Griffith University

Phytochemical study and anticancer potential of high antioxidant Australian native plants

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Statement of Originality

This work, entitled **Phytochemical study and anticancer potential of high antioxidant Australian native plants**, has not previously been submitted for a degree in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Publications Arising From This Thesis

1. Sirdaarta, J., Matthews B., I.E. Cock. : *Kakadu plum fruit extracts inhibit growth of the bacterial triggers of rheumatoid arthritis: Identification of stilbene and tannin components*. Journal of Functional Foods. 06/2015; 17:610-620. doi:10.1016/j.jff.2015.06.019.

2. Sirdaarta, J., Matthews, B., White, A., Cock, I.E.: *The potential of selected Australian medicinal plants with anti Proteus activity for the treatment and prevention of rheumatoid arthritis*. Pharmacognosy Magazine 05/2015; 11(42 (Supp 1)):S190-S208. doi:10.4103/0973-1296.157734.

3. Sirdaarta, J., Cock, I.E.: *The anti-proliferative properties of Australian plants with high antioxidant capacities against cancer cell lines*. Pharmacognosy Communications 10/2014; 4(4):71-82. doi10.5530/pc.2014.4.8.

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Conference Proceedings

1. Sirdaarta, J. Cock, I.E.: *Antioxidant and anticancer activities of Terminalia ferdinandiana (Kakadu Plum)*. 3rd International Symposium on Medicinal Plants (ISMP), Petra, Jordan; 01/2012.

2. Sirdaarta J., Cock, I.E.: *Anticancer activity and phytochemical profiling of Terminalia ferdinandiana (Kakadu Plum) fruit extracts*. The 15th International Congress of The International Society for Ethnopharmacology, Petra Jordan; 05/2015

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List of Abbreviations

●OH	A highly reactive radical species of the Hydroxyl ion OH^- .
ADP	Adenosine diphosphate
AGS	Stomach adenocarcinoma cell line
AKT	An intracellular signalling pathway important in regulating the cell cycle
ANOVA	Analysis of variance
AP-1	Activator protein involved in signal transduction
Apaf-1	Apoptotic protease activating factor 1 involved in apoptosis
APO-1	The FAS receptor (Fas R), also known as apoptosis antigen 1
AR	Analytical grade of reagents with the highest level of chemical purity
Asp	Aspartic acid amino acid
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bax	Apoptosis regulator
Bcl-2	Bcl-2 (B-cell lymphoma 2) ,regulator proteins that regulate cell death (apoptosis)
Bid	BH3 interacting-domain death agonist, a pro-apoptotic member of Bcl-2
Caco-2	Colorectal adenocarcinoma cells
CAT	Catalase
cMyc	A regulator gene coding for protein in cell cycle progression and apoptosis
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2, an enzyme
Cys	Cysteine amino acid
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNI	IC_{50}/LC_{50} were not obtained as % mortality did not exceed 50% highest dose
DPPH	2, 2-diphenyl-1-picrylhydrazyl tetrazolium salt
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane used as solid phase in GC-MS
EDTA	Ethylenediaminetetraacetic acid
Ehc	Reduction potential
ERK	A protein pathway that communicates a signal from cell receptor to the nucleus.
ESI	Electrospray ionisation
FADD	Fas-associated death domain protein
Fas	Cell surface receptor protein of the TNF receptor family known also as CD95
FBS	Foetal bovine serum
G0/G1	Cell cycle resting phase
GC-MS	Gas chromatography–mass spectrometry
GPx	Glutathione peroxidase
GSSG	Glutathione disulphide
GST	Glutathione S transferase
GS-X	ATP-dependent glutathione S-conjugate export pump
HCL	Hydrochloric acid
HDAC	Histone deacetylase
HeLa	Human cervical carcinoma cell line
HPLC-DAD	High-performance liquid chromatography diode array detection
Hras	HRAS protein is involved in signal transduction pathways
IAP-1	Apoptotic inhibitors of the bcl-2 family
IBD	Inflammatory bowel disease
IC_{50}	Inhibitory concentration causing 50% mortality or activity
IFN-γ	Interferon gamma, cytokine
IL-17	Interleukin 17, cytokine

IL-1β	Interleukin 1 beta, cytokine
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JEG-3/JAR	Choriocarcinoma cell lines
JNK	c-Jun N-terminal kinases
$K_2Cr_2O_7$	Potassium dichromate
LC MS	Liquid chromatography–mass spectrometry
LC_{50}	Dose required to kill half the members of a tested population
LC-MS QTOF	High-performance liquid chromatography time of flight detection
LPS	Lipopolysaccharides (LPS), also known as lipoglycans and endotoxins
M,W,E,C,H	Methanol, water, ethyl acetate, chloroform, hexane solvents
MAPK	Mitogen-activated protein kinases
MC3T3-E1	Murine calvarial osteoblasts
MDA	Malondialdehyde
MEK	MEK is a member of the MAPK signalling cascade.
MG-63	Osteosarcoma cell line
mTOR	Mammalian target of rapamycin
MTS/MTT	Colorimetric assay for assessing cell metabolic activity.
NA	Not attempted
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	ND indicates IC_{50}/LC_{50} values could not be determined, below 50% mortality.
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NO	Nitrogen oxide
O^{2-}	Superoxide
p53	Tumour protein
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cell
PES	Phenazine ethosulphate
PGE2	Prostaglandin E2
PGI2	Prostacyclin (also called prostaglandin I2), a prostaglandin member
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PSH	Sulfhydryl groups
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP-HPLC MS	Reverse phase high-performance liquid chromatography
RPMI-1640	Roswell Park Memorial Institute medium 1640
S1P	Sphingosine-1-phosphate
S6K1	Ribosomal protein S6 kinase beta-1
SCID	Severe combined immunodeficiency
SEM	Standard error mean
Smac	Second mitochondria-derived activator of caspases
SOD	Superoxide dismutase
SPME	Solid-phase microextraction
TCC	Total compound chromatograms
TIC	Total ion count
TNF	Tumour necrosis factor
Trx	Thioredoxin reductase
TSG	2, 3, 4, 5-tetrahydroxystilbene-2-O-β-D-glucoside
WHO	World Health Organisation
YAMC	Normal colonic cell line

ABSTRACT

High antioxidant capacities have been linked with a reduction in the incidence of chronic diseases including cardiovascular disease, diabetes, obesity, neurodegeneration, inflammation and cancer. Furthermore, phenolic antioxidants may not only have protective effects against these diseases, but may also have therapeutic potential in reversing them by modulation of the cellular redox state. Thus, plants with high antioxidant capacities have potential in the development of new chemotherapeutic treatments to prevent and treat some cancers. Several native Australian plant species including *Terminalia ferdinandiana* Exell (Kakadu plum), *Tasmannia lanceolata* (Poir.) A.C.Sm., (Tasmannian pepper), *Syzygium australe* (H.L.Wendl. ex Link) B.Hyland (brush cherry), *Syzygium luehmannii* (F.Muell.) L.A.S.Johnson (riberry), *Davidsonia pruriens* F.Muell. (Davidson's plum), *Elaeocarpus angustifolius* Blume (quandong), *Kunzea pomifera* F.Muell. (muntries), *Podocarpus elatus* R.Br.ex Endl., (Illawarra plum) and *Acronychia acidula* F.Muell., (lemon aspen) have recently been reported to have extraordinarily high antioxidant content and were therefore selected to screen for the ability to inhibit proliferation of selected carcinoma cell lines.

The selected plant species were extracted by maceration using solvents of varying polarity. The present study verified the high antioxidant activity of these extracts using DPPH assays. The antiproliferative potential of the extracts was examined in HeLa and Caco-2 cell lines

using MTS based colorimetric assays. Aqueous *S. australe* fruit extract (HeLa IC_{50} of 172 µg/mL; Caco-2 IC_{50} of 27 µg/mL) and aqueous *S. leuhmannii* leaf extracts (HeLa IC_{50} of 128 µg/mL; Caco-2 IC_{50} of 43 µg/mL) were particularly potent. *T. ferdinandiana*, *T. lanceolata* and *D. pruriens* were also good antiproliferative agents against HeLa and Caco-2 cell lines, with IC_{50} values substantially < 1000 µg/mL against both cell lines. The toxicity of the extracts was also examined using an *A. franciscana* nauplii mortality assay. The *Syzygium* spp. were moderately to highly toxic (LC_{50} values < 500 µg/mL). All other plant species were deemed to be non-toxic.

Selection criteria were implemented to narrow the focus of plants for further studies. Extracts which had high antioxidant content (> 100 mg AA equivalents per g extracted plant material), good antiproliferative potential against HeLa and Caco-2 with IC_{50} values substantially < 1000 µg/mL, and were non-toxic (LC_{50}> 1000 µg/mL) were selected for further study. Whilst several of the plant species studied fulfilled 1 or more of these criteria, only *T. ferdinandiana* and *T. lanceolata* fulfilled all 3 criteria and were therefore selected for further studies. *T. ferdinandiana* and *T. lanceolata* extracts were screened against specialised JEG-3/JAR and MC3T3-E1/MG-63 cell line pairings to elucidate mechanisms and pathways by which the extracts inhibit proliferation. These studies demonstrated acute oxidative stress induction by these plant extracts and indicated TNF-induced apoptotic pathways may be implicated. Cell imaging studies in Caco-2 carcinoma cells were consistent with apoptosis induction pathways, with several apoptotic morphological markers observed.

Furthermore, metabolomic profiling studies using HPLC- MS QTOF and GC-MS putatively identified phytochemicals which are consistent with the induction of apoptosis. C17 sphingosine was present in all antiproliferative *T. ferdinandiana* extracts. This lipid induces apoptosis via an inhibition of both shingosine-1-phosphate and NF-κB. Several monoterpenoids (linalool oxide, terpineol, camphor) were also detected in the antiproliferative *T. ferdinandiana* extracts. These compounds have been previously reported to induce TFN-α production and inhibit NF-κB, thus inducing apoptosis. Similarly, polygodial, a major component identified in all antiproliferative *T. lanceolata* extracts, also induces TFN-α production and inhibits NF-κB. The sesquiterpenoid caryophyllene oxide was also identified in the *T. lanceolata* extracts. Caryophyllene oxide induces apoptosis in some carcinoma cells via the induction of increased mitochondrial ROS, leading to the activation of caspase 3 and ultimately apoptosis. Penta-hydroxy stilbene was also putatively identified in these extracts. This compound upregulates ROS generation and depletes intracellular glutathione, leading to the induction of apoptosis via a caspase dependent mechanism. Thus the phytochemistries of both the *T. ferdinandiana* and *T. lanceolata* extracts are consistent with apoptotic mechanisms.

It is noteworthy that the extracts tested in these studies were crude mixtures. Therefore, it is possible that these extracts may also have cytostatic mechanisms and this remains to be rigorously tested. Indeed, the phytochemical profiles have highlighted a number of compounds which have been reported to induce cell cycle arrest. In particular, extracts of both species had significant levels of gallotannins and ellagitannins which inhibit cell cycle progression. Combretastatins (evident in both species) bind intracellular tubulin, thus inhibiting cell

division. It is therefore likely that these extracts act via pleuripotent antiproliferative mechanisms consisting of both cytostatic and cytotoxic components.

In summary, the results of these studies demonstrate the potential of *T. ferdinandiana* and *T. lanceolata* in the prevention and treatment of some cancers. Furthermore, the lack of toxicity indicates that extracts of these species may be well suited to this role. Thus, further studies into the anticancer potential and mechanisms of these plant species is warranted.

CHAPTER 1: INTRODUCTION

1.1 Natural Therapeutics: Historical background

Ancient civilisations confronted with illness and disease discovered a wealth of useful therapeutic agents from plants. These civilisations practiced ethnomedicine/ethnopharmacology, utilising botanical resources to cure diseases. Empirical knowledge of these medicinal preparations and their toxic potential was passed on by oral tradition, and was sometimes recorded in herbal literature. Sumerian clay tablets (4000 BC) detailing 1000 medicinal plants and plant remedies are historic remains of the recorded usage of herbal medications by ancient civilisations [1, 2]. The Pun-tsao (a collection of thousands of herbal cures) by the Chinese 4500 years ago; the Hippocratic Corpus (a collection of Greek medical texts of herbal remedies) by Greek physician Hippocrates in the late 5 century BC and the De Materia Medica (Roman writings that documented more than 600 species of plants with medicinal value) by Dioscorides, are other ancient records of the ethnobotanical use of therapeutic plants [2]. These records are far more valuable than being just an anthropologic or archaeological find. Their value stems from an understanding of the plant and usage of the plant medicines. Traditional knowledge of phytomedicines is still used by more than 65% of the world's population as their primary modality of health care [3]. The majority of the remainder of the world's population also use plant based medicines, either in conjunction with existing allopathic medicines, or as health supplements. Traditionally, plant

based medications have been used as crude formulations including infusions, tinctures, extracts, essential oils, powders and poultices [4, 5].

1.2 Plants and phytochemicals of therapeutic significance

Plants are an abundant source of phytochemicals which have multiple uses as therapeutic agents. Phytochemical research is required for the following reasons:

1. To isolate bioactive compounds for direct use as drugs, e.g., atropine, ephedrine, digoxin, digitoxin, morphine, reserpine, tubocurarine, taxol, vinblastine and vincristine. These compounds have come into use through researching indigenous remedies [6].

2. To produce bioactive compounds of novel/known structures as guide compounds for semi-synthesis (part natural and part synthesised artificially). Such patentable compounds may be manufactured with higher activity, lower toxicity, and enhanced bioavailability e.g. metformin, nabilone, oxycodon, physostigmine, quinidine, emetine (and other narcotic analgesics), taxotere, teniposide, verapamil, and amiodarone [6].

3. Research enables us to use phytochemical agents as pharmacological tools in understanding the normal physiology of the human body. Previous studies have utilised lysergic acid diethylamide (LSD) as a pharmacological tool in investigating the synaptic mechanism in the lateral geniculate of the brain [7]. Acetylcholine, eserine, prostigmine, atropine, adrenaline, noradrenaline, amphetamine, histamine, d-tubocurarine, strychnine, veratrine, veratridine, cevadine, yohimbine, mescaline and

yohimbine are examples of other phytochemicals that have been used in similar investigations involving neurotransmitters [6, 7].

4. The whole plant, or its individual parts, may be directly used as an herbal remedy e.g. the use of cranberry, echinacea, feverfew, garlic, ginkgo biloba and St. John's wort [6].

Current ethnopharmacological research embraces a multidisciplinary (pharmacological, botanical and chemical) approach towards documentation of indigenous medical knowledge, scientific study of indigenous medicines and the search for pharmacologically unique principles from existing indigenous remedies. Phytochemicals of plant origin have provided therapeutic alternatives for a variety of diseases and conditions, including uses as analgesics, cardiotonics, hypertensives, antitumor agents etc. Some examples of important plant medicines are listed in (Table 1). Research requires exhaustive testing to ascertain pharmacological and toxicological mechanisms of action, and if possible, clinical studies on their efficacy.

Table 1.1 Drugs derived from plants with their ethnomedical indications and sources [8]

Drug	Ethnomedical indications	Plant source
Atropine	Anticholinergic	*Atropa belladonna* L
Berberine	Bacillary dysentery	*Berberis vulgaris* L.
(+)-Catechin	Haemostatic.	*Potentilla fragaroides* L
Codeine	Analgesic; antitussive	*Papaver somniferum* L.
Colchicine	Antitumor agent; antigout	*Colchicum autumnale* L.
Curcumin	Choleretic	*Curcuma longa* L.
Digitalin	Cardiotonic.	*Digitalis purpurea* L
Digitoxin	Cardiotonic	*Digitalis purpurea* L.
Digoxin	Cardiotonic	*Digitalis lanata* Ehrh.
Ephedrine	Sympathomimetic	*Ephedra sinica* Stap f.
Etoposide	Antitumour agent	*Podophyllum peltatum* L.
Kawain	Tranquilizer	*Piper methysicum* Forst. f.
Morphine	Analgesic	*Papaver somniferum* L.
Ouabain	Cardiotonic	*Strophanthus gratus* Baill.
Papain.	Proteolytic; mucolytic	*Carica papaya* L
Quinine	Antimalarial	*Cinchona ledgeriana* Moens ex. Trimen
Reserpine	Antihypertensive; tranquilizer	*Rauvolfia serpentina* (L.) Benth ex. Kurz
Salicin.	Analgesic	*Salix alba* L
Scopolamine	Sedative	*Datura metel* L.
Sennosides A & B	Laxative.	*Cassia* spp
Teniposide	Antitumor agent	*Podophyllum peltatum* L.
Theophylline	Diuretic; bronchodilator	*Camellia sinensis* (L.) Kuntze
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum* R. & P.

Numerous examples attest to the tremendous value, importance and diversity of plant based medicines. The leaves of *Wendita calysina* (a Paraguayan plant) are commonly consumed as an infusion (tea beverage) in South America as a traditional anti-rheumatic and anti-inflammatory agent [9]. The leaves yield 14 known phenolic constituents including flavanols, methoxy flavones, methoxy flavanols, phenyl ethanoid glycosides and benzoic derivatives [9]. The phenyl ethanoid glycosides and benzoic acid derivatives are present in the greatest quantities in these infusions [9]. Phenyl ethanoid glycosides have well-documented antibiotic properties [10]. They also inhibit platelet aggregation [11], leukotriene B4 formation [12], and scavenge free radicals to prevent peroxidation in liver microsomes [13]. The polyphenol rich

extracts from the *Wendita calysina* are also DNA protectants, scavenging reactive oxygen species (ROS), and prevent inflammation induced by nitrogen oxide (NO) [9].

Plant based medicines have been particularly useful in the development of anticancer/antineoplastic drugs. Of all plant based medicines, approximately 52% are therapeutics used in treatment of cancer; 14% are natural products, 27% are semi-synthetic natural products derivatives and the remaining 11% are synthetic compounds modelled after natural product leads [14]. Indeed, 74% of all anticancer agents that reached the market between 1981 and 2002 were plant compounds or semi-synthetic analogues based on plant compounds [15].

Green tea is an abundant source of tannins including epigallocatechin and epigallocatechin gallate, as well as multiple flavone compounds [4]. Studies have reported that the potent antioxidant activity of green tea is due to its high content of water-soluble polyphenols and flavonoids [16]. These compounds have increased hydrogen-donating ability, hence excellent anti-oxidative potential in quenching ROS [17]. Thus, green tea is useful in averting oxidative damage of DNA and lipids by ROS species [18]. Therefore, these flavonoids and polyphenolic compounds possess cancer chemopreventive effects related to their antioxidant effect [19] via their ability to scavenge free radicals [20] and induce apoptosis [21].

Asian ginseng and its chemical constituents have been extensively studied since the 1970's for their ability to inhibit human cancer cell proliferation [22]. Ginseng mediates an anti-proliferative effect via the induction of apoptosis [23]. Studies have also reported other

antitumor properties and other pharmacological activities of ginseng, and the ginsenosides Rg3 and Rh2 are recognized as the main active anticancer compounds [24]. Furthermore, ginseng has anti-metastatic effects via the inhibition of tumour cell invasion through the action of the ginsenoside saponin components [23, 25-29]. Ginseng extracts are also protective against the peroxidation of unsaturated fatty acids, preventing free radical induced injury by quelling hydroxyl radicals, and inducing the production superoxide dismutase [30, 31]. Ginsenosides scavenge reactive oxygen intermediates (superoxide, hydroxyl radicals) which play a role in multi-stage carcinogenesis mechanisms [32].

Leaf extracts of *Catharanthus roseus* (L.) G. Don (commonly known as the Madagascar periwinkle) were historically used in the treatment of diabetes [33]. Interestingly, attempts in phytochemical profiling of periwinkle extracts led to the isolation of two complex indole alkaloids, vinblastine and vincristine [33]. These compounds have become mainstays in the clinical treatment of a variety of cancers. These alkaloids, although structurally almost identical, nevertheless differ in the type of tumours that they affect, and in their toxicities [33]. These, and related alkaloids, have been the subject of many pharmacological and biochemical investigations, both *in vivo* and *in vitro* in the search for improved cancer treatments [33]. Mechanistic studies have revealed that these alkaloids arrest cell division, thus inhibiting cancer proliferation [34].

Taxol, pharmaceutically known as paclitaxel, is semi-synthetically manufactured from the bark of the Pacific yew (*Taxus brevifolia*) [35], and is commonly used in the treatment of ovarian and metastatic breast cancer. Other forms of cancer inclusive of lung cancer, cancer

of the head and neck, malignant melanoma and lymphomas have reported much success on account of the cytotoxicity of this drug [36]. At a cellular level, the diterpenoid taxol, binds tubulin rendering it unavailable for polymerisation, thus promoting mitotic G2/M arrest and subsequently apoptosis [37] and remission of the cancerous growth.

Given the complexity of traditional medicines and the wealth of plant derived cancer chemotherapeutic agents, it is likely that plant compounds will be an invaluable source of new cancer treatments in the immediate future. To aid identifying targets, an understanding of cancer aetiology and progression is vital. The following sections describe carcinogenesis and in so doing, identify several processes related to cellular redox state as potential targets for the prevention and treatment of cancer.

1.3 Carcinogenesis

Growth and development of normal cells depends on fine regulation of growth promoting and inhibiting pathways. Mutations caused by genetic damage offset this balance and result in aberrations of cellular behaviour [38]. Proto-oncogenes and tumour suppressor genes are responsible for encoding proteins that regulate the cell division cycle and cell processes regulating programmed death by apoptosis and repair of damaged DNA. However, mutations are also implicated in the onset of cancer [39].

These genes encode seven vital proteins:

(1) extracellular signalling molecules,

(2) signal receptors/intracellular receptors,

(3) signal transduction proteins/intracellular transducers,

(4) transcription factors,

(5) apoptotic proteins

(6) cell cycle control proteins and

(7) DNA repair proteins that are responsible for controlling cell growth and proliferation. The mutant forms often result in the development of cancers [39].

Hanahan and Weinberg [39] have proposed that normal cells must acquire six phenotypes to become malignant (Figure 1.1).

(a) Self-sufficiency in growth signals

(b) Insensitivity to anti-growth signals

(c) Limitless replicative potential

(d) Tissue invasion and metastasis

(e) Sustained angiogenesis

(f) Evasion of apoptosis

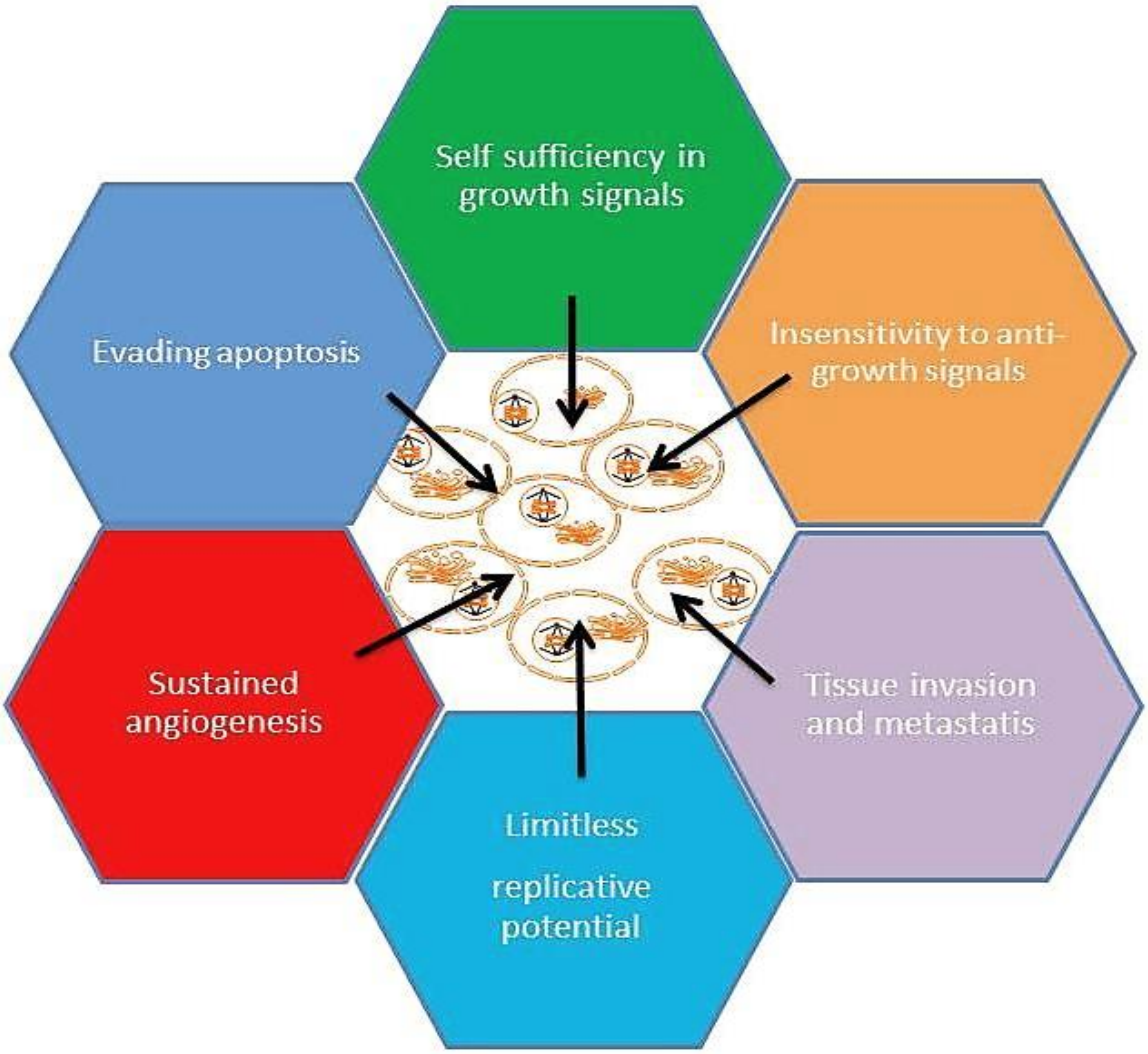

Figure: 1.1 A representation of the Hanahan Weinberg model of carcinogenesis. Normal cell have to acquire these six phenotypes before they are deemed as malignant [39].

Cancer cells do not require external signals to proliferate. Furthermore, they also fail to recognise signals that restrict cell division [39]. In tumorigenesis, multiple genes are altered and transmitted to daughter cells which are clones. These subsequently escape normal growth restraints and form a tumour, which may be either benign or malignant [39]. In the Hanahan and Weinberg model (Figure 1.1), the chronological order of the six phenotypes and the

mechanism by which these phenotypes are acquired may differ in each tumour [39]. Genomic instability provides the driving force for acquiring new phenotypes [39].

1.3.1 Cancer and its relationship to oxidative stress

Pathological diseases that share oxidative stress and affinity towards cancer, such as Fanconi anaemia, Xeroderma pigmentosum, Ataxia telangiectasia, Bloom Syndrome, Down's syndrome and cystic fibrosis are congenital disorders in which cells show evidence of increased oxidative stress (Table 1.2). Interestingly, affected individuals also show an increased incidence of cancer [40]. Chromosome instability is also a common feature of the first four listed disorders, suggesting that increased oxidative stress may contribute to development of genetic instability (i.e. a mutant phenotype that is a hallmark of cancer cells), and the continuous production of oxidants at the site of chronic inflammation may induce cancer.

Table 1.2 : Table of inflammatory conditions linked to cancer [41].

Cancer	Inflammatory condition
Lymphoma	HIV, Epstein-Barr, and Herpes B virus, chronic host vs. graft disease
Colon	Ulcerative Colitis
Lung	Asthma, Chronic Bronchitis, Emphysema
Ovarian	Ovarian Epithelial Inflammation
Bladder	Eosinophilic cystitis, schistosomiasis
Pancreatic	Pancreatitis
Oesophageal-gastric junction carcinoma	Barett's oesophagus
Gastric	*Helicobacter pylori* infection
Liver	Sarcoidosis, Hepatitis B virus
Cervical	Human Papilloma virus
Mesothelioma	Asbestos fibre exposure

Seis [42] views oxidative stress as a disturbance in the anti-oxidant/pro-oxidant balance, which may subsequently result in cell damage (Figure 1.2). Cells are generally equipped with intracellular antioxidant systems including glutathione peroxidise (GSH-Px), catalase (CAT), thioredoxin (THx), superoxide dismutase (SOD) and extracellular antioxidants (vitamin E, vitamin C and uric acid) to neutralise ROS onslaught [43].

