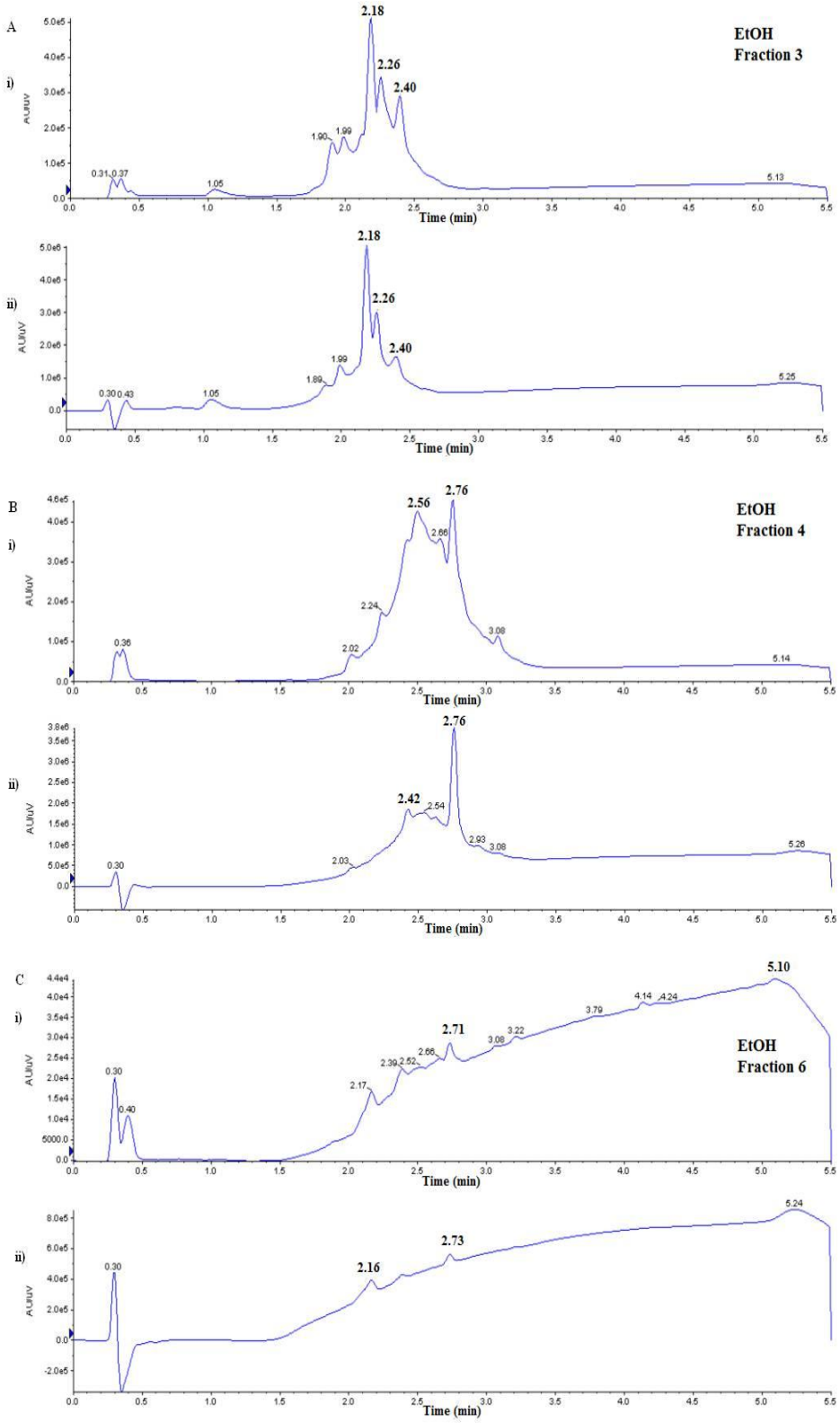


Figure 5-15: HPLC traces of *P. macrophylla* extract fractions using Reversed Phase chromatography. EtOH: F3 (A), F4 (B) and F6 (C) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

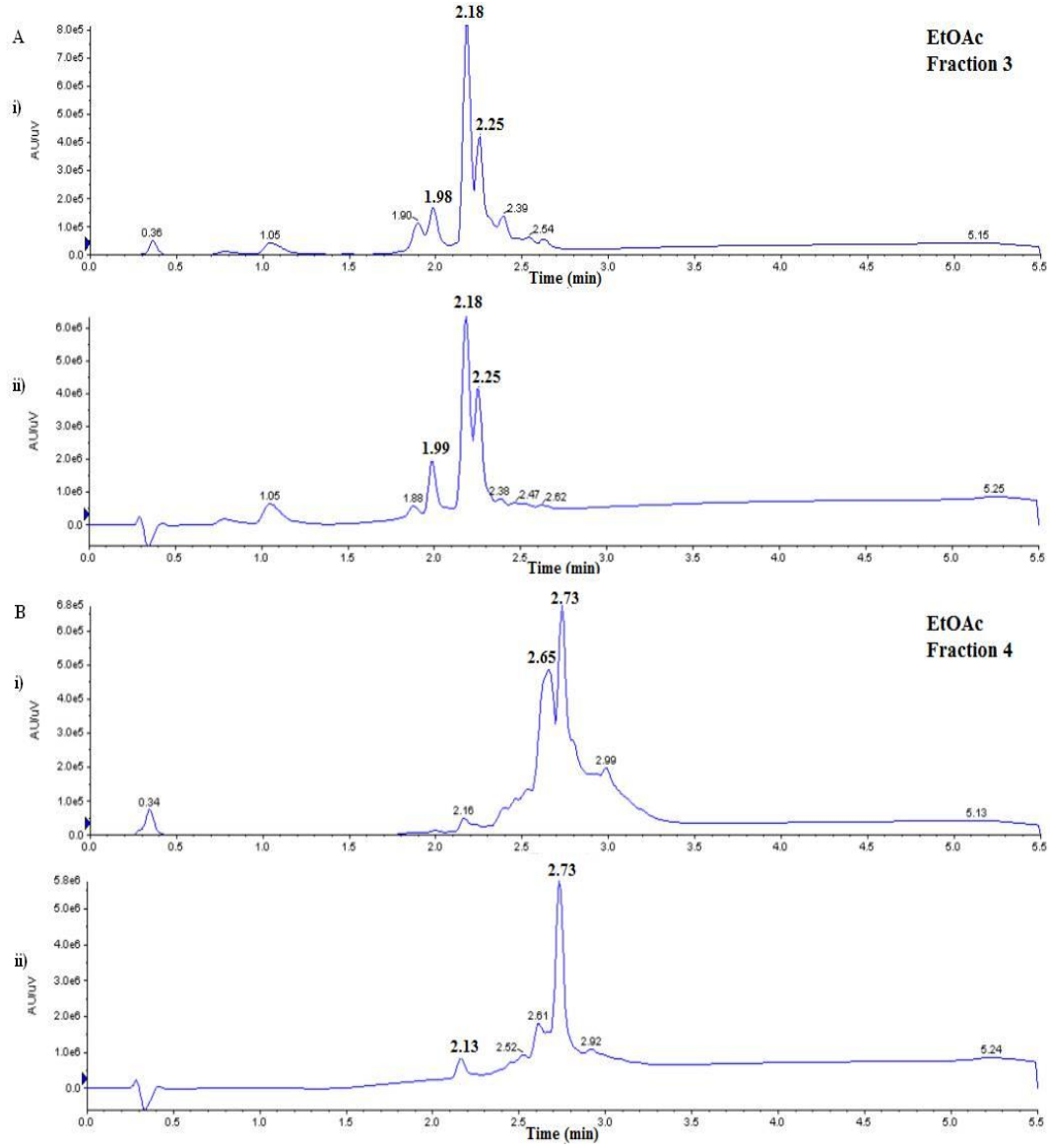


Figure 5-16: HPLC traces of *P. macrophylla* extract fractions using Reversed Phase chromatography. EtOAc: F3 (A) and F4 (B) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

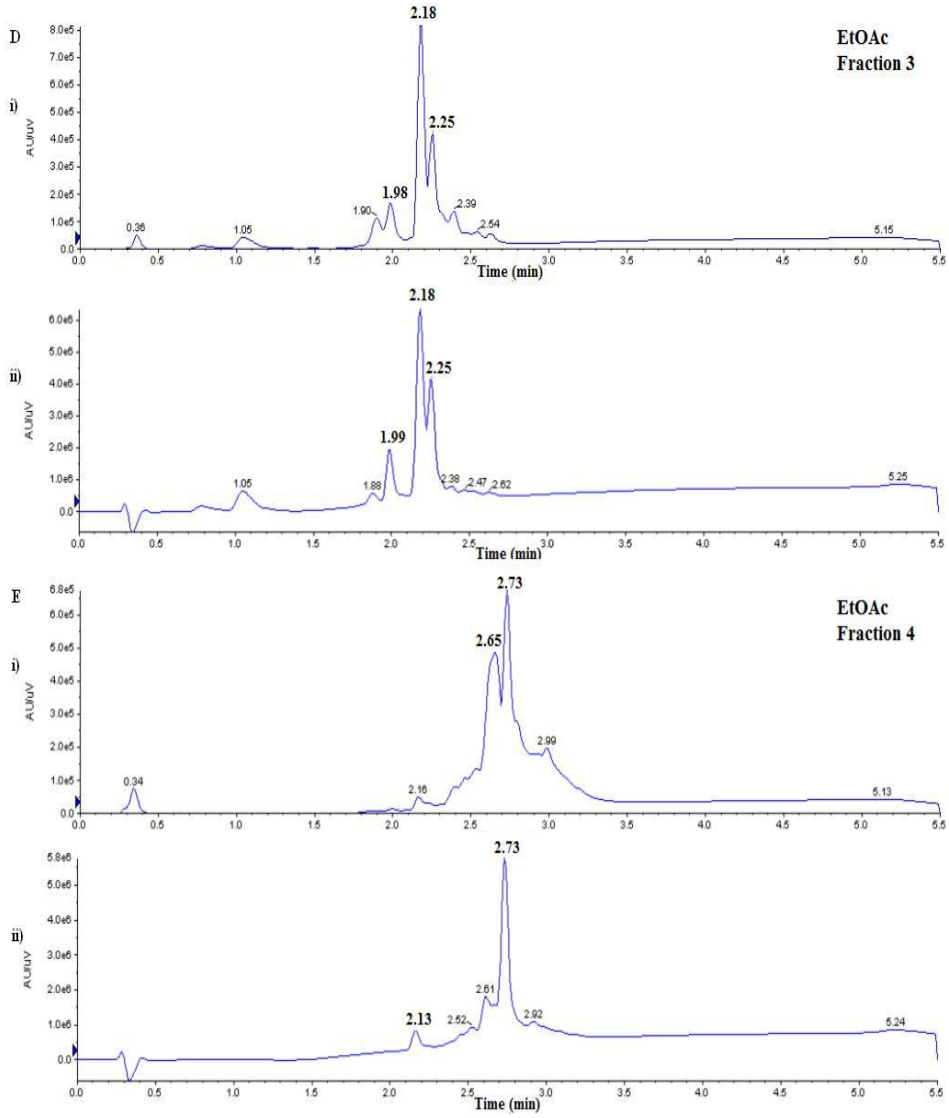


Figure 5-17: HPLC traces of *P. macrophylla* extract fractions using Reversed Phase chromatography. EtOAc: F3 (D) and F4 (E) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

6

Conclusion and future direction

Selection of UNMC plants *Acalypha wilkesiana, Duabanga grandiflora, Archidendron ellipticum* and *Pseuduvaria macrophylla*, for investigation was based on different approaches e.g. random screening of plants with unknown biological activity or selection on the basis of traditional use reported in literature. A range of bioassays and a panel of cancer cell lines were employed in systematic examination of UNMC plants' extracts, starting with examination of any untoward activity (cytotoxicity, cytostasis) against cancer cells. Data from initial stages of this project provided direction to explore extracts'-induced growth inhibition observed and the induction of apoptosis to provide information on the mechanism of action of UNMC extracts. Simultaneously, extracts were also tested for activities against cell migration (important in invasion and metastasis), colony formation ability (responsiveness to cell death signals) and induction of senescence (limiting replicative potential).

Furthermore, preliminary fractionation of UNMC crude extracts was performed alongside *in vitro* examination of crude extracts to further explore anticancer activities of extract fractions and to provide guidance on selection of isolation of compounds in active sub-fractions in earlier stages. Malaysian rainforest plants selected for this project have revealed interesting and valuable information from a series of assays and are worthy of further investigation as summarised overleaf.

Table 6-1: Summary table outlining each activity performed

Assay (indication)	**Description**	**Most active plant(s)**
MTT (viability)	[0 µg/ml - 200 µg/ml]: *A. wilkesiana, A. ellipticum, D. grandiflora* [0 µg/ml - 50 µg/ml]: *P. macrophylla* Exposure: 72 h	*P. macrophylla*
Clonogenic (survival)	GI_{50}: [0X, 0.5X, 1X and 2X] Exposure: 24 h	*D. grandiflora*
PI staining (cell cycle distribution)	GI_{50}: [0X, 1X, 2X and 4X] Exposure: 48 h	*D. grandiflora* and *P. macrophylla*
Annexin V-FITC/PI staining Western blot (Apoptosis)	GI_{50}: [0X, 1X, 2X and 4X] GI_{50}: [0X, 1X and 5X] Exposure: 24 h and 48 h	*D. grandiflora* and *P. macrophylla*
Scratch (Migration)	GI_{50}: [0X, 1X and 4X] Exposure: 52 h and ≤ 64 h	*P. macrophylla*
B-galactodase detection (Senescence)	GI_{50}: [0X, 1X and 2X] Exposure: 7 to 12 days	*A. ellipticum*
Aluminium trichloride Folin-Ciolteau (Chemical content)	Extract concentration [1 mg/ml], total flavonoid content as quercetin equivalent. Extract concentration [1 mg/ml], total phenolic content as gallic acid equivalent.	*D. grandiflora*
DPPH (Free radical scavenging)	Extract concentration [1 mg/ml].	*D. grandiflora*
HPLC (Fractionation)	Fractionation using solvent gradients consisting of Water, MeOH and MeCN, a total of 6 fractions of decreasing polarity Detection wavelengths: 220 and 254	*A. ellipticum*
Broth dilution assay (Antimicrobial activity)	[1 – 128 µg/ml]: *D. grandiflora, P. macrophylla* Exposure: 24 h and 48 h	*D. grandiflora* and *P. macrophylla*

Summary table listing the assays performed and the respective UNMC plant(s) that contributed towards the most favourable result for that assay.

***Acalypha wilkesiana* (Euphorbiaceae)**

Polar (EtOH and EtOAc) extracts of *A. wilkesiana* were able to cause growth inhibitory effects across the cell line panel (cell line panel mean GI_{50} (μg/ml): EtOH = 51.79; EtOAc = 55.99; HEX = > 200). Breast cancer MDA-MB-468 cells without ER, PR and Her-2 expressions revealed the greatest sensitivity towards compounds present in EtOH and EtOAc extracts (GI_{50}: 22.67 and 15.88, respectively) and some selectivity was observed as GI_{50} values against MRC-5 fibroblasts (GI_{50}: 46.60 μg/ml and 53.26 μg/ml, respectively) was observed. Inhibition of colony formation induced by EtOH extracts (at 36 μg/ml and 72 μg/ml) was observed in approximately 70% of MDA-MB-468 cells within 24 h. A similar effect was observed with HEX extracts, however the concentrations used were too high to be considered as 'active' according to the NCI's active crude extract criteria. Detection of increased sub-G1 cell population suggests EtOH extract was able to cause cellular damage leading to cell death; however this effect was not deemed as significant in MDA-MB-468 cell cycle distribution. *A. wilkesiana* illustrated significant growth inhibitory effects in triple-negative MDA-MB-468 cells, therefore, the presence of HER, ER and PR receptors may not be a factor determining sensitivity of cells to these plant extracts suggesting the active constituents may or may not be in the phytochemical class of isoflavonoids or flavonoids.

Collectively, results may suggest impaired proliferation and colony formation inhibition were due to DNA damage leading to cell cycle arrest (for repair) or cell death (irreparable damage); such findings are in agreement with data previously reported of double-strand break (DSB) and single-strand break (SSB) accumulation and execution of apoptosis by *A. wilkesiana*.[234] The effects observed could be due to cytotoxic constituents previously identified in *A. wilkisiana* extracts that have the ability to induce apoptosis such as anthraquinone (via caspase 8), corilagin (via G2/M arrest), gallic acid (via G0/G1 arrest), geraniin (by up-regulation of Fas ligand), kaempferol (by p53 activation).[175] Since cytotoxic compounds have been previously found in this plant, continuing with detailed *in vitro* investigations may not serve much purpose (response will be observed due to known cytotoxic compounds), whereas fractionating extracts from this plant would be of greater interest to isolate potential new compounds and investigate any potential novel effects.

***Archidendron ellipticum* (Leguminosae)**

Polar (EtOH and EtOAc) extracts of *A. ellipticum* contain compounds causing most potent growth inhibition in MDA-MB-468 cells (Bark GI_{50} (μg/ml): EtOH = 1.7; EtOAc = 40.9; HEX = > 200; Leaf GI_{50} (μg/ml): EtOH = 9.3; EtOAc = 9.3; HEX = > 200). On the whole, bark extracts were more active than leaf extracts against the cell line panel, however only leaf EtOAc extract demonstrated selectivity between MDA-MB-468 cells and MRC5 fibroblasts (GI_{50} = 69.7 μg/ml). Separation of bark EtOH extract revealed a single active fraction (F4; GI_{50}: ~1.75 μg/ml) causing growth inhibition at the same concentration of the crude extract (GI_{50}: ~1.73 μg/ml), further fraction separation is justified, towards isolation of single active compounds.

Although, crude HEX extracts failed to inhibit growth at concentrations as great as 200 μg/ml, it is intriguing that extract separation yielded fractions with significantly enhanced (> 50-fold) growth inhibitory activity in the fractions (GI_{50} (μg/ml): F4 = 10.0; F5 = 6.0; F6 = 12.6), which strongly suggests antagonism and/or masking of hydrophobic compounds present in the crude extracts. *A. ellipticum* fractions illustrated comparable or greater activity than their crude extracts, which suggests antagonism or 'masking' of activity. Further fractionation may reveal compounds of greater activity in sensitive cell lines worthy of isolation and testing. *A. ellipticum* illustrated greatest growth inhibition in triple-negative MDA-MB-468 cells, therefore the active constituents may not be in the phytochemical class of isoflavonoids or flavonoids. Polar extracts of bark and leaf were most active against MDA-MB-468 colony formation with ≥ 40% of cells unable to proliferate and form colonies. This could be as a result of apoptosis initiation by compounds present in the extracts, as shown by increased sub-G1 populations and detection of PS on the outer membrane in ≥ 40% of the cells after 48 h treatment. Induction of apoptosis will lead to the inability of cells to proliferate and inhibit colony growth. Bark and leaf HEX extracts were able to induce highest numbers of senescent cells detected in comparison to other extracts examined. Other compounds in the crude mixture, possibly sterols, triterpenes, coumarins and saponins can scavenge free radicals and it appears that extracts' ability to induce senescence is inversely related to their ability to scavenge free radicals.[235]

***Duabanga grandiflora* (Lythracae)**

Sensitivity of cancer cells varied towards *D. grandiflora* extracts. Modest growth inhibitory activity and selectivity between MRC5 fibroblasts and CRC HCT116 cells were seen with polar (Water and EtOH) extracts. Moreover, EtOAc extracts were the most active against the panel of cell lines tested (mean GI_{50} (μg/ml): bark EtOAc = 31.58 and leaf EtOAc = 43.78). Interestingly, non-polar bark HEX extract also demonstrated good growth inhibitory effects against the cell line panel (GI_{50} = 36.36 μg/ml), which is in contrast to leaf HEX extract (GI_{50} = > 200 μg/ml). However, lack of selectivity between cancer cell lines and MRC5 fibroblast was observed with less polar extracts. Separation of crude bark extracts has revealed antagonistic interaction or masking of activity in the crude mixture. It will be of interest to priortise additional fractionation of EtOH and HEX bark fractions in an attempt to identify sub-fractions with greater growth inhibitory activity in HCT116 cells than seen with the crude extracts. While loss of activity seen in all leaf extracts following fractionation suggests separated compounds contained in different fractions may have been interacting in a synergistic manner previously. Unexpectedly, SiHa cells' growth was not affected by low concentrations *D. grandiflora* polar extracts although studies revealed eugeniin activity against Walker carcinosarcoma 256 in rats, which may be due to 'masking' or antagonism of (a) particular compound(s) activity by other compounds which comprise the chemical mixtures of crude extracts. It would be of interest to investigate the expression levels of viral proteins in light of reports of eugeniin's activity hindering HSV RNA and DNA replication. Accumulation of sub-G1 and G1/0 cell populations accompanied by decreased events in S and G2/M suggest cells were unable to progress through G1 phase and as apoptosis was triggered (positive Annexin V^+/PI^- binding and activated caspase 3 protein). These observations imply that damage induced by compounds present in *D. grandiflora* polar extracts were sufficient to affect cell survival and prevent subsequent colony formation; this effect was seen to a lesser extent in less polar extracts.

Previously, a range of compounds has been isolated from *D. grandiflora* extracts and some constituents can induce apoptosis such as ellagic acid (by G1 phase arrest), hentriacontane, hentriacontanol, b-sitosterol, a-amyrin,

epifriedelinol, betulinic acid, acacetin and tetramethylellagic acid. Similarly, *D. grandiflora* EtOH bark fractions and all leaf extract fractions possessed greater activity than the crude extract suggesting antagonistic or masking activity of compounds in crude mixtures.

Furthermore, *D. grandiflora* crude EtOH and HEX bark extracts appear to contain compounds that may inhibit cell migration, possibly *via* TNF-α pathway, which may be due to the detected caspase 3 activation (Figure 6-1). A low level of senescence (< 10%) was present in EtOH treated cells but was comparable to the control. Greatest free radical scavenging (FRS) activity was seen in *D. grandiflora* in comparison to all of the UNMC plants tested. Free radical scavenging activity decreased with extract polarity; highest levels of free radical scavenging (~ 95%) was seen with polar extracts since significant quantities of phenolics and flavonoids were detected. Extraction of plants using polar solvents (Water, EtOH and EtOAc) yielded extracts with good to moderate activity implying compounds such as sugars, amino acids, glycosides, alkaloids, aglycones, glycosides could be present in the crude extracts. *D. grandiflora* bark HEX extracts revealed growth inhibitory effects in HCT116 cells. Compounds of chemical class of waxes, fats and oils could be extracted in non-polar hexane solvent, hence may be responsible for the activity. It is also interesting to note that in agreement with recent studies, *D. grandiflora* extracts were able to inhibit both Gram –ve and Gram +ve bacteria (8 – 32 mg/L), which may be due to ellagic acid and betulinic acid.[189]

***Pseuduvaria macrophylla* (Annonaceae)**

P. macrophylla extracts demonstrated the greatest growth inhibitory and anti-clonogenic effects across the cell line panel (mean GI_{50}: EtOH: 5.05, EtOAc: 2.02 and HEX: 11.99 μg/ml) in the MTT and clonogenic assays.

P. macrophylla HEX extract-treated cells were able to form colonies following extract removal suggesting cellular damage was reversible, therefore different compounds influencing different mechanisms may be responsible for the effects observed or longer exposure time might be required for accumulation of damage that is beyond repair. Collectively, *P. macrophylla* crude extracts may contain compounds that can inhibit cell growth and colony formation by inducing DNA damage. This may result in cell cycle arrest to allow for DNA

repair, activation of apoptosis due to irreparable DNA damage or induction of cellular senescence or impairing cells' proliferative potential. Alkaloids contained in *P. macrophylla* (Annonaceae) are very likely to be accountable for the observed effects such as increased G2/M cell population preceding cell death.[209] In comparison, it is known that vinca alkaloids isolated from *C. roseus* (Apocynaceae) possess anti-microtubule, anti-mitotic activity leading to G2/M block.[10] Subsequently, flow cytometric and Western blotting techniques have confirmed apoptotic cell populations and caspase-3 (cleavage) activation. *P. macrophylla* HEX extracts revealed selective growth inhibitory effects in HCT116 cells, compounds classes comprising of chemical class of waxes, fats and oils could be extracted in non-polar hexane solvent, hence the above mentioned chemicals could be responsible for the observed activity. Compound 38 previously isolated from *P. macrophylla* may also be responsible for the observed effects since similar activity was observed in NCI-H187 cells (3.0 μg/ml).[214] However compounds (possibly compound 38) in F3 yielded greater effects in HCT116 cells (0.75 μg/ml). Similarly low levels (< 10%) of senescent cells were observed following EtOH and EtOAc treatments and were similar to levels observed in control cells. Remarkably, *P. macrophylla* extracts were able to inhibit both Gram –ve and Gram +ve bacteria at very low concentrations (8 – 32 mg/).

In comparison, there is also limited information on the biological activity of a related species *Uvaria Macrophylla* due to its decline in recent years. Nevertheless, *U. macrophylla* bark and leaf (EtOH, CHL and HEX) extracts appeared to be potent against MCF-7 and HCT116 cells (see appendix 4). CHL and HEX extracts were able to inhibit growth of both cell lines by 50% at low concentrations (3.25 to 27.03 μg/ml). These GI_{50} values are comparable to that observed in HCT116 cells exposed to *P. macrophylla* EtOH, EtOAc and HEX extracts (GI_{50}: 5.16, 1.61 and 5.41 μg/ml, respectively). In general, similar inhibition of colony formation was observed with *U. macrophylla* extracts, however EtOH concentrations used were ≥ 5-fold greater than *P. macrophylla* EtOH extract. Preliminary cell cycle distribution data suggest that no changes were induced by *U. macrophylla* EtOH and HEX extracts (Appendix 4, Figure 10-24), whereas increased sub-G1 HCT116 cell population with CHL extracts is indicative of apoptotic cells and should be

confirmed by the Annexin V-FITC assay. Other studies in China revealed EtOH root extract from *U. macrophylla* and isolated a new dihydroflavone, macrophyllol as well as uvamalols and uvarimacrophin, dihydroflavones, acetogenins, polyoxgenated cyclohexenes and (benzylisoquinoline) alkaloids.[268, 269] Since the Annonaceae family is known for possessing alkaloids and both *Uvaria* and *Pseuduvaria* genus have been previously identified to possess new compounds, these two plants are worthy of further investigation such as bio-guided fractionation leading to isolation and structure elucidation of the active compound(s).

Overall, all of the UNMC plants selected for this investigation revealed intriguing anticancer properties worthy for further examination. It is important to continue fractionation of these crude extracts in order to isolate and elucidate structures of active compounds. This could be achieved by using hyphenated methods consisting of e.g. HPLC, MS, NMR, technologies that would provide information of chemical composition of pure active compounds for identification on natural compound databases (de-replication). Analytical results obtained would offer a deeper insight into the class of chemical group present; hence enabling estimation of activity (reaction groups) and hypotheses of mechanism(s) of action(s). It may be considered inefficient to only performing bioassays on crude extracts containing a mixture of compounds; likewise, it maybe inefficient to spend significant time conducting only separation and isolation procedures to identify various compounds present in the crude extracts since there may be no suitable (inactive or already known) compounds present. A selection of *in vitro* tests should be performed in parallel with analytical assays, the efficiency of two processes may be improved and decisions regarding pursuit of investigation could be facilitated based on the preliminary biological and analytical data obtained. This is also of importance for sub-fractions, which demonstrate activity where the whole crude extract failed to show activity.

On isolation of the active compound, detection of pro-apoptotic members of Bcl-2 family expression e.g. BAX, and BAK and regulators of the anti-apoptotic Bcl-2 proteins to promote apoptosis e.g. BAD, NOXA and PUMA, could provide insight into the possible involvement of proteins from the

extrinsic or intrinsic apoptotic pathways e.g. FasL, TNF and TRAIL or mitochondria-caspase interaction leading to apoptosis, respectively.[270] Bid is the common modulator between the intrinsic and extrinsic pathways; cleavage of Bid by caspase 8 (extrinsic pathway) enables communication with the intrinsic pathway by activating Bax/Bak protein leading to cytochrome c release and mitochondrial fragmentation.[270] Hence it is important to consider the detection of caspase 8 and cleaved Bid (tBid) protein expression in UNMC extract-treated cells to distinguish between intrinsic or extrinsic pathway that has led to activation of caspase 3. In addition, expression of other proteins of interest may also provide further mechanistic insight including γH2AX, a marker of DNA double-strand breaks (histone γH2AX is phosphorylated on serine 139 by PI3-family) H2AX protein is also involved in DNA repair and regulation cell cycle division hence possesses a role in genomic stability.[88] Cytosolic cytochrome C levels would distinguish intrinsic apoptotic pathway; cleaved PARP protein could confirm subsequent activation of caspase 3.[271] Inhibition of cell migration following treatment of UNMC extracts may be due to involvement of the TNF-α pathway and subsequently activating caspase 3 activation and inhibition of NF-kB inhibition hence preventing signals to EMT induction (Figure 6-1).[272]

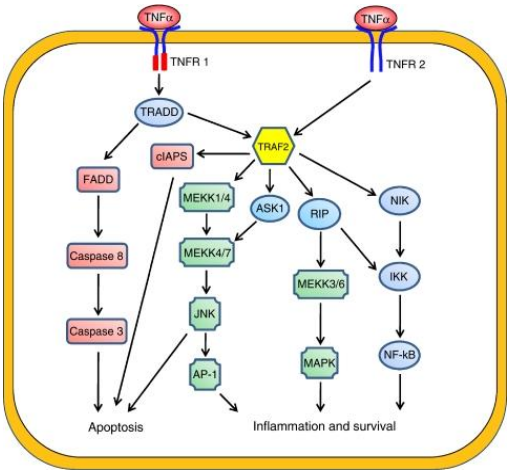


Figure 6-1: The downstream signaling pathways of TNF-α as illustrated by Wu and Zhou (2010). TNF-α can activate two types of TNF receptors (TNFR 1 and 2). TNFR1 can induce apoptosis by binding caspase 8 to Fas-Associated protein with Death Domain (FADD). TNFR2 can promote inflammation and cell survival *via* activation of NF-kB and Akt signaling pathways, which can give rise to EMT and invasion in tumour cells.[272]

Furthermore, another interesting assay to investigate possible targets of active compounds would be using micro-array techniques to enable a range of protein targets to be identified thus generating a fingerprint profile for the plants. Similarly, metabolite profiling could provide information on extracts' influence on metabolism pathways in a biological system.[273] Any similarity with existing profiles could provide suggestions of possible mechanism of action or reveal any unique patterns.

Determination of the type of DNA damage induced by different compounds present in UNMC extracts could provide additional insight into possible mechanisms. For example COMET assay would be able to detect whether single strand breaks or double strand breaks have occurred within the DNA, however there will be a need to distinguish between the causes of DNA strand breaks i.e. a consequence of caspase cleavage (during apoptosis) or extracts per se. Subsequently, it would be of interest to examine isolated compounds' abilities to inhibit invasion, angiogenesis and metastasis by growing cells on wells with a matrigel and measure if cells are able to migrate through this barrier. Additional 'normal' cell lines should be used to determine therapeutic window and any active compounds could be used as a template for structural modification in order to achieve selectivity between cancer cells and normal cells.

There is such a huge variety of assays that are available, however it is important to consider the nature of compound(s) isolated from these plants in order to select purposeful assays to yield more significant results. In addition, compounds testing negative for anticancer properties does not mean they are unsuccessful candidates for other disease areas and it is important to document both positive and negative findings to avoid replication of work and for use by other researchers.

From this body of work, it is concluded that there are several general yet critical points to be made when investigating natural products for therapeutic use, in particularly as anticancer agents.

These points could be broadly categorised into non-practical and practical concerns.

Firstly, one of the main concerns in natural product research surrounds the processes of collection (e.g. random versus literature/ethnopharmacological systems, correct verification of specimens and sustainability of the source) and standardisation of raw material (e.g. location and the environment, developmental stage of the plant, storage conditions of collected material, techniques) as well as the subsequent extraction process used. In addition, publication of results is necessary for de-replication and further information on the plant profile. The lack of supply of *A. wilkesiana* and *P. macrophylla* from their primary collection location has highlighted a non-practical concern experienced in this project as it could mean that the interesting results obtained so are in vain if endangered *P. macrophylla* became extinct; whereas an *A. wilkesiana* specimen was found and collected from a secondary location and it is very likely that variability in compounds of the second batch will affect cells' response and produce different *in vitro* results. Therefore the importance of standardization is raised; use of analytical equipment to determine the variation between batches is essential, and decisions made as to acceptance of identical compound profiles or compromise for the most abundant compounds. This may be overcome by ensuring enough supply prior to selection. Once *in vitro* experiments have verified activity, enough specimens should be collected to perform separation and analytical analyses. Alternatively, if the active compound(s) is identified then analysis of analytical data could be focused on the detection of the active compound(s).

It is important to not cease testing of crude extracts prematurely and give more attention to bio-guided fractionation activity of these extracts; this has been supported by the differences observed in growth inhibition of cancer cells when exposed to crude (inactive) and fractionated *A. ellipticum* HEX extracts ('active'). Collectively, the results revealed that selection of cell lines are dependent on the *in vitro* experiments being performed due to the characteristic variability of cell types, hence their different level of sensitivity towards different compounds, and the indication of the assay. However, it is unnecessary to continuously test crude extracts against different cell lines in the early stages of *in vitro* testing as long as the mixture still proves to be 'active' (with reference to the NCI's guidance) against the cell line(s) being tested. It is important to employ analytical equipment for bio-guided

fractionation process, to separate and elucidate the structure(s) of active compound(s). Natural product research should aim to obtain pure active compounds so that investigation into mechanism of action (defined targets) can be conducted, which would be favourable in obtaining a investigation license approved by regulatory bodies for clinical development in a defined population and indication(s), and subsequently a marketing license. On the contrary, it is difficult to predict the interaction (*in vivo*) for a mixture of compounds (shot-gun effect) and adverse events that may arise - hence inadequate safety profile and poor benefit-risk assessment of the compound. Therefore, the UNMC extracts examined in this project should be subjected to further bio-guided fractionation and reconcile on verified databases (de-replication). If UNMC extracts contain active compound(s), which are subsequently verified to be a novel compounds (or compounds lacking biological data) then it will be interesting to initiate *in vivo* studies.

In general, identification and structure elucidation of active compounds and mechanisms of action are considerably long processes without evening considering antagonistic, masking or synergistic activities of crude extracts, so supply of material is a critical factor. Furthermore it is also important to acknowledge that the 'inactive' fractions may still have a role to play in other therapeutic areas, therefore it is important to analyse the results, which may reveal alternative uses and may be followed. Above all, this work requires the experience and expertise of botanists to correctly verify specimens and vigilant record-keeping as well as international efforts to communicate and maintain an authenticate database.

For potential therapeutic compounds to be identified from the diverse botanic systems, pharmaceutical companies will need to invest significantly into natural product drug discovery programs or find the balance with their investment into high-throughput screening technologies and compound libraries. Therefore there is still a prominent role for natural compounds in drug discovery.

7

Materials and Methods

7.1 Materials

7.1.1 Plant collection and identification

Malaysian rainforest flora *Acalypha wilkesiana* Müll Arg., *Duabanga grandiflora* Walp, *Archidendron ellipticum* (Blume) Hassk and *Pseuduvaria macrophylla* (Oliv.) Merr were collected in Malaysian rainforests and authenticated by Dr. Christophe Wiart (Table 7-1). The extraction of collected plant specimens was conducted by colleagues of the UNMC School of Biosciences and School of Pharmacy (chapter 4.2.1). The UNMC plant extracts were delivered to the University of Nottingham, UK, and kept at – 20 ℃ until use.

7.1.2 Mammalian cell lines

Human–derived cancer cell lines MCF7, MDA-MB-468, SK-OV-3, Caki-1, HCT116 and non-transformed cell line MRC-5 fibroblasts were purchased from the American Type Cell Culture (ATCC), Rockville, U.S.A. Human cancer cell lines IGROV 1, TK10 and HCC 2998 and were purchased from the National Cancer Institute (NCI), Bethesda, U.S.A (Table 7-2).

7.1.3 Bacterial strains

Gram-negative bacteria (*Pseudomonas aeruginosa*, (*P. aeruginosa*) and *Escherichia coli* (*E. coli*)), Gram-positive (*Staphylococcus aureus* (*S. aureas*) and *Enterococcus faecalis (E. faecalis)* and yeast *Candida albicans* (*C. albicans)* were purchased from ATCC.

Table 7-1 UNMC Plant name and reference number, place of collection and UNMC plant extraction solvents.

UNMC Number	Genus	Species	Family	Place of collection (Forest)		Extraction solvent and Yield (%)				
						Chloroform	Hexane	Ethyl acetate	Ethanol	Water
9	*Acalypha*	*Wilkesiana*	Euphorbiaceae	Broga	Whole	-	0.32	0.18	3.29	-
20	*Pseuduvaria*	*Macrophylla*	Annonaceae	Sg. Congkak	Whole	-	n/a	n/a	n/a	-
35	*Archidendron*	*Ellipticum*	Leguminosae	Sg. Congkak	Leaf	-	1.79	1.82	2.33	-
35	*Archidendron*	*Ellipticum*	Leguminosae	Sg. Congkak	Bark	-	n/a	0.10	2.30	-
37	*Duabanga*	*Grandiflora*	Lythraceae	Semenyih Dam	Leaf	-	2.54	4.72	18.27	6.63
37	*Duabanga*	*Grandiflora*	Lythraceae	Semenyih Dam	Bark	-	1.02	0.64	4.11	2.73
-	*Uvaria*	*Macrophylla*	Annonaceae	-	Whole	n/a	n/a	-	n/a	-

Each plant was assigned a UNMC number and was subjected to extraction in solvents of varying polarity: chloroform (CHL), hexane (HEX), ethylacetate (EtOAc), ethanol (EtOH) and water (Water) and the corresponding yield (%) displayed; several extracts' yields could not be determined and indicated as n/a (not available).

Table 7-2 Mammalian cell lines and maintenance conditions

Cell type	Cell line	Reference	Supplement
Breast Adenocarcinoma	MCF7	ATCC no. HTB-22 Brooks *et al.* 1973[219]	RPMI 1640 medium supplemented with 10% FBS in a humidified atmosphere at 37°C in 5% CO_2.
	MDA-MB-468	ATCC no. HTB-132 Cailleau *et al*. 1978[220]	
Ovarian Adenocarcinoma	IGROV1	Bénard *et al.* 1985[221]	
	SK-OV-3	ATCC no. HTB-77 Frogh *et al*. 1975[222]	
Renal cell carcinoma	Caki-1	ATCC no. HTB-46 Frogh *et al*. 1975[222]	
	TK-10	Bear *et al.* 1987[223]	
Colorectal carcinoma	HCT116	ATCC no. CCL-247 Brattain *et al.* 1981[224]	
	HCC2998	Goldwasser *et al.* 1995[225] Kobayashi *et al.* 1999[226]	
Non-transformed foetal lung fibroblast	MRC-5	ATCC no. CCL-171 Jacobs *et al*. 1970[228]	MEME medium supplemented with 10% FBS, 7.5% sodium bicarbonate, 1% 0.1 mM Non essential amino acids, 1 M HEPES, 1% 200 mM L-glutamine and 1% penicillin. Cells are kept in Humidified atmosphere at 37°C in 5% CO_2.

Further information on the human cell lines used can be obtained from the American Type Cell Culture (www.atcc.org) and the National Cancer Institute (http://dtp.nci.nih.gov).

7.1.4 Antibodies

Rabbit polyclonal antibodies (Ab) to histone H2A.X (phosphor S139; product code: ab11174) and rabbit monoclonal Ab to α-Mcl1 (product code: Ab32087) were purchased from Abcam plc, Cambridge, UK. Rabbit monoclonal Ab to PARP1 (46D11, site Gly 523; product code: 9532S), rabbit Ab to Bax (product code: 2772S) and mouse monoclonal Ab to Caspase 3 (product code: 9668S) were purchased from Cell Signaling Technology Inc., Hertfordshire, UK. Anti-rabbit IgG, peroxidase-linked species-specific whole Ab from donkey enhanced chemiluminescence (ECL; product code: NA934) were purchased from GE Healthcare, UK. Mouse IgG1 Ab to αBcl-2 (produce code: mAB827) was purchased from R & D systems. All antibodies were stored at -20 °C in the dark in accordance to the manufacturers' guidance unless otherwise stated.

7.1.5 Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), 2-mercaptoethanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 5-bromo-4-chloro-3-indolylβ-D-galactopyranoside (X-gal), *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES), *N,N,N′,N′*-tetramethylethylenediamine (TEMED), β-mercaptoethanol, aluminium chloride ($AlCl_3$) anhydrous, ammonium persulfate (AMPS), bovine serum albumin (BSA), bromophenol blue, calcium chloride ($CaCl_2$), citric acid, dimethyl sulfoxide (DMSO), foetal bovine serum (FBS), Folin-Ciocalteau (FC) phenol reagent, gallic acid (GA), glycerol, glycine, L-glutamine, Kodak® GBX fixer (for autoradiography films), magnesium chloride ($MgCl_2$), methylene blue, minimum essential medium eagle (MEME), non-essential amino acids (NEAA) solution, penicillin/streptomycin, polyoxyethylenesorbitan monolaureate (Tween® 20), potassium ferricyanide, potassium ferrocyanide, propidium iodide (PI), protease inhibitor cocktail (AEBSF: 104 mM, aprotinin: 80 μM, bestatin, 4 mM: E-64: 1.4 mM, leupeptin: 2 mM, pepstatin A: 1.5mM, in DMSO; product code: P8340), quercetin, ribnonuclease A from bovine pancreas (RNAse A), Roswell Park Memorial Institute (RPMI)-1640 liquid medium (containing 0.3 g/L L-glutamine and 2 g/L sodium bicarbonate), sodium bicarbonate, sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium citrate, sodium dodecyl

sulphate (SDS), sodium deoxycholate, sodium phosphate (Na_3PO_4), sodium pyruvate, tris(hydroxymethyl) aminomethane (Trizma® base), tris(hydroxymethyl) aminomethane hydrochloride (Trizma® hydrochloride) and triton-X-100, trypsin/ethylenediamine tetra-acetic acid (EDTA) solution were purchased from Sigma Aldrich Ltd., Dorset UK.

Phosphate buffered saline Dulbecco A (PBS) tablets (8.0 g sodium chloride, 0.2 g potassium chloride, 1.15 g di-sodium hydrogen phosphate, 0.2 g potassium dihydrogen phosphate per litre at pH 7.1 -7.5) were purchased from Oxoid, Hampshire, UK. Annexin V-FITC reagent was purchased from BD Biosciences, Oxford, UK. Coulter Clenz® Cleaning Agent, Flow Check® beads and IsoFlow™ Sheath Fluid were purchased from Beckman Coulter Ltd., Buckinghamshire, UK. Formaldehyde (16%, MeOH free) solution ampoules were purchased from Thermo Scientific Inc, Rockford, IL, U.S.A. 30% w/v acrylamide-bis acrylamide stock solution was purchased from Severn Biotech Ltd, Worcestershire, UK.

MagicMark™ XP Western protein standard, Novex® ECL kit, Novex® Sharp pre-stained protein standard, NuPAGE® 4-12% bis tris gel 1.5 mm 15-well cassette, NuPAGE® antioxidant, NuPAGE® LDS sample buffer, NuPAGE® MOPS SDS running buffer, NuPAGE® sample reducing agent, NuPAGE® transfer buffer were purchased from Life Technologies-Invitrogen, Paisley, UK. Bio-Rad DC protein assay kit, bovine serum albumin (BSA) standard solution and polyvinylidene difluoride (PVDF) membrane were purchased from Bio-Rad Laboratories, Hertfordshire, UK. X-ray films processing solutions (Developer) was purchased from Champion Photochemistry International Limited (CPIL), Brentwood, UK. Analytical grade acetonitrile Far UV (ACN), ethanol (EtOH), ethylacetate (AcOEt), hexane (HEX), industrial methylated spirits (IMS), methanol (MeOH) were purchased from Fisher Scientific Ltd., Leicestershire, UK. All chemicals and reagents were stored according to the manufacturers' guidance unless otherwise stated.

7.1.6 Equipment and Software

Class II microbiological safety cabinet was supplied by BioMAT2 MDH. Cell culture CO_2 incubator Sterisonic GxP supplied by Sanyo-Panasonic. UV/Vis spectrophotometer Ultraspec 2000, orbital shaker ZLE 164 was supplied by Amersham. Spectrophotometer 2104 multi-label reader and *Wallac Evision* software manager was by PerkinElmer *Wallac Evision*. Allegra® X-15R bench-top centrifuge, Epics XL-MCL™ flow cytometer and EXPO32™ software were purchased from Beckman Coulter. Micro-centrifuge Heraeus Fresco 17 was supplied by Thermo Electron Corporation. XCell *SureLock*™ Mini-Cell electrophoresis chamber system, PowerEase® 500 power supply, Novex® Semi-Dry blotter were products obtained from Invitrogen. Nikon Coolpix 4500 camera was by Nikon. UFLCXR system coupled to an Applied Biosystems API2000 was by Shimadzu. Strata C18-E (55 µm, 70 Å) 1 g/ml tubes were purchased from Phenomenex®, Cheshire, UK. C18 Gemini-NX 3u-110A 50x2 mm column purchased from Phenomenex was kindly donated by Dr. A. Abdallah. Cylchred program was created by Terry Hoy, University of Cardiff, UK. WinMDI program was created by Joe Trotter of the Scripps Institute, La Jolla, U.S.A. GraphPad Prism version 5.0d for Mac OS X was created by GraphPad software Inc. and Software MacKiev. *ImageJ* program was created by the National Institute of Health (NIH).

7.2 Methods

7.2.1 Extraction of UNMC plants

The extraction process was conducted at UNMC, Malaysia, and was previously described in Othman *et al.* (2011) and modified from Spigno and Marco (2007).[205, 274] Collected plant materials were air dried in an enclosed room (25-28 °C) for approximately two weeks. Dried plant samples (1 g) were refined using a Waring® commercial heavy-duty grinder by macerating with organic solvent (1:8 ratio) at room temperature (RT) for 24 h. Plant samples were then subjected to sequential extraction solvents of increasing polarity: HEX > AcOEt > EtOH > Water. Ground samples were macerated with solvents for 24

h before being filtered. The filtrates were then concentrated by evaporation of the solvent in vacuum at 40 °C using a rotary evaporator (Buchi, USA). Concentrated crude extracts were desiccated for 1 - 2 weeks. The dried crude extracts were kept at ≥ – 20 °C in the dark prior to freeze-drying. The known percentage yields of corresponding plant extracts are shown in Table 7-1.

7.2.1.1 UNMC extract stocks

The plant samples were received as dried powder and stored at -20 °C in the dark. Ailquots of UNMC extracts were prepared in DMSO as 100 mg/ml top-stocks and kept at -20 °C, protected from light until use.

7.2.2 General cell culture and maintenance

All cell culture procedures were conducted aseptically in a Class II microbiological safety cabinet with a laminar flow system. The safety cabinet was disinfected with 70% IMS in d.H_2O prior to each use. Cell lines were routinely cultured in tissue culture flasks (25 cm^2 or 75 cm^2) in culture media RPMI-1640 (supplemented with 10% heat inactivated FBS) or MEME (supplemented with 10% FBS, 7.5% sodium bicarbonate, 1% 0.1 mM NEAA, 1% 1 M HEPES, 1% 200 mM L-glutamine and 1% penicillin) and grown in a humidified incubator containing 5% CO_2 in air at 37 °C. FBS was heat inactivated at 56 °C for 30 min prior to addition into culture medium to denature complement proteins and cell growth inhibitors. When the observed cell growth exceeds approximately 80% confluence, the cells were sub-cultured into new culture flasks with fresh culture medium to ensure maintenance of logarithmic growth when used in experiments.

Prior to cell subculture, medium (RPMI-1640 and MEME) stored at 4 °C were warmed in a water bath to 37 °C. To subculture adherent cells, medium was aspirated and cells were washed with 1 ml PBS. The cells were treated with 1 ml trypsin-EDTA solution (1X; 1 in 10 dilution in sterile PBS) and incubated for 2-5 min at 37 °C until the cells were visibly detached from the flask. Cells were re-suspended in 5 ml fresh medium then 0.5 ml cell suspension was transferred to a new flask containing 7 ml of fresh culture medium. The cells

were returned to the incubator until subsequent subculture or usage. Cells were discarded once they reached their recommended passage number to minimise phenotypic drift and assay variation (usually after ~ 25 passages).

7.2.2.1 Cell Preservation

Batches of mycoplasma-free cells were stored in cryovials in the liquid nitrogen cell bank (-180 °C) as stock for future use. Cells were grown in a 75 cm^2 flask until approximately 80% confluent then the medium was aspirated from the cells then the cells were washed with 3 ml sterile PBS, followed by incubation in 2 ml 1X trypsin-EDTA solution at RT for 2 min. The trypsin-EDTA solution was carefully removed and cells were incubated for a further 5 minutes at 37 °C until they had detached. The detached cells were re-suspended in freezing medium (5% DMSO in 1:1 FBS and medium). Subsequently, 1 ml cell suspension was aliquoted into Nalgene cryovials. Cells secured in cryovials were immediately stored at -20 °C for 24 h then transferred to -80 °C storage overnight before permanently stored at -180 °C in the liquid nitrogen cell bank. Frozen cells were retrieved by thawing rapidly in a water bath at 37 °C and re-suspending in 7 ml of fresh culture medium before incubation at 37 °C for 16 h to allow attachment. Thereafter, liquid contents were aspirated from the flask and fresh nutrient medium introduced.

7.2.3 MTT (*in vitro* cell viability) assay

The following method has been adapted from Mosmann (1983), exploiting the colour change upon reduction of MTT by mitchondrial dehydrogenase in living cells, from a yellow tetrazole to purple formazan.[229] Thus the increase in cellular formazan can be quantified and directly related to the activity of mitochondrial dehydrogenase.[245] In apoptotic or necotic cells mitchondrial dehydrogenase activity is lost as a result of irreversible mitchondrial fragmentation.[275] The MTT assay can therefore be used to assess cell viability and determine growth inhibitory or cytotoxic potential of investigative agents.

Cells were harvested at 70-80% confluence and were gently syringed through a 23-gauge needle to obtain a near-single cell suspension. The average number

of cells counted on a haemocytometer was used to calculate the desired cell seeding density for 72 h treatment (2 - 3.5 x 10^3 for MCF7, IGROV1, HCT116, TK-10, MRC-5 cells; 4 - 6 x 10^3 for MDA-MB-468, SK-OV-3, Caki-1, HCC2998 cells). Using a 96-well micro-titre plate, cells were seeded in 180 µl medium per well and incubated overnight at 37 ºC (5% CO_2). A separate time zero (T_0) plate was set-up to measure the initial viability of cells at the time of UNMC extract addition. After 24 h, serial dilutions of UNMC extract stocks in culture medium (concentration at 100 mg/ml in DMSO) were prepared to 10X the final concentrations required. The final treatment concentration ranges used for UNMC 20 [0 µg/ml - 50 µg/ml] and for UNMC 9, 35 and 37 [0 µg/ml – 200 µg/ml] were tested in quadruplicate. To each well, 20 µl extract solution was added to achieve a final volume of 200 µl (DMSO concentration < 1%). To the peripheral lanes, 20 µl medium was added to control cells and cells in the T_0 plate. Cell viability was quantified at the time of extract addition for T_0 plate and following 72 h exposure of cells to test extract for treated plates using the MTT assay. Initially, 50 µl MTT solution (2 mg/ml MTT in sterile PBS; stored at 4 °C in the dark) was added to each well and further incubated for 4 h at 37 °C in the dark. The medium and any unconverted MTT were aspirated then 150 µl DMSO was added to each well and the plates were placed on a plate shaker for 2 min. The optical density (OD) was read on a spectrophotometer at 555 nm using *Wallac Envision* software.

The absorbance intensity is directly proportional to cell viability and there is a linear relationship between cell number and the amount of formazan present. The mean absorbance for treated and control cells at treatment concentration range could be used as a quantitative measure of cell viablity by constructing a dose-response curve using the mean absorbances to enable the estimated extract concentration inducing 50% growth inhibition (GI_{50} value) to be calculated (Equation 1). Additionally, vehicle control (DMSO) was tested to ensure that DMSO did not compromise cell viability in extract-treated wells. Another set of assays using quercetin as a positive control was performed. The GI_{50} values established from the MTT cytotoxicity assay will be used in all the subsequent assays.

$$\frac{\textbf{Control or Treatment } \mathbf{OD_{555}} - \mathbf{T_0\ OD_{555}}}{\mathbf{2}} + \mathbf{T_0\ OD_{555}} = \mathbf{OD_{555}\ at\ GI_{50}}$$

Equation 1: Following MTT assay, concentration which inhibit cell growth by 50%, GI_{50} value was determined from optical density readings of extract treated and control cells absorbancies.

7.2.4 Clonogenic assay *(in vitro* cell sensitivity assay)

The following method was adapted from the method described by Brown and Wouters (1999) and Plumb (2004) to determine cell death or recovery by determining subsequent proliferative and colony formation capacity of cells following brief exposure to investigative agents.[276, 277]

HCT116 cells were harvested at 70-80% confluence and were gently syringed through a 23-gauge needle to obtain a near-single cell suspension. Cells were counted on a haemocytometer and the average number of counted cells was used to calculate the desired cell seeding density for 24 h treatment. HCT116 cells were seeded in 6-well plate at a density of 250 cells per well in 2 ml culture medium and incubated overnight at 37 °C for cellular attachment. Freshly prepared dilutions of UNMC extract in medium were added to wells in triplicate to obtain treatment concentrations of 0.5X, 1X and 2X GI_{50} values. DMSO vehicle was added to the control wells. After 24 h exposure to extracts, culture medium was aspirated from all wells and cells washed with 2 ml PBS to remove any remaining extract in the well; 2 ml fresh culture medium was then introduced to each well. The cells were kept in the incubator at 37 °C to allow recovery and the end-point was considered when control cell colonies size reached ≥ 50 cells, usually 8 to 11 days. To quantify the number of colonies formed, each well was rinsed in 1 ml chilled PBS and fixed with 1 ml chilled MeOH at RT for 10 min. The solvent was removed and the colonies were stained with 500 µl methylene blue solution (0.5% methylene blue in 1:1 MeOH and d.H20 (v/v); stored at 4 °C) for 10 min. The plates were washed gently with $d.H_2O$, air-dried and cell colonies were counted. The plating efficiency for each well was > 90% (Equation 2).

$$\frac{\textbf{\textit{Number of colonies counted}}}{\textbf{\textit{Number of cells seeded}}} \textbf{\textit{x}}\ \mathbf{100} = \textbf{\textit{Plating efficiency}}\ (\%)$$

Equation 2: % of plating efficiency determined by the number of colony (ies) counted in comparison to the total number of cells seeded in clonogenic assay.

7.2.5 Flow cytometric techniques

7.2.5.1 Cell cycle analysis

Analyses of cell cycle distributions were performed by flow cytometric measurement of cellular DNA content using fluorescent dyes such as propidium iodide (PI), which intercalates DNA bases stoichiometrically. Any disruption in the DNA histogram, which is representative of the cell cycle, could indicate a DNA damaging or cytotoxic effect on the cell cycle induced by investigative agents. The following method was adapted from the method described by Nicoletti *et al*. (1991).[249]

MDA-MB-468 and HCT116 cells were harvested at 70-80% confluence and counted on a haemocytometer. The average number of counted cells was used to calculate the desired cell seeding density for 48 h treatment. The cells were seeded in 6-well plates in duplicate at a density of 1.5 x 10^5 in 2 ml of culture medium. The cells were allowed 24 h at 37 ºC to adhere and commence cell division. Freshly prepared dilutions of UNMC extract stock solution in medium were introduced to MDA-MB-468 cells (treated with UNMC 35) and HCT116 cells (treated with UNMC 20 or UNMC 37) at final concentrations 1X, 2X and 4X GI_{50} values. The cells were treated for 48 h at 37 ºC. DMSO vehicle was added to the control cells. Cell cycle analysis preparation was conducted at 4 ºC. Floating and trypsinised cells were pooled and pelleted by centrifugation (306 x g, 5 min at 4 ºC), washed with 2 ml chilled PBS. Cells were pelleted again and the supernatant was discarded. Cells were incubated in 400 µl fluorochrome solution (0.1% sodium citrate, 0.1% triton-X-100, 50 µg/ml PI, 0.1 mg/ml Ribonuclease A in d. H_20; stored at 4 ºC in the dark)

overnight at 4 ºC protected from light. Samples remained protected from light on ice and gently syringed prior to cell cycle distribution analysis. Cell cycle distribution analysis was performed on a Beckman Coulter EPICS-XL MCLTM flow cytometer. The FL3 channel was used to detect propidium iodide (PI) and at least 15,000 events were recorded for each sample. Data were further analysed using *WinMDI* and *Cylchred* softwares.

7.2.5.2 Apoptosis detection

One of the hallmarks of early apoptosis involves the translocation of phosphatidylserine (PS) from the inner to the intact outer plasma membrane (as membrane asymmetry is lost). The membrane integrity of an apoptotic cell diminishes when progressing into late apoptosis/necrosis. A calcium ion (Ca^{2+}) dependent phospholipid-binding protein, annexin V, possesses high PS binding affinity. Analyses of apoptotic cell populations were performed using flow cytometric techniques. Dual staining of annexin V (conjugated to FITC, a green fluorescent dye) and PI to detect early apoptotic (annexin V-$FITC^+/PI^-$) and late/necrotic cell populations (annexin V-$FITC^+/PI^+$) can be analysed using flow cytometric techniques. The method was adapted from the method described by Vermes *et al.* (1995).[94]

MDA-MB-468 and HCT116 cells were harvested at 70-80% confluence and counted on a haemocytometer. The average number of counted cells was used to calculate the desired cell seeding density of 2 x 10^5 and 1.5 x 10^5 for 24 h and 48 h treatment, respectively. MDA-MB-468 and HCT116 cells were seeded in 6-well plates in duplicate at the aforementioned densities in 2 ml culture medium and allowed 24 h to adhere at 37 ºC. Freshly prepared dilutions of UNMC extract stock solution in medium were introduced to MDA-MB-468 cells (treated with UNMC 35) and HCT116 cells (treated with UNMC 20 or UNMC 37) at final concentrations 1X, 2X and 4X GI_{50} values. DMSO vehicle was added to the control cells. Preparation for apoptosis analysis was conducted at 4 ºC. After 24 h and 48 h treatment, floating and trypsinised cells were pooled and wells were washed twice with PBS. Cells were counted and 1 x 10^5 cells were transferred to FACS polyproplene tubes on ice prior to

centrifugation at 306 x g for 5 min at 4 ºC. The supernatant was discarded and the cell pellets were incubated in 100 µl binding buffer (10 mM HEPES/NaOH at pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$ in d. H_20; stored at 4 ºC) with 5 µl Annexin V-FITC (stored at 4 ºC in the dark). Pellets were gently vortexed and kept in the dark for 15 min. Subsequently, 5 µl PI solution (50 µg/ml in d.H_20; stored at 4 ºC) and 400 µl binding buffer were added to the samples, which were gently vortexted and kept at 4 ºC until analysis. Analysis of intact, early apoptotic and late apoptotic/necrotic cells were detected within 1 h on a flow cytometer.