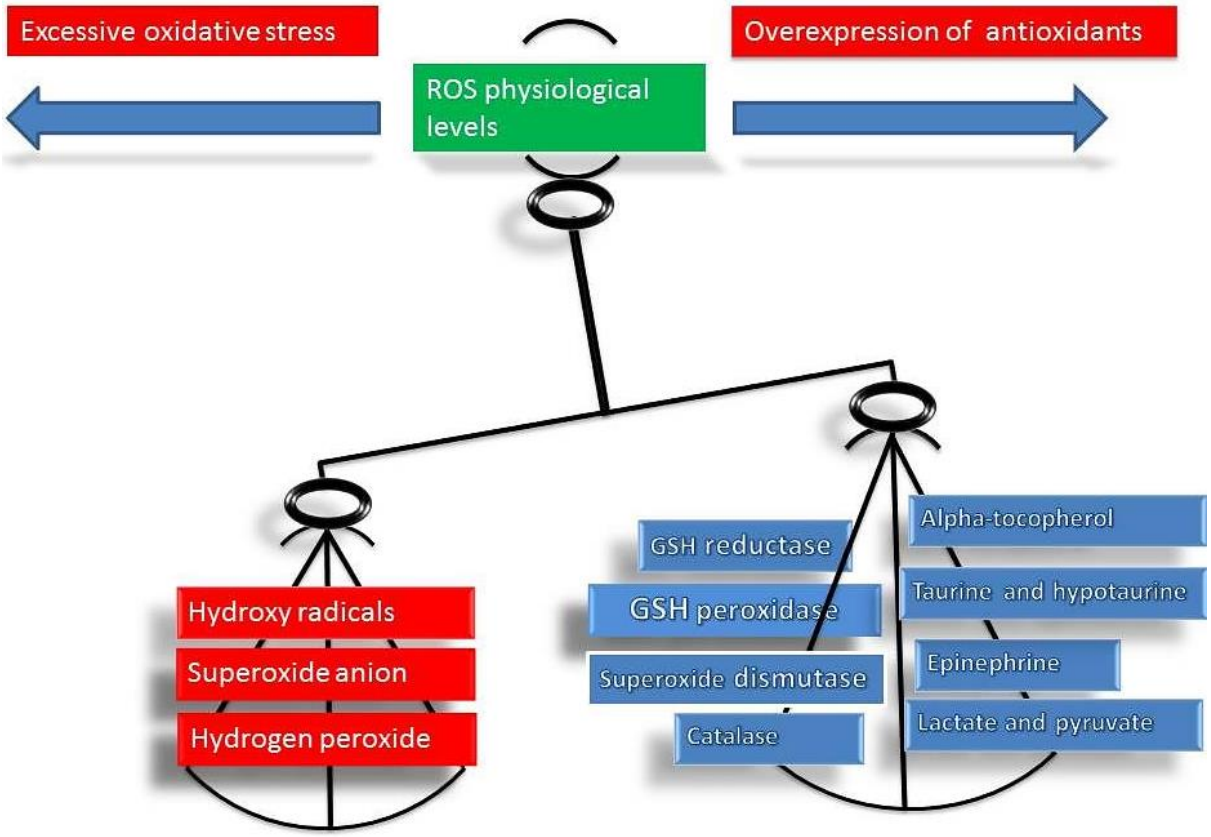

Figure: 1.2 Excessive oxidative stress causes disruption of the balance between anti-oxidants and reactive oxygen species (ROS). The left side of the figure indicates the events leading to the establishment of oxidative stress, whilst the right of the figure shows the antioxidant mechanisms used by the cell to maintain a reducing environment.

From a molecular perspective, cellular processes are invariably dependant on the redox environment of the cell, which is the sum total of the products of the reduction potential and the reducing capacity of the linked redox couples, which entails the flow of electrons [44, 45]. This is described by an adaptation of the Nerst equation:

$$\text{Redox environment} = \sum_{i=1}^{n\,(couple)} E_i\, x_i$$

From a thermodynamic standpoint, a cell is never in equilibrium with the environment unless equilibrium is attained through cellular death. This system favours entropy, and energy is needed for the continuation of life [44]. From a cellular perspective, NADPH is usually a cofactor in reductive biosynthetic reactions and serves as a source of electrons; NAD^+ dependant reactions are usually oxidative (catabolic) reactions, where NAD^+ functions as a sink for electrons. The NADPH/$NADP^+$ and NADH/NAD^+ couples are maintained in concentrations distant from their equilibrium to fulfil their intended function as electron carriers from oxidised substrates to reduced substrates [42]. The reducing potential of $NADP^+$/NADPH couple as a source of electrons, and NADPH as a source of reducing equivalents makes it especially ideal in the glutathione and thioredoxin system to meet the challenge of oxidative stress experienced by a cell. Intracellular antioxidant enzyme systems, particularly glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT), work synergistically to not only scavenge oxygen free radicals, but also aid in the repair of oxidised membrane components [43]. Mechanistic studies *in vivo* indicate that SOD catalyses superoxide to H_2O_2, which is successively detoxified by CAT and GSH-Px [43]. Failure in any of these enzyme systems results in damaged cells/tissue and the generation of free radicals, subsequently inducing the production of intracellular ROS, triggering protein and DNA oxidation, as well as lipid peroxidation [43].

Lipid peroxidation of polyunsaturated fatty acids present on the membrane surface of cells is caused by the deleterious effect of oxygen free radicals [43]. The lipid peroxidate in turn disintegrates, with subsequent liberation of carbonyl fragments, predominantly malondialdehyde (MDA) [43]. MDA is an end product of lipid peroxidation and a reactive electrophilic aldehyde species [46, 47]. MDA can be used a biomarker in measuring levels of oxidative stress in cells and reflects cellular injury. Some studies indicate that increased

oxidative stress and cell damage correlate with decreased of the Na^+ -K^+ –ATPase activity and damage to the α subunit of the pump caused by free radical hydrolysis [48, 49]. Ultimately, the cell and organelles' physiology and osmoregulatory ability is compromised since the ability of the plasma membrane to control the passage of ions and water is disrupted. Oxidative stress has been implicated in a myriad of biological processes and pathological conditions including aging, inflammation, carcinogenesis, ischemic-reperfusion, AIDS, Parkinson's disease, Alzheimer's disease, Huntington's chorea, and cataract formation [43, 50].

The death of a cell may follow either an apoptotic or a necrotic pathway. Oxidative stress levels would be required to reach a certain threshold before induction of cell directed apoptosis may occur, however, beyond this level, necrosis is the result (Figure 1.3). The necrotic pathway is caused by acute cellular dysfunction due to severe stress or toxic injury. It is characterised by ATP depletion before an increase in cellular volume and finally rupture of the cellular membrane [51]. This is destructive as the contents of the dying cell inflicts further damage on neighbouring cells and results in inflammation [52]. Hence, the aim of therapeutic intervention is to remove free radicals and prevent their formation [43].

1.3.2 Cellular antioxidant systems and the relation to redox stress

1.3.2.1 Glutathione system

The glutathione system GSSG (oxidised form)/2GSH (reduced form) couple is the cell's major thiol-disulphide redox buffer and also the major contributor to the overall redox environment of the cell [44]. It is present in cells in concentrations of 1-11mM; hence it ranks

higher in concentration than all other redox active components [44, 53, 54]. GSH and GSSG are also found extracellularly in lower amounts of 100 – 1000 times less than intracellular levels. The GSSG/GSH system is involved in detoxification processes and protection against oxidative injury [54]. The principal location of GSH biosynthesis occurs in the cytosol from L-glutamate and is a result of the actions of two enzymes, γ-glutamyl cysteine synthetase (γ-GCS) and glutathione synthetase (GS) [55]. GSH flux occurs between the cytosol and intracellular compartments [56], with the exception of certain organelles (mitochondria, endoplasmic reticulum and nucleus). The nucleus has stringent requirements to maintain its levels of GSH at all times as critical protein sulfhydryls are required for DNA repair and expression [56-59].

Oxidative stress induces the formation of GSSG at the expense of GSH, resulting in a more positive potential ratio of GSH/GSSG. This is detrimental to the cell. Therefore, the export of GSSG out of the cell serves as a protective mechanism for cells under oxidative stress [60, 61]. Inevitably this would result in a loss of glutathione from the cell, thereby decreasing the reducing capacity. The synthesis of new GSH is required to restore GSH/GSSG when the cell is under oxidative stress [44].

1.3.2.2 Other protein sulfhydryls

Although the sulfhydryl groups of glutathione have the greatest contribution to cellular redox maintenance, other proteins also contain protein sulfhydryl groups (PSH) as a result of the presence of cysteine (Cys) residues in their sequence and also contribute to cellular redox maintenance. These proteins are present in higher concentrations than glutathione and may

therefore also make significant contributions [62]. These sulfhydryl groups may be present as thiols (-SH), disulphides (PS-SP) or as mixed sulphides e.g. when conjugated with GSH (PS-SG) [63]. Enzyme activation and deactivation in a redox setting is dependent on the oxidation of thiols or reduction of disulphides. Hence, protein S-thiolation and dethiolation is a dynamic process, which is reversible and reflects the redox state of the GSH-system in the cell [64-66]. An early response to oxidative stress in a cellular environment involves the oxidation of protein sulfhydryls to form mixed disulphides. Thus, PSH play a vital role in the antioxidant network of the cell [63]. Since the redox environment of the cell is dependent on its reduction potential and reducing capacity, PSH functions as a buffer to maintain GSSG/2GSH reduction potential, enabling the GSH system to cope with oxidative stress [44].

1.3.2.3 Thiols and disulphides

The reduction potential of redox couples in a cell are akin to triggers that activate a cellular switchboard changing reduction potential and activating nano-switches that shift the cell into proliferation, differentiation or apoptosis (Figure 1.3). Alternatively, necrosis is induced, resulting in a complete loss of regulation in the cell where these nano-switches are rendered invalid. GSH exerts the greatest control over these nano-switches as it is the most abundant redox buffer. Although GSSG/2GSH controls the biological state of the cell, signalling events can change the 2GSH/GSSG ratio and initiate their own signalling.

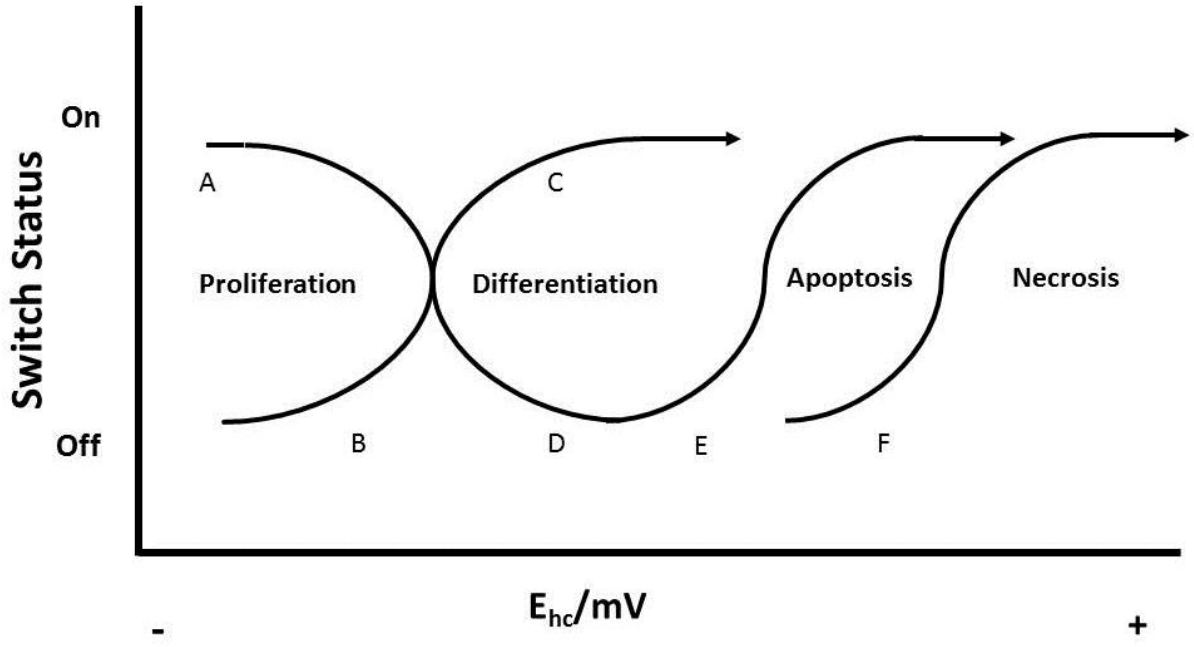

Figure: 1.3 The relationship between nano switches that modulate reduction potential and the cellular redox state.

The redox environment of a cell changes throughout its life cycle. During proliferation, the reduction potential (Ehc) for the GSSG/2GSH couple has the most negative value.

With reference to (Figure 1.3):

(A) The switches for proliferation are on.

(B) When Ehc (GSH) becomes more positive, the differentiation switches can be turned on while proliferation decreases.

(C) All of the differentiation switches are turned on as Ehc (GSH) becomes more positive, until they reach a maximum where nearly all cells are differentiating.

(D) Whilst cells undergo differentiation, proliferation switches are turned down, and finally turned off. Cells that have not differentiated may undergo proliferation with an appropriate signal.

(E) Should Ehc (GSH) becomes overtly positive, death signals are activated and apoptosis is initiated. This mechanism provides for the orderly removal of cells that have lost their ability to control their redox environment normally. It also coincides with normal signalling pathways to purposely dispose of unneeded cells.

(F) Very high values of Ehc (GSH), resulting from severe oxidative stress, leave only necrosis as a path to cell death [44].

Oxidative stimuli may not necessarily shift the cell from proliferation to differentiation [44]. Oxidative stimuli have been observed to be normal regulators of cellular transcription. When buffer limits for detoxification of oxidants have been surpassed, induction into cell death may occur [67, 68]. Therefore, an increase in the oxidative production of superoxide and hydrogen peroxide induces proliferation. It may also be responsible for cellular responses that may result in the production of reducing equivalents, generating a reducing environment [44]. Cancer can be observed as a condition where the balance of cell proliferation and cell death has been pushed into excess proliferation, suggesting the presence of an abnormal reducing environment [69].

1.3.2.4 Thioredoxin system

The thioredoxin system which is comprised of oxidised thioredoxin (TrxSS) and reduced thioredoxin (Trx $(SH)_2$) is another important cellular thiol system. It is involved in the reduction of Cys residues at the DNA binding sites of transcription factors, making it vital in gene expression [70, 71]. Mammalian thioredoxin levels are 100-1000 fold lower than glutathione [72]. The thiols in mammalian thioredoxin function as electron donors for proteins including ribonucleotide reductases and methionine sulphoxide reductases (Figure 1.4). Thioredoxin facilitates the refolding of disulphide containing proteins and regulates the DNA binding activity of transcription factors [72, 73]. In comparison to the smaller antioxidant glutathione molecule which only forms inter-molecular disulphides, thioredoxin is capable of forming intra-molecular disulphides. Thioredoxin reductase (Trx R) is also known to catalyse the reduction of disulphides to thiols, utilising NADPH as an electron source [74, 75].

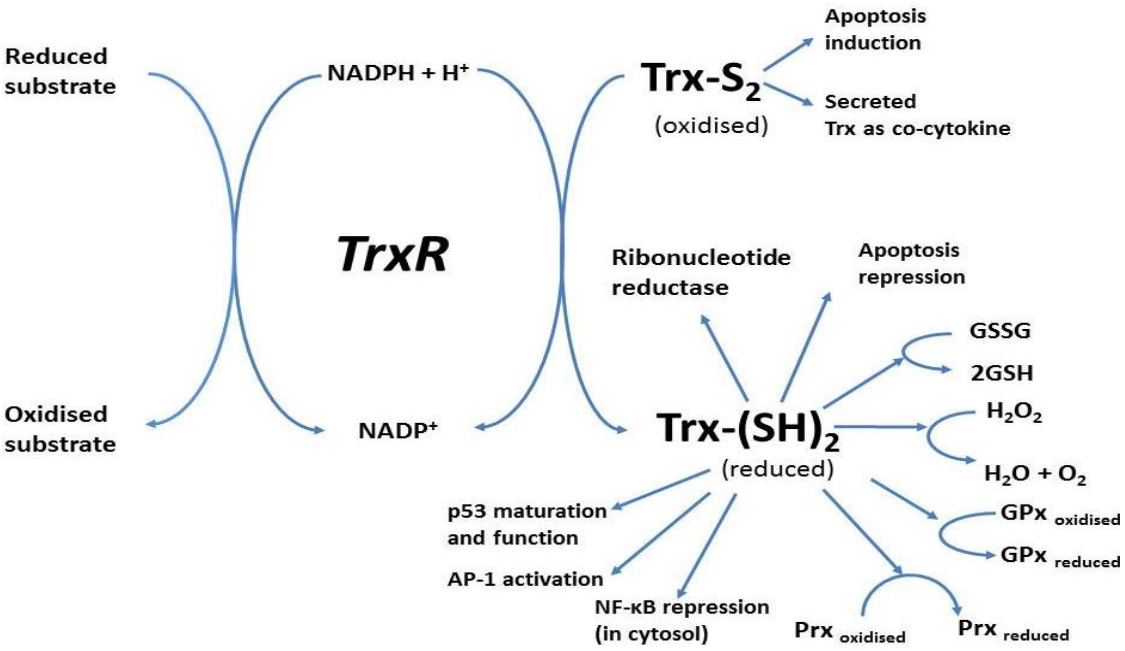

Figure: 1.4 : The mammalian thioredoxin system that functions to modulate the cellular redox state and its relation to proliferation/apoptosis. Adapted from Arner, 2011 [76].

1.3.3 Cell processes governed by cellular redox state

The major pathways involved in cell signalling are dependent on protein phosphorylation and the status of thiol group(s) in proteins under cellular redox control [77]. The thiol status is dependent on redox cascades triggered by oxidative and reductive stresses experienced by the cell. Therefore, any changes in cellular redox environment affects signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, regulation of cell cycle, ligand binding, DNA binding and nuclear translocation [78, 79]. Transcription factors are active in their reduced form and their translocation to the nucleus is redox dependant [80]. Furthermore, high concentrations of thiols stimulate proliferation of cells. This is consistent with a more reducing environment. Conversely, an oxidising environment initiates cell death where pro-oxidants increase the reactive oxygen species, inducing apoptosis [81-84]. Antioxidants such as GSH and thiol containing proteins (e.g. thioredoxin) are reducing agents and have been reported to prevent apoptosis [85-87]. Therefore, the redox environment may determine if a cell will proliferate, differentiate or die [88].

1.3.3.1 Proliferation and differentiation

Cellular proliferation requires high GSH levels and low levels of the anti-oxidant proteins SOD, GPx and CAT, favouring a reducing environment [89-91]. The redox environment of the cell has a significant role in cell proliferation as sequential changes in antioxidant profiles

suggest that GSH levels fluctuate in response to cell cycle progression [44]. Low levels of these antioxidants induce cell proliferation, whilst high levels inhibit it [69, 92-95]. Therefore, proliferation favours a reducing environment whilst differentiation requires an oxidizing environment [96, 97].

1.3.3.2 Cell death

Oxidising environments may predispose a cell to apoptosis or necrosis, depending on the degree and nature of the oxidative stimuli [50]. Moderate lethal oxidative stimuli induce apoptosis, whereas severe oxidative stress may result in necrosis. Apoptosis requires ATP for organised cell death. The stages of apoptosis are:

(a) Initiation – signal is received,

(b) Effector phase – signal is integrated and cell fate is decided,

(c) Irreversible execution phase – digestion of DNA and protein occurs. Severe oxidative stress depletes energy and further damages the mitochondrial machinery.

1.3.3.3 Regulated cell death (apoptosis)

Apoptosis is a regulated cell death programme that involves a mechanism of cell replacement, tissue remodelling and subsequent removal of damaged cells [98, 99]. Apoptosis is characterised by cell shrinkage, chromatin condensation, inter-nucleosomal DNA

fragmentation and the formation of apoptotic bodies [100-102]. Caspases are central to apoptosis and eliminating caspases through mutation or pharmacological inhibitors may slow down or block apoptosis [103].

1.3.3.3.1 Caspase mediation of apoptosis

Caspases are calcium-dependant cysteine proteases that cleave proteins following aspartic acid residues. They exist in the cytosol, mitochondrial inter-membrane space and nuclear matrix as soluble inert zymogens (inactive enzyme precursors) in most cells [104]. Their active sites contain cysteine residue(s). There are twelve caspases of which six are known to participate in apoptosis [103]. Caspases are subdivided into subfamilies based on their substrate preference, extent of sequence identity and structural similarity [105]. The inert caspases (zymogens) have three domains: an N-terminal prodomain, a p20 domain and a p10 domain [105]. Caspases act via selective cleavage of the target proteins following an aspartate acid (Asp) residue, thereby inactivating the target protein [105]. Caspases have also been known to activate proteins by cleaving off negative regulatory domains directly, or by inactivating a regulatory subunit [105].

1.3.3.3.2 Two major apoptotic pathways in mammalian cells

Apoptosis can occur through either an extrinsic or an intrinsic pathway (Figure 1.5). The death-receptor pathway (extrinsic pathway) is triggered by death receptor CD95 and tumour necrosis factor receptor I. Binding of CD95 ligand to CD95 induces the formation of a death inducing signalling complex via the adaptor molecule FADD (Fas-associated death domain

protein) with multiple procaspase-8 molecules, resulting in caspase-8 activation by induced proximity. Caspase-8 activation may in turn be blocked by recruitment of the caspase homologue c-FLIP [105].

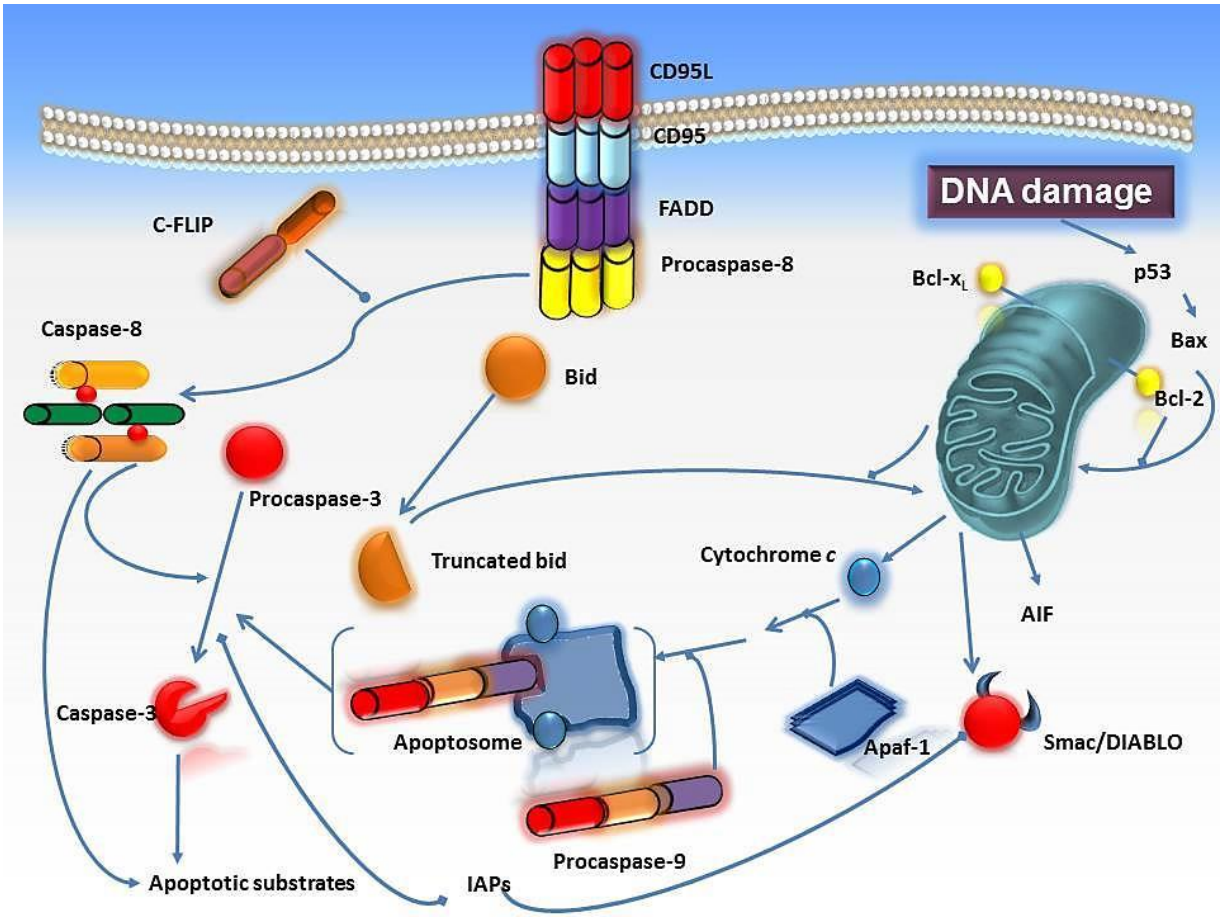

Figure: 1.5 : A representation of the two major apoptotic pathways in mammalian cells adapted from Hengartner. [105]

The mitochondrial pathway (intrinsic pathway) is triggered in response to both extracellular signals and internal insults such as DNA damage. Activation of pro-apoptotic Bcl-2 members can occur through proteolysis, dephosphorylation, as well as several other mechanisms. Pro- and anti-apoptotic Bcl-2 family members at the surface of mitochondria compete to regulate

cytochrome c exit [105]. If the pro-apoptotic numbers are high, cytochrome c is released from the mitochondria and associates with Apaf-1 and then procaspase-9 and other proteins to form the apoptosome [105].

Death-receptor (extrinsic) and mitochondrial (intrinsic) pathways converge at the level of caspase-3 activation [105]. This activation is further regulated by the inhibitor of apoptosis protein (IAP) and by the second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) protein which is released from the mitochondria. Downstream of caspase-3, the apoptotic programme branches into a multitude of sub programmes, the sum of which results in the ordered dismantling and removal of the cell [105]. Signal integration between the death-receptor and mitochondrial pathways is provided by Bid, a pro-apoptotic Bcl-2 family member. Caspase-8-mediated cleavage of Bid greatly increases pro-death activity, resulting in its translocation to mitochondria, where it promotes cytochrome c exit [105].

1.4 Therapeutical intervention in cancer: current cancer targets and chemotherapies

Many commonly used anticancer agents (e.g. doxorubicin, daunorubicin, mitomycin C, etoposide, cisplatin, arsenic trioxide, ionising radiation, photodynamic therapy) rely either exclusively, or partly on reactive oxygen species (ROS) for induction of cytotoxicity. The success of the treatment depends on the sensitivity of the tumour cells to oxidative stress and their ability to undergo apoptosis [106, 107]. WEH17.2 mouse thymoma cells, which either over express catalase (CAT) or thioredoxin (THX), are resistant to glucocorticoid-induced

apoptosis *in vitro* [108-110]. This suggests that glucocorticoid-induced apoptosis occurs by a ROS dependent mechanism. The same study revealed a significant gain in tumour weight of severe combined immune-deficient (SCID) mouse tumour xenografts that overexpress catalase or thioredoxin [109, 111]. Interestingly, the tumours from both transfectants had similar mitotic cell number, but a markedly lower number of apoptotic cells [41]. Therefore, glucocorticoid-induced apoptosis occurs via a ROS dependant mechanism. An increased overexpression of antioxidants leads to a decrease in apoptosis, subsequently resulting in an increase in tumour size/progression.

In summary, carcinogenesis as a result of oxidative stress leads to genetic instability that would cause the emergence of new tumour phenotypes. In such populations, decreases in apoptosis and increases in tumour growth and subsequent tumour progression are observable. ROS based therapy to treat cancer would be expected to induce tumour regression if the tumour is not comprised of apoptotic/oxidant-resistant cells. In contrast, ROS based therapy on tumours with apoptotic resistant/oxidant resistant cells would escalate tumour progression.

Studies into the anti-oxidant or pro-oxidant behaviour of plant extracts from other species to those examined in this project have reported that the ability to function as an antioxidant or pro-oxidant is conditional and is concentration dependent. For example, the (*Aloe barbadensis* Miller) anthraquinone aloe emodin functions as an antioxidant at lower concentrations, yet as a potent pro-oxidant at high concentrations [4]. In contrast, a different *Aloe vera* anthraquinone (aloin) has pro-oxidant effects at lower concentrations, yet an antioxidant effect at higher concentrations. Thus, *Aloe vera* extracts and components may act

as either antioxidants/oxidants, dependant on the concentrations of the various constituents, and on their ratios. The redox environment also affects cellular signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, regulation of the cell cycle, ligand binding, DNA binding and nuclear translocation and therefore, ultimately cell proliferation/death [77, 78]. Transcription factors are active in their reduced form and their translocation to the nucleus is also redox dependent [80].

Conditions favouring antioxidant activity would be expected to favour cellular proliferation whilst conditions favouring pro-oxidant activity would favour cell death. Many phenolic compounds could potentially behave as either antioxidant or pro-oxidant dependant on their concentration, redox state and ratio between compounds [8]. Antioxidant phytochemicals such as flavonoids [112] and tannins,[113] can also function as pro-oxidants when present in high concentrations [114].

Transition metal ions such as copper or iron present in plant extracts could further enhance the conversion of the antioxidant to the pro-oxidant state [112-114]. The balance between the free radical scavenging activities and reducing power of phytochemical components is crucial in the determining of the pro-oxidant/antioxidant effect of plant extracts [115, 116]. This can be illustrated using the antioxidant vitamin ascorbic acid as an example. Ascorbic acid is well characterised by its known antioxidant bioactivities, but has also been reported to function as a pro-oxidant at high concentrations [117, 118]. Furthermore, the presences of transition metal ions facilitate ascorbic acid's function as a reducing agent by its own oxidation, hence transforming into a pro-oxidant. Therefore, in individuals with high iron levels (e.g.

premature infants, hemochromatosis), high dietary intake of ascorbic acid may pose detrimental health effects by induction of oxidative damage to susceptible biomolecules [118-120]. Conflicting reports suggest that ascorbic acid may not be able to function as a pro-oxidant *in vivo*, since transition metal ions (e.g. iron or copper) are normally sequestered by proteins and are unable to induce free radical mediated oxidation, although the reverse is true *in vitro* [121, 122].

ROS based tumour therapy would cause tumour regression only if the tumour cells are not apoptotic/oxidant-resistant cells. The extract would be expected to induce apoptosis and would have anticancer activity if phytochemical antioxidants were present in concentrations and ratios consistent with pro-oxidant activity. In contrast, antioxidant activity would be observed and the extract may have a proliferative activity if the levels of components were consistent with a reducing environment [8].

1.4.1 Polyphenols as antioxidants

Polyphenols (e.g. flavonoids, phenolic acids, tannins), alkaloids, chlorophyll derivatives, tocopherols, carotenoids and ascorbic acid [123] are important bioactive phytochemicals with strong anti-oxidative activity [124-127]. The radical scavenging ability of polyphenolic compounds is attributed to the presence of multiple hydroxyl groups [127-130]. Polyphenols can scavenge free radicals and prevent cellular damage via their interaction with receptors and enzymes involved in signal transduction through the up-regulation of the anti-oxidant/detoxification enzymes, modulation of cell signalling and gene expression [131, 132].

Alternatively polyphenols may influence vital cellular and molecular mechanisms associated with carcinogenic progression. These processes may include the expression of key proteins in signal transduction pathways such as the mitogen activated protein kinases (MAPKs), activator protein (AP-1), nuclear transcription factor (NF-κB), as well as its downstream gene products that are involved in cell cycle regulation and the induction of apoptosis [133]. Subsequently, polyphenols affect cell differentiation, proliferation, apoptosis, immune responses and the metabolism of carcinogens [123]. One of the most prevalent classes of polyphenols is the flavonoids. Flavonoids have important roles in plant biochemistry and physiology, functioning as anti-oxidants, enzyme inhibitors, and precursors of toxic substances, pigments and as light screening compounds [134, 135]. In plants, they play a crucial role in maintaining and coordinating normal physiology via their roles in energy transduction through photosensitisation, as growth hormone effectors and growth regulators, via the regulation of respiration, photosynthesis, morphogenesis, sex determination and via defence against infection [136].

Research has determined a positive correlation between the activation of bacterial (*Rhizobium*) modulation genes involved in the control of nitrogen fixation and the presence of flavonoids responsible for activation and expression of mammalian genes [137-140]. Flavonoids can initiate anti-inflammatory, anti-oxidant, anti-allergic, hepatoprotective, antithrombotic, antiviral, and anti-carcinogenic activity [141-150]. Since flavonoids are phenolic compounds, they act as potent metal chelators and free radical scavengers [151-155]. They display a remarkable array of biochemical and pharmacological actions. Therefore, it is believed that flavonoids may affect multiple important functions of mammalian cellular systems. Polyphenolics inclusive of flavonoids have been linked with the prevention of

chronic degenerative diseases including cancer as well as cardiovascular and neurodegenerative diseases [156-162]. Indeed, positive correlation has been reported between the consumption of high levels of polyphenolic compounds and decreased morbidity. [156, 163-167] The anti-oxidant activity of polyphenolics imbues the mammalian system with anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activity. [127] Furthermore, several plant phenolic compounds have been reported to inhibit carcinogenesis and mutagenesis [168, 169]. Subsequent studies have demonstrated that polyphenols may have both anti-proliferative and cytotoxic activities towards several tumour cell lines [170, 171]. A Finnish study established that there was an inverse correlation between intake of flavonoids and cancer incidence in several organs [167].

1.5 Current research into plants as a therapeutic agents

Despite the therapeutic benefits of plants, only approximately 15% of the estimated 250,000 species of higher plants worldwide have been systematically researched for any therapeutic properties [172]. The number of plants that have been screened for their anticancer potential is significantly lower. However, about 25% of the modern pharmacopoeia is constituted of plant derived medicines, whilst many other drugs are synthetic analogues built on prototype compounds isolated from plants [173].

Although not necessarily founded, the common perception is that natural therapeutics offer a safer alternative than synthetic formulations. The traditional knowledge involved in these medicinal preparations has been refined and passed on over long periods of time. However in many regions internationally, this knowledge is currently being lost through cultural polarisation. Current trends indicate that 65% of the world's population have assimilated plant-based therapies into their primary modality of health care, despite the availability of allopathic medicines. This is particularly evident in India (Ayurveda) and China (Traditional Chinese Medicine (TCM)) [174] where traditional medicines remain ancient yet living traditions. This is in contrast to the many other indigenous systems of medicine around the world which are instead becoming obsolete and much of the traditional knowledge has been lost. The usage of herbal medicines in societies where traditional medicine is still prevalent is primarily driven by the safety, efficacy, cheaper prices and the perception of lower incidence of side effects [175, 176]. The current demand for medicinal plants worldwide is estimated by the World Health Organization (WHO) to be at US $14 billion per year [177], and is predicted to increase to US $5 trillion per year by 2050 [176].

1.5.1 Neglect of Australian flora and medical significance

The lack of research into the phytochemistry and bioactivity of the diverse flora found in Australia is rather unexpected, given the unique and endemic flora, as well as the long history of medicinal plant usage by the first Australians. Recent pharmacognostic and ethnobotanical research has largely targeted plants in Asia, Africa and South America in the hope of finding novel species and possible 'miracle cures'. This could in part be related to the economic

infrastructure of third world countries which have welcomed this research as a means to improve their economic climate, trade or tourism. In contrast, comparatively little interest has been kindled into phytotherapeutic research of the local flora in Australia and with some notable exceptions. Australian flora is generally not viewed as a valued resource. Moreover, all efforts of reviving and sustaining the gross domestic product in Australia have largely been geared towards ecotourism, the mining industry, cattle farming and conventional agriculture.

Australia has the highest degree of endemism of its plants of any region internationally, yet the population is slow to realise the potential of its plants. For example, the *Eucalypt* spp. are a large group of trees endemic to Australia. They have well established antiseptic properties. The market for eucalyptus oil worldwide is huge, yet Australian production only accounts for approximately 5% of the world market, with Spain and Portugal producing the bulk of the world's supply of eucalyptus oil from introduced trees [178]. Another example is the Kakadu plum (*Terminalia ferdinandiana*) which is an endemic species with a huge potential for food and medicinal industries. Much of the interest in this plant lies in its exceptionally high levels of vitamin C. Indeed, recent studies have reported Kakadu plum to be the highest natural source of vitamin C worldwide [179]. Furthermore, that study also reported an extremely high total antioxidant content for this plant. The American company Mary Kay Cosmetics was quick to realise the potential of Kakadu plum and have sought to patent its use for cosmetics, despite the fact that Australian Aborigines have utilised Kakadu plums for thousands of years, both as a food source, and as a medicinal agent. Mary Kay Cosmetics have aggressively attempted to patent the skin protective and regenerative effects of Kakadu plum

as they feel that the intellectual property does not exist to demonstrate that Aborigines used it for anything other than food [180].

This study will attempt to revive, recover and partially document the medicinal potential of a number of species of Australian medicinal plants with high antioxidant activity, based on their ability to inhibit proliferation of human carcinoma cell lines. Much documented evidence suggests that diets rich in antioxidants modulate the redox state of cells thus blocking their oxidative damage. Antioxidants are able to protect cell constituents against oxidative damage through scavenging/quenching of free radicals, thereby averting their deleterious effects on nucleic acids, proteins, lipids [124, 125]. The total phenolic content present in fruit extracts are strongly correlated with antioxidant activity critical in protecting cells, thereby preventing cancer [179]. The present study focusses on plant species which have been reported to have high antioxidant levels, including *Terminalia ferdinandiana* Exell (Kakadu plum), [179] *Tasmannia lanceolata* (Poir.) A.C.Sm., (Tasmannian pepper), [179] *Syzygium australe* (H.L.Wendl. ex Link) B.Hyland (brush cherry), [179] *Syzygium luehmannii* (F.Muell.) L.A.S.Johnson (riberry), [179] *Davidsonia pruriens* F.Muell. (Davidson's plum), [179] *Elaeocarpus angustifolius* Blume (quandong) [181], *Kunzea pomifera* F.Muell. (muntries) [179], *Podocarpus elatus* R.Br.ex Endl., (Illawarra plum) [182] and *Acronychia acidula* F.Muell. (lemon aspen) [183].

1.5.2 Genus *Terminalia*

The genus *Terminalia* (Family Combretaceae) is comprised of approximately 200-250 species which are widely distributed through the tropical and subtropical regions of Asia,

Australia and Africa [8]. Many *Terminalia* spp. have a history of usage in traditional medicinal systems globally. The Asian *Terminalia*'s have extensive documentation of their therapeutic effects and are often regarded as being the most useful on their basis of their traditional uses. Asian *Terminalia* spp. are distributed from the Malaysian peninsula and the Indonesian archipelago in the east, across Southern Asia to Western Asia and into the Middle East. The medicinal uses of *Terminalia arjuna* and *Terminalia chebula*, are particularly well documented due to their myriad of uses in traditional medicine, particularly in the Indian system of Ayurveda [8]. Approximately 30 species occur in Africa, with the majority of these occurring in the southern part of the continent. A further 28 species are known to occur in Australia and the South Pacific region and they are the least studied of the *Terminalia* spp. Of these, *Terminalia ferdinandiana* has been receiving recent attention due to its interesting phytochemistry, its extremely high natural vitamin C content and high anti-oxidant activity [184]. A number of *Terminalia* spp. are also native to the North, Central and South America [8].

Many *Terminalia* spp. worldwide have documented uses for the treatment of a host of medical conditions including yellow fever, pink eye, hepatitis, pain, high blood pressure, cholesteremia, inflammation, fungal infections, cough, constipation, anaemia, malaria, bacterial infections, liver disease, diarrhoea, dysentery, cancer, coughs and colds, worm infestations, digestive disorders, diabetes. [8]

1.5.2.1 Australia *Terminalia* species

Several *Terminalia* spp. are endemic to tropical northern regions of Australia. Further species (e.g. *Terminalia catappa*) have a wide global distribution, being native not only in Australia

but also in other distant regions. The best known of the Australian species include: *Terminalia arenicola*, *Terminalia australis*, *Terminalia bursarina*, *Terminalia grandiflora*, *Terminalia mauritana*, *Terminalia oblongata*, *Terminalia carpentariae*, *Terminalia hadleyana* and *Terminalia latipes*. However, perhaps the most well-known Australian *Terminalia* spp. is *Terminalia ferdinandiana* Exell, commonly known as the Kakadu plum. This species is endemic to the tropical northern areas of Australia, encompassing coastal and inland regions of the Northern Territory and the Kimberley region of Western Australia [185]. The fruit of this species has received considerable interest due to its extremely high antioxidant content [179, 181]. Levels of ascorbic acid in Kakadu plum have been reported as high as 6% of the recorded wet weight, making this the highest natural source of ascorbic acid of any plant globally. Indeed, Kakadu plum contains approximately 900 times higher (g/g) ascorbic acid content compared with blueberries (which are themselves considered to have high ascorbic acid content). As a further comparison, oranges and grapefruit only contain approximately 0.007% wet weight (0.5% dry weight) [186]. Due to its high vitamin C levels, the primary use of *T. ferdinandiana* fruit is currently for production of vitamin C in health food, cosmetic and in the pharmaceutical industry. However, *T. ferdinandiana* fruit also contain many other compounds which also contribute to its overall high antioxidant capacity/activity [179, 181].

In contrast to other regions internationally, the ethnobotanical knowledge of Australian *Terminalia* spp is limited. Reliance on an oral tradition with little written ethnopharmacological records has contributed to an almost complete loss of the first Australians traditional knowledge. The Australian Aborigines existed for thousands of years without interference, subsisting on the continent's unique endemic flora. Over time, the

various tribal groupings developed sophisticated phytochemical systems, enabling them to treat most maladies they encountered. However, the marginalisation of the indigenous population and cultural polarisation has destroyed the indigenous medical systems and the associated use of indigenous materials to such an extent that they are now generally limited to the small tribal and geographical areas [174]. This has been further reinforced by the increase in reliance on Western medicine over traditional plant based therapeutics by the first Australians [4]. There are limited records of the known Australian species that have been used as medicinal plants [4] [178].

1.5.2.2 Phytochemistry and anti-oxidant capacity of genus *Terminalia*

Terminalia spp. share several phytochemical commonalities. Many species have been reported to have high anti-oxidant contents, as well as high flavonoid and tannin levels. However, interspecies differences occur within the genus. All *Terminalia* spp. contain a number of compounds (both phenolic and non-phenolic) that can act as anti-oxidants/pro-oxidants. The individual components themselves may function either as an anti-oxidant/pro-oxidant, dependant on their concentrations/ratios, and thus may be effective in the treatment/prevention of cancer and other conditions.

The powdered tree bark of the Indian species *Terminalia arjuna* has been known to alleviate angina [187]. It is believed that this is due, at least in part, to its high anti-oxidant content [188]. Phytochemical evaluations of *T. arjuna* extracts report the presence of glycosides, flavonoids, tannins and inorganic minerals. In addition, *T. arjuna* aqueous bark extracts contain β-

sitosterol and β-sitosterol glucosides [189, 190] in a reduced form. These have anti-oxidative potential and may contribute to the therapeutic properties of this plant. β-sitosterol stimulates the release of prostacyclin (PGI_2) from smooth muscle cells, [191] and it blocks release of PGI_2 and prostaglandin E_2 (PGE_2) from macrophages [191]. Thus, β-sitosterol treatment could decrease inflammation by affecting vasodilation, and it subsequently has uses in wound management and promotion of wound healing. Conversely oxidised sterols are pro-oxidants and they induce cell death [8]. Furthermore, unusually high levels of hydrolysable tannins have been reported in *T. arjuna* extracts. In particular, pyrocatechols, punicallin, punicalagin, terchebulin, terflavin C, castalagin, casuariin and casuarinin have all been identified. All of these are strong antioxidants and therefore would affect cellular redox state and cell function [192].

Interestingly, the tannin casuarinin (Figure 1.6) isolated from bark extracts of *T. arjuna*, induces apoptosis in human A549 lung cancer cell lines [193]. Casuarinin's apoptotic property was postulated to be due to an enhanced Fas/APO-1 ratio and increased levels of the membrane bound and soluble Fas ligands [193]. In addition, casuarinin arrests cell cycle progression in the G_0/G_1 phase, possibly due to a p53 dependent induction of p21/WAF1 [193]. Thus, casuarinin has multipotent anticancer activities, acting by both cytocidal and cytostatic mechanisms.

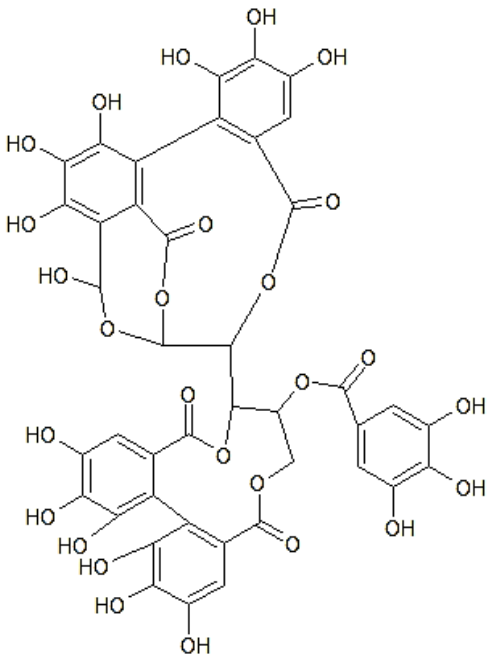

Figure: 1.6 : The chemical structure of casuarinin, an anticancer compound isolated from *T. arjuna* (reproduced with permission from the author and the publisher) [8].

Similarly, Triphala an Ayurvedic medication, is cytotoxic towards thymic lymphoma cells [194], human breast cancer cells [194, 195], human prostate cancer cells and human pancreatic cancer cells [196]. Interestingly, negligible cytotoxicity was observed in the corresponding normal cell lines [194, 196, 197]. The Triphala Ayurvedic medication is itself a combination of fruits from three different plants, of which two belong to the *Terminalia* genus (*Terminalia chebula* and *Terminalia belliricia*). It is noteworthy that whilst characterisations of the cytotoxic compounds of Triphala were not achieved, the *Terminalia* spp. fruits are very rich in antioxidants, particularly gallic acid (Figure 1.7 b) [198]. Furthermore, pure gallic acid and Triphala have similar cytotoxicity effects towards cancer cell lines [195, 199, 200]. Thus gallic acid may be a contributing factor to the observed cytotoxicity in Triphala. Further studies into

the *T. belliricia* extracts have reported growth inhibitory effects towards human A549 lung cancer and HepG2 hepatocarcinoma cell lines in the absence of the other plant compounds found in the Triphala mixture [201]. Other studies have demonstrated the ability of *T. belliricia* extracts to modulate cytotoxicity by enhancing the cytotoxicity of cisplatin and doxorubicin towards the cancer cell lines, indicating its potential to act synergistically with conventional anticancer chemotherapeutics [201]. Thus these extracts have therapeutic potential, both as a treatment in their own right, and through the potentiation of current, conventional chemotherapies.

Phytochemical analysis of *Terminalia chebula* extracts have also identified compounds with high antioxidant activity including gallic acid, ellagic acid, tannic acid, ethyl gallate, chebulic acid, chebulagic acid, corilagin, mannitol and ascorbic acid [202]. The presence of these compounds is believed to be responsible for the anti-inflammatory, cell protective and cell proliferative potential of these extracts [203-205]. Chebulagic acid displays potent anti-inflammatory activity in LPS-stimulated RAW 264.7 (mouse macrophage) cells [206]. Other studies have reported increased transcription, translation and DNA replication rates in cells exposed to chebulagic acid, [207, 208] suggesting that these effects may have been due to its very high antioxidant content. In a different study, PC12 cells were subjected to H_2O_2 induced cell death whilst exposed to chebulagic acid. Neuro-protective and cell proliferative effects were observed due to the synergistic effects of the high tannin content and polyphenolic compounds via radical scavenging activities of •OH and H_2O_2 [204]. This suggests that whilst a reducing environment favours cellular proliferation, an oxidising environment favours production of reactive oxygen species, hence initiating cell death [83, 84].

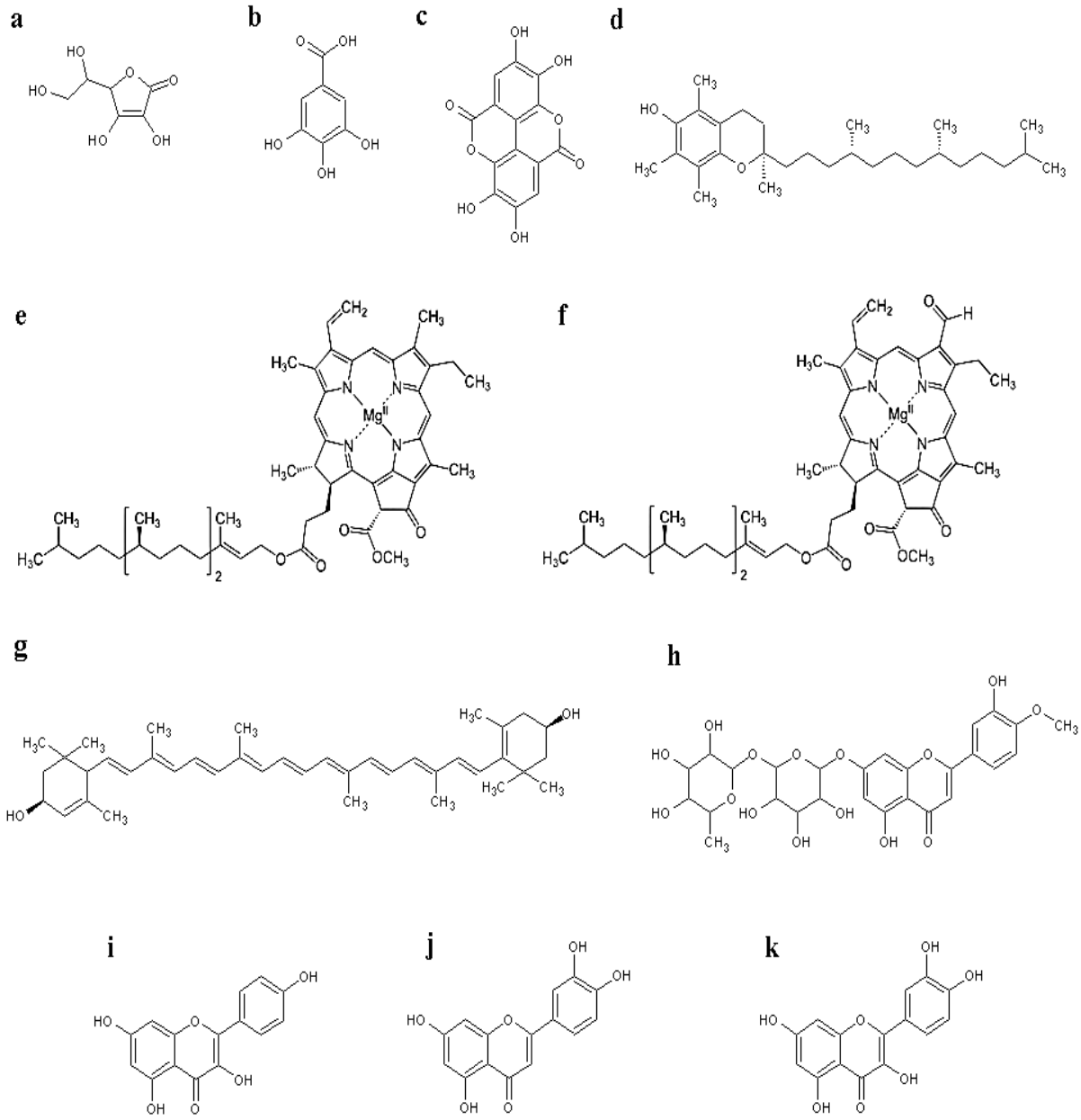

Figure: 1.7 : Chemical structures of molecules with antioxidant potential identified in *T. ferdinandiana* fruit: (a) ascorbic acid (vitamin C), (b) gallic acid, (c) ellagic acid, (d) α-tocopherol (vitamin E), (e) chlorophyll a, (f) chlorophyll b, (g) lutein, (h) hesperitin, (i) kaempferol, (j) luteolin, (k) quercetin. Reproduced with permission of the publishers and the corresponding author. [8]

Terminalia sericea, a southern African species has been found to contain sericoside (a triterpenoidal saponin) which has anti-inflammatory activity [209, 210]. Sericoside pre-treatment *in vivo* decreases the extent and severity of colon inflammation colitis in an induced inflammatory bowel disease (IBD) experimental model [209]. Some studies indicate that superoxide (O_2^-) radicals are implicated in IBD, and that sericoside may be actively involved in the quelling/scavenging of these radicals, resulting in a lessening of the observed symptoms [211]. A further study reported strong lipolytic activity following sericoside treatment of 3T3-L1 preadipocytes [212]. Similar effects have been reported for the antioxidant (-) -epigallocatechin-3-gallate isolated from green tea [213]. This suggests that lipolytic activity is a redox related phenomena. In other investigations, toxicity screenings for anticancer activity using *Artemia franciscana* lethality assays [214] revealed that *T. sericea* leaves induced a more potent cytotoxicity than the potent anticancer agent cyclophosphamide [215]. Similarly, *T. sericea* leaf ethyl acetate extracts inhibit topoisomerase II enzyme activity. This presupposes that certain phytochemicals (possibly tannins) may be responsible for its possible anticancer activity [216].

In a similar study examining *Terminalia brownii* stem wood extracts, significant cytotoxic activity (comparable to the anticancer drug cyclophosphamide) was observed [217]. In addition, toxicity screenings for anticancer activity were reported utilising a brine shrimp assay [214]. The authors reported that the stem wood extracts were responsible for significant cytotoxic activity and postulated that the extracts may have anticancer potential [217]. There was no characterisation of the active components, or reference to antioxidant activity in that study.

1.5.2.3 *T. ferdinandiana* phytochemistry and antioxidant properties

Terminalia ferdinandiana Exell, Combretaceae (Kakadu plum) has been described as a 'super fruit' with an extraordinarily high ascorbic acid [218] (Table 1.3) and polyphenolic content. [219] Indeed, the ascorbic acid content has been reported to be as high as 6% of the recorded wet weight [220, 221] (900 times that of blueberries higher (g/g), which are generally accepted as a premium standard) [8]. It is not surprising that the first Australians have used *T. ferdinandiana* as a functional food and medicine for thousands of years [222, 223].

Table 1.3 : The comparison of the ascorbic acid content of high antioxidant Australian plants against blueberry.

Fruit	**ascorbic acid (µmol/g fruit)**	**times higher than blueberry**	**TEAC (µmol/g fruit)**	**times higher than blueberry**
Blueberry (Standard)	0.076	1.0	39.5	1.0
Syzygium australe (Brush Cherry)	0.72	22.0	26.9	0.7
Syzygium leuhmannii (Riberry)	1.77	23.3	28.1	0.7
Terminalia ferdinandiana (Kakadu Plum)	71.30	938.2	204.8	5.2
Tasmannia lanceolata (Tasmannia Pepper berry)	ND	-	123.2	3.1
Kunzea pomifera (Muntries)	0.91	12	123.8	3.1
Podocarpus elatus (Illawarra plum)	ND	-	122.8	3.1
Davidsonia pruriens (Davidson's plum)	ND	-	36.5	0.9

ND: Not Detected, - : Not Determinable

T. ferdinandiana fruit contains other interesting compounds in addition to ascorbic acid, many of which may also contribute to its high anti-oxidant potential [179, 218]. High anti-oxidant activities of many plants have been linked to their polyphenolic contents [224]. Tannins and flavonoids are largely responsible for the high polyphenolic content of *T. ferdinandiana* fruit and are a distinguishing characteristic of the genus *Terminalia*. These may be partially responsible for the high reported antioxidant capacity of the fruit [224, 225]. It is believed that the exceptionally high anti-oxidant content of *T. ferdinandiana* fruit may be responsible (at least in part) for its therapeutic effects [226]. *T. ferdinandiana* fruit has also been shown to contain benzoic acids, oxalic acid, flavonols (catechins, proanthocyanidins) and flavonones [184, 218]. The fruit is also a good source of gallic acid (Figure 1.7 b) and ellagic acid (Figure 1.7 c) [227, 228], all of which demonstrate strong antioxidant activity *in vitro* [229, 230]. *T. ferdinandiana* fruit is also rich in chlorophyll a (Figure 1.7 e) and chlorophyll b (Figure 1.7 f). Chlorophyll a has been shown to be capable of relieving oxidative stress and it is anti-carcinogenic and has anti-inflammatory properties [231]. Lipophilic *T. ferdinandiana* fruit extracts are rich in lutein (a carotenoid antioxidant compound (Figure 1.7 g) associated with eye health) and with vitamin E and vitamin E analogues including α-tocopherol (Figure 1.7 d) [218]. Hesperitin (Figure 1.7 h) and the glycosides kaempferol (Figure 1.7 i), luteolin (Figure 1.7 j), and quercetin (Figure 1.7 k) are some of the other antioxidants present in *T. ferdinandiana* fruit [218]. Furthermore, *T. ferdinandiana* fruit has also been reported to have an impressive 13-fold increase of total reducing capacity and 4.1-fold increase in oxygen radical-scavenging capacity in comparison to a blueberry control [218]. The fruit is also a good source of the minerals magnesium, zinc, calcium, potassium, sodium, iron, phosphorous, manganese, copper, and molybdenum which may further potentiate the anti-oxidant capacity of *T. ferdinandiana* fruit [218].