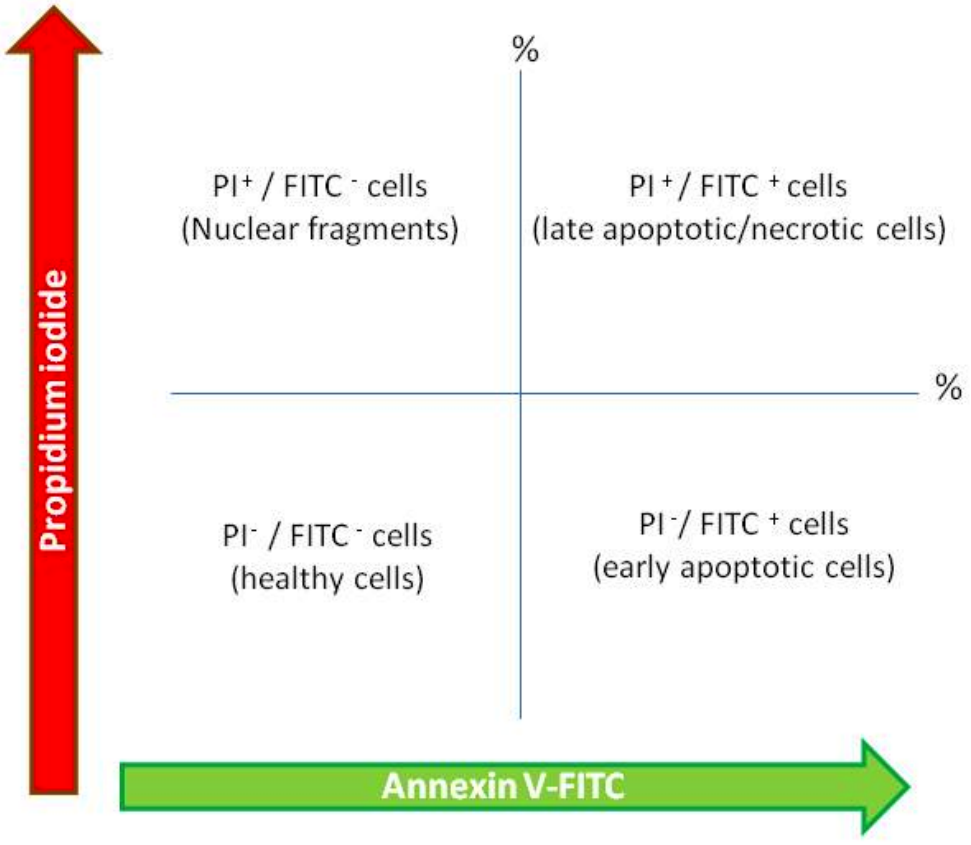


Figure 21: An example of flow cytometry diagram (dot plot) of double-staining with Annexin V-FITC and Propidium iodide. The dot plot is representation of annexin V-FITC log fluorescence versus PI log fluorescence. Cells in the top left quadrant ($PI^+/FITC^-$) represent cells with nuclear fragments, cells in the lower left quadrant ($PI^-/FITC^-$) represent living cells, cells in the lower right quadrant ($PI^-/FITC^+$) are early apoptotic cells and cells in the top right quadrant ($PI^+/FITC^+$) are late apoptotic/necrotic cells.

7.2.6 Western blot procedures

Western blot analysis is a widely used quantitative method for investigating presence of proteins within cellular extracts. Proteins extracted from samples, denatured and separated according to molecular weight by gel electrophoresis. The proteins are then electrophoretically transferred to a membrane to allow protein(s) of interest to be probed using specific primary antibodies, which can be recognised by a secondary antibody conjugated with horseradish peroxidase (HRP) enzyme. The light released (from illuminating substrate 3-aminophthalate) in chemiluminescence reaction is captured on film. Protein size can be determined from the bands on the film against molecular weight markers of proteins of known molecular weight. Camptothecin was chosen as a positive control for apoptosis. A series of procedures adapted from Towbin *et al*. (1979) were performed to obtain the protein-bound membrane for detection.[278]

7.2.6.1 Cell lysis

MDA-MB-468 and HCT116 cells were seeded in 10 cm plates at a density of 1.5 x 10^6 in 5 ml of culture medium and incubated for 24 h to adhere at 37 ºC to allow cells to adhere and reach ~70 % confluency. Freshly prepared dilutions of UNMC extract stock solution in medium were introduced to MDA-MB-468 cells (treated with UNMC 35) and HCT116 cells (treated with UNMC 20 or UNMC 37) at final concentrations 1X, 2X and 4X GI_{50} values. Camptothecin (300 nM stock solution) and DMSO vehicles were added to the control cells. Cell lysis preparation was conducted at 4 ºC. At elected time points, 24 and 48 h, floating and scraped cells were pooled and wells were washed with 3 ml chilled PBS. The procedure was repeated 4X before the cell suspension was transferred to FACS polypropylene tubes prior to centrifugation (306 x g for 5 min at 4 ºC). Cell pellets were retained and incubated with 80 µl NP-40 cell lysis buffer (150 mM NaCl, 1% Triton-X-100 and 50 mM Tris at pH 8.0) supplemented with protease inhibitor cocktail (AEBSF: 104 mM, aprotinin: 80 µM, bestatin, 4 mM: E-64: 1.4 mM, leupeptin: 2 mM, pepstatin A: 1.5mM, in DMSO; Sigma Aldrich product code: P8340). The cell pellets in NP-40 lysis buffer were mixed gently by pipetting

at 4 ºC was performed to separate the cell pellet from the supernatant. The cell pellet was discarded and the supernatant lysate containing protein content were kept in labeled eppendorfs on ice for immediate protein concentration determination or stored at –20 ºC.

7.2.6.2 Protein determination

The principle of Bio-Rad DC protein assay kit is that reduction of the phosphomolybdic-tungstic mixed acid chromogen (present in Folin-Ciocalteau phenol reagent) by proteins. The level of reduction is proportional to the amount of protein present in the sample, which is determined by measuring the absorbance on a spectrophotometer at 750 nm. The principle of the assay is based on the method described in Lowry *et al.* 1951.[279] Protein concentrations in the lysates were determined using the Bio-Rad DC protein assay kit in 96-well plates according to the manufacturer's instructions. Briefly, 5 µl lysate sample, 25 µl $reagent_{A+S}$ and 200 µl $reagent_{B}$ were added into triplicate wells. Triplicates of BSA standards (concentration range: 0.1-10 mg/ml) were measured with every set of samples requiring protein determination. The OD was analysed by a spectrophotometer at 750 nm using *Wallac Envision* software. The protein content in each sample was determined using a standard curve using BSA standards against the lysate protein concentration.

7.2.6.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The separation of protein mixtures could be achieved by loading the protein sample in an anionic sodium dodecyl sulfate (SDS) gel and passing an electric current through the gel. SDS denatures proteins into their peptide chains and binds to them at a constant ratio of 1.4 g of SDS per 1 g of protein, which gives the same charge-to-mass ratio of SDS and protein therefore the ability to migrate through the gel is dependent on the molecular weight of the protein.[280] Protein samples were prepared using NuPAGE® 4X LDS sample buffer (3.75 - 7.50 µl), NuPAGE® reducing agent (1.5 – 3.0 µl) and a calculated volume of cell lysate equivalent to 100 µg of protein; the total sample volume is ≤ 30 µl. The samples were denatured for 5 min at 95 ºC then briefly centrifuged and

kept at 4 ºC. Samples were loaded on a polyacrylamide gel (8 - 12%) depending on the size of the protein of interest. Molecular weight markers were also loaded onto the gel before submersion in running buffer (25 mM Trizma base, 200 mM glycine, 1.00 g SDS, pH 8.5 HCl in 1L of d.H_2O). Proteins were then separated by electrophoresis using 125 V on a polyacrylamide gel for 90 min at RT until the dye front of the loading buffer reached the bottom of the gel. Pre-made NuPAGE® Bis-Tris gel (4-12%) and pre-made NuPAGE® MOPS SDS running buffer were used at times in place of the corresponding materials according to manufacturer's manual.

Table 7-3 Receipe for Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Material	**Gel**			
	Resolving (ml)			**Stacking (ml)**
	8%	10%	12%	-
10% AMPS	0.20	0.20	0.20	0.10
10% SDS	0.20	0.20	0.20	0.10
30% Acrylamide mix	5.30	6.70	8.00	1.70
TEMED	0.012	0.010	0.008	0.01
Tris	5.0 (1.5M, pH 8.8)			1.25 (1M, pH 6.8)
d. H_2O	9.3	7.9	6.6	6.8

7.2.6.4 Western Blot

The PDVF membrane was calibrated prior to electroblotting by being fully immersed in 100% MeOH for 20 min then in 10 ml of transfer buffer (25 mM Trizma base, 200 mM glycine, 0.375 g SDS, pH 7.6, 20% MeOH, HCl in 1L of d.H_2O) with blotting paper for a further 10 min. Gels were washed with 10 ml transfer buffer for 2 min. Protein bands on the gels were electroblotted onto PDVF membranes at 100 V for 90 min at RT using Novex® Semi-Dry Blotter. The membranes were washed using 20 ml TBST (25 mM Tris, 113 mM NaCl, 0.05% Tween 20 (v/v) at pH 7.4. To reduce non-specific binding, membranes were kept in blocking solution (10 % fat-free milk powder in TBST) overnight at 4 ºC on a roller. Subsequently, blocked membranes were washed to remove

excess blocking solution with TBST for 3 min, which was repeated 5 times. To enable any specific binding of Ab, the membranes were incubated with 10 ml primary (1º) Ab dilution (Table 7-4) overnight at 4 ºC on a roller.

Table 7-4 Primary and secondary antibodies used for western blotting.

	Antibody (Ab)	Polyacrylamide gel (%)	Concentration of Antibody (in 3% milk-TBST)	Blocking
Primary	Caspase 3	10 - 14	1:1000	10% milk-TBST
	Mcl-1	10 - 12	1:1000	
Secondary	Donkey Anti rabbit Goat Anti mouse	-	1:5000	

The membrane was washed again with 20 ml of TBST for 5 min, which was repeated 6 times. After washing, the membrane was incubated with 15 ml secondary (2º) Ab (Table 7-4) for overnight at 4 ºC on a roller. The membrane was washed with 20 ml TBST for 3 min and repeated 3 times. Novex® ECL Chemiluminescent Substrate Reagent Kit was used to wash the PDVF membranes and developed onto x-ray film. The reaction was stopped after 2 min by washing the membrane with TBST and stored at 4 ºC.

7.2.7 Senescence assay (β-galactosidase staining)

Cellular senescence describes the irreversible growth arrest, which is the underlying mechanism cause of aging in normal somatic cells upon reaching their Hayflick replicative limit. Hence senescence is also a tumour suppression mechanism. A biomarker, β-galactosidase, is present in the lysosome of senescent cells. Several chemotherapeutic agents are able to induce senescence in tumours.[257] The assay is based on detecting an increased abundance of β-galactosidase using X-gal where senescent cells are stained blue-green. The following method was adapted from the method described by Dimri *et al.* (1995).[257]

MDA-MB-468 cells were seeded at 1 x 10^4 and 5 x 10^4 for vehicle control and treatment cells, respectively, in 6-well plates in duplicates. HCT116 cells were seeded at 5 x 10^3 and 1 x 10^4 for vehicle control and treatment cells, respectively, in 6-well plates in duplicates. MDA-MB-468 and HCT-116 cells were seeded into wells containing 2 ml culture medium and allowed to attach overnight at 37 °C. Freshly prepared dilutions of UNMC extract fraction stock solution were introduced to MDA-MB-468 cells (treated with UNMC 35) and HCT116 cells (treated with UNMC 20 or 37) at final concentrations of 1X and 2X GI_{50} values. DMSO vehicle was added to the control cells and 5 μM of RHPS4 was used as the positive control.

At day 7 and 12 of treatment for HCT116 and MDA-MB-468 cells, respectively, culture media were aspirated from the wells and cells were washed twice with 2 ml PBS to remove any remaining extracts in the well. The cells were fixed using 1 ml fixative solution (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 10 min then the fixative solution was removed and 1 ml senescence buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM $MgCl_2$, 40mM Na_3PO_4/citric acid buffer pH 6.0, 1 mg/ml X-gal in DMF in 1L of d. H_20) was added to each well. The plates were protected from light and incubated at 37 °C for 24 h. The number of senescent (blue-green stained) cells and total number of cells in the sample were counted (observation under phase contrast light microscope) and photographs of the cells were captured using a Nikon camera at 200X - 400X

magnification. To quantify the proportion of senescent cell population, cells were scraped from the wells, syringed and counted on a haemocytometer.

7.2.8 Cell migration assay (Wound healing)

The ability of malignant cells to invade and migrate is crucial to cancer metastasis. This simple *in vitro* assay mimics *in vivo* cell migration during wound healing. A 'wound' is created in a cell monolayer and observations (images captured) at timed intervals enable analysis of the migration wound-healing capacity rate of the cells. The following method was adapted from the method described by Liang *et al*. (2007).[253]

The under side of 6-well culture plates was marked with two crosses 'x' aligned in the center. HCT116 cells were harvested and seeded at 3 x 10^5 in 6-well plate containing 2 ml culture medium with reduced 7.5% FBS. The cells were incubated overnight at 37 ℃ to allow cellular attachment. The scratch was created when cells were ~80% cellular confluent by applying a sterile 200 µl pipette tip through the center, aligned with 'x'. Dilutions of UNMC extract stock solution were freshly prepared and introduced to the cells at final concentrations 1X and 4X GI_{50} values. DMSO vehicle was added to the control wells. Each extract and control sample were tested in duplicate. The scratch was evaluated under phase contrast microscopy and images of the scratch were captured with a Nikon camera (100X magnification) at 0, 24, 48, 52 and 64 h. Images of the wound were taken just above and below the 'x' mark to ensure consistency. Occassional replenishment of the medium (with control or extracts) is required to capture clear images of the wound. The level of wound healing was analysed using *ImageJ* software by measuring the difference in total percentage wound area (%) of treated cells and untreated control cells.

7.3 Phytochemical composition of plant extracts

UNMC plant extracts were tested for phytochemical composition using colorimetric assays.

7.3.1 Total flavonoid content

The aluminium trichloride ($AlCl_3$) colorimetric method was adapted from Meda *et al.* (2005) and Chang *et al.* (2002).[263, 264] $AlCl_3$ could form acid stable complexes with reactive groups of flavones and flavonols and form acid labile complexes with the flavonoids. Quercetin is a widely distributed flavonoid hence employed as a standard to measure against the UNMC extracts for total flavonoid content determination.

UNMC extract stock solution (100 mg/ml in DMSO), quercetin stock solution (0.5 mg/ml in DMSO) and 2% $AlCl_3$ working solution (20 mg/ml in MeOH (w/v)) were freshly prepared prior to the assay. Due to the natural pigment of the extract samples, a set of UNMC control extract samples were prepared in glass vials to account for baseline absorbance. Sets of control UNMC extract samples were prepared in triplicate in glass vials and consisted of 1998 µl MeOH to which 2 µl UNMC extracts stock solution was added to achieve concentration of 1 mg/ml in a final volume of 2 ml. The UNMC extract test samples were also prepared in triplicates in glass vials, which consisted of 1.98 ml of 2% $AlCl_3$ working solution to which 20 µl UNMC extracts stock solution was added to achieve a concentration of 1 mg/ml in a total volume of 2 ml. Both sets of UNMC extract control and UNMC extract samples were incubated for 10 min at RT protected from light.

Quercetin stock solution was diluted with MeOH (total volume of 2 ml) and prepared at a range of [0-100 µg/ml] to construct the calibration curve. A 'blank' cuvette containing 1 ml 2% $AlCl_3$ and 1 ml MeOH was used for calibration prior to each set of readings. The OD of quercetin standard solutions, UNMC extract controls and UNMC extract samples solutions were measured at 415 nm using UV/Vis spectrophotometer. The mean absorbances of control UNMC extract control samples were determined and deducted from

the mean UNMC extract sample absorbances. The total flavonoid content in UNMC extracts was calculated using a quercetin standard curve and expressed as quercetin equivalents (QE)/100 g of extract.

7.3.2 Total phenolic content

The Folin-Ciocalteau colorimetric method was adapted from Singleton *et al.* (1999) and Meda *et al.* (2005).[263] FC reagent contains phosphomolybdate and phosphotungstate, which react with reducing substances such as polyphenolic compounds. Gallic acid (GA) is a widely distributed phenolic compound and is the chosen standard used to measure against the UNMC extracts for total phenolic content determination.

UNMC extract stock solution (100 mg/ml in DMSO), GA stock solution (500 µg/ml in DMSO), 0.2 N FC working solution (0.40 ml of 20 N FC reagent in 19.60 ml of d.H_20) and sodium carbonate solution (75 mg/ml in d.H_20, total volume of 50 ml) were freshly prepared prior to the assay. UNMC extract control samples were prepared in glass vials in triplicates, of which, each vial consisted of 985 µl d.H_2O, 500 µl $NaCO_3$ and 15 µl UNMC extract stock solution in a total volume of 1.50 ml. The UNMC extract test samples were prepared in triplicate in glass vials by mixing 140 µl d.H_2O and 15 µl UNMC extract solutions then added to 845 µl 0.2 N FC reagent and incubated for 5 min at RT protected from light. Subsequently, 500 µl $NaCO_3$ was added to all UNMC extract test samples. Both sets of UNMC extract control and UNMC extract test samples were incubated for 2 h at RT in the dark. The final extract concentration was 1 mg/ml in 1.50 ml final volume.

GA stock solution was diluted with d.H_20 (total volume 1.50 ml) and prepared at a concentration range of [0-100 µg/ml] to construct the calibration curve. A 'blank' cuvette containing 155 µl d.H_2O, 845 µl 0.2N FC reagent and 500 µl $NaCO_3$ was used for calibration prior to each set of reading. The OD of GA standard solutions and UNMC extract sample solutions were measured at 760 nm using UV/Vis spectrophotometer. The mean OD of UNMC extract control samples was deducted from the mean UNMC extract test sample absorbances. The total phenolic content in UNMC extracts was calculated using a GA standard curve and expressed as GA equivalents (GAE)/100 g of extract.

7.4 *In vitro* free radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) is a stable free radical, which can be used to assess the radical scavenging activity of plant extracts. In the presence of an antioxidant such as quercetin, $DPPH^{\cdot}$ is reduced to the molecular form (DPPHH) resulting in a colour change from yellow to purple. This change in absorbance can be used to measure the radical scavenging potency of the plant extracts. The following method was adapted from the method described by Amic *et al*. (2003)[265] and Nara *et al.* (2006).[266]

UNMC extract stock solution (100 mg/ml in DMSO), quercetin stock solution (500 mg/ml of quercetin in DMSO) and DPPH stock solution (1 mg/ml of DPPH in MeOH) were freshly prepared prior to the assay. DPPH stock solution was further diluted in MeOH to a 4 % working solution. Two 96-well micro-titre plates were labeled '$sample_{+DPPH}$' or '$sample_{-DPPH}$'. To determine UNMC extract test samples' radical scavenging activity, the $sample_{+DPPH}$ plate was prepared with wells containing 180 µl 4% DPPH working solution to which 20 µl of UNMC extract stock solution were added. To account for the pigmented UNMC extract control samples, the $sample_{-DPPH}$' plate was prepared with wells containing 180 µl MeOH ($sample_{-DPPH}$) to which 20 µl UNMC extract stock solution were added. All tests were conducted in quadruplicate wells. The baseline absorbance was considered by measuring wells containing solvents only ($solvent_0$), such as MeOH, DMSO or MeOH and DMSO. Quercetin stock solution was diluted with MeOH in order to construct a calibration curve at a concentration range of [0 - 100 µg/ml] ($sample_{+DPPH}$). Plates were wrapped in foil and incubated for 15 min at 37 °C then placed on an orbital plate shaker for 2 min. Absorbances were analysed by spectrophotometry at 517 nm using *Wallac Envision* software. The absorbances for plates: $sample_{-DPPH}$ and $solvent_0$, were taken into consideration when calculating the free radical scavenging activity of the UNMC extracts. The average $solvent_0$ values were subtracted from the all UNMC extract test sample and quercetin measurements. The radical scavenging activity could be calculated using the following equation to create a graph of radical scavenging (%) against UNMC concentration:

Equation 3: % of radical scavenging ability of extracts determined from optical density readings obtained from the DPPH assay.

$$\frac{Solvent_0-(Sample_{+DPPH}-Sample_{-DPPH})}{Solvent_0} \text{ x } 100 = \text{Radical scavenging (\%)}$$

7.5 Broth microdilution method (anti-microbial assay)

Assessment of UNMC extracts in broth microdilution method, described below, was performed by Dr. Jaroslav Havlík and his group from the Department of Microbiology, Nutrition and Dietetics, Czech Universitiy of Life Sciences, in Prague.

The minimum inhibitory concentrations (MICs) were determined by the broth microdilution method according to the CLSI guidelines (2009) using 96-well microtitre plates. Briefly, extract samples were two-fold diluted in Mueller Hinton broth (Oxoid, Basingstoke, UK), inoculated with bacterial suspension at a density of $5x10^7$ cfu/ml ($5x\ 10^4$ for *C. albicans*) and then incubated in thermostat at 37° C for 24 h (48 h for *C. albicans*). Growth turbidity was measured by Multiscan Ascent Microplate Reader (Thermo Fisher Scientific, Waltham, USA) at 405 nm. Extract concentration range used was 1 – 128 µg/ml. MICs were calculated based on the density of the growth control and were expressed as the lowest extract concentrations that resulted in 80% growth reduction compared to that of the extract-free growth control. The bacterial strains tested were obtained from Oxoid (Basingstoke, UK) and all reference antibiotics used were purchased from Sigma-Aldrich (Prague, CZ). DMSO (Lach-Ner, Neratovice, CZ) was used as a solvent for all extracts; except for *C. albicans*, MICs of reference antibiotics used as positive controls were in range of acceptable limits as published by CLSI guidelines (2009).[281]

7.6 Fractionation and analysis of UNMC extract fractions using High Performance Liquid Chromatography (HPLC)

The mixture of compounds embedded within UNMC extracts can be separated using a mobile and a stationary phase. Reversed solid-phase extraction (SPE) can separate crude extracts using different eluents (of varying polarity) and a non-polar stationary phase into phytochemicals groups with similar physicochemical characteristics such as solubility, size, shape and electrical charge.[18]

Crude fractionations of UNMC 20, 35 and 37 extracts were prepared in d.H_2O and MeOH at a ratio of 50:50 in a total volume of 1 ml. Separation was performed using Strata extraction cartridge. Prior to fractionation, each cartridge was equilibrated using 4 ml MeOH and 4 ml d.H_2O. The crude UNMC extracts were weighed then loaded on a Strata C18-E extraction cartridge (C18-E (55 µm, 70 Å) 50 mg/ml tube). Glass vials used to collect the fractions were also weighed. Employing solvents of increasing polarity MeOH > MeCN > d.H_20, UNMC extracts were fractionated using different solvent gradients. Fractions were collected in clearly labeled glass vials. A new cartridge was used for loading a different UNMC extract to prevent contamination. Solvents were removed using nitrogen and d.H_2O was removed by freeze-drying to obtain dried powder compounds (for yield (%) and description of compounds, see Appendix II). The dried compounds were kept protected from light at – 20 °C until further examination of the isolated analytes using HPLC. Extract eluents were dissolved in DMSO (1 mg/ml) and 1:1 d.H_2O and MeOH before analysis using a Phenomenex C18 Gemini-NX 3u-110A 50x2 mm column in a HPLC (Shimadzu UFLCXR system coupled to an Applied Biosystems API2000) system. The solvents used for the gradient were solvents A (0.1% formic acid in d.H_2O) and B (0.1% formic acid in MeCN). Samples were run on a solvent gradient of 10% B for 1 min, 10-98% B over 2 min, isocratic elution at 98% for 2 min, re-equilibrated to 10% B over 0.5 min (0.5 ml/min). The samples were detected with a UV light at λ = 220 and 254 nm.

Table 7-5 Solvent gradient used in crude extract separation.

Fraction	Solvent (%)		
	Water	**MeOH**	**MeCN**
1	100	0	0
2	75	25	0
3	50	50	0
4	25	75	0
5	0	100	0
6	0	0	100

Strata C18-E extraction cartridge was equilibrated with equal volume of d. H_20 and MeOH prior to loading the cartridge with UNMC extract. The solvents were passed through the extract and collected then removed to obtain the extract fraction for analysis.

Fractions collected required solvent evaporation via freeze-drying and/or nitrogen gas to separate solvents from extract fractions. Once solvent is removed, the vials were weighed again to calculate the difference in weight, hence determine the % yield of extract fraction material collected.

7.7 Statistical Analysis

Data from the assays are presented as the means ± SEM. Data analysis was conducted using GraphPad Prism software (Version 6.0 c, San Diego, CA, USA). The differences between treatment groups and control groups were deteremined by one-way ANOVA for the following assays: clonogenic, cell cycle distribution, apoptosis detection, migration, senescence; $p < 0.05$ was considered to be statistically significant. The correlation of chemical contents in the extract samples was measured against a reference compound (standard cruve) and analysed using the Pearson coefficient.

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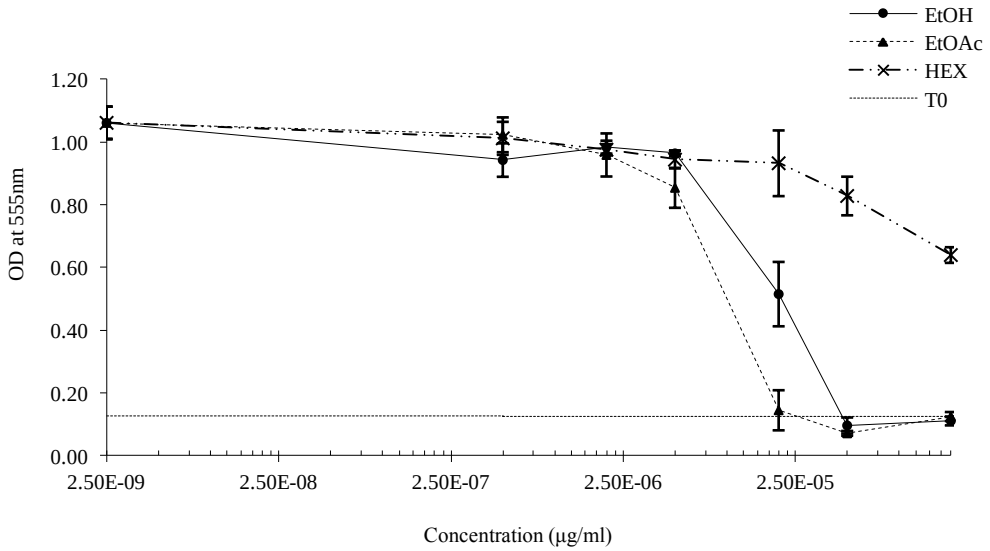
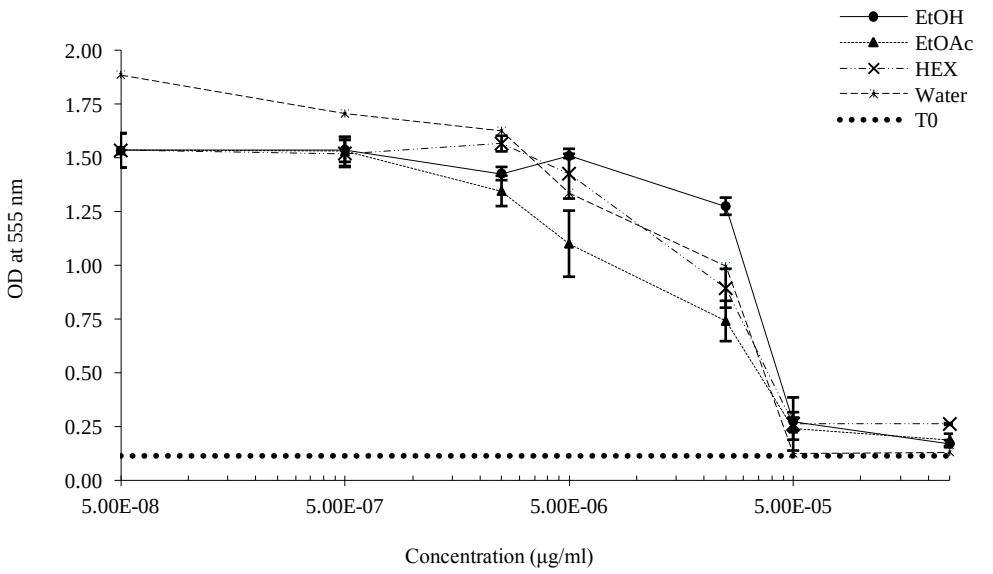
Appendices

Appendix I

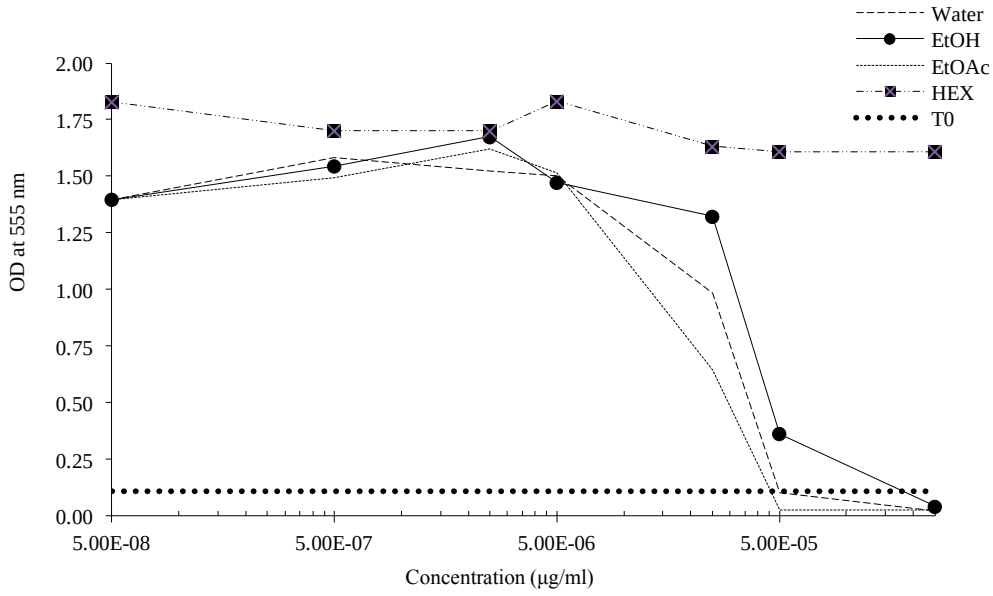
The MTT assay was used to determine cell viability after exposure to UNMC extracts by measuring the conversion of MTT to formazan crystals in viable cells. The optical density readings were used to construct dose-response curves hence determination of the extract concentration (µg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h exposure.