In a recent study, a *T. ferdinandiana* fruit extract displayed anti-proliferative activity against a limited panel of cancer cell lines [184]. That study reported that several cancer lines (gastric and colorectal cancer lines) showed significant sensitivity to *T. ferdinandiana* fruit extracts compared to the corresponding normal cell lines. However, the proliferation of normal cell lines was also seen to be inhibited in this study. The same study examined the induction of apoptosis to understand the cancer cell viability suppression mechanisms of the *T. ferdinandiana* fruit extract. Apoptotic induction was reported to follow a mitochondrial intrinsic pathway as the extracts were shown to activate caspase-7, caspase-9 and poly (ADP-ribose) polymerase (PARP) [232]. However, that study was inconclusive due to the insufficient number and variety of cancer cell lines trialled. A larger panel of cancer lines encompassing different organ systems is required to ascertain sensitivity to *T. ferdinandiana* extracts. This is a major focus of my study. Furthermore, cell viability has not been fully established for all normal cell lines so as to deem the extract as being safe before commencement of phase 1 trials for use in nutraceutical or pharmaceutical products. The previous study used a directed fingerprinting approach and only partially characterised and identified five out of the thousand compounds present in the extracts, and almost certainly missed identifying many important compounds. Our studies will employ a non-targeted metabolomic profiling approach, using RP-HPLC MS/MS and GC-MS, to characterise the extracts as completely as possible to gain further insights into the possible antiproliferative properties and mechanisms of *T. ferdinandiana*.

1.5.3 Genus *Tasmannia* family Winteraceae

Winteraceae is a small family of flowering plants consisting of approximately 90 species of trees and shrubs divided into 5 genera (*Drimys*, *Pseudowintera*, *Takhtajania*, *Tasmannia* and *Zygogynum*) with limited geographic range [233]. The *Winteraceae* are believed to be of Gondwanan origin and have developed almost exclusively as southern hemisphere plants. Their current distribution ranges from the cool climate regions of southern Australia (particularly Tasmania) and New Zealand through to the temperate and tropical regions of Borneo, Madagascar, Molucca, New Caledonia, Papua New Guinea, the Philippines and Southern and Central America, with the majority concentrated in Australasia and Melanesia [234]. Many *Winteraceae* spp. are fragrant and are often used to produce essential oils. *Tasmannia* is one of the largest genus of the family, with approximately 30 species [235].

Members of family Winteraceae have been used for a broad range of dietary and medicinal purposes by a variety of ethnic and cultural groupings. The best documented of these is the South American species *Drimys winteri*. The stem and bark of this species has been used as a stimulant and as a tonic in traditional Brazilian medicinal systems.[236] They are also used for the treatment of a wide variety of diseases and medicinal conditions including use as an analgesic, and to treat diarrhoea, inflammation, and ulcers.[236, 237] This species also has widespread usage in the treatment of scurvy due to its high antioxidant content [238]. Several other *Winteraceae* spp. also have a history of ethnobotanical usage, usually for purposes related to their high antioxidant contents and as flavourants. Indeed, high levels of the compound polygodial (which gives the *Winteraceae* a characteristic peppery flavour) and high antioxidant contents are characteristic of several *Winteraceae* spp [237].

1.5.3.1. *Tasmannia lanceolata*

Tasmannia lanceolata (syn. *Drimys lanceolata*) (commonly known as Tasmanian pepper berry or mountain pepper berry) is a shrub which is endemic to the woodlands and cool temperate rainforests of Tasmania and the south-eastern region of the Australian mainland. As with many other Winteraceae species, *T. lanceolata* berries, leaves and bark have historical uses as a food and as a medicinal plant [239] by the first Australian people, and more recently by European settlers. Historically, the leaves have been used as an herb and the berries have been used as a spice. The first Australians also used *T. lanceolata* as a therapeutic agent to treat stomach disorders and as an emetic, as well as general usage as a tonic [4, 178]. Reports also exist of the use of *T. lanceolata* by the first Australians for the treatment and cure of skin disorders, venereal diseases, colic, stomach ache and as a quinine substitute [4, 240, 241]. Later, European colonists also recognized the therapeutic potential of *T. lanceolata* and the bark was used as a common substitute for other herbal remedies (including those derived from the related South American *Winteraceae* spp., *Drimys wintera* (winter bark) [242] which is used to treat scurvy due to its high antioxidant activity) [4] [178].

Recent studies have documented the exceptionally high antioxidant content of *T. lanceolata* [179, 181]. These studies have reported that *T. lanceolata* leaves have antioxidant contents more than four-fold higher than those reported for blueberries (which themselves are considered to have high antioxidant content). Interestingly, ascorbic acid (which itself makes a significant contribution to the antioxidant content of many fruits) was reported to be below the threshold of detection in this study and therefore would not contribute significantly to the high antioxidant content of *T. lanceolata*. Furthermore, the levels of *T. lanceolata* leaf phenolic

antioxidants were reported in the same study to be over 3 fold higher than the levels in blueberries [179]. The antioxidant phenolic contents of *T. lanceolata* berries are also high, although these levels are significantly lower (less than 20%) compared with the leaf phenolic antioxidant levels.

T. lanceolata leaves and berries also contain numerous other compounds which contribute to their high antioxidant activities [179, 181]. Whilst many of these compounds are yet to be identified, the berry has been shown to contain benzoic acids, flavonols, or flavonones [181]. *T. lanceolata* is a good source of eugenol (Figure 1.8 a), methyl eugenol (Figure 1.8 b) and gallic acid (Figure 1.7 c) [227, 228] all of which demonstrate strong antioxidant activity *in vitro* [229, 230] *T. lanceolata* fruit extracts are also rich in lutein (Figure 1.8 g) a carotenoid antioxidant compound associated with eye health) and with vitamin E (Figure 1.8 h), vitamin A (Figure 1.8 i) and folic acid (Figure 1.8 j) [181]. The glycosides quercetin (Figure 1.8 e) and rutin (Figure 1.8 f) are some of the other antioxidants present in substantial levels in *T. lanceolata* berries and leaves [181]. *T. lanceolata* berry has a very high antioxidant content and it is possible that the individual components may act as either antioxidants or as oxidants, and thus may also be effective in the treatment of cancer, as well as in its prevention at different concentrations. Preliminary studies in our group have reported the ability of *T. lanceolata* to affect inflammatory processes (e.g. cytokine release) [243].

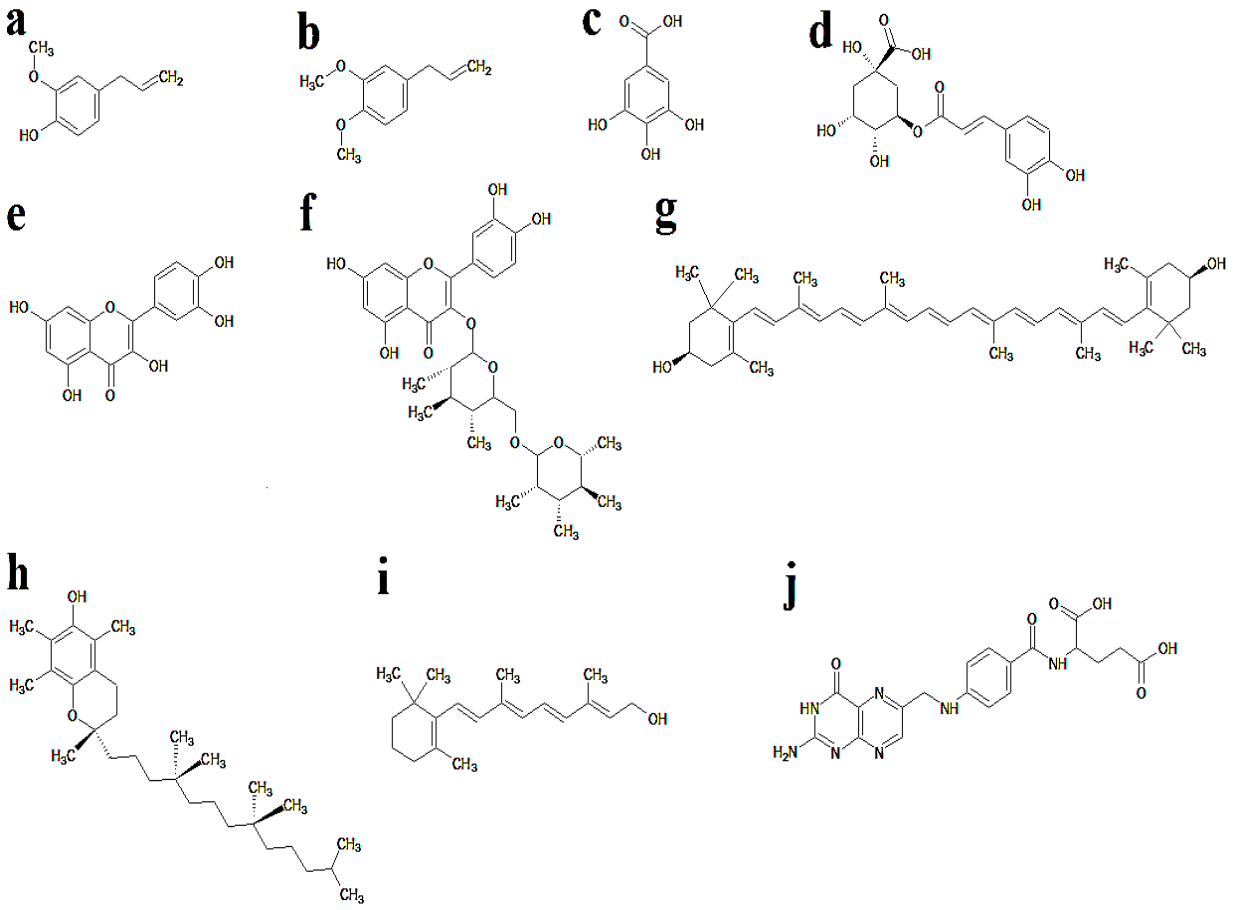

Figure: 1.8 : Chemical structures of selected antioxidant molecules identified in *T. lanceolata*: (a) eugenol, (b) methyl eugenol, (c) gallic acid, (d) chlorogenic acid, (e) quercetin, (f) rutin, (g) lutein, (h) α-tocopherol (vitamin E), (i) vitamin A, (j) folic acid.

A recent study used a metabolomic finger printing approach to examine the phytochemistry of *T. lanceolata* extracts [243]. That study aimed to screen the extracts for presence of stilbene compounds, particularly the common anti-inflammatory stilbene agent 3,5,4'-trihydroxy-trans-stilbene (resveratrol) (Figure 1.9 a) [244, 245]. This compound can block cytokine production and inflammation via its inhibition of NF-κB activation [244]. Studies have reported resveratrol's ability to act as a potent specific inhibitor of NF-κB activation through its

induction by TFN-α and IL-1β [244]. Thus, resveratrol (if present) may block cytokine production and inflammation via its inhibition of NF-κB activation. Whilst that study did not detect resveratrol, the presence of piceid (resveratrol glycoside) (Figure 1.9 b) was interesting as it is likely that it may have similar properties [243]. Interestingly, this study also reported the presence of several combretastatins in the extracts (Figure 1.9 c-d). Combretastatins are well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin at the colchicine binding site, thereby disrupting microtubule formation [246, 247]. Thus, any stilbenes (if present) may contribute to the anti-inflammatory activities of *T. lanceolata* fruit extracts by blocking cytokine production and inflammation, via its inhibition of NF-κB activation.

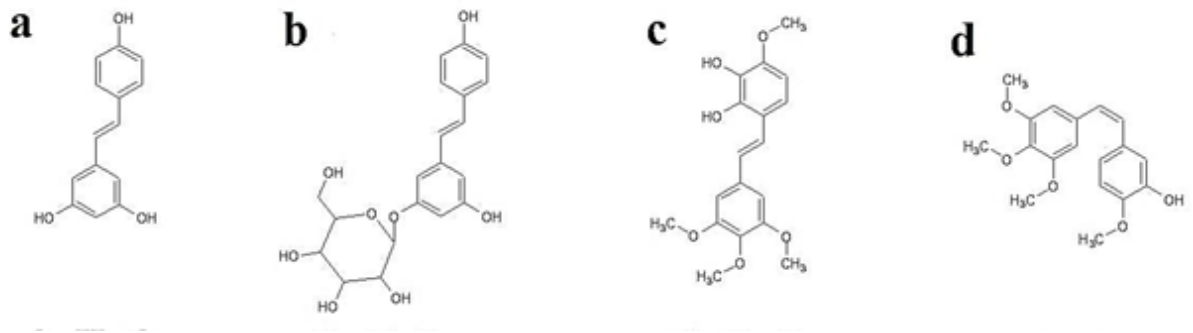

Figure: 1.9 : Chemical structures of (a) resveratrol and the stilbenes and stilbene glycosides identified in *T. lanceolata* extracts: (b) piceid, (c) combretastatin A-1, (d) combretastatin A-4.

Several terpenoids have also been identified in *T. lanceolata* fruit extracts. Several of these are known suppressors of NF-κB signalling, which plays a major role in progression of inflammatory diseases and cancer [248]. NF-kB signalling represents the link between inflammation and cancer development and progression and is therefore a potential anticancer target [249]. In particular, the monoterpenes limonene and α-pinene have been isolated in the *T. lanceolata* fruit extracts. Limonene [250, 251] and α-pinene [252] have been reported to inhibit NF-κB signalling pathways. Furthermore, other sesquiterpenes and sesquiterpene lactones present in *T. lanceolata* also have well established anti-inflammatory activities [248]. Whilst much work is still needed to characterize the mechanisms of action of these compounds, it appears that NF-κB inhibitory activities may be responsible [243].

1.5.4 Genus *Syzygium*

The genus *Syzygium* (family Myrtaceae) is a large genus of evergreen flowering plants which consists of approximately 1100 species [253]. Plants of this genus are widespread internationally, occurring in tropical and subtropical regions of South-East Asia, Australia, New Zealand and Africa [254, 255]. Many *Syzygium* species produce edible fruits and berries (e.g. *Syzygium jambos*, commonly known as rose apple). Past studies have revealed that *Syzygium* spp. have high antioxidant contents [181, 183]. In the commercially most important species *Syzygium aromaticum* (clove), the unopened flower bud is used as a spice [256]. In addition, it has been used to treat respiratory and digestive ailments because of its antibiotic properties [256]. This plant has ancient ethnopharmacological uses in traditional medicine as a traditional mouthwash and for the treatment of toothaches due to its anaesthetic properties

[256, 257]. The microbial activity of *S. aromaticum* is also well known. Numerous studies have reported on the antibacterial [258], antifungal [259], anticancer [256] and anti-inflammatory [256] activities of oils and extracts from this plant.

Phytochemical analyses of clove extracts have indicated the presence of the sesquiterpenes eugenin and eugenol in relative abundance [260, 261]. Zheng reported a significant increase in glutathione S transferase levels (GST) in the livers of laboratory animals exposed to clove terpenes, which is believed to be a primary mechanism for carcinogen detoxification. This increased activity of the detoxifying enzyme system may aid in inhibiting carcinogenesis. Recent studies revealed that the *Syzygium aromaticum* L. aqueous extracts possess chemopreventive potential against cancer due to their ability to induce apoptosis and its anti-proliferative effects [256]. *S. aromaticum* extracts function via a downregulation of the expression of certain growth-promoting proteins through the COX-2, cMyc and Hras pathways [256]. The inhibition of COX-2 is also likely to contribute to its anti-inflammatory and anti-carcinogenic potential in the chemoprevention of lung carcinogenesis [256].

Syzygium cumini L**.** seed extracts contain high amounts of ellagic acid/ellagitannins [262]. In contrast, the berry has been reported to contain high amounts of anthocyanidins: malvidin, petunidin, delphinidin, cyanidin, and peonidin [263]. Interestingly, the same extracts were shown to have significant anti-proliferative activity against human lung cancer A549 cells [263], indicating that these phytochemicals may contribute to the anticancer properties of *S. cumini*.

1.5.4.1 Australian species: *Syzygium australe* (brush cherry) and *Syzygium leuhmannii* (riberry)

Fifty-two species of *Syzygium* have been reported in Australia and are generally known as lillipillies, brush cherries or satinash. Much of our knowledge about the medicinal application of Australian *Syzygium* species is anecdotal, although Australian Aborigines are known to have used them for a wide range of therapeutic purposes [4]. In a recent study by Netzel, the *Syzygium australe* (H.L. Wendl. ex Link) B. Hyland (Brush cherry) and *Syzygium leuhmannii* (F. Muell.) L. A. S. Johnson (Riberry) were reported to have higher levels of phenolic compounds, and more potent scavengers of free radicals than blueberries [179]. Despite this, reports of the antiproliferative activity of the Australian *Syzygium* spp. are lacking. In a recent study, a *Syzygium paniculatum* extract was reported to have potent antioxidant activity and to induce significant antiproliferative activity against MiaPaCa-2 and ASPC-1 pancreatic cancer cells [264]. This activity was reported to be comparable to the first line chemotherapeutic agent gemcitabine.

1.5.5 Genus *Davidsonia*

The genus *Davidsonia* (family Cunoniaceae) is comprised of three species; *Davidsonia jerseyana* Bailey (Davidson's plum), *Davidsonia johnsonii* J. B. Williams & G. J. Harden (smooth Davidson's plum), and the common species *Davidsonia pruriens* F. Muell., family Cunoniaceae [265, 266]. *Davidsonia jerseyana* grows in the subtropical rainforests of northern New South Wales (NSW). In contrast, *Davidsonia pruriens* grows in north-east Queensland

[267], whilst *Davidsonia johnsonii* is found in southeast Queensland [265]. *Davidsonia* spp. develop into small trees [268] with each species producing an edible fruit similar in form, colour, size and substantially more astringent in flavour to the European plum [269].

The *Davidsonia* genus has been studied for both antibacterial [183, 270, 271] and antioxidant properties [183, 272], yet studies into their anticancer properties are lacking. *Davidsonia pruriens* is rich in polyphenolic compounds and is particularly high in flavonoids [273]. A recent study identified ellagic acid in *D. pruriens* extracts by high performance liquid chromatography-diode array detection (HPLC-DAD) utilising a targeted approach [273-276]. The same study reported *D. pruriens* extracts to have comparable anti-proliferative activity to blueberries against the HT-29 colon adenocarcinoma and HepG2 hepatic carcinoma cells. The extracts were not effective against stomach adenocarcinoma cells (AGS), non-transformed colon cells (CCD-18Co) and mixed stomach/ intestine (Hs 738.St/Int) cells. However, that study targeted only a specific group of compounds. Thus, many novel compounds of interest may have not been identified. It is possible that most potent antiproliferative compounds were overlooked.

1.5.6 Genus *Elaeocarpus*

The *Elaeocarpus* genus comprised of approximately 200 tropical and subtropical species, with approximately 20 species endemic to Australia [268]. Considered important more for its valuable timber [277] and rainforest restoration functionality [278] than for nutritional and medicinal properties, there is little evidence in existing literature of any reported antioxidant

Elaeocarpus spp. linked with antiproliferative activities. Furthermore, no extensive phytochemical studies of the Australian *Elaeocarpus* spp. exist. Perhaps the most well-known *Elaeocarpus* species is the blue Quandong (*Elaeocarpus angustifolius* Blume (family Elaeocarpaceae [279, 280], formerly known as *Santalum acuminatum* A.D.C., Santalaceae [273]. Studies by Netzel and Konczak have reported that *E. angustifolium* possesses high antioxidant and polyphenolic contents [179, 273]. *E. angustifolium* extracts efficiently reduce the H_2O_2 damage in HepG2 cells by reason of it polymeric compounds. Anti-proliferative studies have reported that *E. angustifolium* extracts were effective against HT-29 (human colorectal adenocarcinoma) cells [273].

1.5.7 Genus *Kunzea*

Genus Kunzea family Myrtaceae is native to the Oceania region, with species predominantly distributed in south Western Australia. A total of 30 known species are found between Australia and New Zealand. Indigenous people from New Zealand have used essential oils from these species for the treatment of diarrhoea, colds, inflammation and wounds [281]. *Kunzea* spp. are rich in terpenoids, tannins, flavonoids and phloroglucinol derivatives [282]. *Kunzea pomifera* F. Muell., (family Myrtaceae) commonly known as muntries, [184] possesses moderate to high levels of phenolics, high antioxidant contents and also a relatively high abundance of anthocyanins [232]. The same study tested the *Kunzea pomifera* extracts against CCD-18Co (colon normal), HT-29 (colorectal adenocarcinoma), AGS (gastric adenocarcinoma), Hs 738.St/Int (mixed stomach and intestine normal), HL-60 (promyelocytic leukaemia), and PBMC (peripheral blood mononuclear cell) [232]. The *K.*

pomifera extracts were antiproliferative and pro-apoptotic against the respective cancer cells compared to the normal cell lines. Further, it was also noted that *K. pomifera* inhibited HL-60 cells significantly compared to PBMC indicating immunomodulatory and anticancer potential [232].

1.5.8 Genus *Podocarpus*

The *Podocarpus* genus is native to many areas due to its Gondwanan origin [283, 284] . Several species of this genus are known for their application in a variety of traditional medicine systems for the treatment of fever, asthma, cough, arthritis, cholera and some venereal diseases [285]. Studies have identified the presence of bioflavonoids and epicatechin in 4 of the *Podocarpus* spp. [285]. Furthermore, these species showed potent antioxidant, anti-inflammatory and tyrosinase inhibitory activities. The Illawarra Plum *Podocarpus elatus* Endl., (family Podocarpaceae) [184] is endemic to the eastern New South Wales and south eastern Queensland regions of Australia [286]. It is a medium to large evergreen tree, bearing cones that are berry-like in appearance. Recent studies have reported high antioxidant and moderate phenolic content as well as high levels of anthocyanins in the fruit extracts [184, 272]. Another study reported the testing of the normal colonic cell line (YAMC) and a tumorigenic human colonic cell line (HT-29) against the *P. elatus* fruit extracts. A significant decrease in HT-29 proliferation and increased apoptosis was noted, whilst no changes were observed in the YAMC normal cells [287]. The study postulated that high level of anthocyanins was responsible for this activity. However, the study was limited as it did not trial carcinoma cells from other organ systems. In addition, its directed approach focussed on a particular phytochemical class (anthocyanins), and subsequently omitted phytochemicals that may have contributed to the activity observed.

1.5.9 Genus *Acronychia*

The genus *Acronychia* (family Rutaceae) consists of approximately 49 species of shrubs and trees which are predominantly distributed over rainforests of Southern Asia, Australia and New Caledonia [288]. Most species of this genus are endemic to New Guinea and Australia, belonging to the citrus family (Rutaceae), and are not related to true aspens. A total of 27 species of this genus are endemic to New Guinea, and a further 19 species are endemic to eastern Australia. The Rutaceae family is characterised by the presence of cytotoxic alkaloids, which have use as DNA intercalating agents [289]. Many species in the genus *Acronychia* have documented uses as traditional medicines for the treatment of asthma, cough, itchy skin, scales, sores and rheumatism [290]. Furthermore, several *Acronychia* spp. have antifungal, anti-spasmodic, anti-pyretic, and anti-haemorrhagic activities [291]. Recent studies isolated phytochemicals from *Acronychia* spp. These comprised multiple phytochemical classes inclusive of coumarins, alkaloids, flavonoids and triterpenes. These phytochemical classes have a multitude of reports of biological activities including anti-plasmodial, anti-cancer, anti-oxidant, anti-inflammatory, anti-fungal, and neuroprotective effects. The Australian species *Acronychia acidula* F. Muell., (family Rutaceae), commonly known as lemon aspen has high antioxidant contents [292]. A number of interesting compounds including kaempferol, luteolin hexoside, quercetin hexoside, rutin, chlorogenic acid, caffeic acid, coumaric acid and ferulic acid have been isolated from *A. acidula* [275, 292]. Whilst a thorough literature search failed to reveal reports of *A. acidula* antiproliferative activity, the related species *Acronychia baueri* displays potent antiproliferative activity against leukemic and solid tumour cell lines [293].

1.6 Aims

1. Selected plants with high antioxidant capacities will be tested for antioxidant, anti-proliferative and toxicity properties.
2. Plants will be screened for their ability to induce of antiproliferative activity/cell proliferative activity, since plants with high antioxidant content have been previously correlated with both.
3. The antioxidant capacity of selected Australian plants will be determined by DPPH to determine their antioxidant potential and to confirm previous results from literature search.
4. The cytostatic/cytotoxic potential of these high antioxidant plants will require an initial screen using a panel of cervical carcinoma (HeLa) and colorectal carcinoma (Caco-2) cells testing for their potent antiproliferative activity.
5. The toxicity of bioactive plant extracts will be determined utilising the *Artemia* nauplii toxicity assay to benchmark their toxicities.
6. Selection criteria will also be imposed to narrow the focus to a smaller group of plants which would possess high antioxidant content, are potent antiproliferative agents and of low toxicity.
7. Plant extracts that have been deemed to the initial selection criteria will be trialled against an extended panel of choriocarcinoma and osteosarcoma cell lines (JEG-3/JAR and MC3T3-E1/MG-63) that have been well characterised and have diagnostic potential.
8. The antiproliferative potential of against these diagnostic cell lines will provide information as to the pathways and mode of action of these plant extracts.

9. The morphological changes of a selected and well characterised cell line (Caco-2) on exposure to the extracts of known antiproliferative activity will be observed and correlated with screening studies.
10. Cytostatic/cytotoxic phenomena or both will used to explain observations noted in cell lines tested.
11. Unbiased metabolomic profiling utilising Liquid Chromatography MS-MS and Gas chromatography MS will be used to determine the full complement of compounds present in each of the extracts of the selected plants. Metabolomic comparison will be utilised to narrow the focus and gain insight into which compounds are responsible of the observed bioactivity. This will provide much needed mechanistic detail into pathways and mechanisms by which these compounds induce antiproliferative activity in cancer cell lines.
12. Plants deemed to contain bioactivity will undergo bioassay guided fractionation, their components isolated and, purified. Phytochemical compounds of interest will be structurally identified to provide information for structural activity studies. The mechanisms of these bioactive molecules will be further examined and elucidated.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant material

Davidsonia pruriens, *Elaeocarpus angustifolium*, *Syzygium leuhmannii* and *Syzygium australe* fruit and leaves were obtained from the Mt Coot-tha Botanical Gardens, Brisbane, Australia. All plant materials were identified by Philip Cameron, chief botanist, Mt Cootha Botanical Gardens, Brisbane. *Kunzea pomifera*, *Podocarpus elatus*, *Acronychia acidula* and *Tasmannia lanceolata* fruit and leaf materials were acquired from Taste Australia Bush Food as verified fruits. Voucher samples have been stored in the School of Natural Sciences, Griffith University. *Terminalia ferdinandiana* plum pulp and leaf samples were a gift from David Boehme of Wild Harvest, Northern Territory, Australia. The pulp was frozen for transport and stored at -10°C until processing.

2.2 Solvent extraction

All plant materials were thoroughly dried in a Sunbeam food dehydrator and the dried plant materials were subsequently stored at -30 °C. Prior to use, the plant materials were thawed and freshly ground to a coarse powder. Individual 1 g quantities of the ground leaves and fruit were weighed into separate tubes and 50 mL of methanol, deionised water, ethyl acetate,

chloroform and hexane were added. All solvents were obtained from Ajax, Australia and were AR grade. The ground fruit and leaves were individually extracted by maceration in each solvent for 24 hours at 4 °C with gentle shaking. All solvents were supplied by Ajax, and were AR grade. The extracts were subsequently filtered through filter paper (Whatmann No.54) under vacuum, followed by drying by a rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10 mL deionised water containing 1% Dimethyl sulfoxide (DMSO). The extracts were passed through 0.22 µM filter (Sarstedt) and stored at 4 °C until use.

2.3 Qualitative phytochemical studies

Phytochemical analysis of the crude extracts was conducted by modified versions of the Salkowski, Keller-Kiliani, Kumar, Ajaiyeoba and Leiberman-Buchard methods as described in earlier studies [294-296]. All chemicals were of AR grade and were obtained from Ajax, Australia.