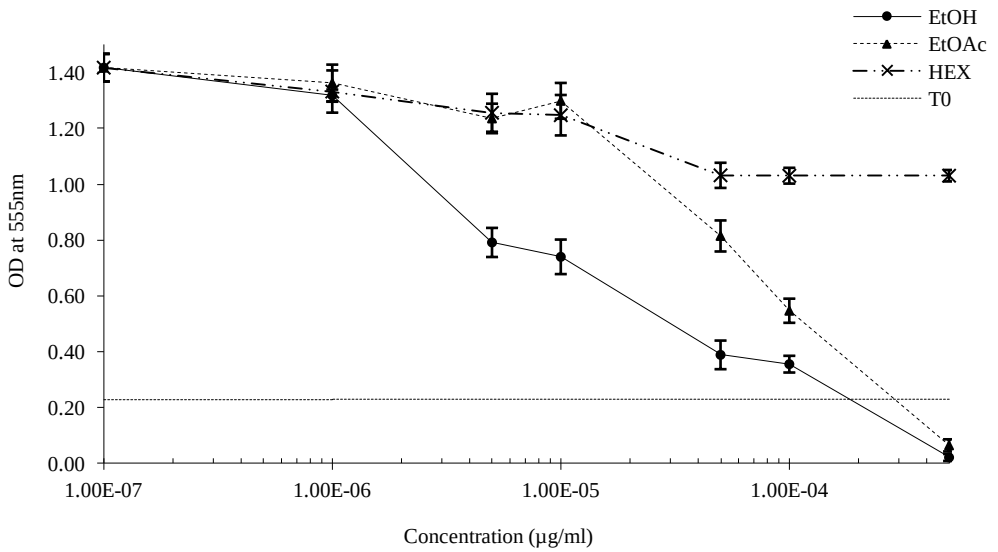
Appendix I-A: Dose-response curve of MDA-MB-468 cells after exposure to *Acalypha Wilkesiana* extracts for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of EtOH, EtOAc and HEX extracts are: 17.38, 10.51 and >200 µg/ml, respectively.



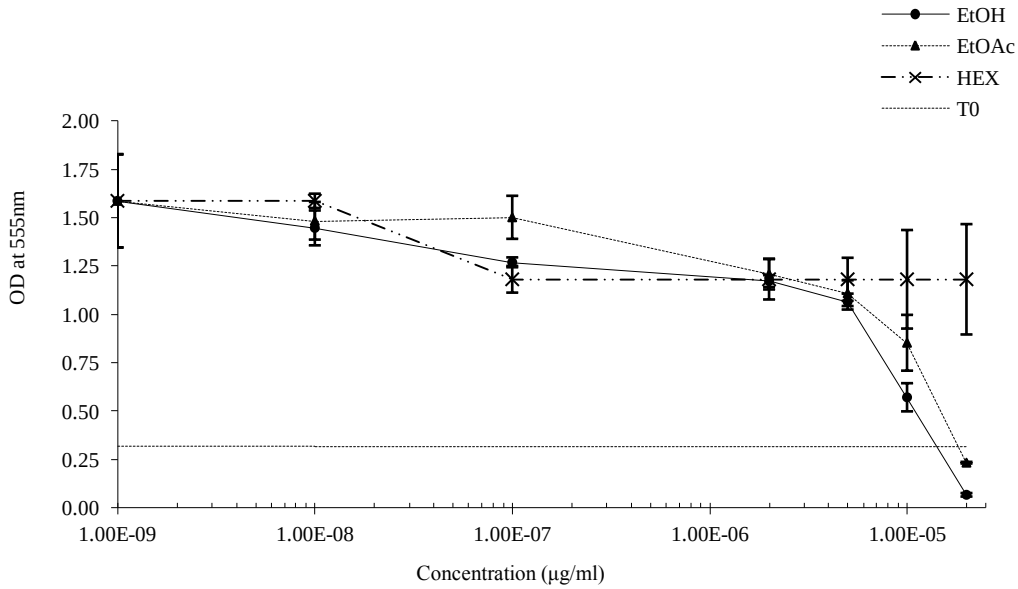
Appendix I-B: Dose-response curve of HCT116 cells after exposure to *Duabanga grandiflora* bark extracts for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of Water, EtOH, EtOAc and HEX and extracts are: 49.00, 36.29, 20.43, 27.74 and >200 µg/ml, respectively.



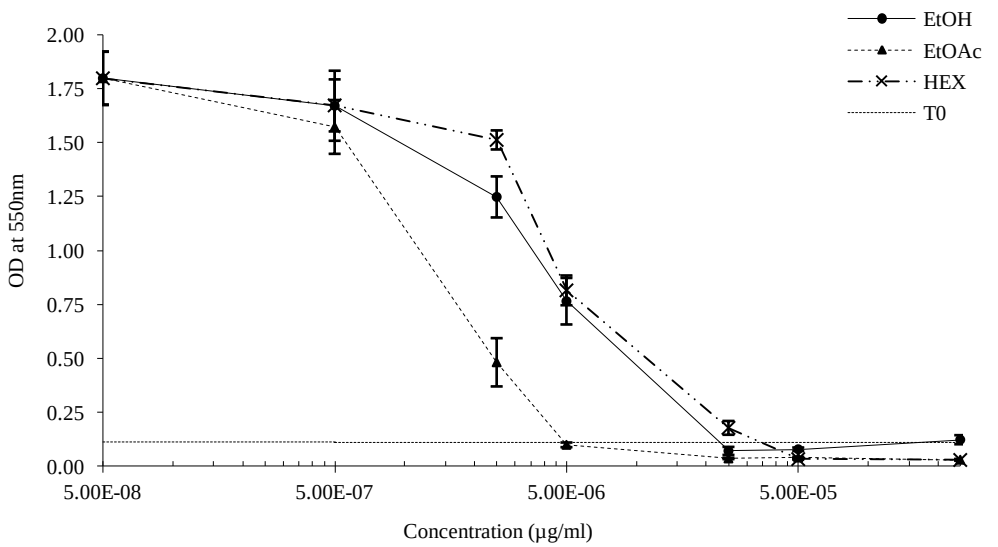
Appendix I-C: Dose-response curve of HCT116 cells after exposure to *Duabanga grandiflora* leaf extracts for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of Water, EtOH, EtOAc and HEX and extracts are: 31.53, 39.81, 22.51 and >200 µg/ml, respectively.



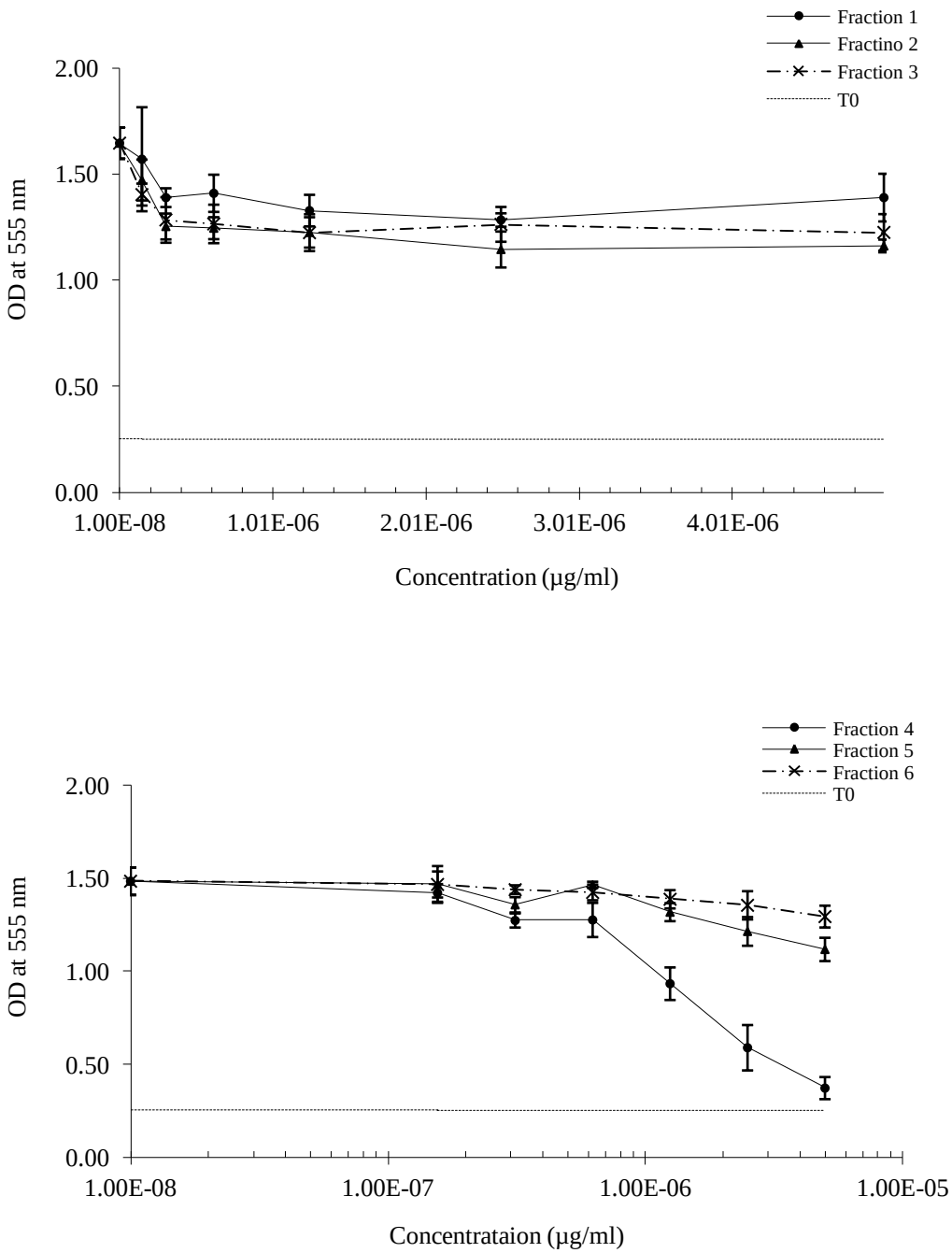
Appendix I-D: Dose-response curve of MDA-MB-468 cells after exposure to *Archidendron ellipticum* bark extracts for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of EtOH, EtOAc and HEX extracts are: 4.76, 49.31 and >200 µg/ml, respectively.



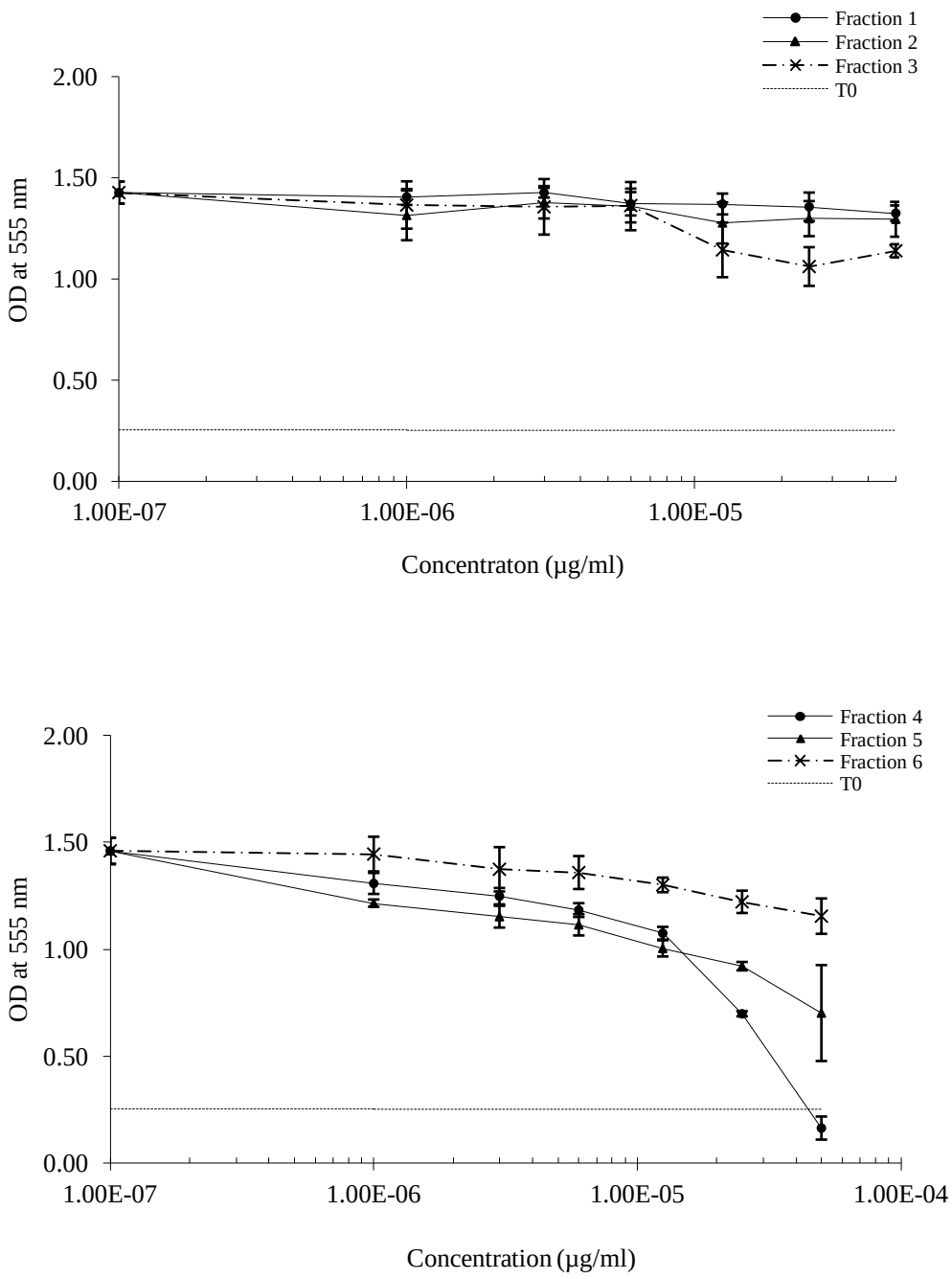
Appendix I-E: Dose-response curve of MDA-MB-468 cells after exposure to *Archidendron ellipticum* leaf extracts for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of EtOH, EtOAc, HEX and Water extracts are: 6.12, 8.06 and >200 µg/ml, respectively.



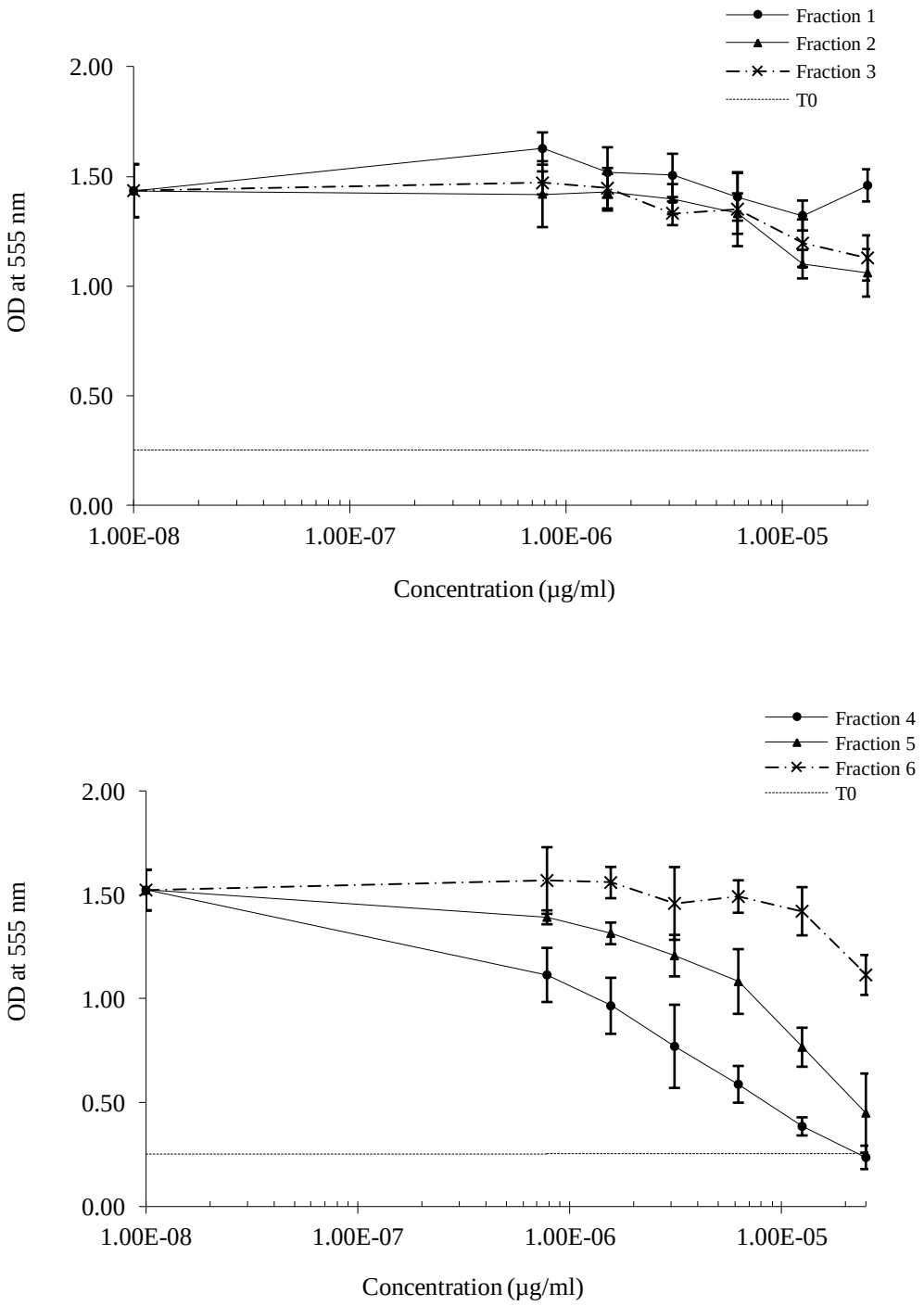
Appendix I-F: Dose-response curve of HCT116 cells after exposure to *Pseuduvaria macrophylla* extracts for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of EtOH, EtOAc and HEX extracts are: 4.02, 1.63 and 4.50 µg/ml, respectively.



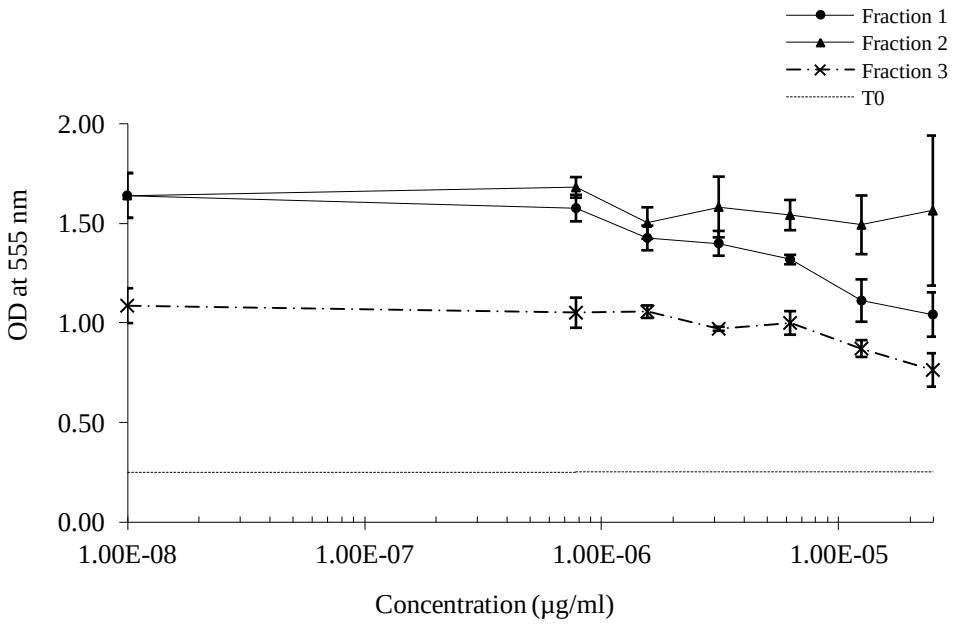
Appendix I-G: Dose-response curve of MDA-MB-468 cells after exposure to *Archidendron ellipticum* EtOH bark extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, > 50, > 50, 1.49, > 50 and > 50 µg/ml, respectively.

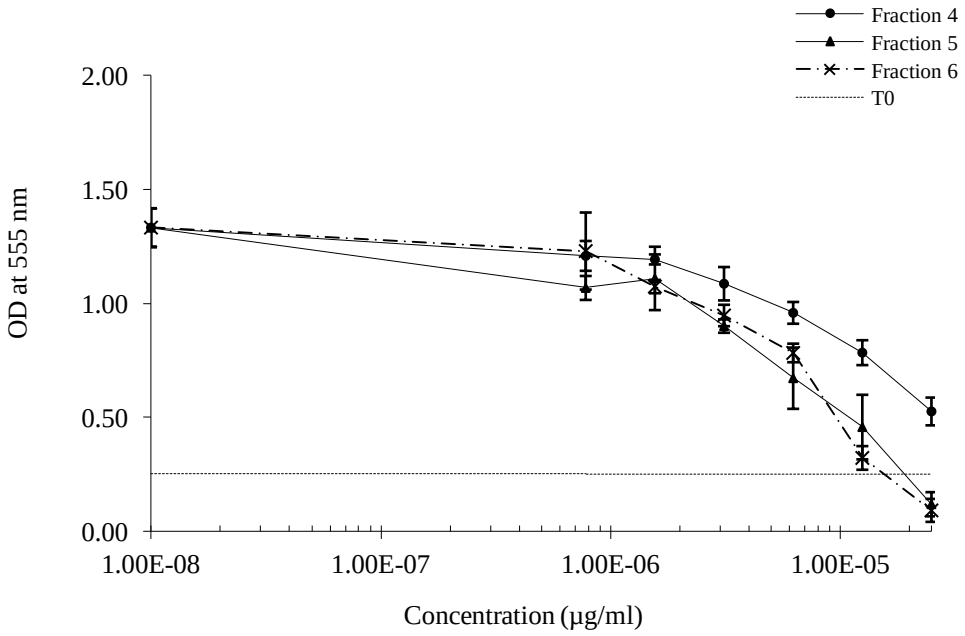


Appendix I-H: Dose-response curve of MDA-MB-468 cells after exposure to *Archidendron ellipticum* EtOAc bark extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, > 50, > 50, 19.74, 32.32 and > 50 µg/ml, respectively.

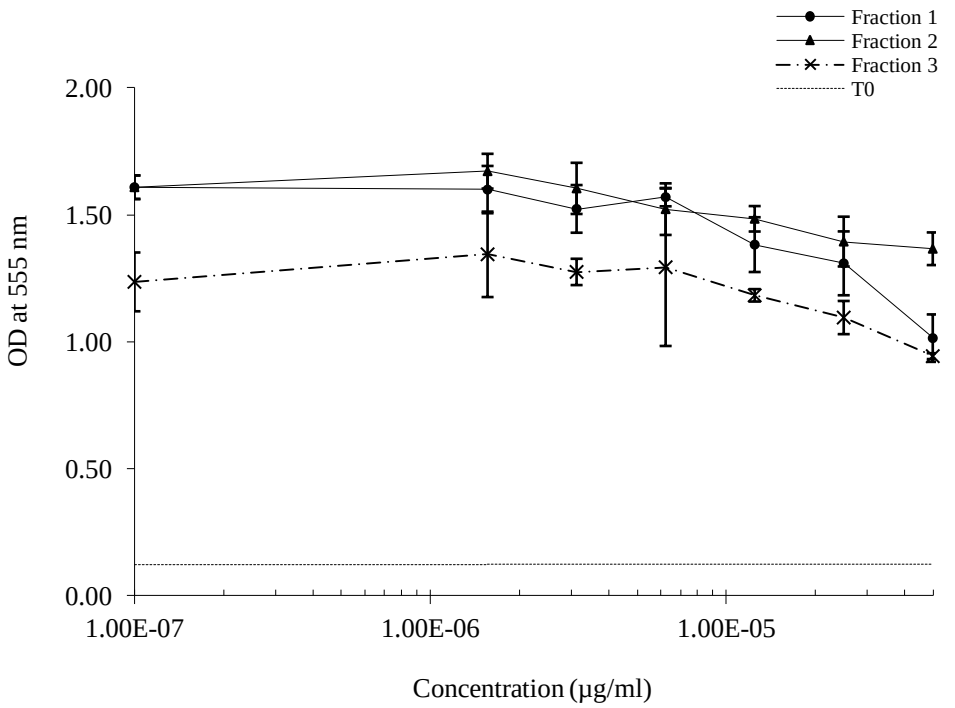


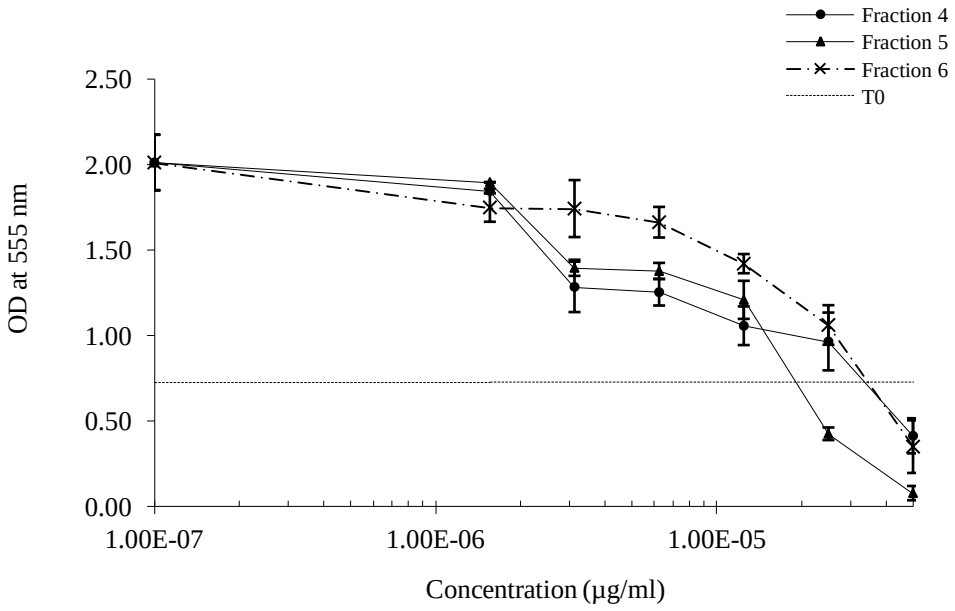
Appendix I-I: Dose-response curve of MDA-MB-468 cells after exposure to *Archidendron ellipticum* EtOH leaf extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, > 50, > 50, 2.20, 10.11 and > 50 µg/ml, respectively.



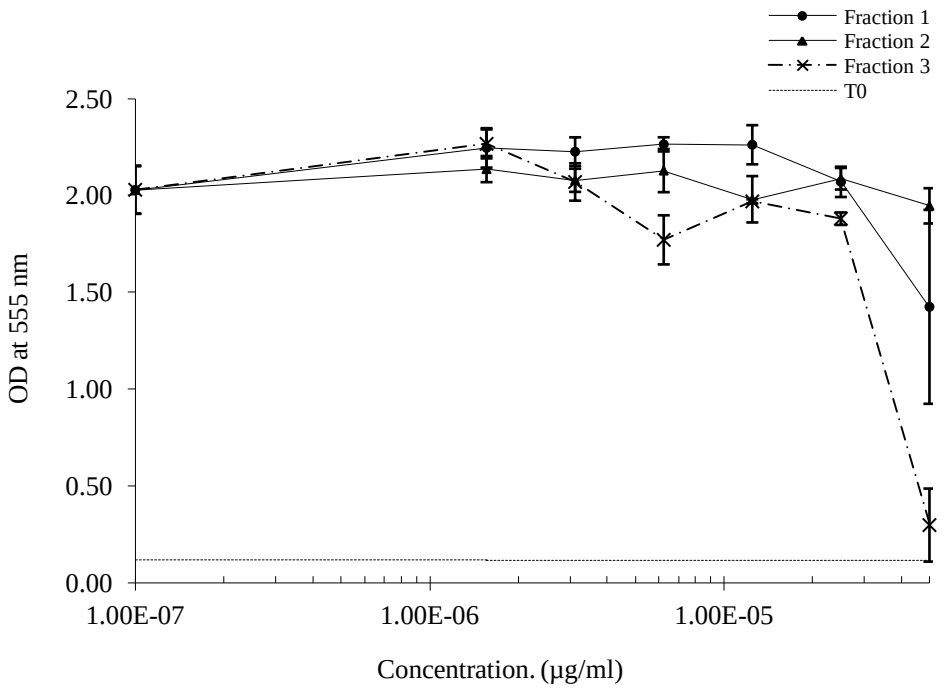


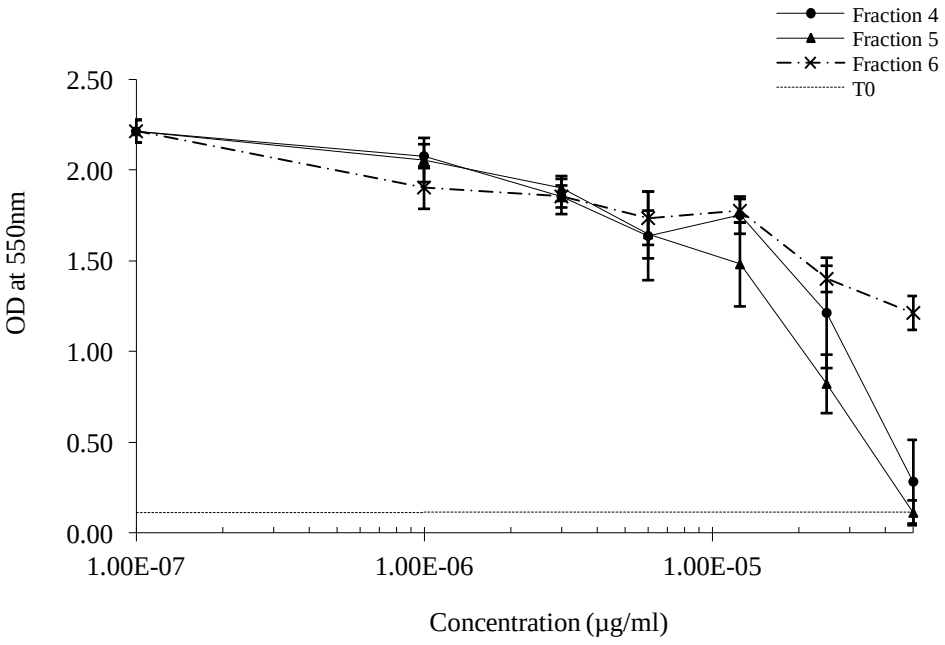
Appendix I-J: Dose-response curve of MDA-MB-468 cells after exposure to *Archidendron ellipticum* EtOAc leaf extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, > 50, > 50, 12.22, 4.61 and 6.08 µg/ml, respectively.



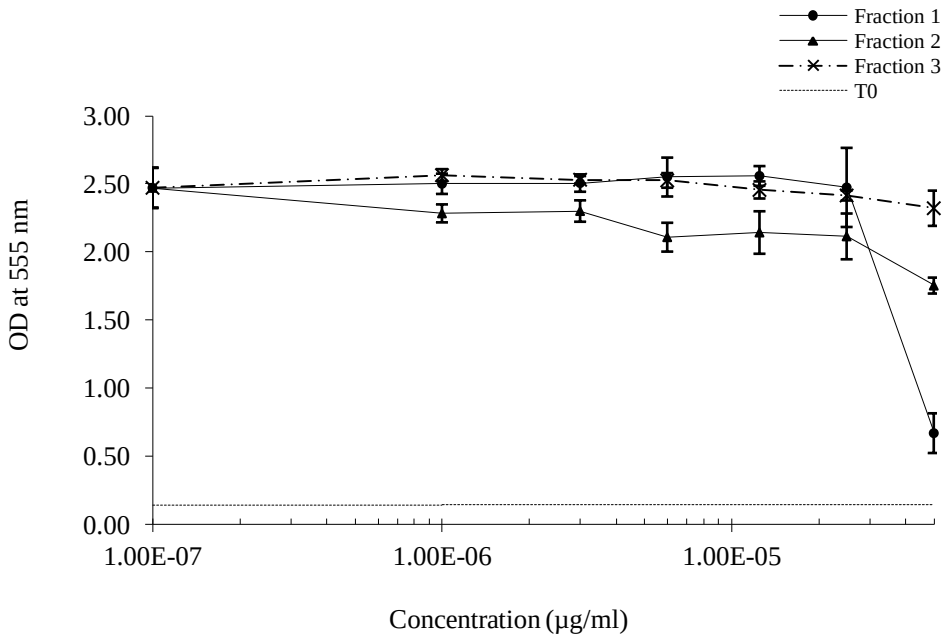


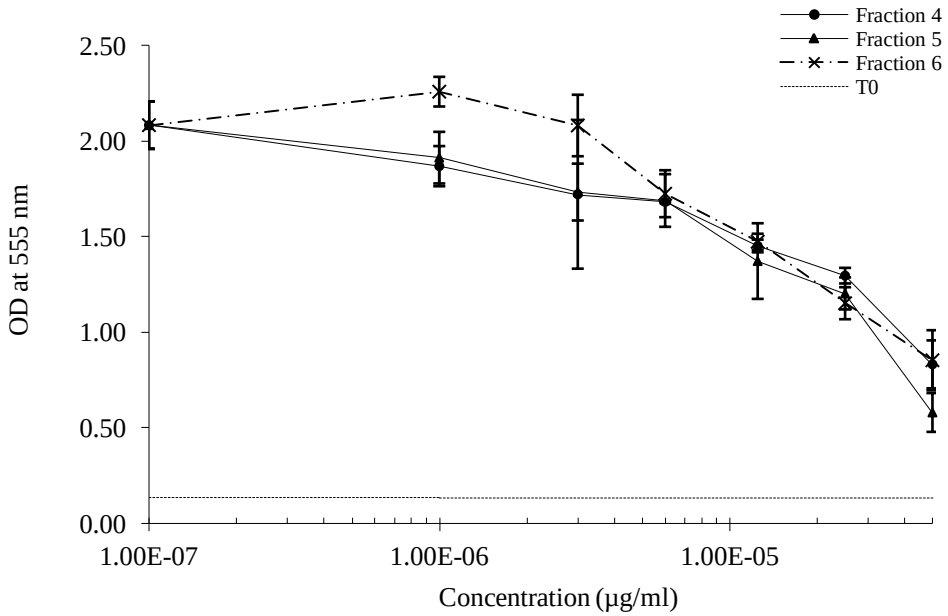
Appendix I-K: Dose-response curve of MDA-MB-468 cells after exposure to *Archidendron ellipticum* HEX leaf extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, > 50, > 50, 2.89, 6.50 and 14.23 µg/ml, respectively.



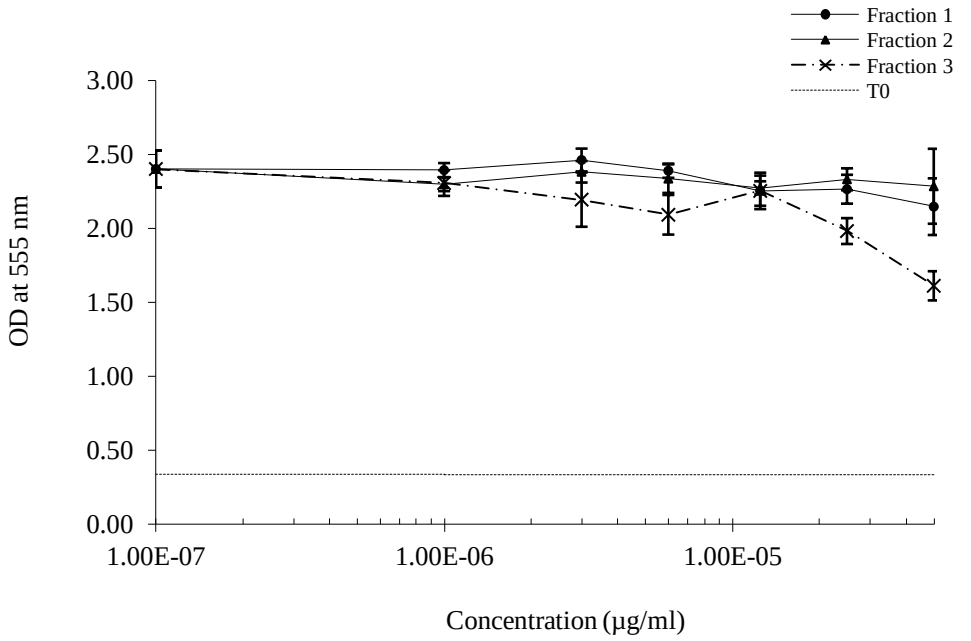


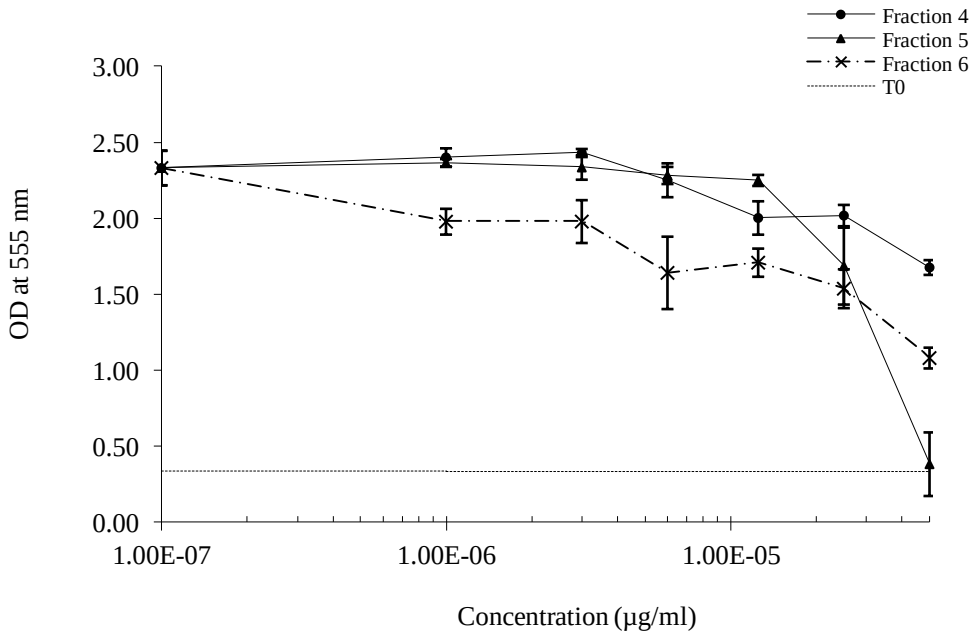
Appendix I-L: Dose-response curve of HCT116 cells after exposure to *Duabanga grandiflora* EtOH bark extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, > 50, 37.75, 26.31, 18.50 and > 50 µg/ml, respectively.



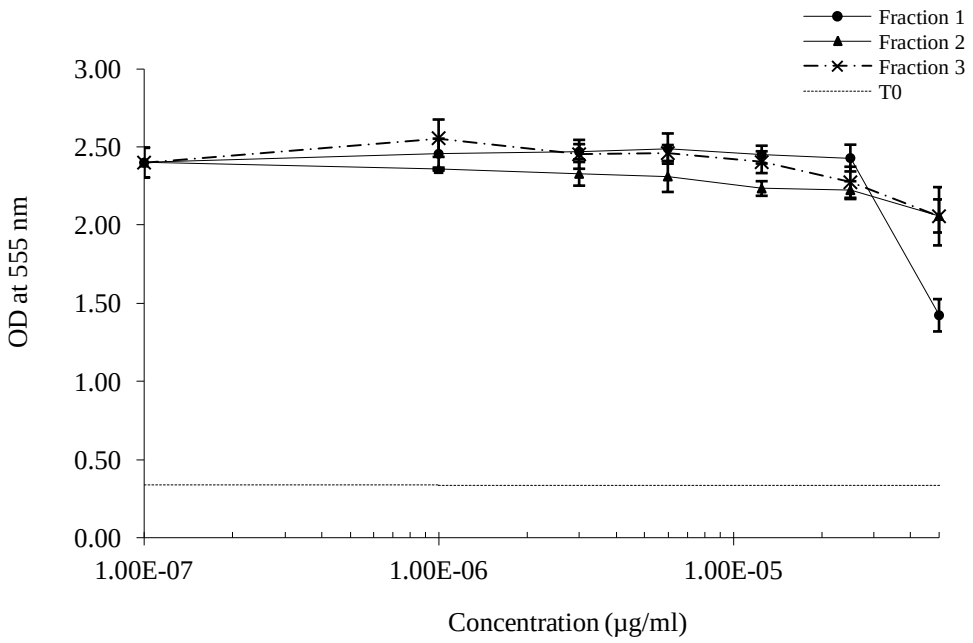


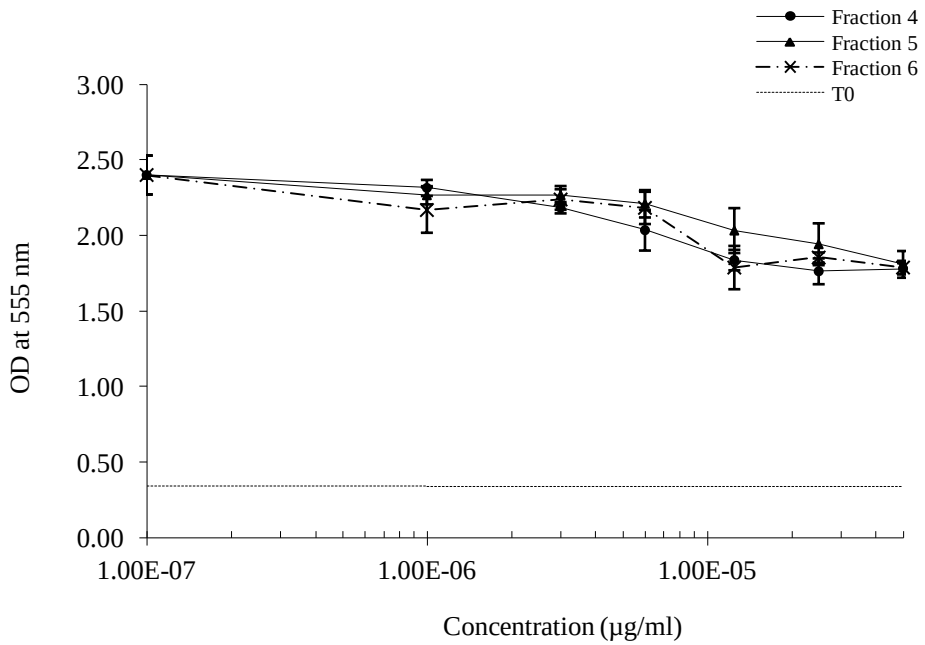
Appendix I-M: Dose-response curve of HCT116 cells after exposure to *Duabanga grandiflora* EtOAc bark extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: 41.17, > 50, > 50, 35.08, 28.78 and 28.60 µg/ml, respectively.



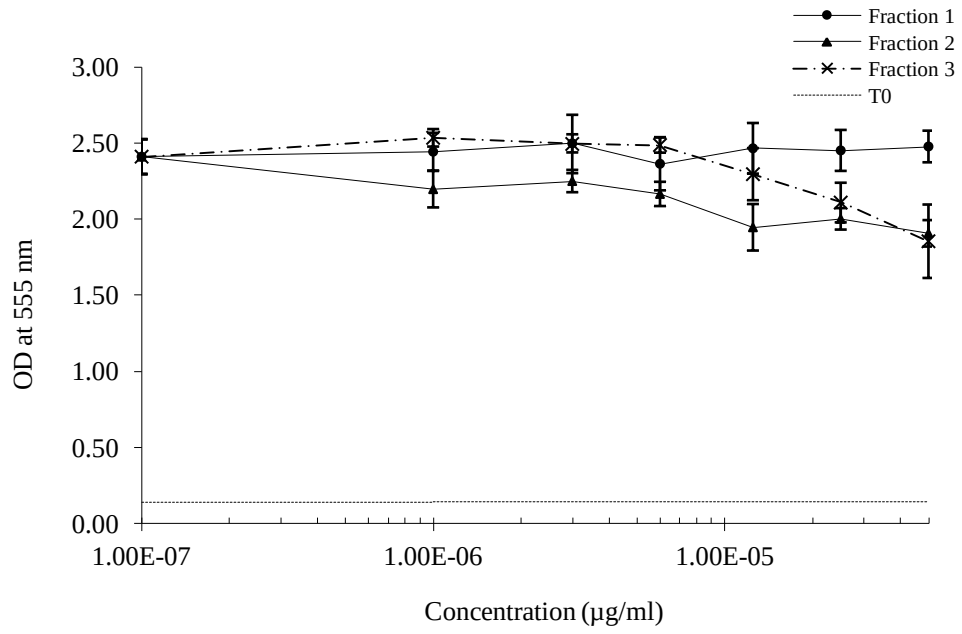


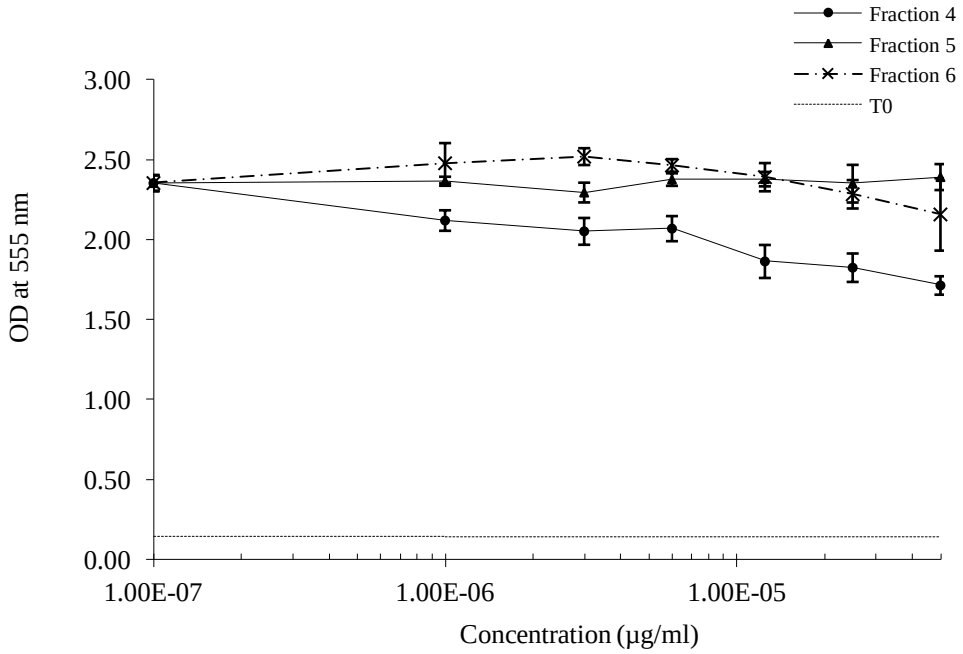
Appendix I-N: Dose-response curve of HCT116 cells after exposure to *Duabanga grandiflora* HEX bark extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, >50, >50, > 50, 31.77 and 36.18µg/ml, respectively.



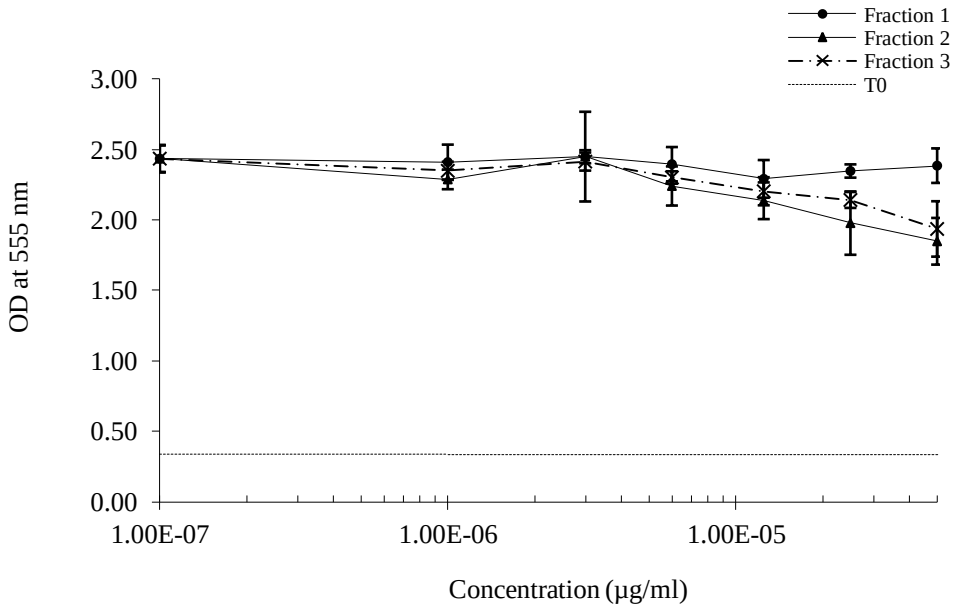


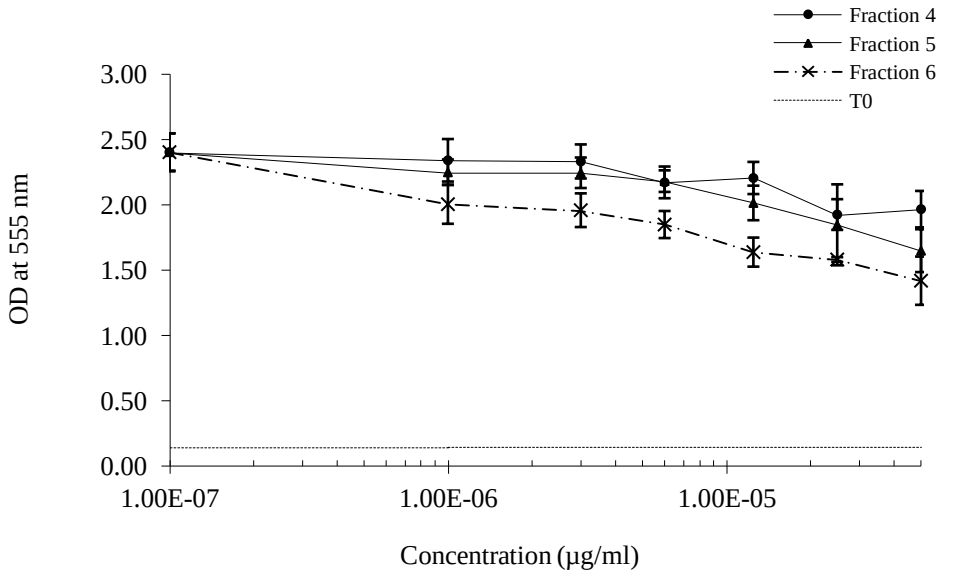
Appendix I-O: Dose-response curve of HCT116 cells after exposure to *Duabanga grandiflora* EtOH leaf extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, >50, >50, > 50, >50 and > 50 µg/ml, respectively.



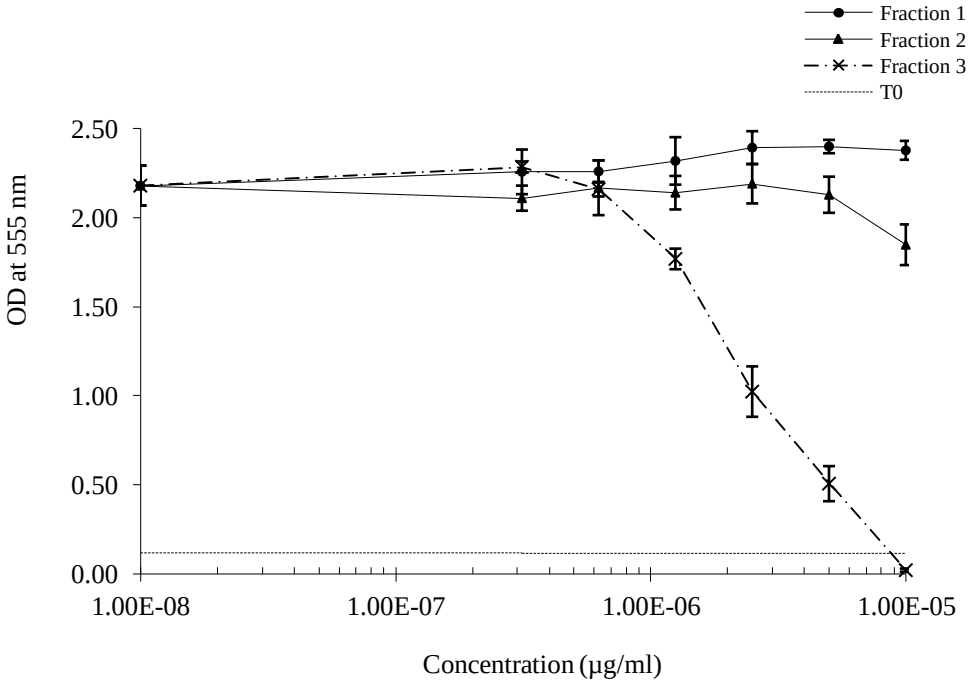


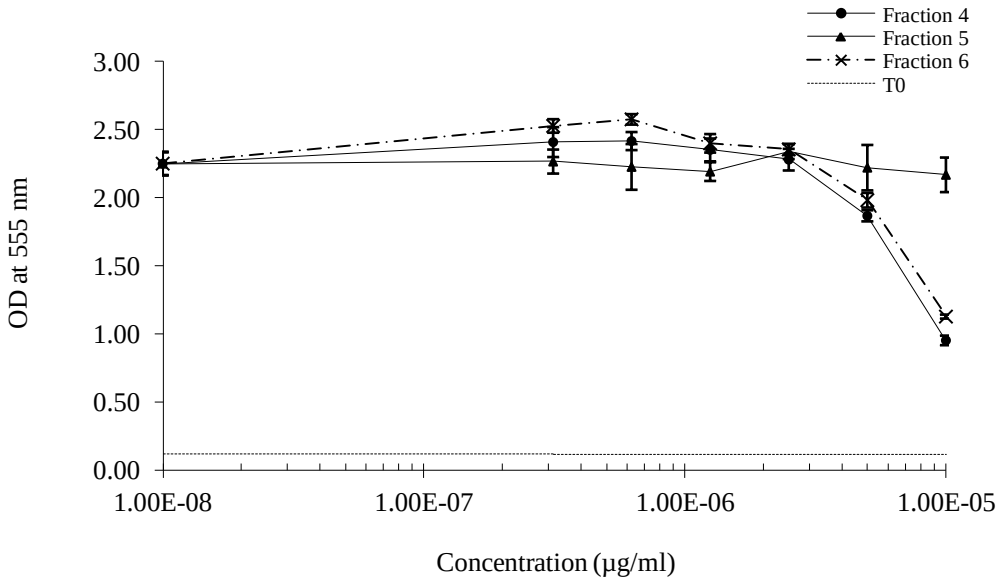
Appendix I-P: Dose-response curve of HCT116 cells after exposure to *Duabanga grandiflora* EtOAc leaf extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, >50, >50, > 50, >50 and > 50 µg/ml, respectively.



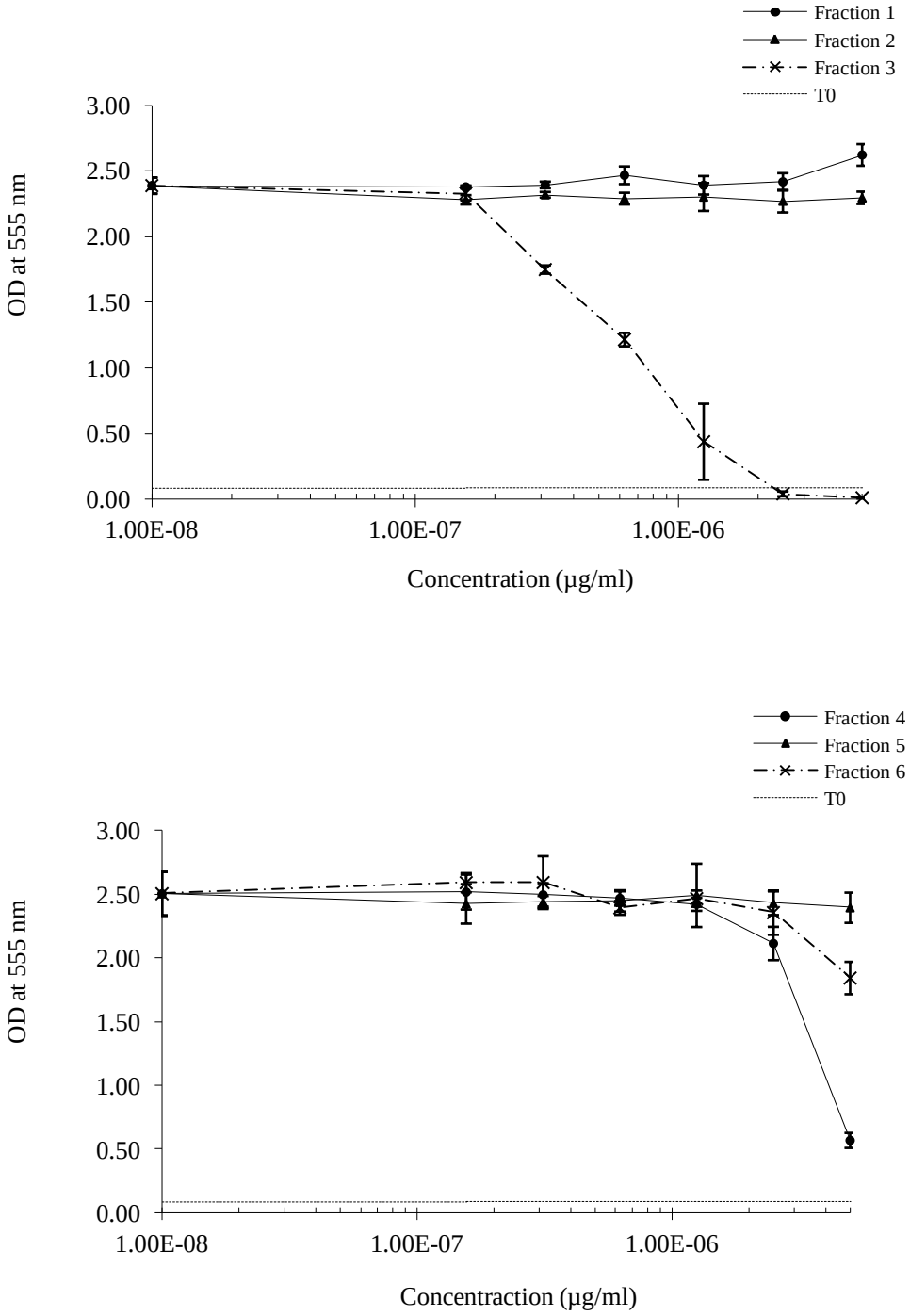


Appendix I-P: Dose-response curve of HCT116 cells after exposure to *Duabanga grandiflora* HEX leaf extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 50, >50, >50, > 50, >50 and > 50 µg/ml, respectively.