2.3.1 Alkaloids

Two methods were used to test for the presence of alkaloids:

(i) A volume of 200 µL of pure extract was treated with a few drops of aqueous solution of hydrochloric acid and 500 µL Mayer's reagent. Formation of white

precipitate indicated the presence of alkaloid. Mayer's reagent was prepared as follows: Mayer's reagent [mercuric chloride (1.358 g) dissolved in 60 mL deionised water; potassium iodide (5.0 g) dissolved in 10 mL deionised water]. The two solutions were mixed and made up to 100 mL with deionised water.

(ii) A volume of 200 µL of pure extract was treated with a few drops of an aqueous solution of hydrochloric acid and 500 µL Wagner's reagent. A reddish-brown flocculent precipitate indicated the presence of alkaloid. The Wagner's reagent was prepared as follows: Wagner's reagent [iodine (1.27 g) and potassium iodide (2 g) in 5 mL were dissolved in deionised water] and brought up to final volume 100 mL with deionised water.

2.3.2 Saponins

Saponins were screened using two methods:

(i) A volume of 1 mL of pure extract was added to 1 mL deionised water and shaken vigorously for 30 seconds. The tubes were allowed to stand for 15 minutes and the presence or absence of persistent frothing was noted. Persistent frothing indicates the presence of saponins.

(ii) A few drops of olive oil (Aurora) were added to 1 mL of pure extract. The development of an emulsion indicates the presence of saponins.

2.3.3 Tannins

Tannins were detected using a modified version of the ferric chloride test [296]. Two drops of 1% aqueous ferric chloride reagent were added to 500 µL of crude extract. The mixture was observed for the formation of blue, blue-black, green or green-black colouration which indicates the presence of tannins.

2.3.4 Phenolic compounds

Phenolic compounds were detected using a modified version of the Folin-Ciocalteu procedure [296]. A 200 µL volume of crude extract was added to 2 mL of 3% aqueous sodium carbonate, followed by the addition of 200 µL Folin-Ciocalteu reagent. The mixture was allowed to stand for 30 minutes at room temperature. The formation of blue/grey colour indicates the presence of phenolic groups.

2.3.5 Terpenoids

Terpenoids were detected using a modified version of the Salkowski test [295]. A volume of 1 mL of extract was added dropwise to 400 µL chloroform, followed by the careful addition of 400 µL concentrated sulphuric acid. Formation of a red/brown/purple colour at the interface indicates the presence of terpenoids.

2.3.6 Cardiac glycosides

Cardiac glycosides were detected using a modified version of the Keller-Kiliani test [296]. A volume of 500 µL of extract was added to 500 µL glacial acetic acid. A few drops of 1% aqueous iron chloride and concentrated sulphuric acid were then added dropwise. The presence of a red/brown ring of the interface or the formation of a green/blue colour throughout the solution indicates the presence of cardiac glycosides.

2.3.7 Anthraquinones

Anthraquinones were detected using modified versions of the Kumar and Ajaiyeoba tests [294, 296]. The modified Kumar test involves the addition of a few drops concentrated sulphuric acid to 500 µL pure extract, followed by careful addition of 500 µL of ammonia. A rose pink colour indicates the presence of anthraquinones. For the Ajaiyeoba test, 450 µL of crude extract was added to 50 µL concentrated HCL and allowed to stand at room temperature for several minutes. A volume of 500 µL chloroform was then added dropwise. The formation of a rose pink colour indicates the presence of anthraquinones.

2.3.8 Flavonoids

Flavonoids were detected using a modified Kumar test [294, 297]. A volume of 100 µL of aqueous 10% sodium hydroxide was added to 1 mL pure extract. The development of an intense yellow colour indicates the presence of flavonoids. A volume of 100 µL of concentrated HCl was then added to the solution, resulting in reversion to the original colour, confirming the presence of flavonoids.

2.3.9 Phytosteroids

Phytosteroids were detected using a modified version of the Leiberman-Buchard test [294]. Three drops of acetic anhydride was added to 500 µL of crude extract, followed by the addition of a few drops of concentrated sulphuric acid. The solution was allowed to sit at room temperature for 5 minutes. Formation of a blue/green colour indicates the presence of phytosteroids.

2.4 Toxicity screening

2.4.1 Reference toxins for toxicity assay

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/mL solution in distilled water and serially diluted in artificial seawater for use in the *A. franciscana* nauplii bioassay.

2.4.2 Evaluation of toxicity

Toxicity was measured using the *Artemia franciscana* nauplii lethality assay originally developed by Meyer [214] for screening of phytotoxins. The assay was modified as previously described [298, 299]. Briefly, *Artemia franciscana* Kellogg cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/L distilled water were prepared prior to use. A mass of 2 g of *A. franciscana* cysts were incubated in 1 L synthetic seawater under artificial light at 25 °C, 2000 Lux with continuous aeration.

Hatching commenced within 16-18 hours of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 hours. Nauplii were separated from the shells and remaining

cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. Seawater (400 µL) containing approximately 40 - 60 nauplii were added to wells of a 48 well plate and immediately used for bioassay. Plant extracts and the reference toxin were diluted in artificial seawater for toxicity testing. A 400 µL volume of the samples and the reference toxin were transferred to the wells and incubated at 25 ± 1 $^{\circ}$C under artificial light (1000 Lux). A negative control (400 µL seawater) was run in at least triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead nauplii counted. The nauplii were considered dead if no movement of the appendages was observed within 10 seconds. After 24 hours all nauplii were sacrificed by adding a few drops of acetic acid and counted to determine the total number per well. The lethal concentration (LC_{50}) with 95% confidence limits for each treatment was calculated using probit analysis [300].

2.5 Antioxidative Assays

2.5.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant capacity of each sample was assessed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method [279] with modifications. Briefly, DPPH solution was prepared fresh each day as a 400 µM solution by dissolving DPPH (Sigma) in AR grade methanol (Ajax, Australia). The initial absorbance of the DPPH solution was measured at 517 nm using a Molecular Devices, Spectra Max M3 plate reader, and did not change significantly throughout the assay period. A 2 mL aliquot of each extract was evaporated and the residue resuspended in 2 mL of methanol. Each extract was added to a 96-well plate in amounts of 5, 10, 25, 50, 75 µL in triplicate. Methanol was added to each well to give a volume of 225 µL. A volume of 75 µL of the fresh DPPH solution was added to each well for a total reaction volume of 300 µL. A blank of each extract concentration, methanol solvent, and DPPH was also performed in triplicate. Ascorbic acid was prepared fresh and examined across the range 0 - 25 µg per well as a reference and the absorbances were recorded at 517 nm. All tests were performed in triplicate, and triplicate controls were included on each plate. The antioxidant capacity based on DPPH free radical scavenging ability was determined for each extract and expressed as µg ascorbic acid equivalents per gram of original plant material extracted.

2.6 Cell proliferation assays

2.6.1 Cell lines

All cell lines used in these studies (Table 2.1) were obtained from the American Type Culture Collection (ATCC).

Table 2.1 : Mammalian and animal cell lines used in study by tissue/organ system.

ATCC Line	Tissue Origin	Cell Type	Morphology	Animal Source
MC3T3-E1	Neonatal Calvarial Bone	Pre-osteoblast	Fibroblast-like adherent	*Mus musculus* (mouse)
MG-63	Osteogenic sarcoma of adult bone	Osteosarcoma	Epithelial like, adherent	*Homo sapiens* (Human)
HeLa	Adenocarcinoma of cervix	Adenocarcinoma	Epithelial like, adherent	*Homo sapiens* (Human)
Caco-2	Colorectal	Colorectal carcinoma	Epithelial like, adherent	*Homo sapiens* (Human)
JEG-3	Placental	Chorionic carcinoma	Epithelial like, adherent	*Homo sapiens* (Human)
JAR	Placental	Chorionic carcinoma	Epithelial like, adherent	*Homo sapiens* (Human)

2.6.2 Culture and passaging of cell lines

The bone cell lines (MC3T3-E1 and MG-63) were grown in Dulbecco/Vogt modified Eagle's minimal essential medium: nutrient media (DMEM). The HeLa, Caco-2, JEG-3 and JAR were cultured in Roswell Park Memorial Institute medium 1640 (RPMI-1640) (Invitrogen). Both DMEM and RPMI-1640 media required the additional supplementation of 10% foetal bovine serum (FBS) (Gibco) and 200 IU/mL penicillin/200 µg/mL streptomycin (Invitrogen). Tissue culture assays and passages were performed under strict aseptic conditions in a biological safety cabinet Class II (Clyde Apac). All cell lines were grown in sterile, vented, 25 cm^2 tissue culture flasks (Becton Dickinson) in a humidified incubator (Sanyo model MCO-15AC) at 37 °C in 5% carbon dioxide (CO_2/95% air). Prior to passaging, the cell monolayer in each culture flask was examined under an inverted microscope (Olympus CK2) to verify cell confluence. Spent tissue culture media was removed and the monolayer was washed with 3 - 5 mL of fresh tissue culture media warmed to 37 °C. This media was then aspirated and the monolayer trypsinised by the addition of 1 mL trypsin/EDTA (0.25% trypsin in 90 mM EDTA in PBS Invitrogen). The flask was then incubated at 37 °C until all the cells had lifted (5 - 15 minutes, dependant on the cell lines). After trypsinisation, a volume of 4 mL of fresh tissue culture media (37 °C) was added to each flask and the contents were aspirated into a centrifuge tube. The cell suspensions were centrifuged using MSE Minor centrifuge (BioRad) for 1 minute at 1000 G to pellet the cells. The supernatant containing trypsin was removed and the cells resuspended in 8 mL in sterile tissue culture medium. Aliquots of 1 mL of cell suspension were then used to seed new sterile 25 cm^2 culture flasks, each containing fresh tissue culture media at 37 °C.

2.6.3 Cryopreservation and cryoretrieval of cell lines

For cryopreservation, confluent cell monolayers were trypsinised, washed and pelleted by centrifugation for 1 minute at 1000 G in a MSE Minor centrifuge (BioRad). The cell pellet was resuspended in 900 µL of fresh tissue culture media containing 50 µL of dimethylsulphoxide (DMSO) (BDH Chemicals) and 50 µL of FBS (Gibco), and then placed into cryovials (Sarstedt). Vials were then gradually frozen at a temperature decrease of approximately 1 °C minute, via suspension in the neck of a liquid nitrogen dewar (Taylor-Wharton) for approximately one hour. Cryovials were finally placed in a cryotube storage cane (Nalge Nanc International) and submerged in liquid nitrogen for storage. Cell lines were retrieved by rapid thawing in 37 °C water bath, washing twice in 4 mL of fresh culture media and pelleting by centrifugation for 1 minute at 1000 G. Cells were then resuspended in 9 mL of fresh media at 37 °C and transferred to 25 cm^2 tissue culture flasks. Flasks were then incubated at 37 °C in 5% CO_2/95% air in a humidified incubator.

2.6.4 MTS based assays for cell proliferation

The Cell Titer 96®AQ_{ueous} one solution for cell proliferation (MTS) assay (Promega) was used. This is colorimetric assay for determining the number of viable cells in proliferation/cytotoxicity assays. The assay utilises a novel tetrazolium compound 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron coupling reagent (phenazine ethosulphate PES). All tests were performed on a 96 well micro-titre plate (Sarstedt) in triplicate.

A volume of 70 µL of viable cells suspended in their respective cell media were seeded into 96 well micro-titre plates and incubated for 12 hours. The plant extracts were initially syringe filtered with a 0.22 µm Millex-HV PVDF (Merck) syringe driven filter prior to usage and subsequently stored at 4°C. A volume of 30 µL of the individual plant extracts was aspirated and introduced into the wells containing the cells and appropriate media. Three sets of controls were included on each plate. A set of negative controls with 1% DMSO with cells and media was included on each plate. A second set of controls were set up identically without cells, to correct for background absorbance from the extracts and reagent. A set of positive controls was also included, with cells exposed to Cisplatin (Sigma). Plates were subsequently incubated for a further 12 hours at 37 °C in a humidified 5% CO_2/95% air atmosphere. Proliferation was measured by adding a 20 µL of Cell Titer 96® AQ_{ueous} reagent to the wells. The plates were subsequently re-incubated for a 4 hour period and the absorbance read at 490 nm using Spectra Max 250 96 well plate reader (Molecular Devices) and analysed using SOFTmax PRO 2.6 computer software.

2.7 Statistics

IC_{50} and LC_{50} values, with ± 95% confidence limit, were calculated by Probit analysis [300]. One way paired analysis of variance (ANOVA) was used to calculate statistical significance between the control and treated groups, with a *P* value < 0.05 considered to statistically significant.

2.8 Metabolomics profiling studies

2.8.1 Quadrupole time of flight mass spectrometry (QTOF MS/MS)

Initial chromatographic separations were performed using 2 µL injections of sample onto an Agilent 1290 HPLC fitted with a Zorbax Eclipse plus C18 column (2.1 x 100 mm, 1.8 µm particle size filters). The mobile phases consisted of (A) ultrapure water and (B) 95:5% acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% v/v glacial acetic acid for mass spectrometry analysis in positive mode and with 5 mM ammonium acetate for analysis in negative mode. The chromatographic conditions utilised for the study consisted of the first 5 minutes run isocratically at 5% B, a gradient of (B) from 5% to 100% was applied from 5 - 30 minutes, followed by 3 minutes isocratically at 100%. Mass spectrometry analysis was performed on an Agilent 6530 quadrupole time-of-flight

spectrometer fitted with a jetstream electrospray ionisation source in both positive and negative mode [301].

Data was analysed using the Masshunter Qualitative analysis software package (Agilent Technologies). Blanks using each of the solvent extraction systems were analysed using the Find by Molecular Feature algorithm in the software package to generate a compound list of molecules with abundances greater than 10,000 counts. This was then used as an exclusion list to eliminate background contaminant compounds from the analysis of the extracts. Each extract was then analysed using the same parameters using the Find by Molecular Feature function to generate a putative list of compounds in the extracts. Compound lists were then screened against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (800 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds). Empirical formula for unidentified compounds was determined using the Find Formula function in software package [301].

2.8.2 Non-Targeted GC-MS Head Space Analysis

Separations were performed with a Shimadzu GC-2010 Plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass spectrometer. The system was also equipped with a Shimadzu auto-sampler AOC-5000 Plus (USA) fitted with a solid phase micro-extraction fibre (SPME) handling system utilising a divinyl benzene/carbowax/polydimethyl siloxane (DVB/CAR/PDMS) from Supelco (USA). Chromatographic separation was accomplished using a (5% phenyl, 95% dimethylpolysiloxane, 30 m x 0.25 mm x 0.25 µm) capillary column, obtained from (Restek USA). Helium (99.999%) was employed as a carrier gas at a flow rate of 0.79 mL/min. The injector temperature was set at 230 °C [10]. Sampling utilised a SPME cycle which consisted of an agitation phase at 500 revolutions per minute (rpm) for a period of 5 seconds. The fibre was exposed to the sample for 10 minutes to allow for absorption and then desorbed in the injection port for a minute at 250 °C [302].

The initial column temperature was held at 30 °C for 2 minutes, increased to over 140 °C over a period of 5 minutes, then ramped to 270 °C over a period of 3 minutes and held at that temperature for the remainder of the chromatography. The GC-MS interface was maintained at 200 °C with no signal acquired for 1 minute after injection in splitless mode. The mass spectrometer was operated in the electron ionisation mode at 70 eV. The analytes were then recorded in total ion count (TIC) mode. The TIC was acquired after a minute and for the duration of 45 minutes utilising a mass range of 45 – 450 m/z [302].

2.9 Cell Imaging Studies

Cell morphological changes were measured by using bright field images without staining. Briefly, Caco-2 cells were seeded into individual wells of a 96 well plate at a density of 5 x 10^3 cells/100 µL in a 96 well plate. Cells were allowed to adhere overnight. Selected plant extracts were added to the respective wells at a concentration defined as sub-lethal dosage in chapter 4. Cells were incubated for 24 hours at 37 °C in a humidified 5% CO_2/95% air atmosphere. Each assay was performed in triplicate, and the cell morphology was qualitatively appraised after 24 hour treatment by using an inverted phase microscope with camera (Olympus IX70) with an optical zoom of 20X. The images were captured via computer and analysed to scale.

CHAPTER 3: SCREENING HIGH ANTIOXIDANT AUSTRALIAN PLANTS FOR ANTIPROLIFERATIVE ACTIVITY

Several plant species were selected for screening for the ability to block cancer cell proliferation in this study. These plants were chosen as all have previously been reported to have extremely high levels of antioxidant capacities [179, 181-183]. An extensive search of the literature databases identified *Acronychia acidula*, *Davidsonia pruriens*, *Elaeocarpus angustifolius, Kunzea pomifera, Podocarpus elatus*, *Syzygium australe*, *Syzygium leuhmannii* as well as *Terminalia ferdinandiana* and *Tasmannia lanceolata* as being particularly interesting.

The selected plants were extracted and the antioxidant capacity was determined to verify their high antioxidant status. The extracts were subsequently tested for their ability to block cellular proliferation against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) and Caco-2 (human colorectal carcinoma cancer cell line Caco-2 ATCC ® HTB-37™) cell lines as a preliminary screening step to determine the suitability as anticancer agents, and to narrow the focus for subsequent studies. This initial screening study utilised these two cell lines as they are prevalent cancers afflicting high mortality and morbidity in human populations with little or no remission [303]. Furthermore, both cell lines are widely used for anticancer screening studies, allowing for easy comparison and benchmarking with other reports. The present study attempted to determine if a possible correlation exists between

antioxidant content present in the plants and their potencies as antiproliferative agents as past studies have revealed a correlation between redox state of a cell and carcinogenesis. Hence, a major aim of this study was to test the hypothesis that a change in the redox state of a cell might affect and induce cytostatic or cytotoxic effects.

3.1 Extraction yields and qualitative phytochemical studies

Extracts prepared from the selected plant species were evaluated by qualitative phytochemical determination to obtain an understanding of the phytochemical classes present in each extract (Table 3.1).

3.1.1 Extraction yields

Solvents of varying polarities including methanol, deionised water, ethyl acetate, chloroform and hexane were used to extract phytochemicals from all the selected plants. The yields are reported in Table 3.1. The yields ranged from a low of 3 mg (*P. elatus* ethyl acetate fruit extract) to a high of 589.6 mg (*T. ferdinandiana* aqueous fruit extract) per gram of plant material extracted. The higher polarity solvents (methanol and water) generally extracted greatest yields of material. In contrast, the lower polarity chloroform solvent extracted moderate yields, albeit substantially less in comparison to the highly polar solvents. The mid-

polarity ethyl acetate and low polarity hexane solvents generally yielded significantly lower masses in comparison to the other solvents.

Qualitative phytochemical screening studies (Table 3.1) demonstrated that methanol and water extracted the widest range of phytochemicals for the *Syzygium leuhmannii, Syzygium australe, Davidsonia pruriens* and *Elaeocarpus angustifolius* plant materials. Chloroform and hexane extractions were not attempted for both *Davidsonia pruriens* (fruit and leaf) and *Elaeocarpus angustifolius* (fruit) species due to the lack of availability of the raw plant materials and our belief that the other solvents were likely to yield more promising extracts. Indeed, the high to mid polarity (methanol, aqueous and ethyl acetate) extracts were generally determined to have the greater antiproliferative activity in subsequent studies described later in this chapter.

3.1.2 *Syzygium* species (*Syzygium leuhmannii, Syzygium australe*) (fruit and leaf) phytochemical analysis

Phytochemical analysis of the *Syzygium leuhmannii* fruit methanolic and aqueous extracts showed moderate to high levels of phenolics (both water soluble and insoluble phenolics) and flavonoids, as well as low levels of saponins, triterpenoids and tannins (Tables 3.1). The ethyl acetate fruit extracts generally only contained moderate levels of phenolics and low levels of flavonoids, with no other classes of phytochemicals evident. Low levels of phenolics and flavonoids were also detected in the chloroform fruit extract. Also noteworthy was the lack of detectable levels of any of the screened phytochemical classes in the *S. leuhmannii* fruit chloroform extract despite the large amounts of extracted material in these extract. Chloroform would be expected to extract large amounts of lipids. The current study did not test for lipid levels. It is thus possible that much of this extracted material may consist of

lipids, hydrocarbons and other nonpolar compounds. The *S. leuhmannii* fruit hexane extract contained only low levels of saponins and an absence of all other phytochemical classes. Similarly qualitative phytochemical screening of the *Syzygium leuhmannii* leaf methanol and aqueous extracts detected moderate to high levels of phenolics (both water soluble and insoluble phenolics) and flavonoids, as well as low levels of saponins, triterpenoids and tannins (Table 3.1). The leaf ethyl acetate extract possessed similar levels of phenolic compounds, a moderate level of flavonoids and no other phytochemical classes present. Alkaloids, phytosteroids, cardiac glycosides and anthraquinones were not detected in any of the extracts (Tables 3.1).

Similar trends were observed with *S. australe* with respect to the distribution of phytochemical classes throughout the extracts. Both the fruit and leaf methanolic and aqueous extracts contained moderate to high levels of phenolics (both water soluble and insoluble phenolics). Low levels of triterpenoids and tannins were also evident in both the fruit and leaf methanol extracts. Low levels of saponins were detected in the aqueous fruit extract, but were absent in the aqueous leaf extract. Low levels of triterpenoids were detected in the aqueous leaf extract but were absent in the aqueous fruit extracts. Low to moderate levels of phenolics were present in the fruit and leaf ethyl acetate extracts and in the *S. australe* chloroform fruit extract. The lack of detectable levels of any phytochemical classes in the fruit chloroform extract despite the large amounts of extracted material in the extract is noteworthy. As previously discussed for *S. leuhmannii*, it is likely that this extract instead contains substantial levels of lipids, hydrocarbons and other nonpolar compounds.

3.1.3 *Davidsonia pruriens* (fruit and leaf) phytochemical analysis

Qualitative phytochemical screening of the methanolic and aqueous *D. pruriens* extracts showed moderate to high levels of phenolics (both water soluble and insoluble phenolics) and flavonoids in both the fruit and leaf extracts (Tables 3.1). The fruit ethyl acetate extract generally only had moderate levels of phenolics, low levels of saponins and no other classes of phytochemicals evident. Low levels of saponins were present in both the methanolic and aqueous extracts. Low levels of saponins were also present in the methanolic and ethyl acetate fruit extracts, but were absent in the aqueous extract. Triterpenoids and tannins were present in low levels in the higher polarity methanolic and aqueous fruit extracts. Alkaloids, phytosteroids, cardiac glycosides and anthraquinones were not detected in any of the fruit extracts.

Similarly, *D. pruriens* leaf ethyl acetate extract contained only low to moderate levels of phenolics, with no other classes of phytochemicals evident. Low levels of saponins were present in both the methanolic and aqueous leaf extracts. Triterpenoids and tannins were present in low levels in the higher polarity methanolic and aqueous leaf extracts. Alkaloids, phytosteroids, cardiac glycosides and anthraquinones were not detected in any *D. pruriens* leaf extracts.

3.1.4 *Elaeocarpus angustifolius* (fruit) phytochemical analysis

Methanolic and aqueous *E. angustifolius* fruit extracts contained moderate to high levels of phenolics, (both water soluble and insoluble phenolics), moderate levels of flavonoids and low levels of triterpenoids (Table 3.1). The ethyl acetate fruit extracts generally only had moderate levels of phenolics (only water insoluble phenolics) and low levels of flavonoids. In addition, low levels of saponins were present in the methanolic and ethyl acetate extracts but were absent in the aqueous extracts. No other classes of phytochemicals were evident (Table 3.1).

3.1.5 *Kunzea pomifera* (muntries) fruit phytochemical analysis

K. pomifera methanolic and aqueous fruit extracts contained high levels of water soluble phenolics (Table 3.1). The aqueous and ethyl acetate extracts also contained high levels of insoluble phenolics, although these were absent in the fruit methanolic extract. High levels of flavonoids were observed in the methanolic and aqueous fruit extracts, although lower levels were detected in the ethyl acetate and chloroform fruit extracts. Saponins were present in high levels in both methanolic and aqueous fruit extracts, whilst moderate levels were also present in the hexane extract. Triterpenoids and tannins were present in the methanolic and aqueous extracts in moderate levels, with moderate levels of tannins also detected in the ethyl acetate extract. Cardiac glycosides, alkaloids and anthraquinones were not detected in any of the extracts.

3.1.6 *Podocarpus elatus* fruit phytochemical analysis

High levels of phenolics were detected in both the methanolic and aqueous *P. elatus* fruit extracts (Table 3.1). Water soluble phenolics were present in the methanolic extract at high levels, moderate levels in the aqueous extract, but were absent in the mid-polarity ethyl acetate extract and the low polarity (chloroform and hexane) extracts. Flavonoids were also evident in the methanolic, aqueous and ethyl acetate extracts, albeit in moderate levels. Saponins were present in high levels in the methanolic and aqueous fruit extracts, and in moderate levels in the hexane extract. Moderate levels of triterpenoids and tannins were present in the methanolic and aqueous fruit extracts, whilst low to moderate levels of soluble and free anthraquinones were detected in the methanolic and aqueous fruit extracts. Phytosteroids, cardiac glycosides and alkaloids were absent in all *P. elatus* fruit extracts.

3.1.7 *Acronychia acidula* fruit phytochemical analysis

High levels of phenolics were detected across all *A. acidula* fruit extracts (Table 3.1). Low levels of water insoluble phenolics were also present in the ethyl acetate and chloroform fruit extracts. There was a total absence of water soluble phenolics in all *A. acidula* fruit extracts tested. High levels of flavonoids were only present in the methanolic and aqueous fruit extract. Saponins were present in high levels in only the aqueous fruit extract. Moderate levels of triterpenoids were noted in methanolic, aqueous and ethyl acetate fruit extracts. Despite high levels of cardiac glycosides detected in the ethyl acetate fruit extract, low to moderate levels were present in all other *A. acidula* fruit extracts.

3.1.8 *Terminalia ferdinandiana* (fruit and leaf) phytochemical analysis

Qualitative phytochemical studies showed that *T. ferdinandiana* fruit and leaf methanolic and aqueous extracts had the widest range of phytochemicals of all the plants tested, testing positive for phenolics, saponins, triterpenoids, cardiac glycosides, tannins and alkaloids (Table 3.1). All *T. ferdinandiana* fruit extracts had high to moderate insoluble phenols with the exception of the low polarity chloroform and hexane extracts. Both the methanolic and ethyl acetate extracts contained low to moderate levels of triterpenoids. The *T. ferdinandiana's* fruit methanolic extract had cardiac glycosides present, albeit at barely detectable levels. Cardiac glycosides were absent in all the other *T. ferdinandiana* fruit extracts. The *T. ferdinandiana* fruit methanolic and aqueous extracts also contained moderate level of tannins. Low levels of alkaloids were present in the methanolic extract, and were absent in all other *T. ferdinandiana* fruit extracts. The methanolic, aqueous, and ethyl acetate fruit extracts of *T. ferdinandiana* extracts contained moderate to high levels of flavonoids. None of the *T. ferdinandiana* fruit extracts contained phytosteroids or anthraquinones. Similar studies undertaken for *T. ferdinandiana* leaves (Table 3.1) revealed that methanol and water also extracted a wide range of phytochemicals. Both extracts had high levels of phenolics (both water soluble and insoluble phenolics) and tannins, as well as moderate to high levels of cardiac glycosides, saponins and flavonoids. Triterpenes and anthraquinones were also present in low levels in both leaf extracts, whilst alkaloids were detected only in the methanolic leaf extract. The leaf ethyl acetate extract also had high levels of phenolics and moderate levels of flavonoids and tannins. Only low levels of phenolics and tannins were detected in the lower polarity chloroform and hexane leaf extracts. Moderate levels of flavonoids were noted in the *T. ferdinandiana* hexane leaf extract.

3.1.9 *Tasmannia lanceolata* (fruit and leaf) phytochemical analysis

Qualitative phytochemical studies of *Tasmannia lanceolata* fruit extracts (Table 3.1) revealed that methanol and water extracted the widest range of phytochemicals of all *T. lanceolata* fruit extracts. High levels of phenolics (both water soluble and insoluble phenolics) and flavonoids, as well as low to moderate levels of saponins, were detected in both extracts. The ethyl acetate fruit extract contained low to moderate levels of phenolics, triterpenes and flavonoids. Low levels of saponins were also detected in the *T. lanceolata* ethyl acetate fruit extract. Neither tannins nor alkaloids were detected in any of the fruit extracts. None of the phytochemical classes were evident in *T. lanceolata* chloroform and hexane fruit extracts. Similarly, qualitative phytochemical studies (Table 3.1) of *T. lanceolata* leaf extracts showed that methanol and water extracted a wide range of phytochemicals from the *T. lanceolata* leaves. Both showed moderate to high levels of phenolics (both water soluble and insoluble phenolics) and flavonoids, as well as low levels of saponins, triterpenoids and tannins. The ethyl acetate extracts generally only had moderate levels of phenolics and low levels of flavonoids, with no other classes of phytochemicals evident. Alkaloids, cardiac glycosides and anthraquinones were absent in all of the *T. lanceolata* leaf extracts. Low levels of phenolic compounds, flavonoids and triterpenoids were present in the *T. lanceolata* chloroform leaf extracts, whilst all other phytochemical classes were not detected. Similarly, *T. lanceolata* leaf extracts had detectable levels of flavonoids and triterpenoids although all other phytochemical classes were absent.