Appendix I-P: Dose-response curve of HCT116 cells after exposure to *Pseuduvaria macrophylla* EtOH extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 10, >10, 2.29, 8.73, > 10 and 9.67µg/ml, respectively.



Appendix I-Q: Dose-response curve of HCT116 cells after exposure to *Pseuduvaria macrophylla* EtOAc extract fractions for 72 hour. The optical readings (n= 4) ± SE obtained from a single representative experiment are shown. The GI_{50} value (µg/ml) of F1, F2, F3, F4, F6 and F6 extracts are: > 10, >10, 0.61, 3.83, >10 and > 10 µg/ml, respectively.

Appendix II

Fractionation of extracts were performed using a Strata C18-E extraction cartridge equilibrated with equal volume of d. H_20 and MeOH prior to loading the cartridge with UNMC extract. The solvents were passed through the extract and collected in a pre-weighed glass vial followed by evaporation of solvent to obtain the extract fraction. The yield was determined by the difference in weight of glass vial before fraction collection and after solvent evaporation then subjected to HPLC analysis as described in Chapter 7.5.

Appendix II-A: The yield (%) of *Duabanga grandiflora* extract fractions.

	***D. grandiflora* extracts**	**% Yield of *D. grandiflora* extract fractions**						
		F1	F2	F3	F4	F5	F6	Total % (mg)
Bark	Water	-	-	-	-	-	-	-
	EtOH	13.0	15.5	19.3	3.4	0.5	1.9	53.6 (11.1)
	EtOAc	20.3	2.1	5.7	6.0	14.9	2.9	51.8 (19.1)
	HEX	2.3	0.9	3.5	6.0	23.4	13.9	50.2 (14.5)
Leaf	Water	-	-	-	-	-	-	-
	EtOH	71.6	4.6	4.4	1.2	1.7	1.4	84.8 (23.5)
	EtOAc	25.6	3.8	2.4	1.2	24.4	2.3	59.6 (16.8)
	HEX	21.8	0.5	6.2	1.1	1.2	6.4	47.3 (3.12)

Strata C18-E extraction cartridge was equilibrated with equal volume of d. H_20 and MeOH prior to loading the cartridge with *D. grandiflora* extracts. The solvents were passed through the extract and collected then removed to obtain the extract fraction for analysis. The weigh (mg) used to determine % yield of extracts were of the glass vial prior to collection and after solvent evaporation.

Appendix II-B: The yield (%) of *Archidendron ellipticum* extract fractions.

A. ellipticum extracts		% Yield of *A. ellipticum* extract fractions						
		F1	F2	F3	F4	F5	F6	Total % (mg)
Bark	EtOH	39.1	4.4	7.8	14.9	2.6	3.2	24.6 (72.0)
	EtOAc	10.5	1.5	8.6	3.9	10.0	15.3	9.9 (49.8)
	HEX	-	-	-	-	-	-	-
Leaf	EtOH	10.4	3.3	3.0	6.5	2.7	1.2	21.7 (27.2)
	EtOAc	0.0	0.3	2.0	2.8	7.8	6.2	10.6 (19.0)
	HEX	1.0	0.0	3.1	2.1	8.7	1.2	10.72 (16.2)

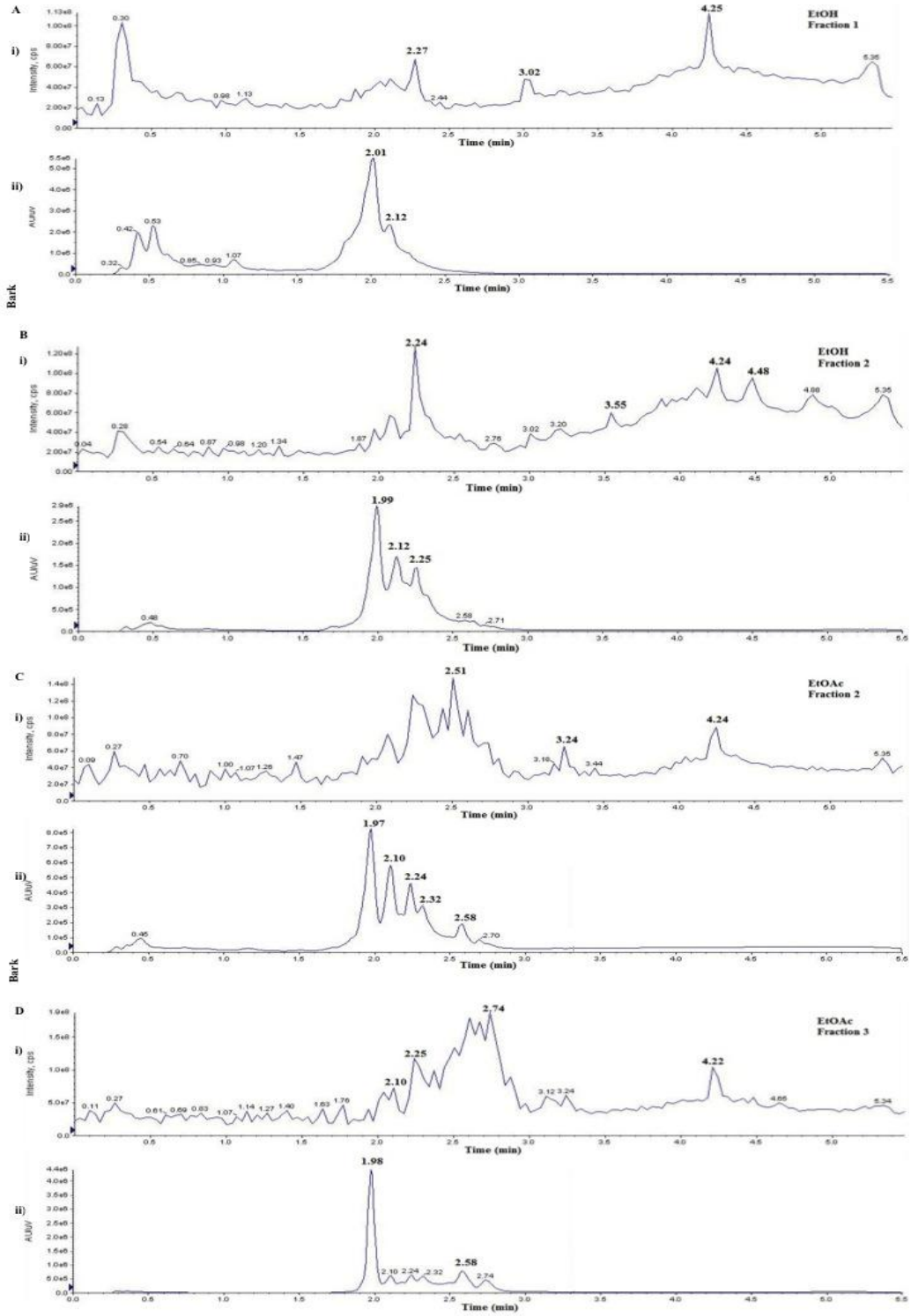
Strata C18-E extraction cartridge was equilibrated with equal volume of d. H_20 and MeOH prior to loading the cartridge with *A. ellipticum* extracts. The solvents were passed through the extract and collected then removed to obtain the extract fraction for analysis. The weigh (mg) used to determine % yield of extracts were of the glass vial prior to collection and after solvent evaporation.

Appendix II-C: The yield (%) of *Pseuduvaria macrophylla* extract fractions.

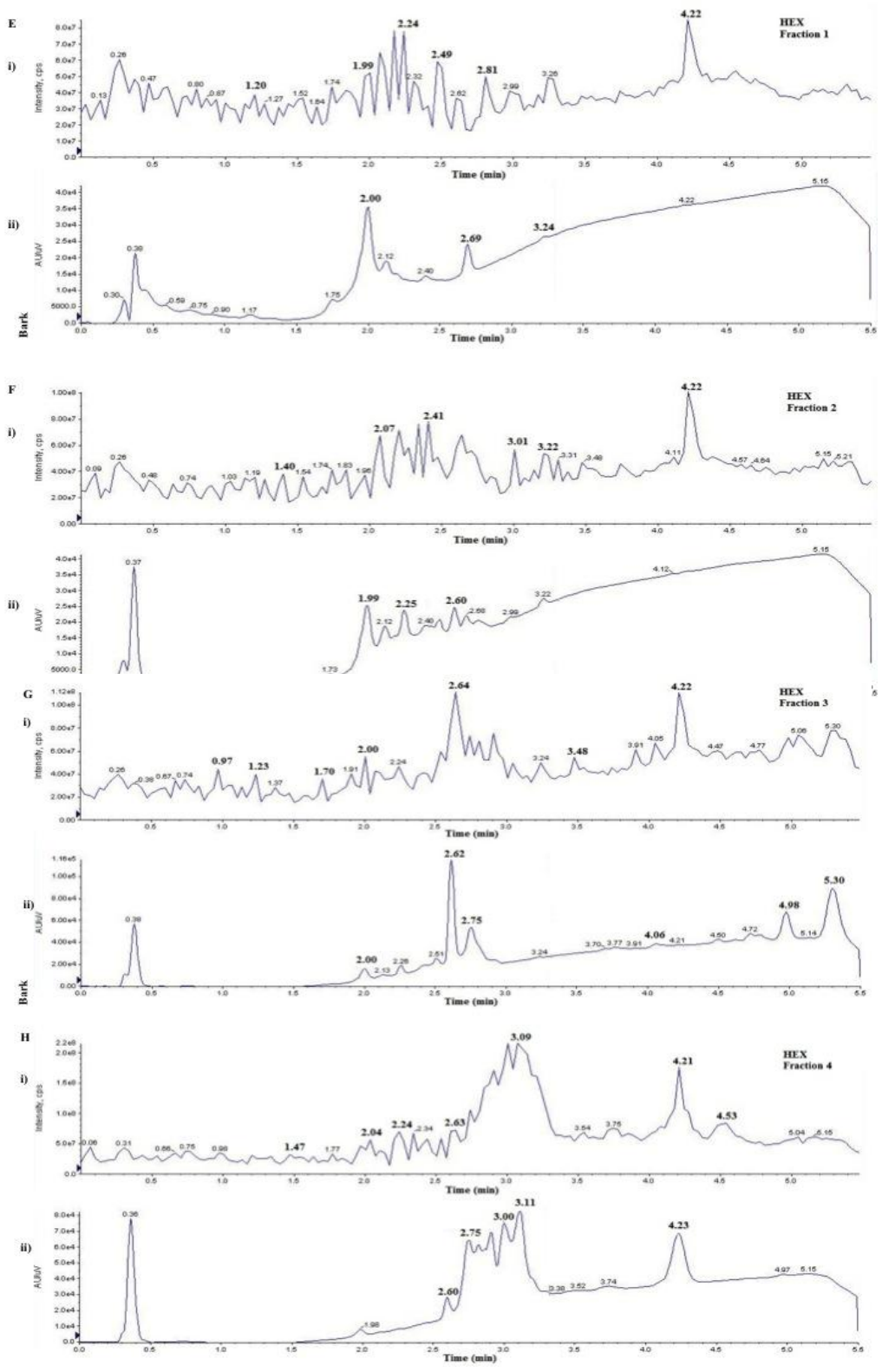
P. macrophylla extracts		% Yield of *P. macrophylla* extract fractions						
		F1	F2	F3	F4	F5	F6	Total % (mg)
Whole	EtOH	2.8	1.1	9.1	3.8	1.7	0.4	18.82 (76.5)
	EtOAc	0.2	1.3	8.5	6.5	6.8	0.6	23.8 (27.9)
	HEX	-	-	-	-	-	-	-

Strata C18-E extraction cartridge was equilibrated with equal volume of d. H_20 and MeOH prior to loading the cartridge with *P. macrophylla* extracts. The solvents were passed through the extract and collected then removed to obtain the extract fraction for analysis. The weigh (mg) used to determine % yield of extracts were of the glass vial prior to collection and after solvent evaporation.

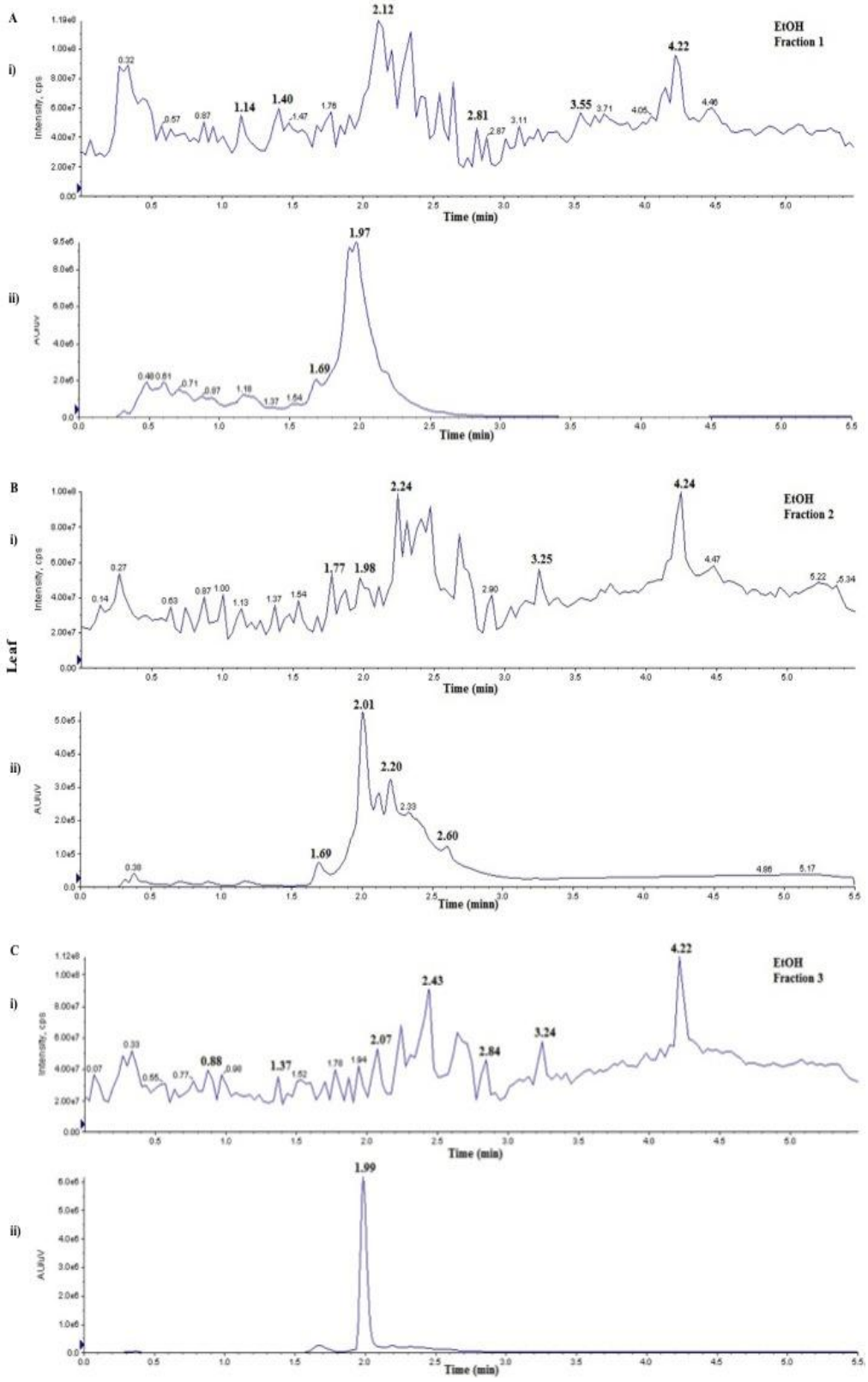
Appendix III



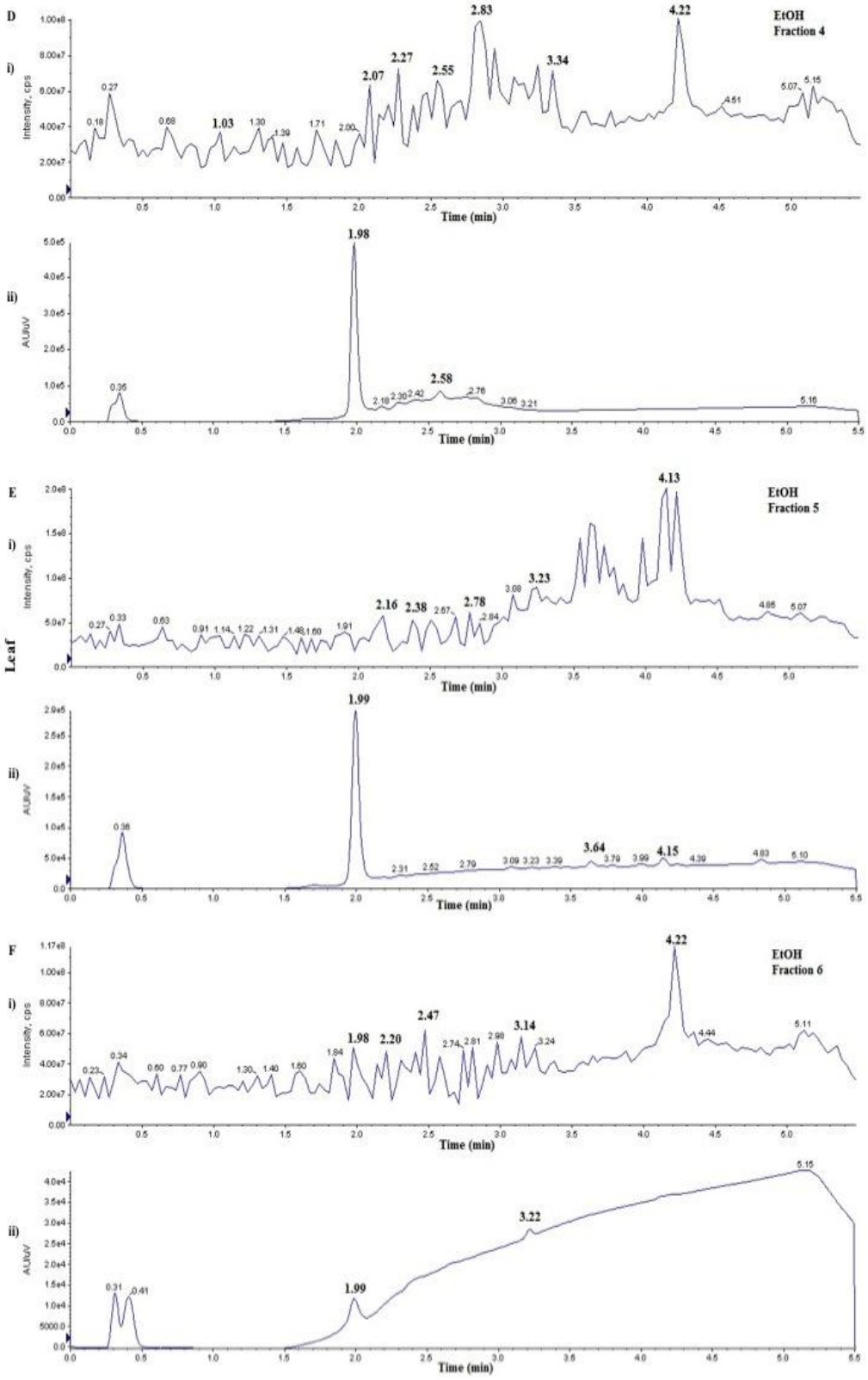
Appendix III-A: HPLC trace of *Duabanga grandiflora* bark extract 'inactive' fractions using Reversed Phase chromatography. EtOH: F1 (A), and F2 (B) and EtOAc F2 (C) and F3 (D) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



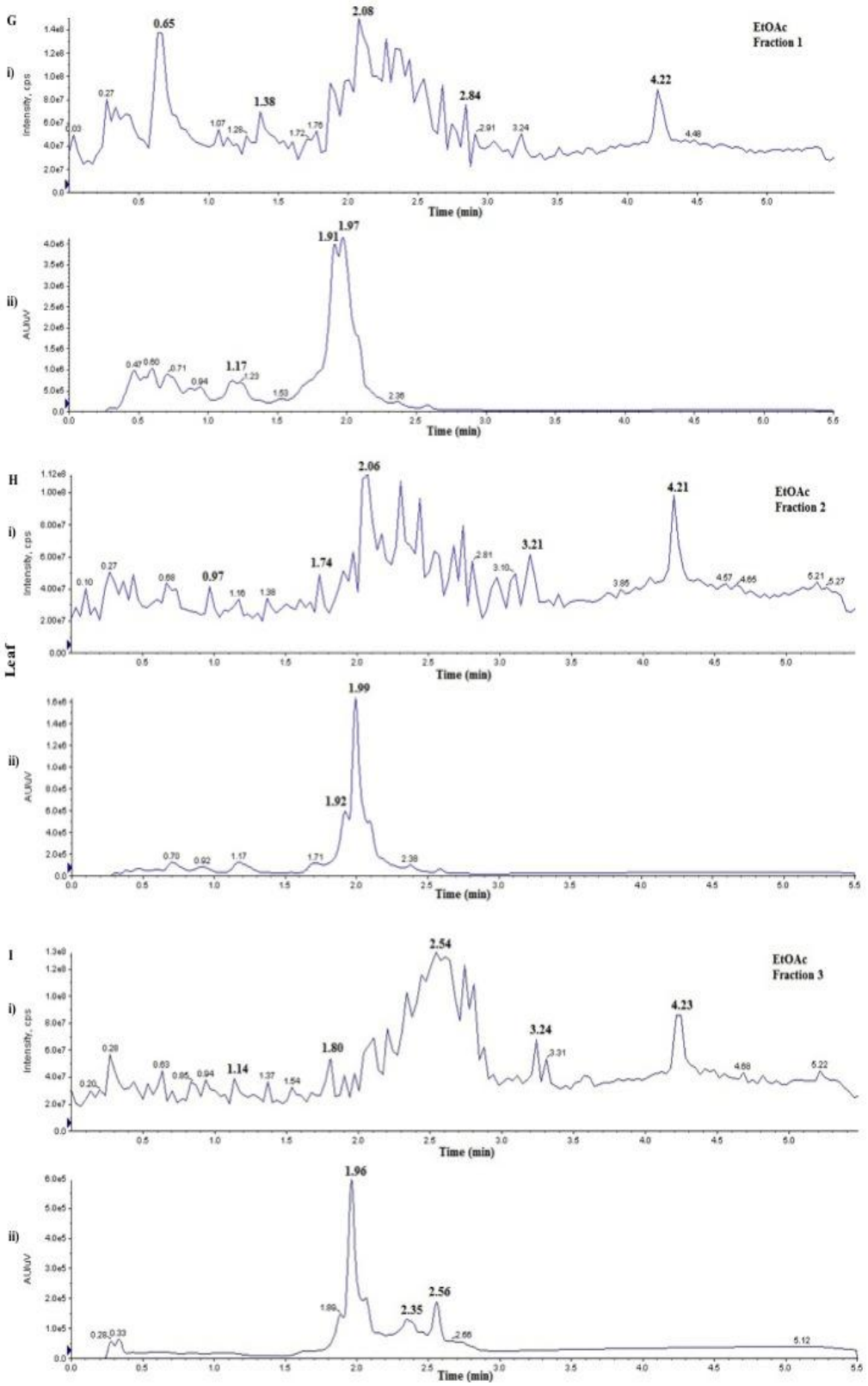
Appendix III-B: HPLC trace of *Duabanga grandiflora* bark extract 'inactive' fractions using Reversed Phase chromatography. HEX: F1 (E), F2 (F), F3 (G) and F4 (H) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



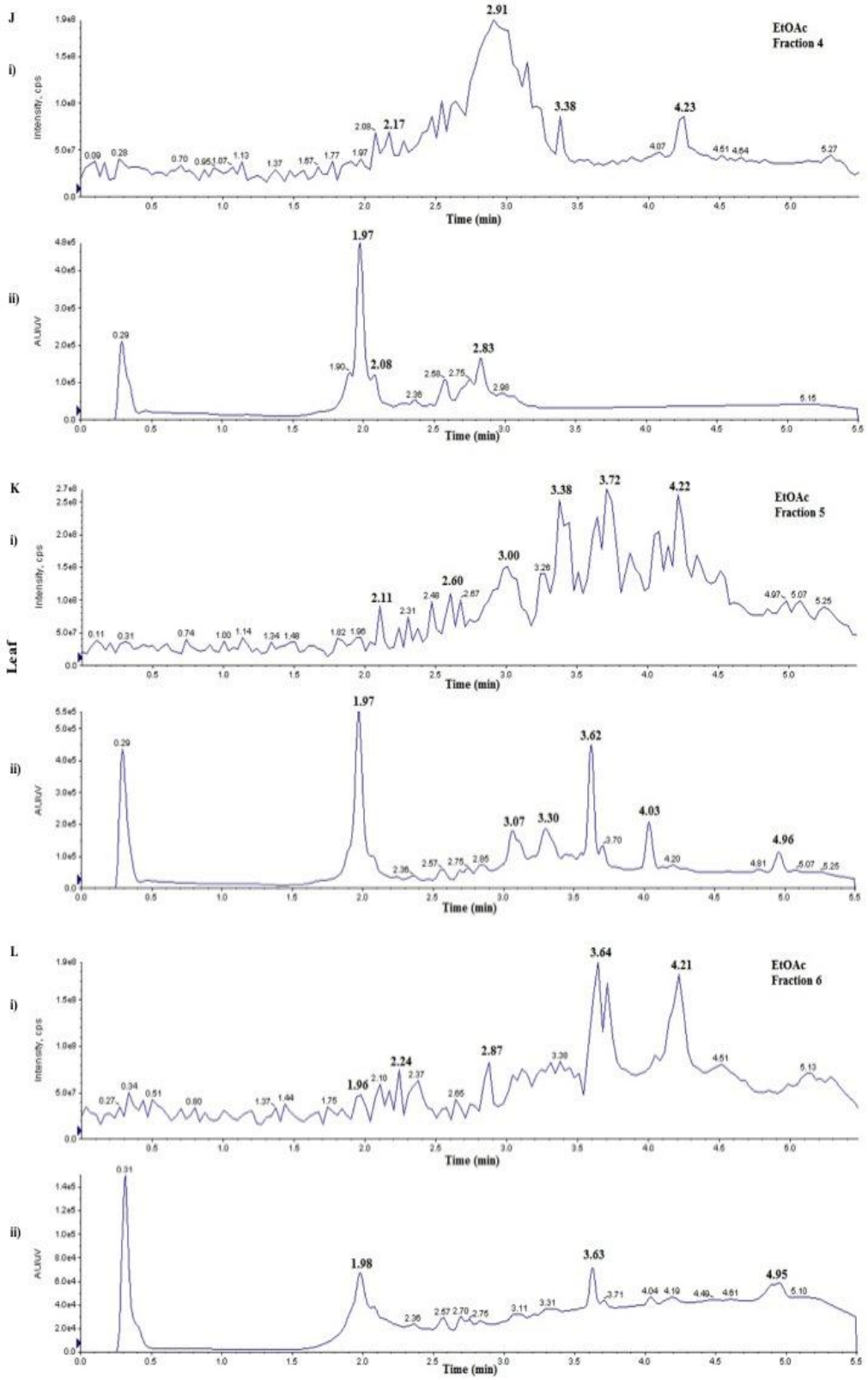
Appendix III-C: HPLC trace of *Duabanga grandiflora* leaf extract 'inactive' fractions using Reversed Phase chromatography. EtOH: F1 (A), F2 (B) and F3 (C) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