Table 3.1 Phytochemical profiles of the Australian plant extracts.

species	part	extracts	mass (mg)	extract concentration (mg/mL)	total phenolics	water soluble phenolics	water insoluble phenolics	flavonoids	phytosteroids	saponins	triterpenoids	cardiac glycosides	tannins	alkaloids (Meyer)	alkaloids (Wagner)	anthraquinones (modified Kumar)	anthraquinones (ajaiyeoba)	extract mg/ml	DPPH mg AA equivalents per g of original plant material
S. leuhmannii	fruit	M	560	56	***	***	***	***	-	*	*	-	*	-	-	-	-	56	95
		A	120	12	***	***	**	***	-	*	*	-	*	-	-	-	-	12	59
		E	130	13	**	-	**	*	-	-	-	-	-	-	-	-	-	13	1.5
		C	382	38.2	*	-	-	*	-	-	-	-	-	-	-	-	-	NA	NA
		H	83	8.3	-	-	-	-	-	*	-	-	-	-	-	-	-	NA	NA
	leaf	M	190	19	***	***	***	***	-	*	*	-	*	-	-	-	-	19	43
		A	88	9	***	***	**	**	-	*	*	-	*	-	-	-	-	9	45
		E	62	6	**	-	**	**	-	-	-	-	-	-	-	-	-	6	5.5
		C	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		H	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*** = Large response, ** = Moderate response, * = Fair response; (-) = Absence of compound tested for in the extract; NA = Not attempted.
M=methanol, A=aqueous, E=ethyl acetate, C=chloroform, H=hexane.

Table 3.1 Phytochemical profiles of the Australian plant extracts (cont'd).

species	part	extracts	mass (mg)	extract concentration (mg/mL)	total phenolics	water soluble phenolics	water insoluble phenolics	flavonoids	phytosteroids	saponins	triterpenoids	cardiac glycosides	tannins	alkaloids (Meyer)	alkaloids (Wagner)	anthraquinones (modified Kumar)	anthraquinones (ajaiyeoba)	extract mg/ml	DPPH mg AA equivalents per g of original plant material
S. australe	fruit	M	360	36	***	***	***	***	-	-	*	**	*	-	-	-	-	36	55
		A	240	24	***	***	**	***	-	*	-	-	*	-	-	-	-	24	41
		E	110	11	**	-	**	*	-	-	-	-	-	-	-	-	-	11	9.2
		C	247	24.7	*	-	*	-	-	-	-	-	-	-	-	-	-	NA	NA
		H	62	6.2	-	-	-	-	-	*	-	-	-	-	-	-	-	NA	NA
	leaf	M	360	36	***	***	***	***	-	*	*	-	*	-	-	-	-	36	40
		A	180	18	***	***	**	**	-	*	*	-	*	-	-	-	-	18	25
		E	110	11	**	-	**	**	-	-	-	-	-	-	-	-	-	11	2.6
		C	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		H	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*** = Large response, ** = Moderate response, * = Fair response; (-) = Absence of compound tested for in the extract; NA = Not attempted.
M=methanol, A=aqueous, E=ethyl acetate, C=chloroform, H=hexane.

Table 3.1 Phytochemical profiles of the Australian plant extracts (cont'd).

species	part	extracts	mass (mg)	extract concentration (mg/mL)	total phenolics	water soluble phenolics	water insoluble phenolics	flavonoids	phytosteroids	saponins	triterpenoids	cardiac glycosides	tannins	alkaloids (Meyer)	alkaloids (Wagner)	anthraquinones (modified Kumar)	anthraquinones (ajaiyeoba)	extract mg/ml	DPPH mg AA equivalents per g of original plant material
D. pruriens	fruit	M	530	53	**	***	**	***	-	*	*	-	*	-	-	-	-	53	23
		A	220	22	***	***	**	**	-	-	*	-	*	-	-	-	-	22	16
		E	290	29	**	**	*	-	-	*	-	-	-	-	-	-	-	29	1.5
		C	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		H	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	leaf	M	230	23	**	**	**	***	-	*	*	-	*	-	-	-	-	23	35
		A	36	4	**	**	*	***	-	*	*	-	*	-	-	-	-	4	39
		E	44	4	**	-	*	-	-	-	-	-	-	-	-	-	-	4	5.5
		C	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		H	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*** = Large response, ** = Moderate response, * = Fair response; (-) = Absence of compound tested for in the extract; NA = Not attempted. M=methanol, A=aqueous, E=ethyl acetate, C=chloroform, H=hexane.

Table 3.1 Phytochemical profiles of the Australian plant extracts (cont'd).

species	part	extracts	mass (mg)	extract concentration (mg/mL)	total phenolics	water soluble phenolics	water insoluble phenolics	flavonoids	phytosteroids	saponins	triterpenoids	cardiac glycosides	tannins	alkaloids (Meyer)	alkaloids (Wagner)	anthraquinones (modified Kumar)	anthraquinones (ajaiyeoba)	extract mg/ml	DPPH mg AA equivalents per g of original plant material
E. angustifolius	fruit	M	490	49	***	**	**	***	-	*	*	-	-	-	-	-	-	49	20.8
		A	140	14	***	**	**	**	-	-	*	-	-	-	-	-	-	14	22.6
		E	25	3	**	-	**	*	-	*	-	-	-	-	-	-	-	3	1.2
		C	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		H	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
K. pomifera	fruit	M	524	52	***	***	-	***	-	***	**	-	**	-	-	-	-	52	6.9
		A	350	35	***	***	***	***	-	***	**	-	**	-	-	-	-	35	2.9
		E	19	2	*	-	***	*	-	-	-	-	**	-	-	-	-	1.9	1.2
		C	120	12	*	-	-	*	-	-	-	-	-	-	-	-	-	12	0.4
		H	20	2	-	-	-	-	-	**	-	-	-	-	-	-	-	2	0.2

*** = Large response, ** = Moderate response, * = Fair response; (-) = Absence of compound tested for in the extract; NA = Not attempted. .
M=methanol, A=aqueous, E=ethyl acetate, C=chloroform, H=hexane**.**

Table 3.1 Phytochemical profiles of the Australian plant extracts (cont'd).

species	part	extracts	mass (mg)	extract concentration (mg/mL)	total phenolics	water soluble phenolics	water insoluble phenolics	flavonoids	phytosteroids	saponins	triterpenoids	cardiac glycosides	tannins	alkaloids (Meyer)	alkaloids (Wagner)	anthraquinones (modified Kumar)	anthraquinones (ajaiyeoba)	extract mg/ml	DPPH mg AA equivalents per g of original plant material
P. elatus	fruit	M	314	31	***	***	***	**	-	***	**	-	**	-	-	*	**	31.4	6.8
		A	195	20	***	**	***	**	-	***	**	-	**	-	-	**	**	19.5	2.7
		E	3	0.3	*	-	*	**	-	-	-	-	*	-	-	-	-	0.3	1.2
		C	140	14	-	-	-	-	-	-	-	-	-	-	-	-	-	14.0	0.3
		H	50	5	-	-	-	-	-	**	-	-	-	-	-	-	-	5.0	0.2
A. acidula	fruit	M	360	36	***	-	-	***	-	-	**	*	-	-	-	-	-	36	15.9
		A	162	16	***	-	-	***	-	***	**	*	-	-	-	-	-	16.2	7.2
		E	66	7	***	-	*	-	-	-	**	***	-	-	-	-	-	6.6	6.4
		C	180	18	***	-	*	-	-	-	-	*	-	-	-	-	-	18	6
		H	70	7	*	-	-	-	-	-	-	**	-	-	-	-	-	7	3.2

*** = Large response, ** = Moderate response, * = Fair response; (-) = Absence of compound tested for in the extract; NA = Not attempted. M=methanol, A=aqueous, E=ethyl acetate, C=chloroform, H=hexane

Table 3.1 Phytochemical profiles of the Australian plant extracts (cont'd).

species	part	extracts	mass (mg)	extract concentration (mg/mL)	total phenolics	water soluble phenolics	water insoluble phenolics	flavonoids	phytosteroids	saponins	triterpenoids	cardiac glycosides	tannins	alkaloids (Meyer)	alkaloids (Wagner)	anthraquinones (modified Kumar)	anthraquinones (aiaiveoba)	extract mg/ml	DPPH mg AA equivalents per g of original plant material
T. ferdinandiana	fruit	M	364	36	***	***	***	***	-	**	*	*	**	*	*	-	-	35.9	660
		A	589.6	59	***	***	***	***	-	*	-	-	**	-	-	-	-	48.3	264
		E	24.8	3	**	**	*	**	-	*	**	-	-	-	-	-	-	3.0	39
		C	102.1	10	*	-	-	-	-	-	-	-	-	-	-	-	-	6.2	7
		H	10.9	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8	1
	leaf	M	331	33	***	***	***	**	-	**	*	***	***	*	*	*	*	33.1	150
		A	471	47	***	***	***	**	-	***	**	**	***	-	-	*	*	47.1	340
		E	59	6	***	***	***	**	-	-	-	-	**	-	-	-	-	5.9	22
		C	59	6	*	-	-	-	-	-	-	-	-	-	-	-	-	5.9	5
		H	58	6	*	-	-	**	-	-	-	-	*	-	-	-	-	5.8	0.4

*** = Large response, ** = Moderate response, * = Fair response; (-) = Absence of compound tested for in the extract; NA = Not attempted. M=methanol, A=aqueous, E=ethyl acetate, C=chloroform, H=hexane.

Table 3.1 Phytochemical profiles of the Australian plant extracts (cont'd).

species	part	extracts	mass (mg)	extract concentration (mg/mL)	total phenolics	water soluble phenolics	water insoluble phenolics	flavonoids	phytosteroids	saponins	triterpenoids	cardiac glycosides	tannins	alkaloids (Meyer)	alkaloids (Wagner)	anthraquinones (modified Kumar)	anthraquinones (ajaiyeoba)	extract mg/ml	DPPH mg AA equivalents per g of original plant material
T. lanceolata	fruit	M	171	17	***	***	***	***	-	**	*	-	-	-	-	-	-		
		A	111	11	***	***	***	***	-	-	-	-	-	-	-	-	-		
		E	56.7	6	*	*	**	**	-	*	**	-	-	-	-	-	-		
		C			-	-	-	-	-	-	-	-	-	-	-	-	-		
		H			-	-	-	-	-	-	-	-	-	-	-	-	-		
	leaf	M	144	14	***	***	***	***	-	***	*	-	-	-	-	-	-		
		A	295	30	***	***	***	***	-	**	-	-	-	-	-	-	-		
		E	17	2	*	*	**	**	-	-	*	-	-	-	-	-	-		
		C			*	-	-	*	*	-	*	-	-	-	-	-	-		
		H			-	-	-	*	-	-	*	-	-	-	-	-	-		

*** = Large response, ** = Moderate response, * = Fair response; (-) = Absence of compound tested for in the extract; NA = Not attempted
M=methanol, A=aqueous, E=ethyl acetate, C=chloroform, H=hexane

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3.2 DPPH assays for the determination of antioxidant content.

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to determine the antioxidant capacity of each plant extract and compared to an ascorbic acid standard curve. Antioxidant capacity (expressed as ascorbic acid equivalence) for the fruit and leaf extracts are shown in (Table 3.1). The antioxidant capacity ranged from a low of 0.2 mg ascorbic acid equivalents per gram of dried plant material extracted (*K. pomifera* and *P. elatus*) hexane extracts to the highest level of 660 mg ascorbic acid equivalents per gram of dried plant material extracted (*T. ferdinandiana* methanolic fruit extract). Whilst significantly lower than the methanolic extract, the *T. ferdinandiana* aqueous fruit extract also had a high antioxidant capacity with 264 mg ascorbic acid equivalents per gram of dried plant material extracted. The methanolic extracts generally had higher antioxidant capacities than the aqueous or ethyl acetate extracts, with the exceptions of the *E. angustifolius* (fruit), *K. pomifera* (fruit), *T. ferdinandiana* leaf and *P. elatus* (fruit) extracts. For these plant materials, the aqueous extract displayed a higher antioxidant capacity than the methanolic extract. Generally, a higher antioxidant capacity was detected in the fruit extracts compared to the leaf extracts for all plant species except in the *D. pruriens* extracts. For the *Syzygium* species, a higher antioxidant capacity was measured in both the fruit and leaf *S. leuhmannii* extracts compared to the corresponding *S. australe* extracts. The chloroform and hexane extracts generally contained the lowest antioxidant content, whilst the ethyl acetate extracts were generally mid-range. The antioxidant capacities were in the order of *T. ferdinandiana* (fruit and leaf) > *T. lanceolata* (fruit and leaf) > *S. leuhmannii* (fruit and leaf) > *S. australe* (fruit and leaf) > *D. pruriens* (fruit and leaf) > *E. angustifolius* (fruit) > *A. acidula* (fruit) > *K. pomifera* (fruit) > *P. elatus* (fruit).

3.3 Antiproliferative MTS assays of Australian plant extracts against cancer cell lines.

The MTS proliferation assay was modified and standardised for use with all plant extracts and tested to quantify their antiproliferative potential of the plant extracts against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) and Caco-2 (human colorectal carcinoma cancer cell line Caco-2 ATCC ® HTB-37™) cell lines.

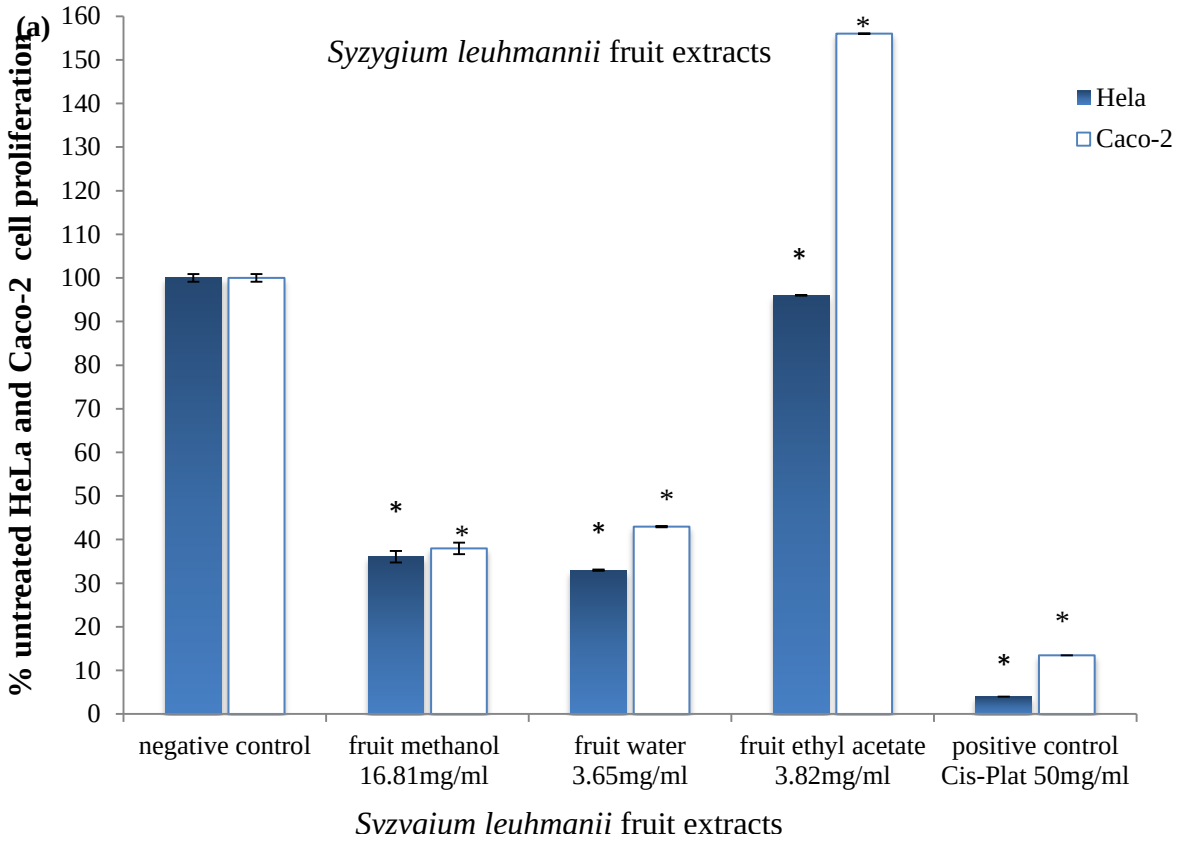
3.3.1 Antiproliferative potential of *Syzygium* species extracts.

Methanolic and aqueous extracts of both *S. leuhmannii* and *S. australe* (fruit and leaf) strongly inhibited carcinoma cell growth with > 50% inhibition determined against both HeLa (Figure 3.1 a) and Caco-2 (Figure 3.1 b) cells. The *S. leuhmannii* ethyl acetate fruit extract induced minor inhibition of HeLa cell proliferation (Figure 3.1a), although this inhibition was not significant (4% inhibition compared to the negative control cell proliferation). In contrast, the *S. leuhmannii* ethyl acetate leaf extract induced significant HeLa cell proliferation by approximately 68% compared to the negative control (Figure 3.1 b). Cisplatin, an agent commonly used in chemotherapy treatments against a variety of carcinomas, was used as a positive control at a concentration of 50 mg/mL. This dosage significantly inhibited HeLa proliferation by 96% compared to the untreated control. It is noteworthy that Cisplatin is a pure compound tested at a relatively high dosage in this study, compared to the concentrations of the tested crude extracts. As these are crude extracts, any bioactive components would be expected to be present in considerably lower concentrations.

Neither, the *S. leuhmannii* fruit or leaf ethyl acetate extracts inhibited Caco-2 cell proliferation. Indeed, proliferation was noted in both the fruit and leaf extracts increasing cellular proliferation by approximately 56% and 72% respectively. In contrast, the *S. leuhmannii* fruit aqueous extract (Figure 3.1 a) inhibited Caco-2 cell proliferation by approximately 47% inhibition of the untreated control value. The *S. leuhmannii* aqueous leaf extract was an even more potent antiproliferative agent, with an inhibition of approximately 90% of Caco-2 cell proliferation. Hence, all methanolic and aqueous extracts were deemed to be effective inhibitors of HeLa and Caco-2 cellular proliferation. In comparison the Cisplatin control at a concentration of 50 mg/mL inhibited Caco-2 proliferation by approximately 86%.

S. australe methanolic and aqueous fruit (Figure 3.2 a) and leaf extracts (Figure 3.2 b) also strongly inhibited HeLa cell growth. The high to mid-polarity (methanol, aqueous, ethyl acetate) *S. australe* fruit extracts were particularly potent HeLa inhibitors. Methanolic *S. australe* fruit extract inhibited HeLa proliferation by 82%; the aqueous fruit extract inhibited by 72%; and the ethyl acetate inhibited proliferation by 70% compared to the untreated control respectively (Figure 3.2 a). A similar trend was observed with the higher polarity methanolic and aqueous *S. australe* leaf extracts. The methanolic extract was very potent in inhibiting HeLa proliferation by 84% compared to the negative control. Similarly, the *S. australe* aqueous leaf extract inhibited HeLa by 58% (Figure 3.2 b). Interestingly, conflicting trends were noted between the results of the *S. australe* mid-polarity ethyl acetate fruit extract and the *S. australe* ethyl acetate leaf extract (Figure 3.2 a, b). The *S. australe* mid-polarity fruit ethyl acetate extract inhibited proliferation of HeLa by 70%. In contrast, the ethyl acetate leaf extracts induced increased proliferation with a 29% increase in proliferation of HeLa cells observed above that of the untreated control.

As seen for HeLa cell proliferation, *S. australe* methanolic and aqueous fruit and leaf extracts, (with the exception of the ethyl acetate fruit and leaf extracts) significantly inhibited Caco-2 cell proliferation (Figure 3.2 a). Indeed, the aqueous fruit extract inhibited Caco-2 proliferation by 98% compared to the negative control, whilst the methanolic fruit extract inhibited by 58% respectively (Figure 3.2 a). The *S. australe* methanolic leaf extract inhibited Caco-2 proliferation by 55%, whilst the leaf aqueous extract inhibited by 58% compared to the negative control (Figure 3.2 b). When exposed to the *S. australe* ethyl acetate fruit and leaf extracts, Caco-2 cells displayed a similarly proliferative potential as observed with the leaf ethyl acetate extract against HeLa cells. Indeed, the *S. australe* fruit ethyl acetate extract induced Caco-2 proliferation by 20% above that of the untreated control cell proliferation. Even more dramatic, the *S. australe* leaf ethyl acetate induced an 82% Caco-2 proliferation compared to the negative control. Hence, Caco-2 cells were not inhibited by the ethyl acetate fruit and leaf extracts, instead significant proliferation was noted. In summary, it is apparent that all methanolic and aqueous extracts (fruit and leaf) were effective inhibitors of HeLa and Caco-2 cellular proliferation, whilst the ethyl acetate extracts had opposing effects.


(a)
Syzygium leuhmannii fruit extracts
% untreated HeLa and Caco-2 cell proliferation
Hela
Caco-2
negative control
fruit methanol 16.81mg/ml
fruit water 3.65mg/ml
fruit ethyl acetate 3.82mg/ml
positive control Cis-Plat 50mg/ml
Svzvaium leuhmanii fruit extracts

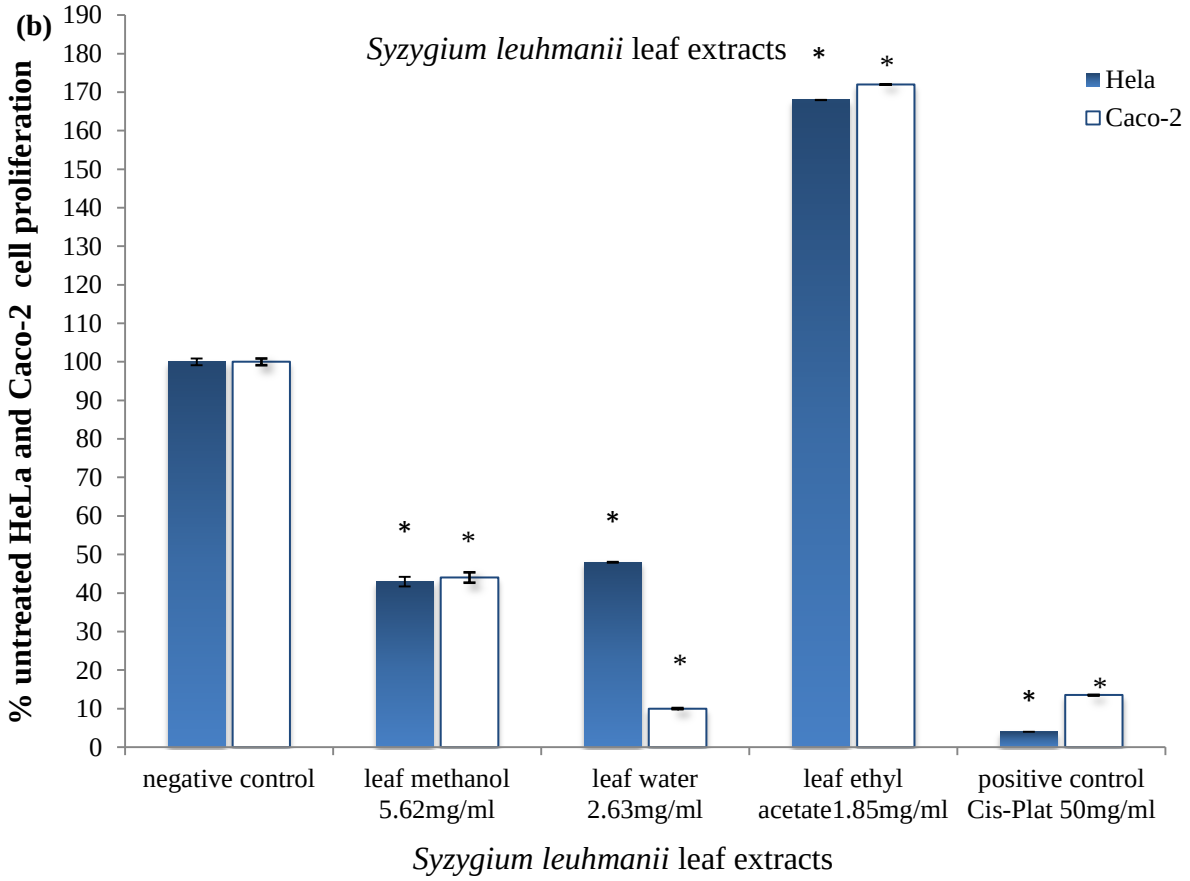
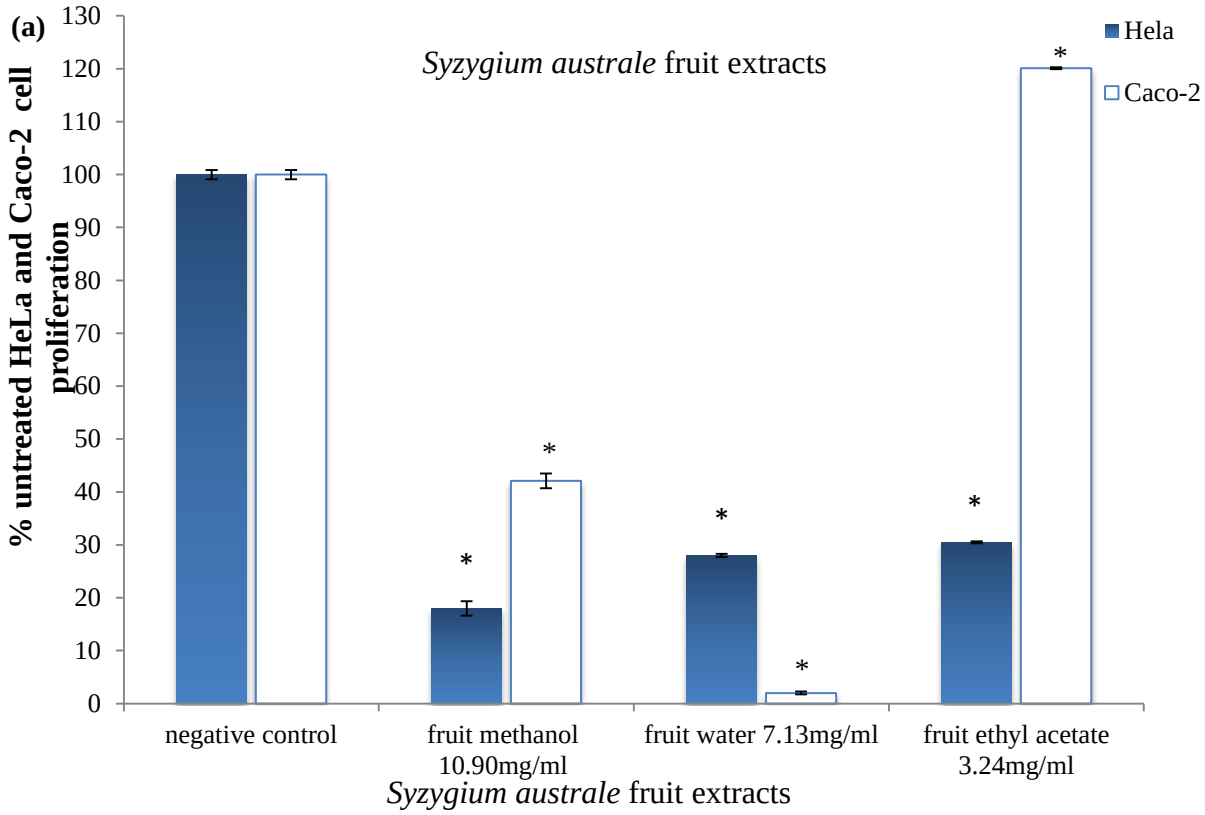

(b)
Syzygium leuhmanii leaf extracts
% untreated HeLa and Caco-2 cell proliferation
Hela
Caco-2
negative control
leaf methanol 5.62mg/ml
leaf water 2.63mg/ml
leaf ethyl acetate1.85mg/ml
positive control Cis-Plat 50mg/ml
Syzygium leuhmanii leaf extracts

Figure 3.1 Cell proliferation (MTS) assays of *S. leuhmannii* (a) fruit and (b) leaf extracts, untreated controls and positive control (Cisplatin) against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.