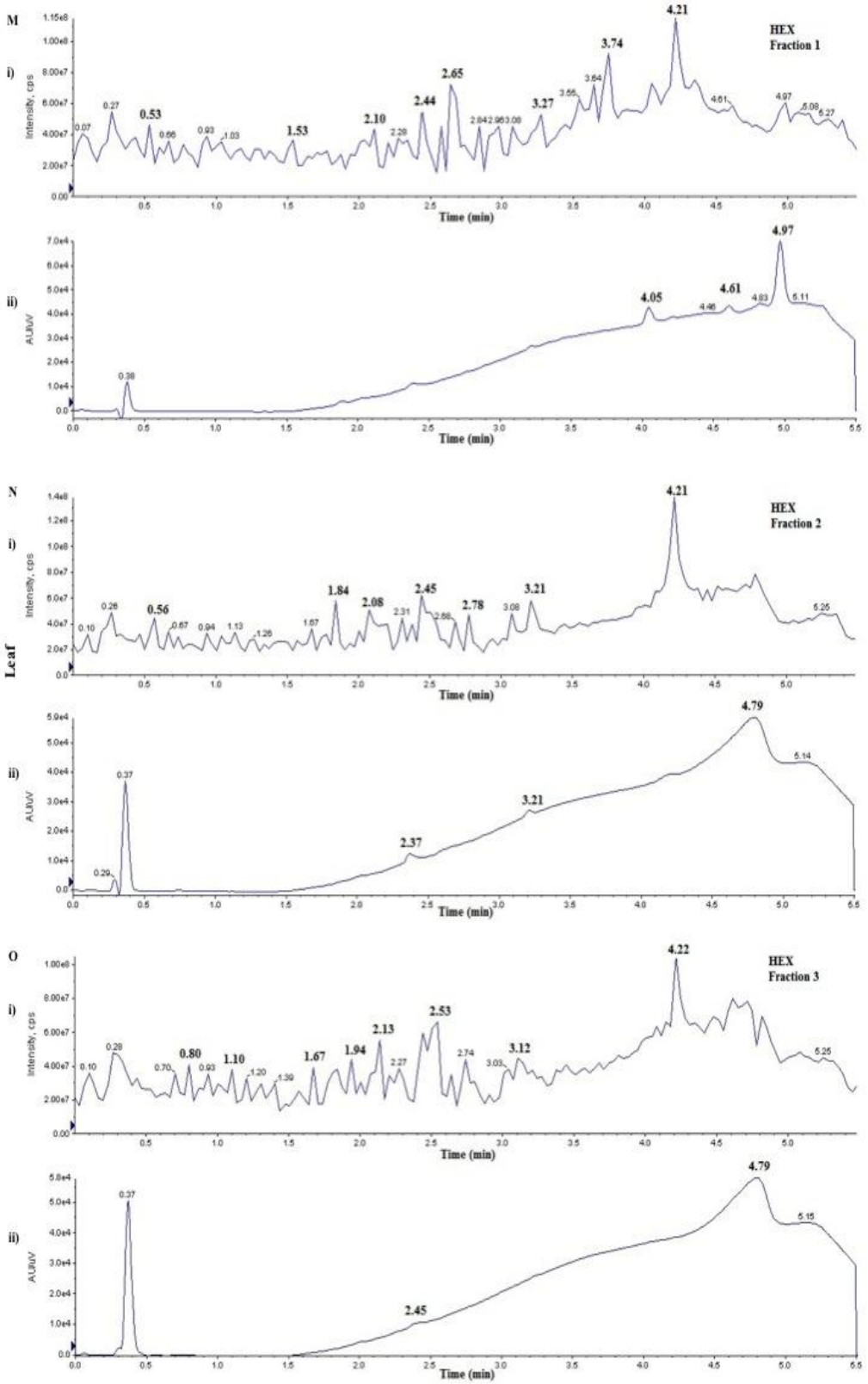
Appendix III-D: HPLC trace of *Duabanga grandiflora* leaf extract 'inactive' fractions using Reversed Phase chromatography. EtOH: F4 (D), F5 (E) and F6 (F) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



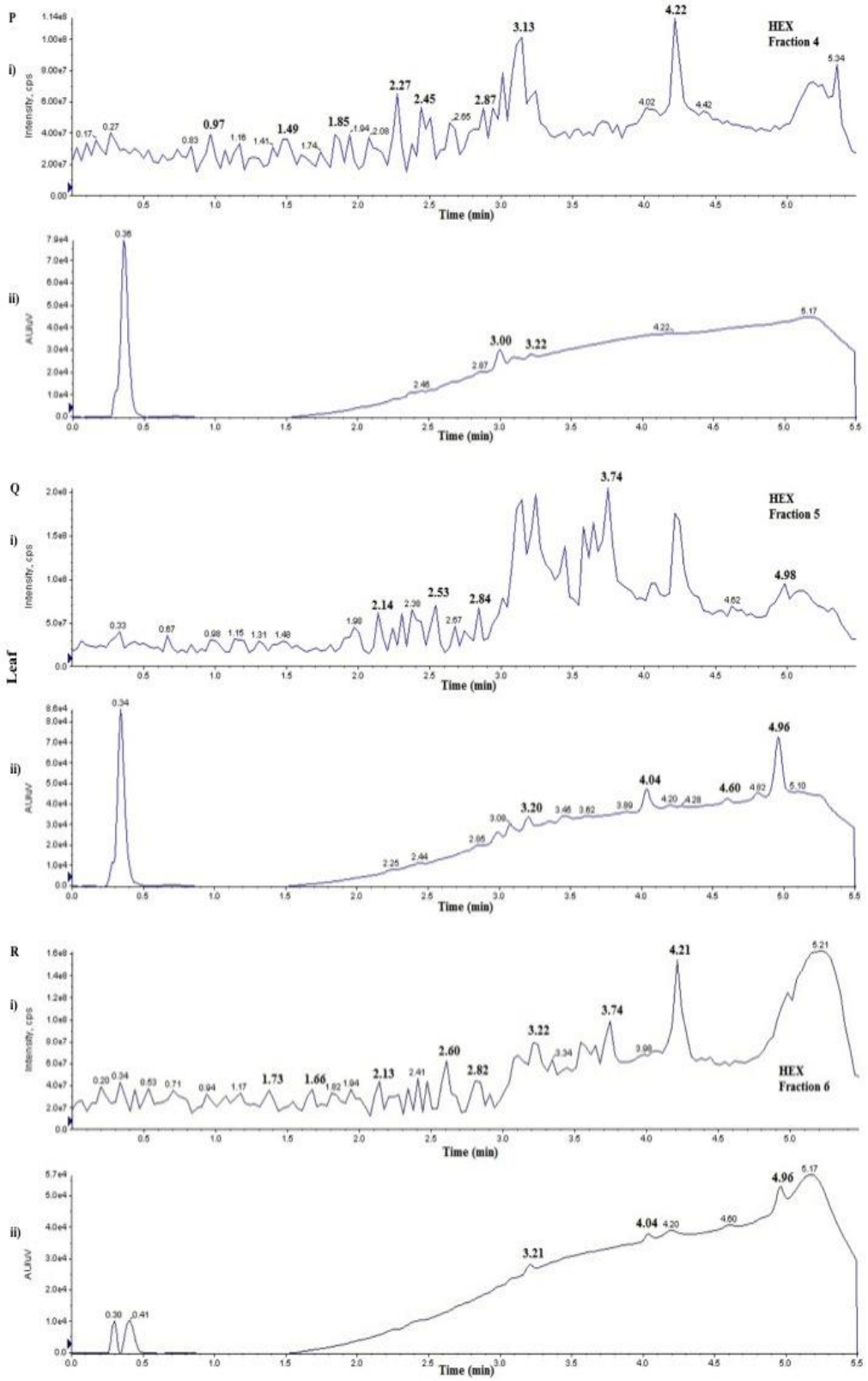
Appendix III-E: HPLC trace of *Duabanga grandiflora* leaf extract 'inactive' fractions using Reversed Phase chromatography. EtOAc: F1 (G), F2 (H) and F3 (I) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



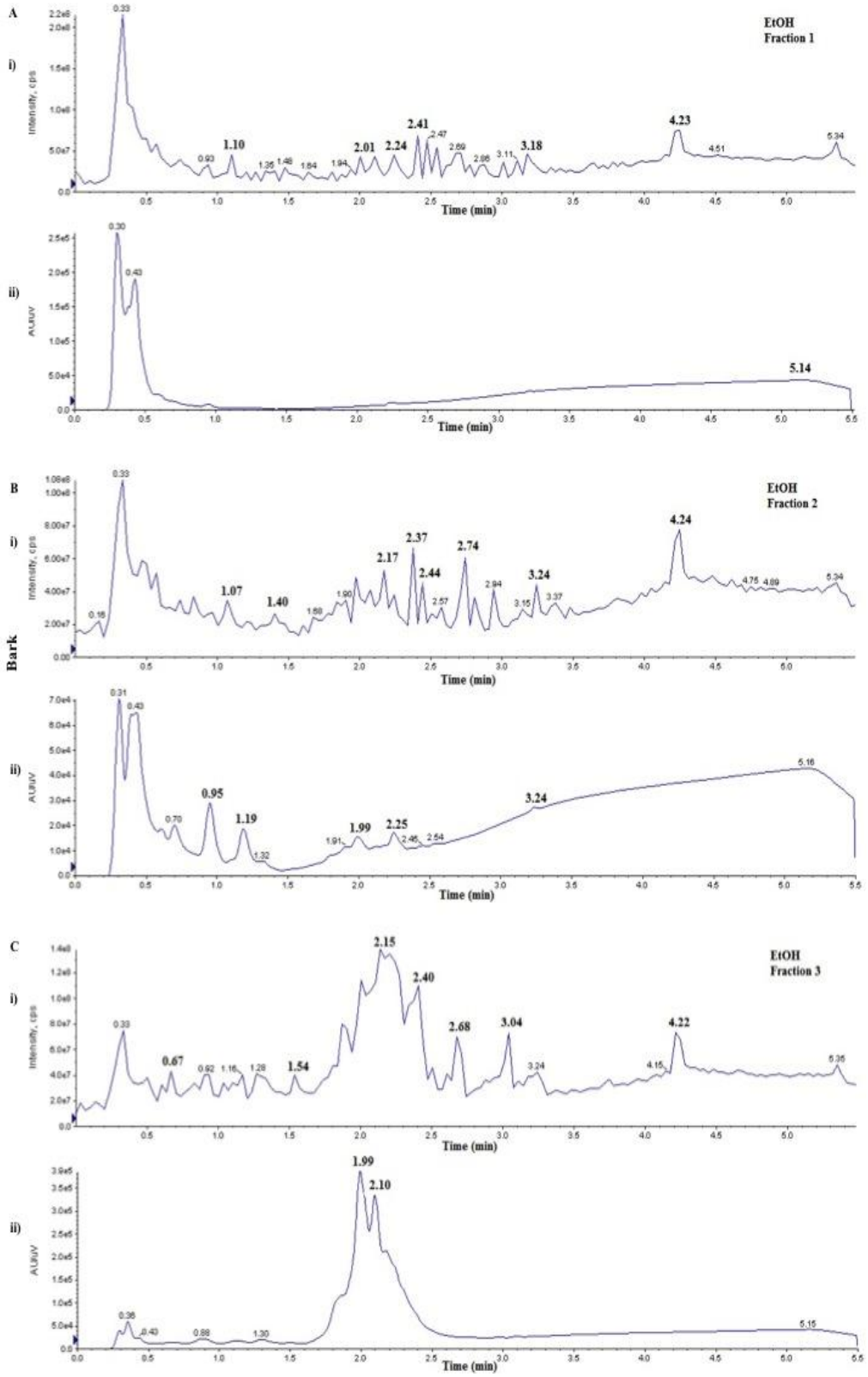
Appendix III-F: HPLC trace of *Duabanga grandiflora* leaf extract 'inactive' fractions using Reversed Phase chromatography. EtOAc: F4 (J), F5 (K) and F6 (L) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



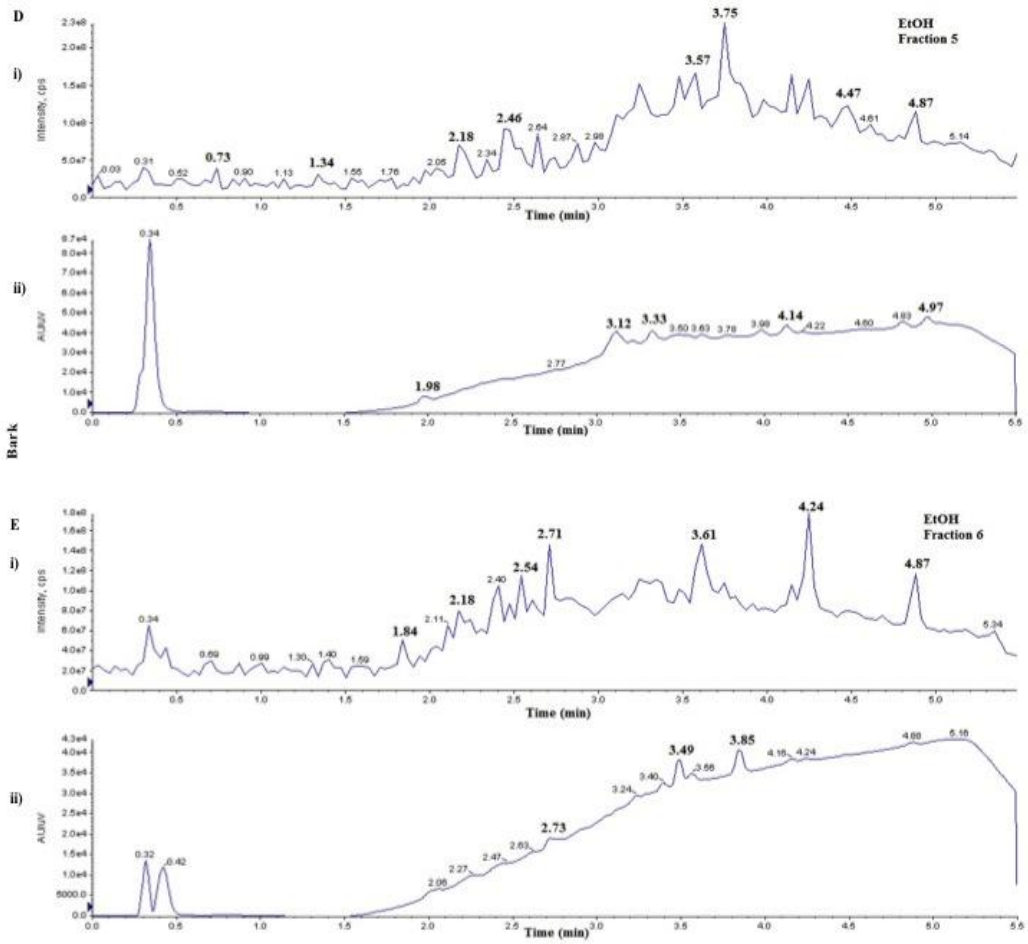
Appendix III-G: HPLC trace of *Duabanga grandiflora* leaf extract 'inactive' fractions using Reversed Phase chromatography. HEX: F1 (M), F2 (N) and F3 (O) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



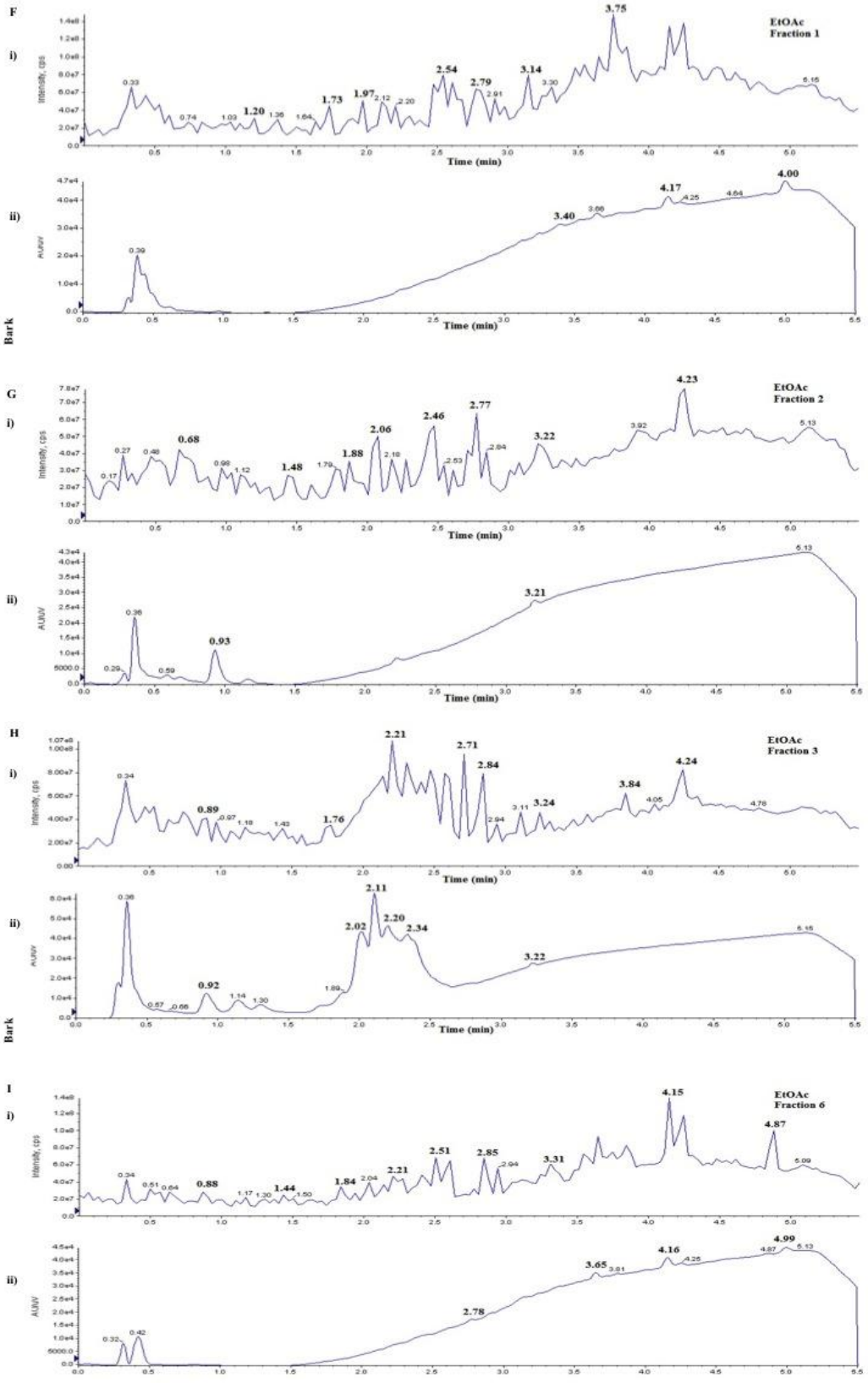
Appendix III-H: HPLC trace of *Duabanga grandiflora* leaf extract 'inactive' fractions using Reversed Phase chromatography. HEX: F4 (P), F5 (Q) and F6 (R) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



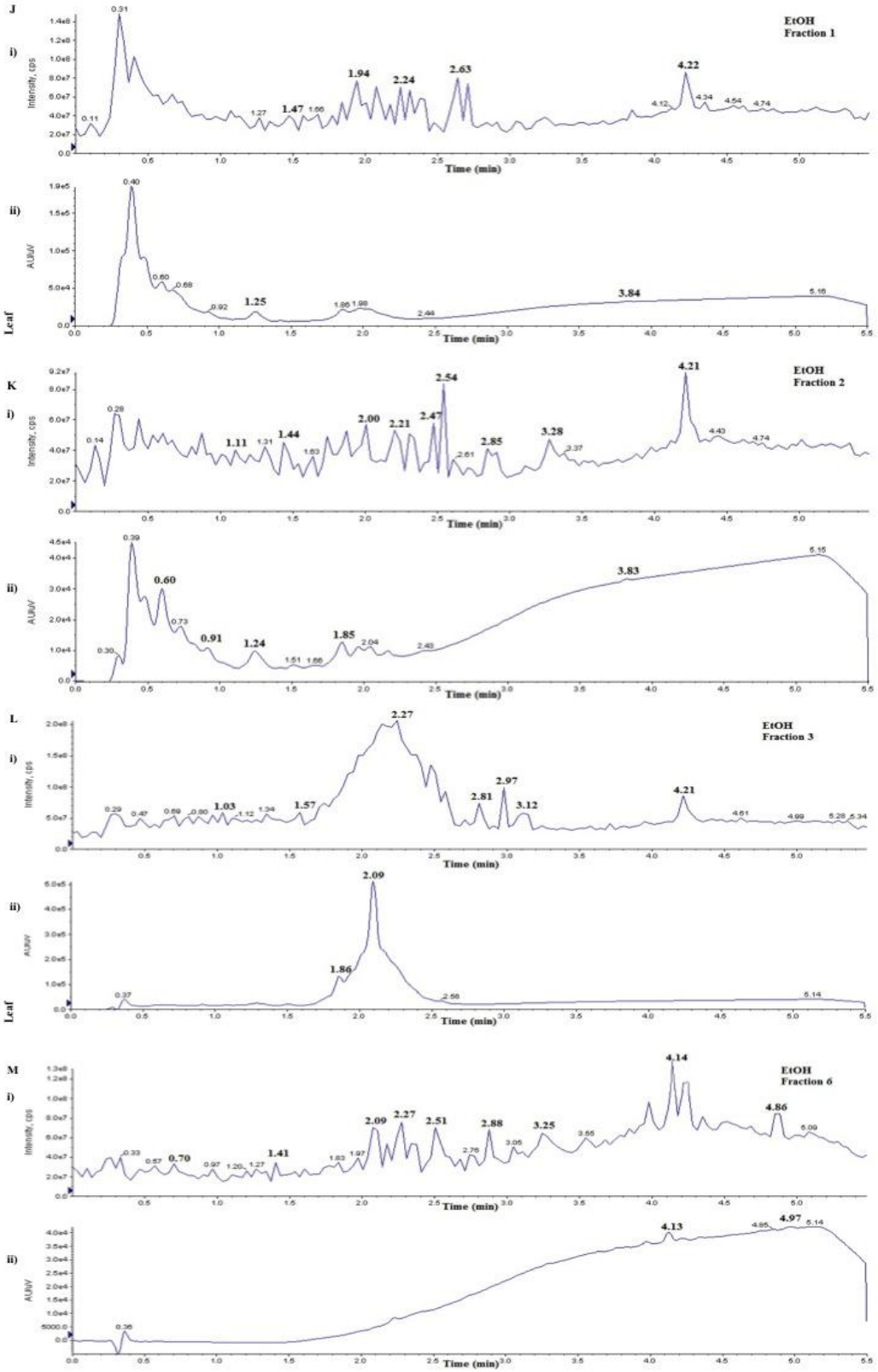
Appendix III-I: HPLC trace of *Archidendron ellipticum* bark extract 'inactive' fractions using Reversed Phase chromatography. EtOH: F1 (A), F2 (B) and F3 (C) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



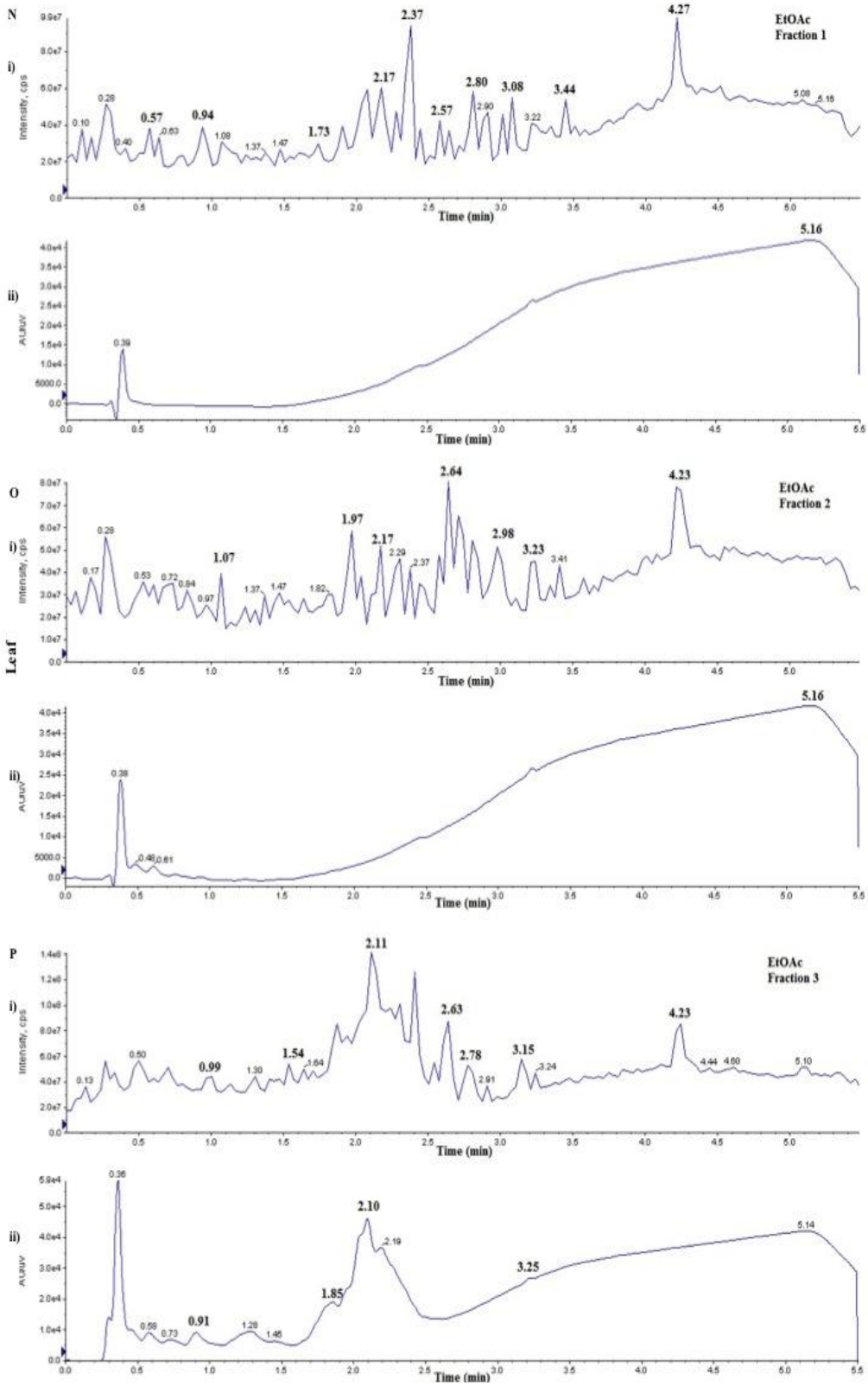
Appendix III-J: HPLC trace of *Archidendron ellipticum* bark extract 'inactive' fractions using Reversed Phase chromatography. EtOH: F5 (D) and F6 (E) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



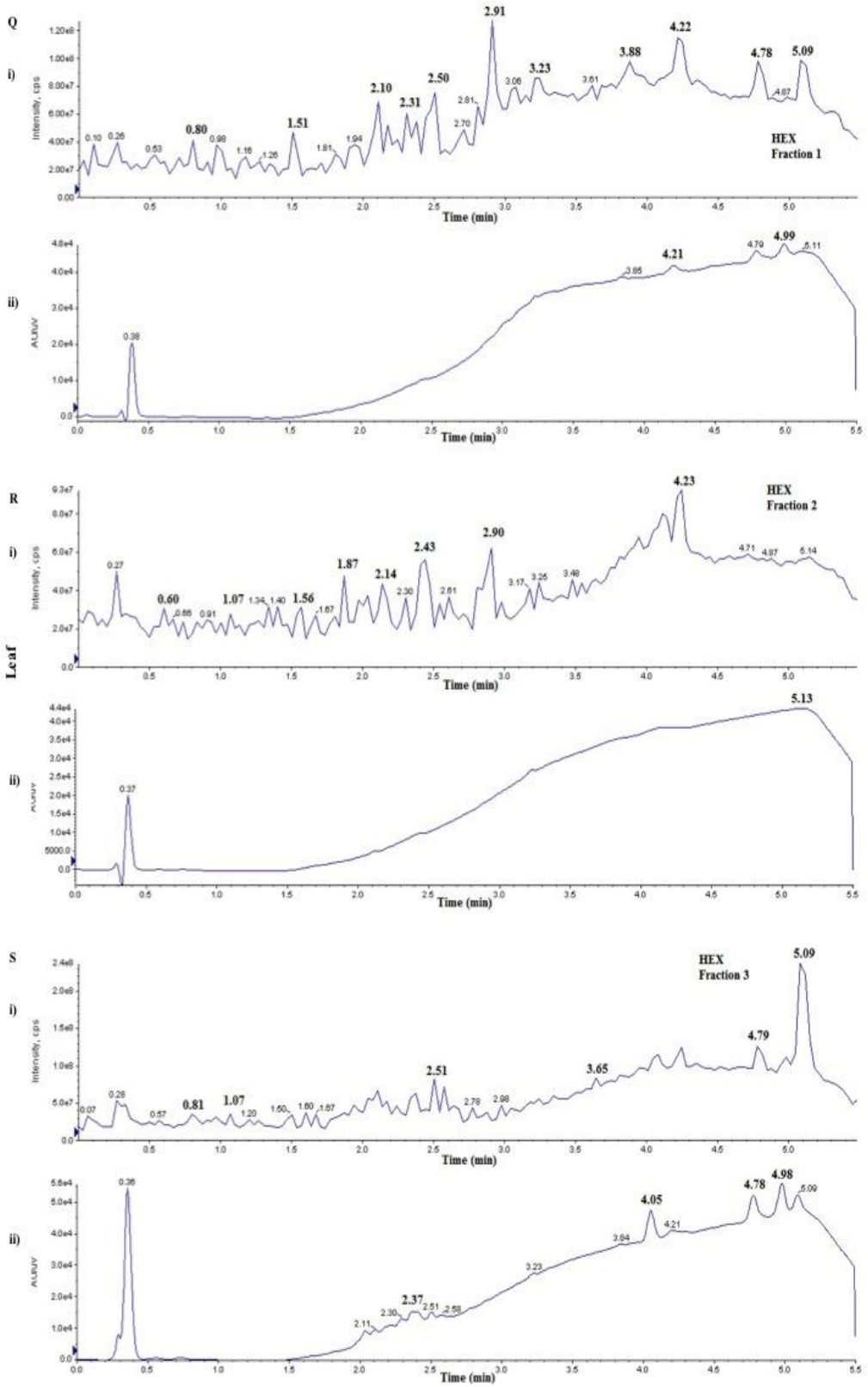
Appendix III-K: HPLC trace of *Archidendron ellipticum* bark extract 'inactive' fractions using Reversed Phase chromatography. EtOAc: F1 (F), F2 (G), F3 (H) and F6 (I) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



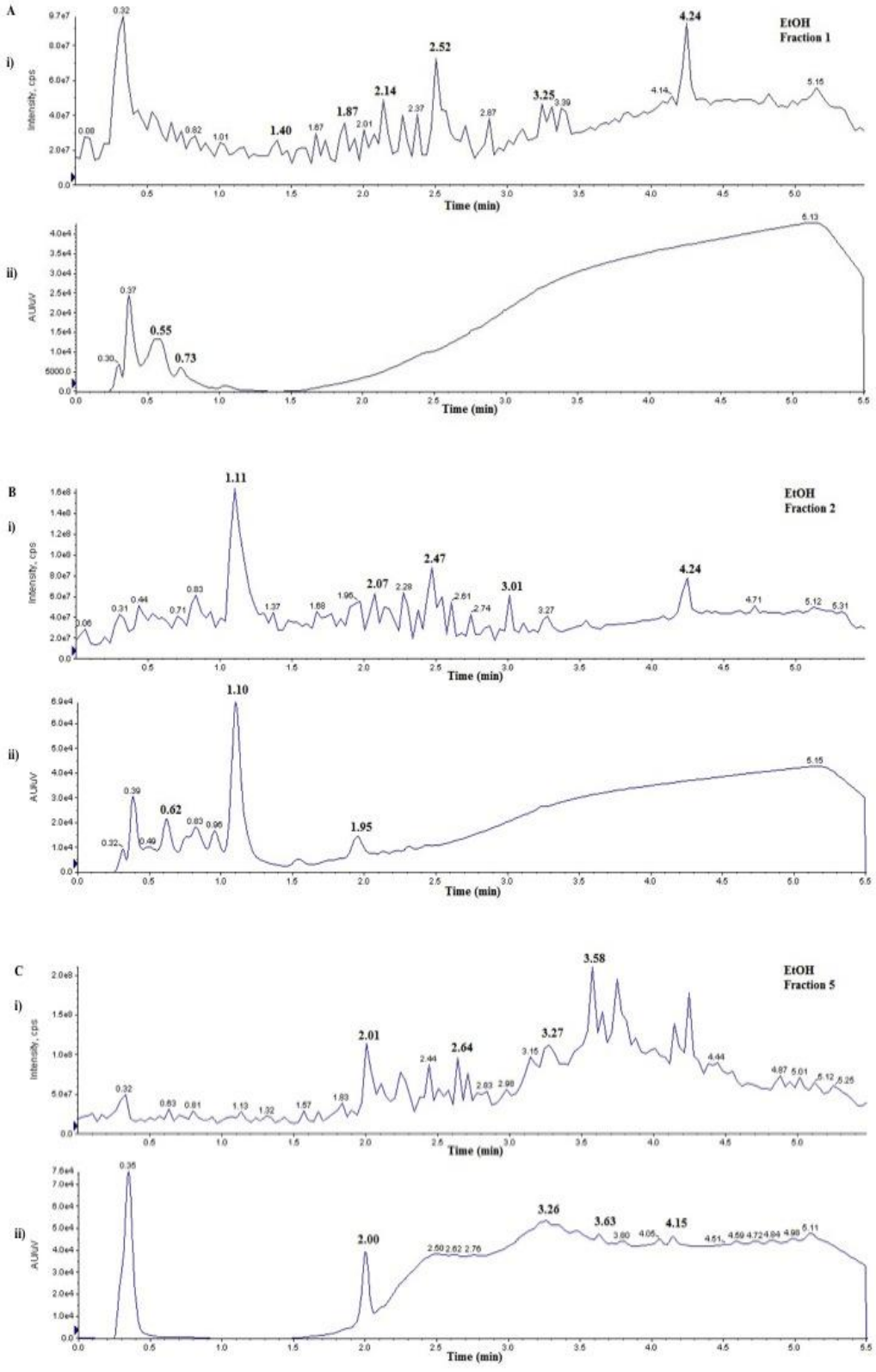
Appendix III-xii: HPLC trace of *Archidendron ellipticum* leaf extract 'inactive' fractions using Reversed Phase chromatography. EtOH: F1 (J), F2 (K), F3 (L) and F6 (M) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



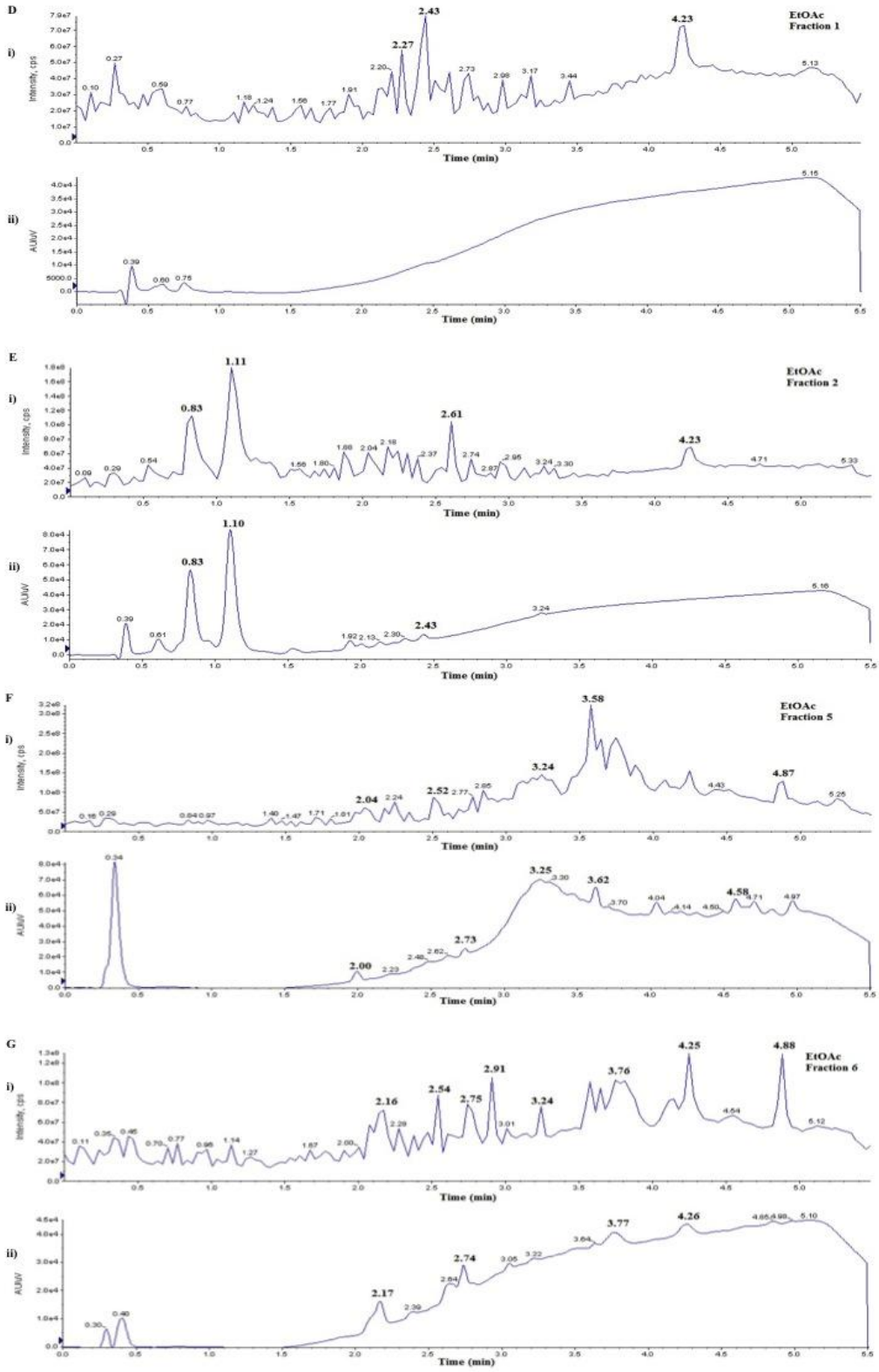
Appendix III-L: HPLC trace of *Archidendron ellipticum* leaf extract 'inactive' fractions using Reversed Phase chromatography. EtOAc: F1 (N), F2 (O) and F3 (P) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



Appendix III-M: HPLC trace of *Archidendron ellipticum* leaf extract 'inactive' fractions using Reversed Phase chromatography. HEX: F1 (Q), F2 (R) and F3 (S) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



Appendix III-N: HPLC trace of *Pseuduvaria macrophylla* extract 'inactive' fractions using Reversed Phase chromatography. EtOH: F1 (A), F2 (B) and F5 (C) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.



Appendix III-O: HPLC trace of *Pseuduvaria macrophylla* extract 'inactive' fractions using Reversed Phase chromatography. EtOAc: F1 (D), F2 (E), F5 (F) and F6 (G) (1 mg/ml in DMSO) in 1:1 distilled water and MeOH were analysed using 10% B for 1 min, 10-98% B over 2 min, at 98% for 2 min, (Phenomenex C18 Gemini-NX 3µ-110A 50x2 mm column). The eluent compounds were detected at a wavelength of 220 nm (i) and 254 (ii) nm.

Appendix IV

IV-1 Antimicrobial activity of UNMC crude extracts

Rapamycin is a well-known antibiotic used in organ transplant with serine/threonine kinase inhibitory effects that can also affect protein translation, cell proliferation and transcription via inhibition of mTOR (mammalian target of rapamycin).[78] Therefore compounds embedded in UNMC extracts may have multiple functions. Aporphinoid (38) present in *P. macrophylla* has been reported to demonstrate anti-tuberculosis activity against *Mycobacterium tuberculosis* with MIC of 100 μg/ml, however it is inactive against *Plasmodium falciparum* in antimalarial assays.[214] Previously, an anti-herpesvirus compound, eugeniin, was isolated from *D. grandiflora* EtOAc extracts and has demonstrated antiviral effects on HSV strains (at 20 μg/ml), inhibition of viral DNA synthesis of HSV strains in Vero cells (at 20 μg/ml) and dose-dependent inhibition of HSV DNA and late HSV proteins.[198] Additionally, *D. grandiflora* extracts have been shown to inhibit accumulation of fat in melanoma cells and increase type II collagen production in dermal fibroblasts.[196] Therefore, further anti-microbial properties of these species are justified.

Antimicrobial properties of UNMC extracts were determined using broth microdilution assay performed by Dr. Jaroslav Havlík and his team from the Department of Microbiology, Nutrition and Dietetics, Czech Universitiy of Life Sciences, in Prague.

The bacterial panel selected consists of both Gram-negative (*S. aureus* and *E. faecalis*) and Gram-positive bacteria (*P. aeruginosa and E. coli*), cocci and rods, as well as one strain of yeast (*C. albicans*). Reference antibiotics were used as positive controls. *A. ellipticum* extracts were not assessed in this assay due to insufficient stock. Two-fold dilutions of each sample tested were tested in the concentration range of 1–128 mg/L. Each well was inoculated with 5 μl of microbial suspension at a density of 10^7 CFU/ml (10^8 for the yeast strain). The microtitre plates were incubated overnight at 37 °C. DMSO was assayed as the negative control and has been shown not to affect bacterial growth.

Growth turbidity was measured at 405 nm and the minimum extract concentration required to inhibit bacterial growth (MICs) was calculated based on the density of the growth control and were expressed as the lowest extract concentrations that resulted in 80% growth reduction compared to that of extract-free growth control. All samples were tested in triplicate.

IV-2 Antimicrobial activity of *Duabanga grandiflora* crude extracts

D. grandiflora bark and leaf extracts revealed comparable MIC values against *E. faecalis* (16 mg/L), *P. aeruginosa* (8 mg/L), *E. coli* (16 mg/L) and *C. albicans* (8 mg/L) in the broth microdilution assay (). Activity against *S. aureus* varied amongst the extracts, with polar (Water and EtOH) bark extracts and leaf EtOH and EtOAc extracts yielding the lowest MIC (16 mg/L). Growth inhibition of Gram-negative bacteria possessing a good natural barrier suggests that these extracts contain compounds that could diffuse through the bacterial wall and impair its cellular function affecting its growth.

In a recently published screening of Malaysian antimicrobial plants, both *D. grandiflora* extracts showed highest activity in an agar diffusion assay, revealing sensitivity of *S. aureus* to EtOH bark and EtOAc leaf extracts (0.094 mg/ml) and less sensitivity to EtOAc bark and EtOH leaf extracts (0.75 and 0.38 mg/ml, respectively).[205] Higher concentrations of bark and leaf extracts were need to inhibit *E. coli* growth (0.75 – 1.5 mg/ml). The activity was among the most potent plants in the test and was non-specific against both Gram-negative and Gram-positive bacteria. Recently, a time-dependent insecticidal study demonstrated *D. grandiflora* MeOH extract-treated rice was toxic to *Sitophilus oryzae* adults and toxicity increased significantly with increasing concentrations (0 - 32 mg/ml).[206]

Appendix IV-A: Antimicrobial activity of extracts against selected bacterial strains.

D. grandiflora extracts/ Reference bacteria		**Minimum Inhibitory Concentration** (mg/L)				
		Gram-positive		**Gram-negative**		**Yeast**
		S. aureus	*E. faecalis*	*P. aeruginosa*	*E. coli*	*C. albicans*
Bark	Water	16	16	8	16	8
	EtOH	32	16	8	16	8
	EtOAc	16	16	8	16	8
	HEX	32	16	8	16	8
Leaf	Water	32	16	8	16	8
	EtOH	16	16	8	16	8
	EtOAc	16	16	8	16	8
	HEX	32	16	8	16	8
Reference antibiotic	Oxacillin	0.25	-	-	-	-
	Vancomycin	-	2	-	-	-
	Ciprofloxacin	-	-	0.25	-	-
	Tetracycline	-	-	-	1	-
	Nystatin	-	-	-	-	2

The bacterial panel consisted of *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922) and yeast *C. albicans* (ATCC 10231). The minimum extract concentration (mg/L; MIC) required to inhibit growth of selected bacteria and yeast were determined in the broth microdilution assay according to the CLSI guidelines (2009).[281] MICs of reference antibiotics used as positive controls were in range of acceptable limits.

IV-C Antimicrobial activity of *Pseuduvaria macrophylla* crude extracts

Similarly, *P. macrophylla* extracts possess comparable antimicrobial activity against *E. faecalis* (16 mg/L), *P. aeruginosa* (8 mg/L), *E. coli* (16 mg/L) and *C. albicans* (8 mg/L) in a broth microdilution assay (). Activity against *S. aureus* varied amongst extracts, and the lowest MIC (16 mg/L) was seen with EtOH extract.

Appendix IV-B: Antimicrobial activity of extracts against selected bacterial strains.

UNMC extracts/ Reference bacteria		**Minimum Inhibitory Concentration** (mg/L)				
		Gram-positive		**Gram-negative**		**Yeast**
		S. aureus	*E. faecalis*	*P. aeruginosa*	*E. coli*	*C. albicans*
P. macrophylla	EtOH	16	16	8	16	8
	EtOAc	32	16	8	16	8
	HEX	32	16	8	16	8
Reference antibiotic	Oxacillin	0.25	-	-	-	-
	Vancomycin	-	2	-	-	-
	Ciprofloxacin	-	-	0.25	-	-
	Tetracycline	-	-	-	1	-
	Nystatin	-	-	-	-	2

Bacterial panel consisted of *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922) and yeast *C. albicans* (ATCC 10231). The minimum extract concentration (mg/L; MIC) required to inhibit growth of selected bacteria and yeast were determined in the broth microdilution assay according to the CLSI guidelines (2009).[281] MICs of reference antibiotics used as positive controls were in range of acceptable limits.

Overall, UNMC extracts were potent at very low concentrations against the panel of Gram-positive and Gram-negative bacteria and yeast strains. Antimicrobial results concurred with previously reports that very low concentration of *D. grandiflora* and *P. macrophylla* extracts could inhibit the growth of Gram-negative bacteria even though they have a good natural barrier against antimicrobial compounds.

Appendix V

Investigating i*n vitro* anticancer activity of *Uvaria macrophylla* (Annonaceace, related to *P. macrophylla*) performed by Mr Vijay Raja under my supervision. Extraction of *U. macrophylla* bark and leaf extracts using solvents (in order of polarity): EtOH > chloroform (CHL) > HEX was prepared by UNMC colleagues. Activity of these extracts has been tested against MCF-7 and HCT116 cancer cells in several bioassays.

V-1 *In vitro* growth inhibitory and cytotoxicity effects of *Uvaria macrophylla* extracts in a cell line panel

The growth inhibitory properties of *U. macrophylla* extracts in MCF-7 and HCT116 cells were assessed using the MTT assay following 72 h exposure (as outlined in section 7.2.3). Both breast and colorectal carcinoma cell lines did not demonstrate comparable level of sensitivity towards *U. macrophylla* EtOH extracts as observed4 with *P. macrophylla* extracts (Table 8-4).

Appendix IV-A: *In vitro* growth inhibitory effects of *Uvaria macrophylla* extracts in breast and colorectal carcinoma cells revealed by the MTT assay.

Cell line	*U. macrophylla* extracts	Mean GI_{50} Values (µg/ml)	
		Bark	Leaf
MCF-7	EtOH	79.08 ± 18.58	102.3 ± 12.88
	CHL	***11.69 ± 4.45***	***5.89 ± 2.12***
	HEX	***4.72 ± 1.50***	*27.03 ± 7.23*
HCT116	EtOH	68.20 ± 21.80	97.23 ± 19.97
	CHL	***3.25 ± 0.66***	***4.55 ± 0.90***
	HEX	***5.59 ± 1.16***	*21.75 ± 8.60*

Extract concentration (µg/ml) at which cell growth is inhibited by 50% (GI_{50}) after 72 h exposure. Mean GI_{50} values (µg/ml) ± SEM was obtained from ≥ 3 (n = 4) individual experiments where active crude extracts (GI_{50} values ≤ 20 µg/ml) are highlighted in **bold**, extracts ± SEM values that could be considered active are highlighted in *italics*.

HEX bark extracts demonstrated greater potency than leaf extracts by 4 to 6 folds. CHL bark and leaf extracts were the most active against both cell lines at low concentrations between (3.25 to 11.69 µg/ml). Extracts of CHL (used in *U. macrophylla* extraction) and EtOAc (used in *P. macrophylla* extraction)

reveal greatest activity against MCF-7 and HCT116 compared to other extraction solvents used, therefore it is likely that similar 'active' compounds in this plant family are extracted by solvents this polarity range. On the whole, *P. macrophylla* extracts exerted greater growth inhibition activity against cancer cells than *U. macrophylla* extracts. Unexpectedly, EtOH and HEX *U. macrophylla* extracts did not yield comparable GI_{50} values as that seen with *P. macrophylla* extracts.

V-2 Influence of *Uvaria macrophylla* extracts on MCF-7 and HCT116 cells' survival and clonogenicity

The clonogenic assay was employed to assess the influence of *U. macrophylla* extracts on MCF-7 and HCT116 cells' survival and colony formation ability (outlined in section 7.2.4). *U. macrophylla* extracts inhibited colony formation ability of both cells at similar or lower concentrations than determined in the MTT assay (Table 8-5).

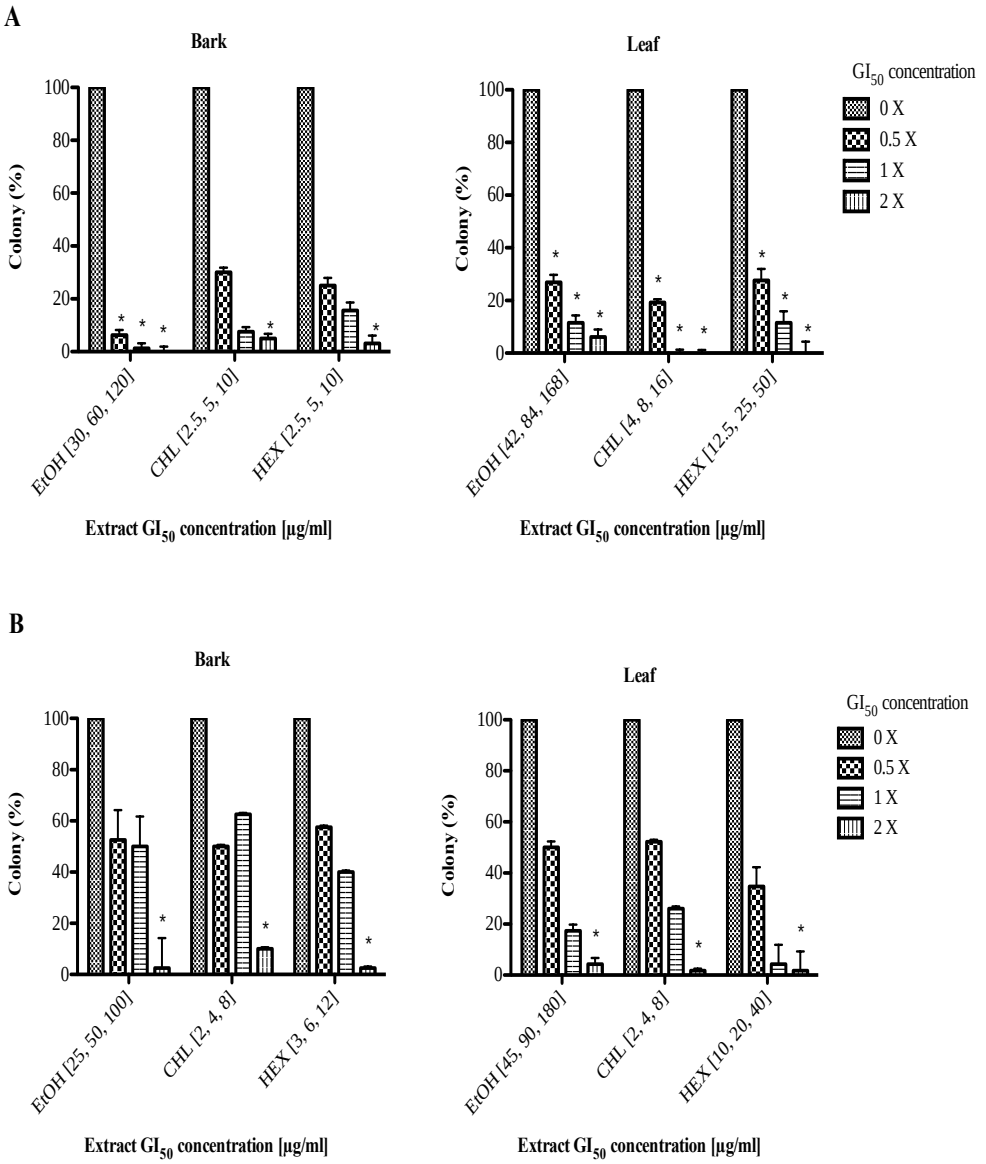
Appendix IV-B: Effects of *Uvaria macrophylla* extracts to MCF-7 and HCT116 cells' survival and clonogenicity.

Cell line	*U. macrophylla* extracts	Mean IC_{50} Values (µg/ml)	
		Bark	Leaf
MCF-7	EtOH	19.61 ± 1.94	29.60 ± 2.81
	CHL	4.72 ± 1.77	3.44 ± 1.23
	HEX	6.60 ± 3.00	12.83 ± 4.34
HCT116	EtOH	42.62 ± 11.70	94.66 ± 22.41
	CHL	3.01 ± 0.67	5.17 ± 0.87
	HEX	3.68 ± 0.63	28.30 ± 7.57

Extract concentration (µg/ml) at which colony formation is inhibited after 24 h exposure. Mean IC_{50} values (µg/ml) ± SEM was obtained from ≥ 3 (n = 4) individual experiments.

It is possible that due to low seeding density, EtOH bark and leaf extracts were able to inhibit colony formation at lower extract concentration than expected than expected with the exception of leaf bark extract against HCT116 cells. Whereas CHL extracts were able to inhibit clonogenicity of MCF-7 and HCT116 cells at similar concentrations seen in the MTT assay (<11 µg/ml). Overall, *U. macrophylla* bark extracts were more active against MCF-7 cells'

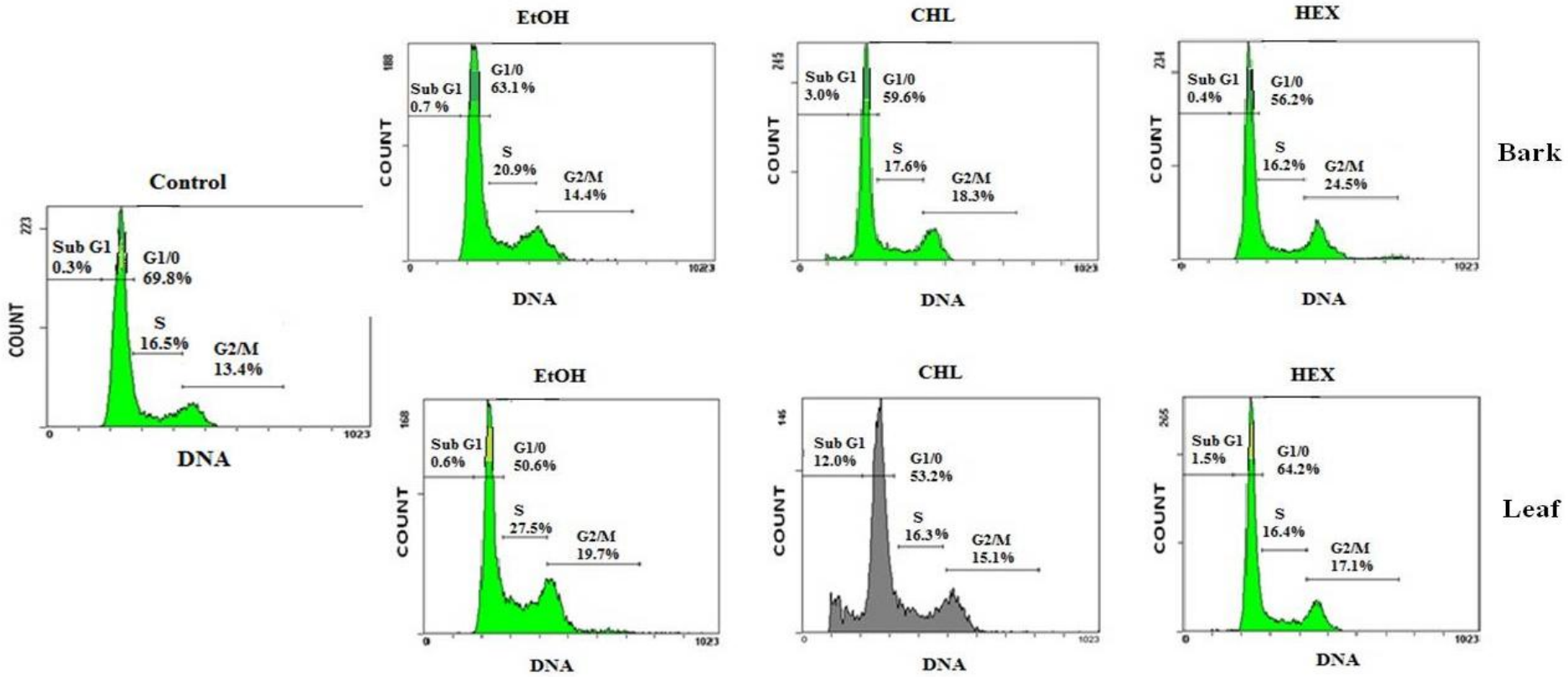
survival and clonogenicity (Figure 8-24), even though greater GI_{50} results were seen in the MTT assay, this may suggest cells remain viable but unable to proliferate following exposure to *U. macrophylla extracts.* Whereas similar GI_{50} and IC_{50} values for *U. macrophylla* extracts against HCT116 cells suggests that extracts are potent to HCT116 cells causing lost in viability and colony forming abilities.



Appendix V-C: Effects of *U. macrophylla* extracts to HCT116 (A: bark and leaf) and MCF-7 (B: bark and leaf) cells' survival and clonogenicity. Bars (error bar = SEM) denote the mean % of MCF-7/HCT116 colonies formed after 24 h exposure to extracts from ≥ 3 (n = 4) individual experiments; plating efficiencies were ≥ 80% per experiment. ANOVA univariate analysis was used to compare each extract-treated group to the control group. Any statistically significant values are denoted: * = 0.05< p.

V-3 *Uvaria macrophylla* extracts effects on HCT116 cell cycle distribution

Flow cytometric technique was employed to determine HCT116 cell cycle distribution following treatment to *U. macrophylla* extracts as outlined in section 7.2.5.1. In general, cell cycle distribution of HCT116 cells appeared to be comparable to that of DMSO control cells (Figure 8-25). Cell cycle histograms of cells exposed to EtOH extracts revealed subtle differences when compared to that of the control, such as decrease in G1/0 phase accompanied by increased S phase levels. Whereas HEX bark extract treatment suggests a possible increase in G2/M cell population accompanied by a decrease in G1/0 cell population. Interestingly, CHL extract treated cells revealed increased sub-G1 cell population, which is indicative of apoptotic cells; CHL leaf extract appeared to be most active in increasing % of sub-G1 HCT116 cells. In summary, preliminary data suggests low concentration of *U. macrophylla* CHL extracts could possess compounds causing cells to undergo apoptosis, however further analysis are required to confirm this finding and enable the use of statistical analysis to reveal if the cell cycle distribution of extract-treated cells are indeed different to the control population.



Appendix V-D: Detection of propidium iodide staining of HCT116 cellular DNA content after exposure to *U. macrophylla* extracts at 4X GI_{50} concentration for 48 hour. Data is represented as histogram of cellular DNA content representation of cell population % in each cell cycle phase; images from a single representative experiment are shown. The samples were kept chilled overnight in a solution containing buffer, detergent and ribonuclease A prior to analysis. Results were established from three experiments of ≥ 15,000 cells per sample (n = 2) and processed using EXPO32 software.