(a)
% untreated HeLa and Caco-2 cell proliferation
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Syzygium australe fruit extracts
Hela
Caco-2
*
negative control
fruit methanol 10.90mg/ml
fruit water 7.13mg/ml
fruit ethyl acetate 3.24mg/ml
Syzygium australe fruit extracts

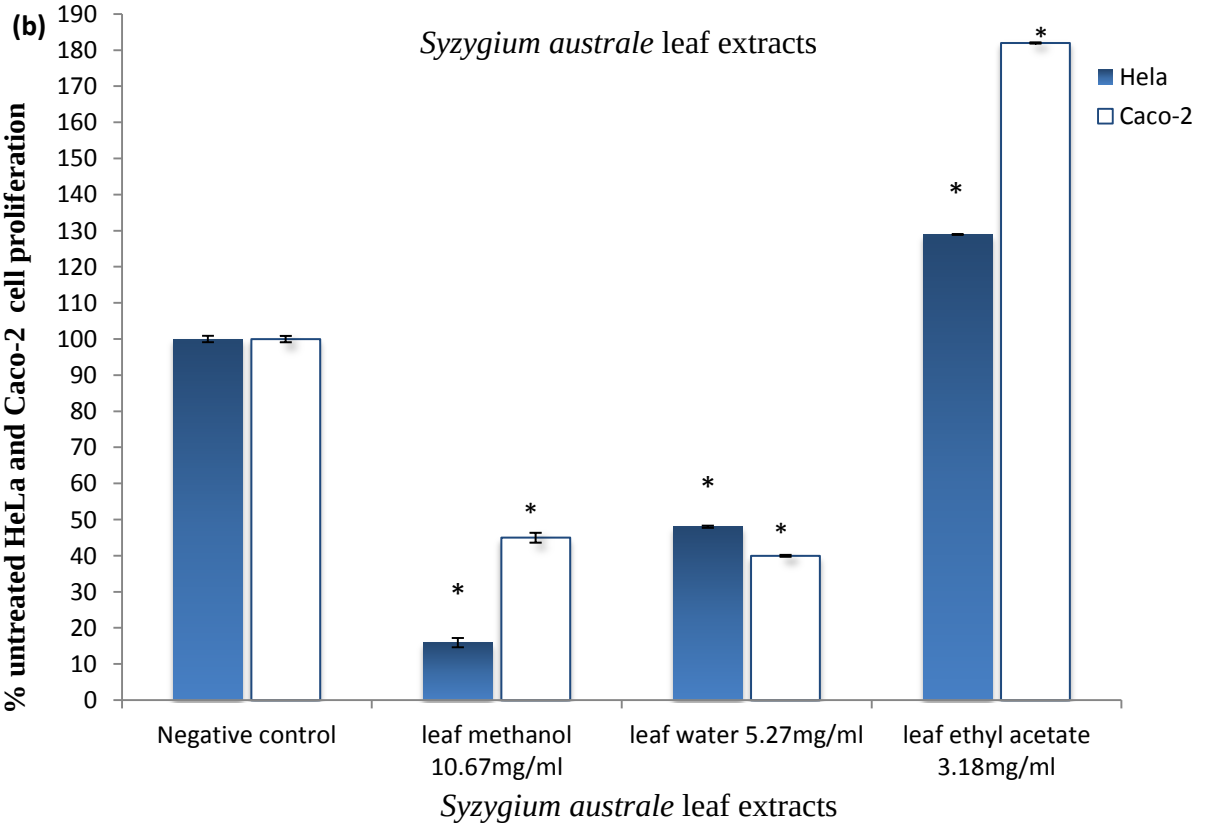

(b)
% untreated HeLa and Caco-2 cell proliferation
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Syzygium australe leaf extracts
Hela
Caco-2
*
Negative control
leaf methanol 10.67mg/ml
leaf water 5.27mg/ml
leaf ethyl acetate 3.18mg/ml
Syzygium australe leaf extracts

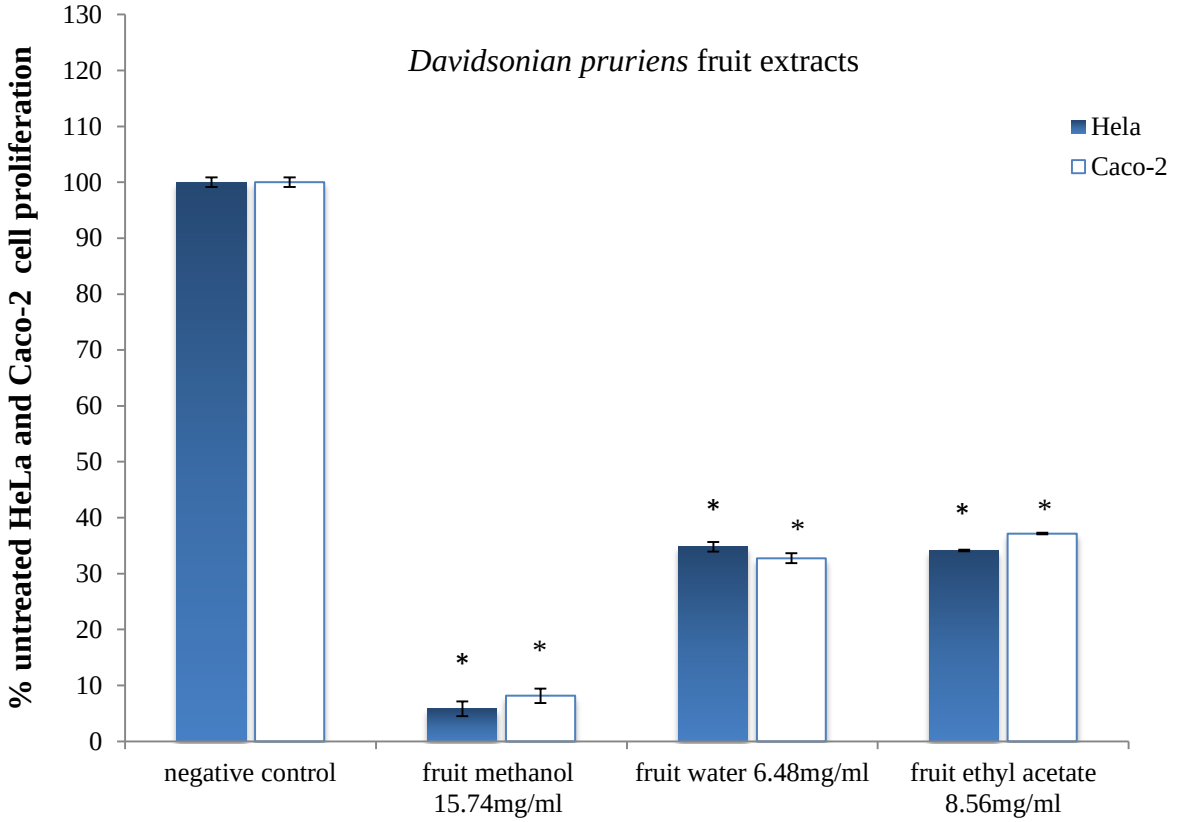
Figure 3.2 Cell proliferation (MTS) assays of *S. australe* (a) fruit and (b) leaf extracts and untreated controls against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

3.3.2 Antiproliferative potential of *Davidsonia pruriens* extracts.

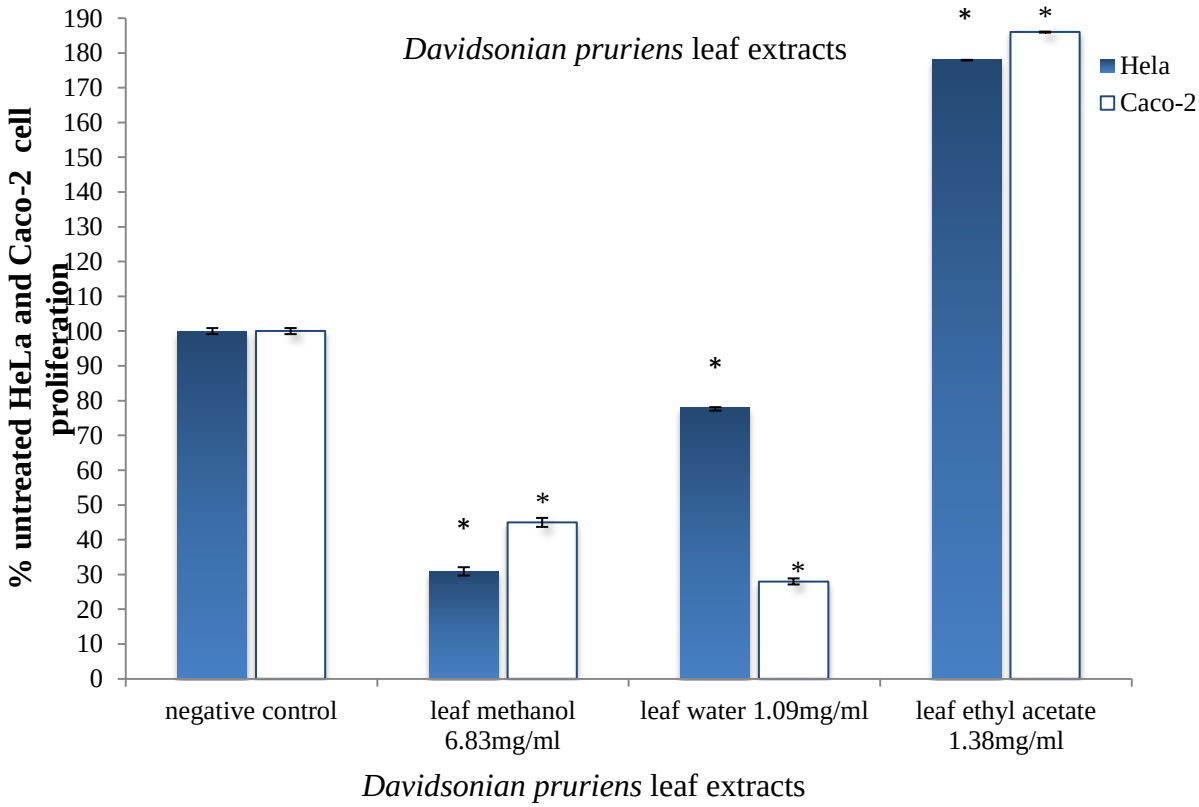
Methanolic and aqueous *D. pruriens* fruit (Figure 3.3 a) and leaf (Figure 3.3 b) extracts significantly inhibited HeLa and Caco-2 cell growth. The higher and mid-polarity (methanolic, aqueous and ethyl acetate) *D. pruriens* fruit extracts were particularly potent growth inhibitors, with the fruit methanolic extract achieving 92% inhibition of HeLa proliferation compared to the negative control. Likewise, the aqueous fruit extract inhibited 66% proliferation, whilst the fruit ethyl acetate inhibited 67% proliferation of HeLa (Figure 3.3 a). Similarly, the higher polarity methanol and aqueous leaf extracts inhibited HeLa proliferation by 69% and by 22% respectively compared to the negative control (Figure 3.3 b). In contrast, the *D. pruriens* fruit ethyl acetate induced a 78% increase in cell proliferation.

As with HeLa cell proliferation, *D. pruriens* methanolic and aqueous fruit and leaf extracts induced significant inhibition of Caco-2 cell proliferation by approximately 95% and 70% respectively. The *D. pruriens* methanolic and aqueous leaf extracts significantly inhibited Caco-2 proliferation by 55% and 72% respectively compared to the negative control. In contrast, the mid polarity leaf ethyl acetate extract did not inhibit HeLa and Caco-2 cells.

Instead, both cell lines had induced proliferation; HeLa proliferation increased by 78% and Caco-2 proliferation increased by 86% compared with the negative control. In summary all *D. pruriens* fruit and leaf extracts were deemed to be effective at inhibiting both cell lines with the exception of the ethyl acetate leaf extract which was a potent inducer of HeLa and Caco-2 cellular proliferation.



Davidsonian pruriens fruit extracts



Davidsonian pruriens leaf extracts

Figure 3.3 Cell proliferation (MTS) assays of *D. pruriens* (a) fruit and (b) leaf extracts and untreated controls against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

3.3.3 Antiproliferative potential of *Elaeocarpus angustifolius* extracts.

Methanolic and aqueous extracts of *E. angustifolium* fruit significantly inhibited HeLa cell proliferation (Figure 3.4). The higher polarity methanolic *E. angustifolium* fruit extract was particularly potent, inhibiting HeLa proliferation by 70% (Figure 3.4). Similar, albeit less potent inhibition was observed for the aqueous extract (22% compared with the untreated control). In contrast, *E. angustifolium* ethyl acetate fruit extract induced significant cell proliferation. Indeed, a 70% increase in cellular proliferation was observed compared to the negative control. *E. angustifolium* leaf extracts were not available for testing.

As was evident for HeLa cell proliferation (Figure 3.4), *E. angustifolium* methanolic and aqueous fruit extracts (with the exception of the ethyl acetate) significantly inhibited Caco-2 cell proliferation. The aqueous fruit extract inhibited Caco-2 by approximately 44%. The methanolic extract was a substantially less potent inhibitor of Caco-2 cell proliferation (5% compared to the negative control). Similar trends described for HeLa proliferation were also noted for Caco-2 cells, with *E. angustifolium* ethyl acetate fruit extract inducing significant

cell proliferation (78% increase in cell proliferation was observed compared to the negative control). Hence, all fruit extracts were deemed effective antiproliferative agents with the exception of the *E. angustifolium* ethyl acetate fruit extract, which was a potent inducer of both HeLa and Caco-2 proliferation.

3.3.4 Antiproliferative potential of *Kunzea pomifera* extracts.

The methanolic and aqueous extracts of the *K. pomifera* fruit extracts strongly and significantly inhibited HeLa cell growth (Figure 3.5). Similarly the chloroform and hexane *K. pomifera* fruit extracts also inhibited HeLa, albeit to a lesser degree. The methanolic and aqueous *K. pomifera* fruit extract inhibited 49% HeLa cell proliferation, compared to 30% inhibition by the chloroform extract. Likewise, the hexane fruit extract inhibited HeLa proliferation by 15% (Figure 3.5). In contrast, the *K. pomifera* ethyl acetate fruit extract induced increased HeLa cellular proliferation compared to the negative control. Indeed, a 48% increase in cell proliferation was observed for HeLa cells exposed to the *K. pomifera* ethyl acetate fruit extract.

All *K. pomifera* fruit extracts (except the hexane fruit extract) significantly inhibited Caco-2 cell proliferation (Figure 3.5). The methanolic extract inhibited Caco-2 proliferation by 25%, whilst the aqueous fruit extract inhibited 19% Caco-2 cellular proliferation compared to the negative control (Figure 3.5). Interestingly, the *K. pomifera* ethyl acetate and chloroform fruit extracts were more potent than the aqueous fruit extract, defying the trends for the previously

described plants. Notably, the *K. pomifera* ethyl acetate fruit extract which induced the highest HeLa proliferation (48%), was the strongest inhibitor of Caco-2 proliferation (35%) (Figure 3.5). Whilst less potent, the *K. pomifera* chloroform fruit extract inhibited Caco-2 proliferation by 24% compared to the negative control. In summary, *K. pomifera* methanolic, aqueous and chloroform fruit extracts were effective inhibitors of HeLa and Caco-2 cellular proliferation.

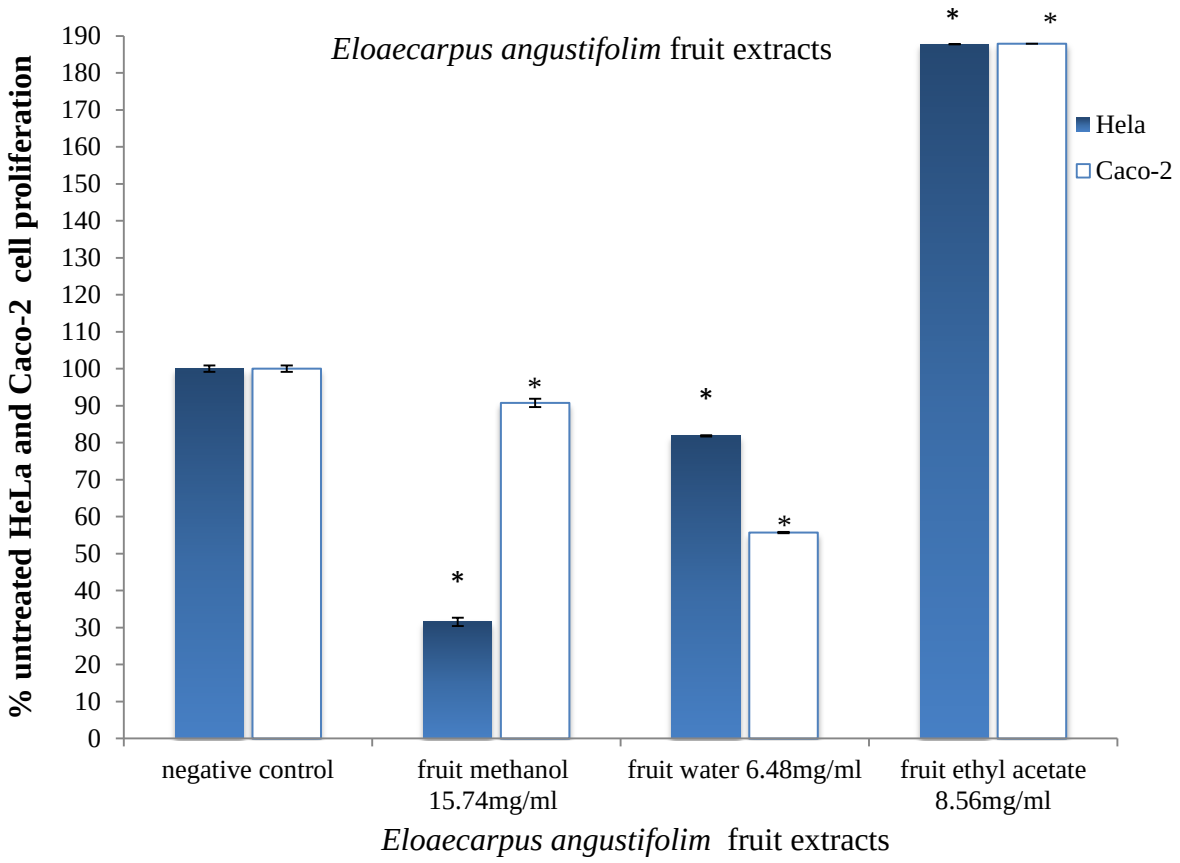


Figure 3.4 Cell proliferation (MTS) assays of *E. angustifolium* fruit extracts and untreated controls against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

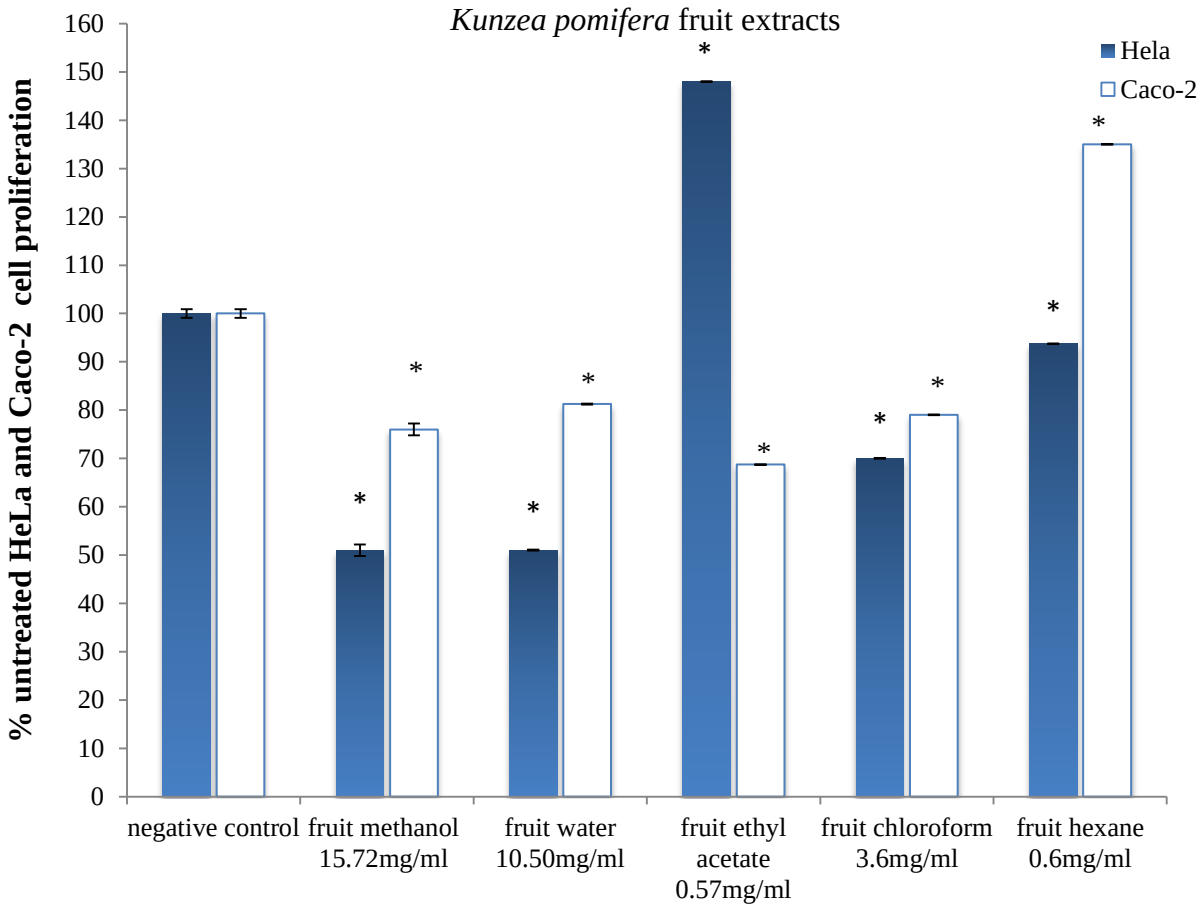


Figure 3.5 Cell proliferation (MTS) assays of *K. pomifera* (muntries) fruit extracts and untreated controls against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

3.3.5 Antiproliferative potential of *Podocarpus elatus* extracts.

Methanolic and aqueous *P. elatus* fruit extracts inhibited HeLa cell growth by 47% inhibition compared to the untreated controls (Figure 3.6). The mid-polarity *P. elatus* ethyl acetate fruit extract also significantly inhibited HeLa cell proliferation by 16%. The lower polarity *P. elatus* fruit extracts (chloroform and hexane) also inhibited HeLa (chloroform, 22% inhibition; hexane, 10% inhibition). Overall, *P. elatus* fruit extracts were effective against HeLa cell proliferation. The most effective were the higher polarity (methanolic, aqueous) fruit extracts.

The converse trend applied for the exposure of Caco-2 cells to the *P. elatus* fruit extracts (with the exception of the hexane fruit extract). The higher and mid polarity methanolic, aqueous and ethyl acetate *P. elatus* fruit extracts inhibited HeLa by 13%, 27% and 33% respectively (Figure 3.6). Similarly, the lower polarity *P. elatus* chloroform fruit extract inhibited Hela proliferation by approximately 40%. However, the *P. elatus* hexane fruit extract induced Caco-2 cell proliferation by 28%. Hence, all *P. elatus* fruit extracts were deemed to be effective inhibitors of both HeLa and Caco-2 cellular proliferation, with the exception of the *P. elatus* hexane fruit extract.

3.3.6 Antiproliferative potential of *Acronychia acidula* extracts.

Methanolic and ethyl acetate *Acronychia acidula* fruit extracts strongly inhibited HeLa cell proliferation (Figure 3.7). The *A. acidula* aqueous fruit extract induced a minor inhibition of HeLa cells, although this inhibition was not significant (5% inhibition compared to the negative control cell proliferation). The lower polarity *A. acidula* chloroform and hexane fruit extracts were effective at inhibiting HeLa cell proliferation. The *A. acidula* chloroform fruit extract inhibited HeLa proliferation by 33%, whilst the hexane fruit extract inhibited HeLa proliferation by 16% compared to the untreated controls. Although all *A. acidula* fruit extracts inhibited HeLa cell proliferation, the strongest inhibition of HeLa proliferation was determined for the *A. acidula* methanolic and ethyl acetate fruit extracts. Indeed, the *A. acidula* methanolic fruit extract inhibited HeLa proliferation by 99% and the ethyl acetate fruit extract by 84% HeLa, compared with the negative control (Figure 3.7).

Similar trends that were observed with HeLa proliferation were also noted with Caco-2 cells. The *A. acidula* methanolic, aqueous and ethyl acetate fruit extracts significantly inhibiting Caco-2 cell proliferation (Figure 3.7). The *A. acidula* methanolic fruit extract was particularly potent, blocking Caco-2 cell proliferation by 96% inhibition. The *A. acidula* aqueous fruit and ethyl acetate fruit extracts both inhibited approximately 81% Caco-2 proliferation compared to the untreated control. The lower polarity *A. acidula* chloroform and hexane fruit extracts also inhibited Caco-2 proliferation, albeit with a lower potency when compared to the high polarity extracts (Figure 3.7). The *A. acidula* chloroform fruit extract inhibited Caco-2 proliferation by approximately 35%, whilst the hexane fruit extract inhibited Caco-2

proliferation by 41% (Figure 3.7). Hence, all fruit extracts were deemed to be effective inhibitors of both HeLa and Caco-2 cellular proliferation.

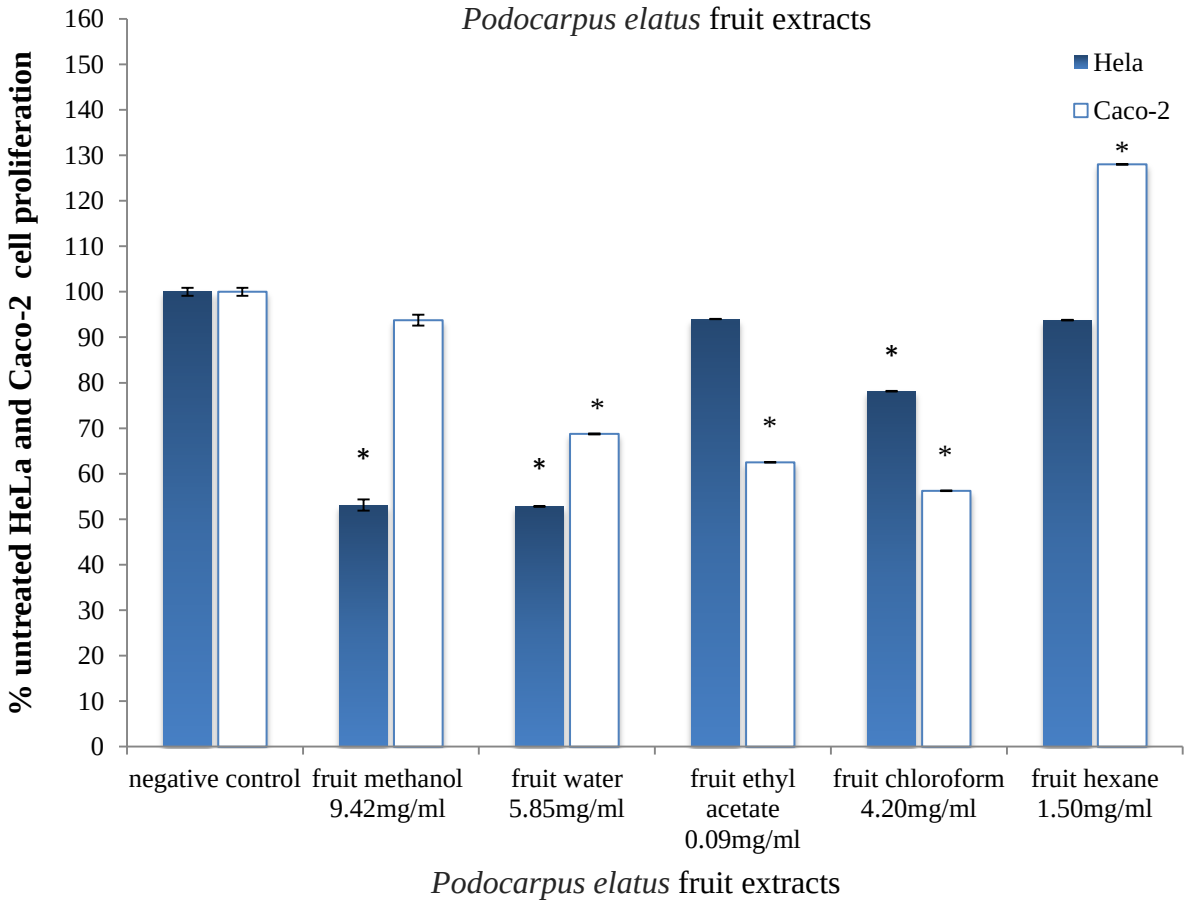


Figure 3.6 Cell proliferation (MTS) assays of *P. elatus* fruit extracts and untreated controls against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

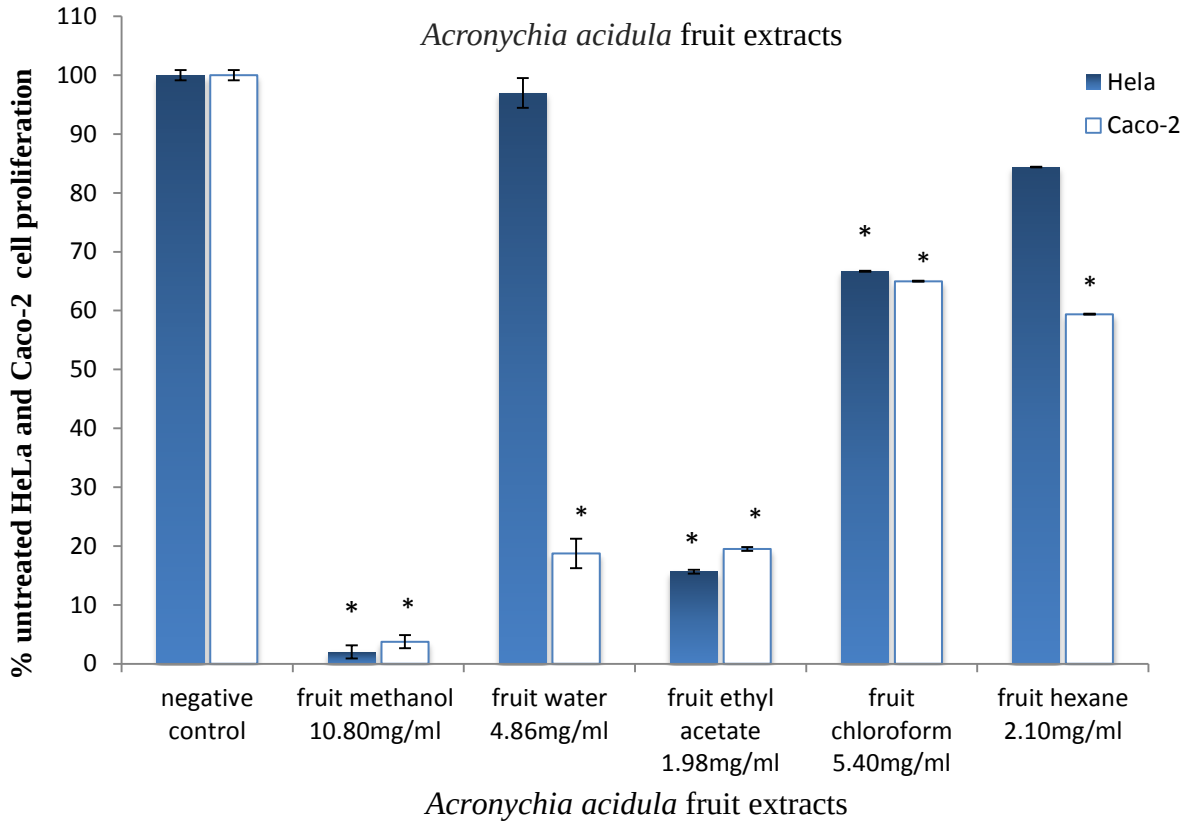


Figure 3.7 Cell proliferation (MTS) assays of *A. acidula* fruit.extracts and untreated controls against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

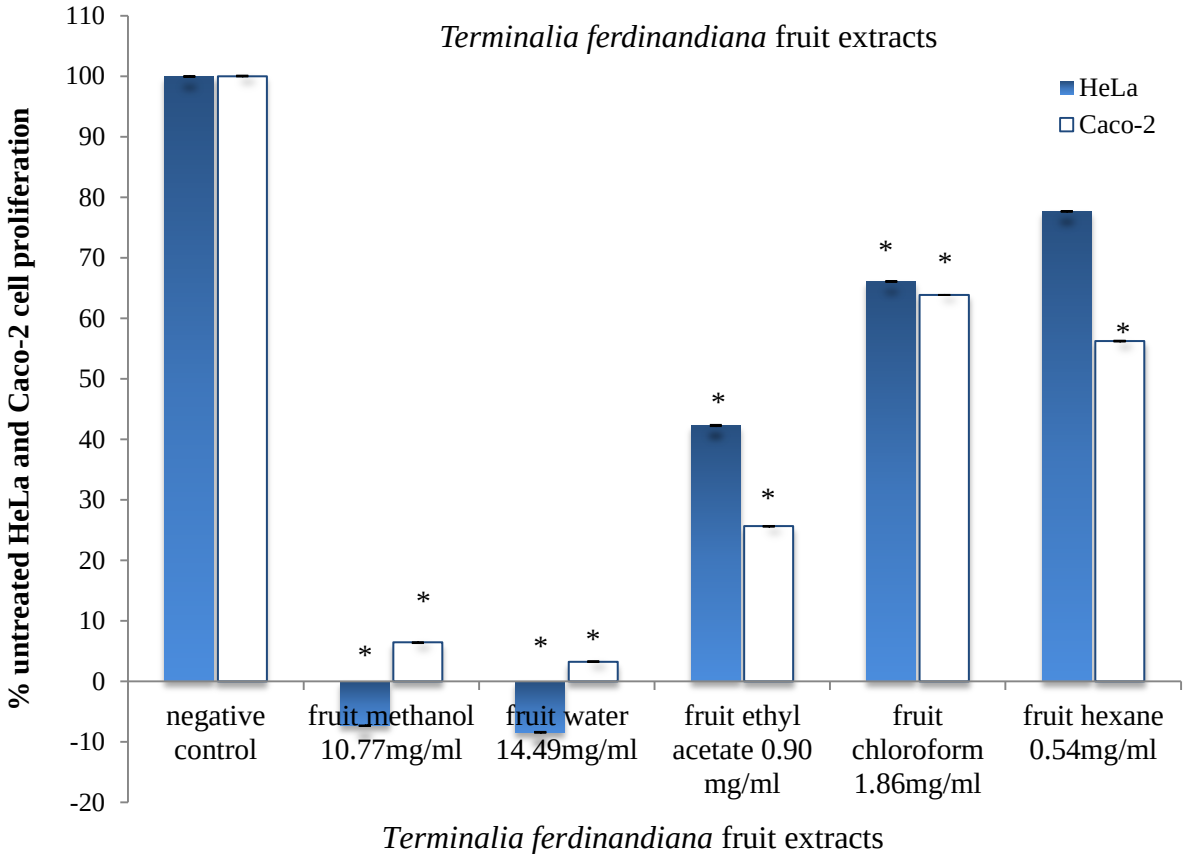
3.3.7 Antiproliferative potential of *Terminalia ferdinandiana* extracts.

The methanolic and aqueous *T. ferdinandiana* fruit extracts were particularly potent inhibitors of HeLa cell proliferation. Indeed, negative HeLa proliferation was noted for some extracts, suggesting that the antiproliferative activity was not only cytostatic in nature, but was possibly cytotoxic (Figure 3.8 a). The mid to lower polarity *T. ferdinandiana* ethyl acetate, chloroform and hexane fruit extracts inhibited HeLa cell proliferation by 58%, 34% and 22.4% respectively (Figure 3.8 a).

Consistent with the trends observed with HeLa cell proliferation for *T. ferdinandiana* fruit extracts, Caco-2 cells proliferation was also inhibited by these extracts. The higher polarity *T. ferdinandiana* fruit methanolic extract blocked 94% Caco-2 cell proliferation; the fruit aqueous extract inhibited by 97% Caco-2 cellular proliferation compared with the untreated control (Figure 3.8 a). The mid polarity and lower polarity *T. ferdinandiana* ethyl acetate, chloroform and hexane fruit extracts also inhibited Caco-2 cell proliferation. The *T. ferdinandiana* ethyl acetate fruit extract inhibited Caco-2 proliferation by 74%, compared to the negative control. The *T. ferdinandiana* chloroform and hexane fruit extracts were lower in potency, inhibiting Caco-2 cellular proliferation by 36% and 44% respectively. Hence, all *T. ferdinandiana* fruit extracts were considered to be effective inhibitors of both HeLa and Caco-2 cellular proliferation.

A different trend was apparent with *Terminalia ferdinandiana* leaf extracts. All extracts were effective inhibitors of HeLa cell proliferation (Figure 3.8 b). The higher polar methanolic and aqueous *T. ferdinandiana* leaf extracts strongly inhibited HeLa cell proliferation by 75% and the 69% HeLa cell proliferation respectively compared to the untreated control (Figure 3.8 b). In comparison with inhibition by the corresponding fruit extracts, the mid-polarity *T. ferdinandiana* leaf ethyl acetate extract produced the strongest inhibition against HeLa cell proliferation with a 92% decrease in proliferation. The lower polarity *T. ferdinandiana* chloroform and hexane leaf extracts also inhibited HeLa cell proliferation, albeit with lower efficacy. The *T. ferdinandiana* chloroform leaf extract induced minor inhibition of HeLa cells, although this inhibition was not significant (2% inhibition compared to the negative control cell proliferation).

The *T. ferdinandiana* hexane leaf extract inhibited HeLa cell proliferation by 34% compared to the negative control (Figure 3.8 b). Notably, the *T. ferdinandiana* methanolic and ethyl acetate leaf extracts not only strongly inhibited Caco-2 cell proliferation, but negative proliferation was noted, suggesting not only cytostatic, but also significant cytotoxic mechanisms may be involved. The *T. ferdinandiana* aqueous, chloroform and hexane leaf extracts also inhibited Caco-2 cell proliferation, albeit with lower potency. The aqueous *T. ferdinandiana* leaf extract inhibited Caco-2 proliferation by 73%; the chloroform and hexane *T. ferdinandiana* leaf extracts inhibited by 49% (Figure 3.8 b). The *T. ferdinandiana* fruit and leaf extracts were potent inhibitors of HeLa and Caco-2 (Figure 3.8 a - b). In summary, the *T. ferdinandiana* fruit aqueous and methanolic extracts were the most potent inhibitors of HeLa cell proliferation, whilst the methanolic and ethyl acetate leaf extracts were most potent at reducing Caco-2 proliferation.


Terminalia ferdinandiana fruit extracts
% untreated HeLa and Caco-2 cell proliferation
HeLa
Caco-2
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
negative control
fruit methanol 10.77mg/ml
fruit water 14.49mg/ml
fruit ethyl acetate 0.90 mg/ml
fruit chloroform 1.86mg/ml
fruit hexane 0.54mg/ml
Terminalia ferdinandiana fruit extracts

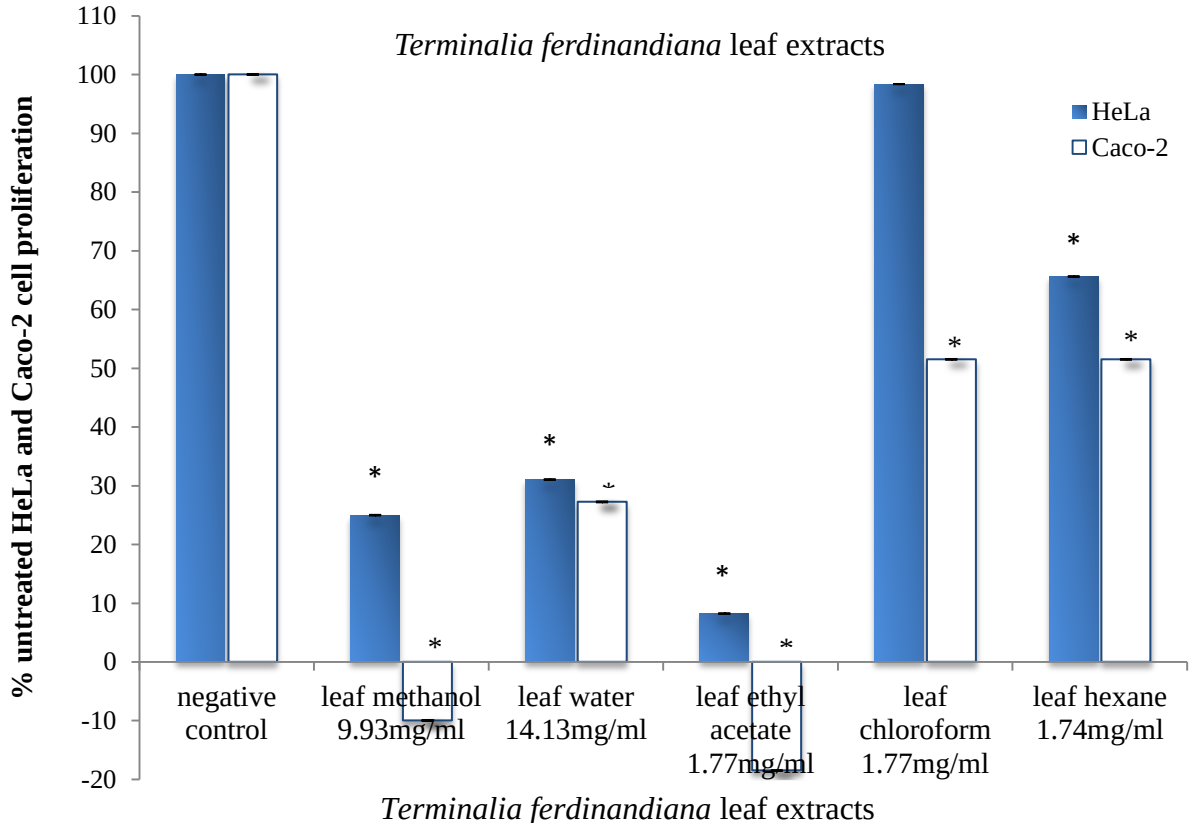

Terminalia ferdinandiana leaf extracts
% untreated HeLa and Caco-2 cell proliferation
HeLa
Caco-2
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
negative control
leaf methanol 9.93mg/ml
leaf water 14.13mg/ml
leaf ethyl acetate 1.77mg/ml
leaf chloroform 1.77mg/ml
leaf hexane 1.74mg/ml
Terminalia ferdinandiana leaf extracts

Figure 3.8 Cell proliferation (MTS) assays of *T. ferdinandiana* (a) fruit and (b) leaf extracts and untreated controls against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

3.3.8 Antiproliferative potential of *Tasmannia lanceolata* extracts.

Methanolic, aqueous and ethyl acetate *Tasmannia lanceolata* fruit extracts strongly inhibited HeLa proliferation (Figure 3.9 a). The *T. lanceolata* methanolic fruit extract inhibited 81% HeLa cellular proliferation, whilst the aqueous fruit extract inhibited 94% proliferation. Similarly, the *T. lanceolata* ethyl acetate fruit extract inhibited 69% of HeLa cell proliferation. The lower polarity *T. lanceolata* chloroform and hexane fruit extracts did not inhibit HeLa proliferation and instead induced proliferation by 10% and 15% respectively compared to the negative control (Figure 3.9 a).

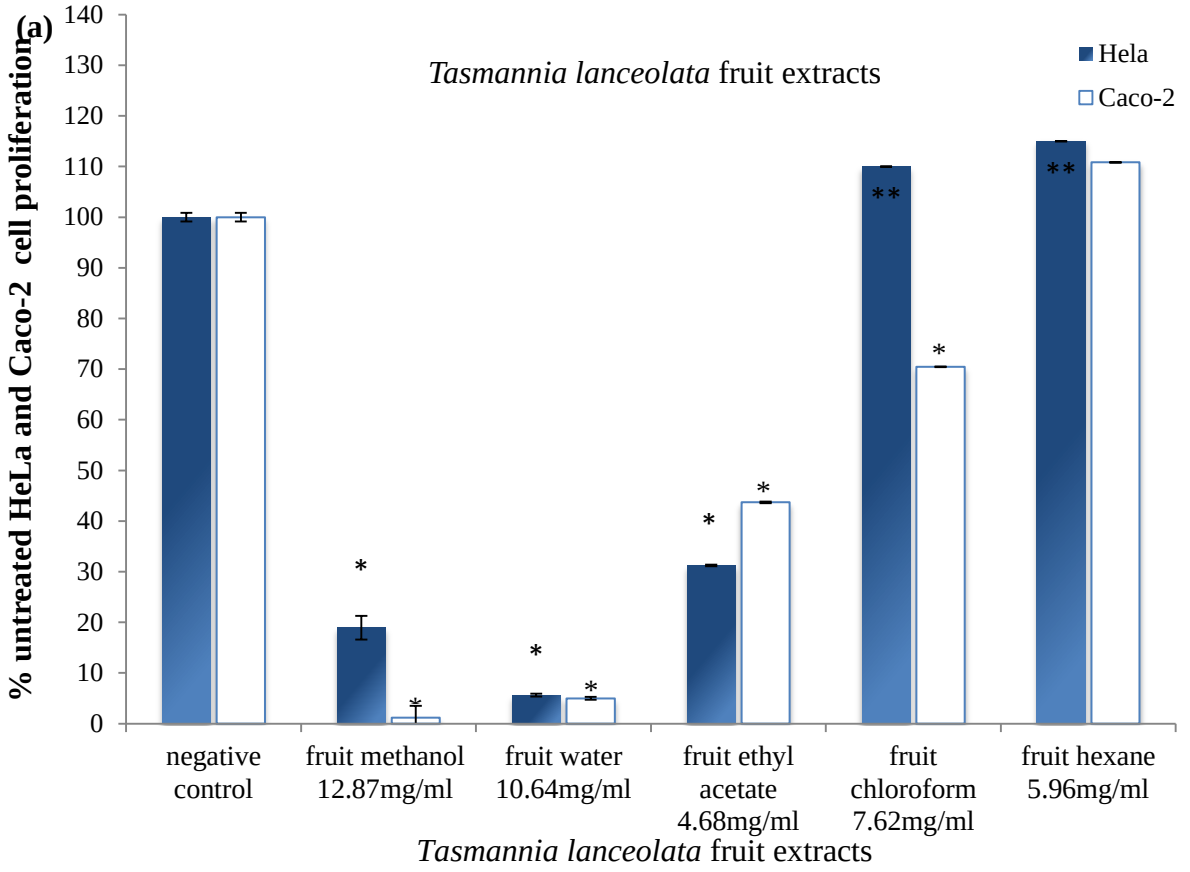
All *T. lanceolata* fruit extracts also inhibited Caco-2 proliferation (with the exception of the hexane fruit extract). The methanolic, aqueous and ethyl acetate fruit extracts strongly inhibited Caco-2 proliferation by 95%, 99% and 60% respectively (Figure 3.9 a). The lower polarity *T. lanceolata* fruit extracts were substantially less potent than the higher polarity extracts. However, it is noteworthy that these levels would still be classed as amongst the strongest for several of the other fruits. It is testament to the potency of the *T. lanceolata* fruit

extracts that they are classed as mid to low potency for this species. The *T. lanceolata* chloroform fruit extract also inhibited Caco-2 by 30%, whilst the lower polarity hexane fruit extract was devoid of inhibitory activity. Instead, the *T. lanceolata* hexane extract induced Caco-2 proliferation by 10% above the level recorded for the untreated control (figure 3.9 a). In summary, the *T. lanceolata* methanolic, aqueous, ethyl acetate fruit extracts were highly effective against both HeLa and Caco-2 cell lines. Hence, all *T. lanceolata* fruit extracts were deemed to be effective inhibitors of both HeLa and Caco-2 cellular proliferation, with the exception of the lower polarity hexane fruit extracts.

A similar trend was noted with *T. lanceolata* leaf extracts. All *T. lanceolata* leaf extracts were effective inhibitors of HeLa cell proliferation. The methanolic *T. lanceolata* leaf extract was particularly potent, inhibiting HeLa cell proliferation by 93% (Figure 3.9 b). Similarly, the *T. lanceolata* aqueous leaf extract inhibited 95% HeLa cell proliferation, whilst the *T. lanceolata* ethyl acetate leaf extract inhibited 77% HeLa proliferation (Figure 3.9 b). The lower polarity *T. lanceolata* chloroform and hexane leaf extracts also inhibited HeLa cell proliferation, albeit to a lesser degree. The *T. lanceolata* chloroform leaf extract induced minor inhibition of the HeLa cells (11% inhibition), although this inhibition was not significant. The *T. lanceolata* hexane fruit extract was marginally more potent, inhibiting HeLa cell proliferation by 20% compared to the untreated control.

The *T. lanceolata* methanolic, aqueous and ethyl acetate leaf extracts also strongly inhibited Caco-2 cell proliferation. The *T. lanceolata* methanolic leaf extract was particularly potent, inhibiting approximately 93% Caco-2 cellular proliferation (Figure 3.9 b). Likewise, the *T.*

lanceolata aqueous leaf extract inhibited 98% Caco-2 proliferation (Figure 3.9 b). The *T. lanceolata* ethyl acetate leaf extract was comparatively less potent, inhibiting 51% Caco-2 cellular proliferation compared to the negative control. The lower polarity *T. lanceolata* chloroform leaf extract showed only a minor inhibition of Caco-2 cell proliferation, (7% inhibition compared to the negative control cell proliferation) although this inhibition was not significant. Conversely, the *T. lanceolata* leaf hexane extract did not inhibit Caco-2 cell proliferation and instead induced Caco-2 proliferation by 13% above the level compared to the untreated control. Thus, the *T. lanceolata* fruit and leaf, high and mid-polarity extracts were deemed effective at inhibiting both HeLa and Caco-2 compared to the lower polarity extracts (Figure 3.9 a - b).


(a)
% untreated HeLa and Caco-2 cell proliferation
Tasmannia lanceolata fruit extracts
Hela
Caco-2
negative control
fruit methanol 12.87mg/ml
fruit water 10.64mg/ml
fruit ethyl acetate 4.68mg/ml
fruit chloroform 7.62mg/ml
fruit hexane 5.96mg/ml
Tasmannia lanceolata fruit extracts

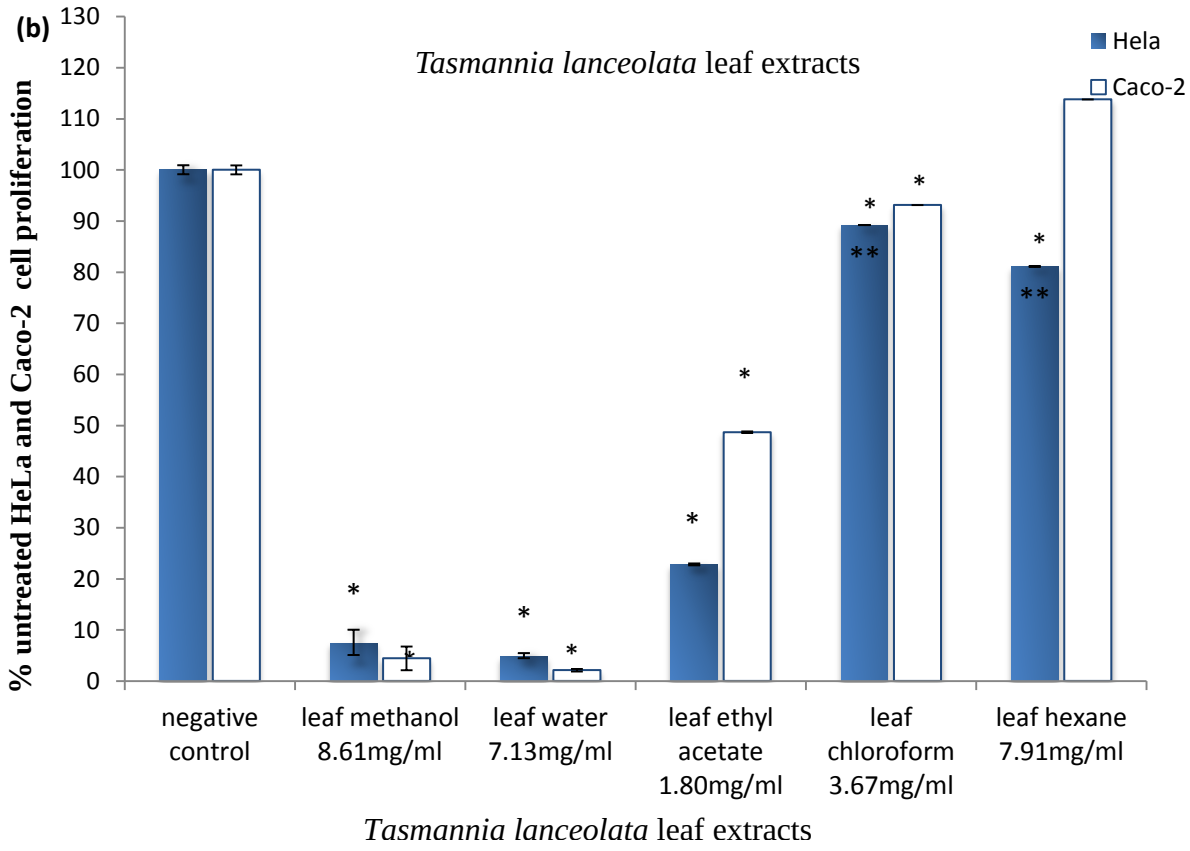

(b)
% untreated HeLa and Caco-2 cell proliferation
Tasmannia lanceolata leaf extracts
Hela
Caco-2
negative control
leaf methanol 8.61mg/ml
leaf water 7.13mg/ml
leaf ethyl acetate 1.80mg/ml
leaf chloroform 3.67mg/ml
leaf hexane 7.91mg/ml
Tasmannia lanceolata leaf extracts

Figure 3.9 Cell proliferation (MTS) assays of *T. lanceolata* (a) fruit and (b) leaf extracts and untreated controls against HeLa (human cervical carcinoma cancer cell line ATCC CCL-2) (solid bars) and Caco-2 (human colorectal carcinoma cancer cell line ATCC ® HTB-37™) (clear bars) carcinomas cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

3.3.9 Quantification of antiproliferative efficacy by IC_{50} determination

The antiproliferative efficacies of the plant extracts were further quantified by IC_{50} determination. The inhibition of HeLa/Caco-2 proliferation for all plant extracts was dose dependant, with the level of inhibitory activity decreasing at lower concentrations (Table 3.2). Selection criteria were implemented to grade the effectiveness of plant extracts. IC_{50} values < 200 µg/mL were deemed to be very potent antiproliferative agents; values < 500 µg/mL were classified as potent antiproliferative agents, values < 1000 µg/mL were classed as good antiproliferative agents, whilst values above 1000 µg/mL were categorised as moderate to weak antiproliferative agents. Generally, the methanolic and aqueous extracts were generally the most promising antiproliferative agents against HeLa and Caco-2 cells with some notable exceptions. Many of these extracts had IC_{50} values substantially < the 1000 µg/mL. In comparison, most ethyl acetate fruit and leaf extracts were relatively weak antiproliferative agents, with some exceptions.

Forty-nine of the 56 plant extracts tested displayed significant ($p<0.01$) anti-proliferative effects against HeLa carcinoma cells (Fig 3.1 - 3.9). Of the 49 extracts tested, the HeLa antiproliferative IC_{50} values varied in range from 58 µg/mL (*S. australe* ethyl acetate fruit extract), to a high of 6550 µg/mL (*K. pomifera* methanolic fruit extract). Only nineteen out of the 49 extracts inhibited HeLa proliferation by 50%. Subsequently, IC_{50} values were not determined for the remainder of the extracts. The *S. australe* ethyl acetate fruit extract was the most potent antiproliferative agent in this category and of all the 49 extracts tested against HeLa with an IC_{50} 58 µg/mL (< 200 µg/mL). The *S. leuhmannii* aqueous fruit extract was

also very potent, with an IC_{50} value of 86 µg/mL. The *S. australe* methanolic, aqueous fruit and methanolic leaf extracts IC_{50} values of 134 µg/mL, 172 µg/mL and 187 µg/mL were also indicative of very potent antiproliferative agents. The *S. leuhmannii* aqueous fruit, methanolic and aqueous leaf extracts with IC_{50} values of: 86 µg/mL, 165 µg/mL, 128 µg/mL were also classified in this category. A total of eight extracts were determined to be in the potent antiproliferative agents category with IC_{50} values < 500 µg/mL. Notably, the *D. pruriens* (methanolic, aqueous, ethyl acetate) fruit and methanolic leaf extracts were categorised as potent antiproliferative agents (IC_{50} values: 276 µg/mL, 305 µg/mL, 316 µg/mL and 376 µg/mL respectively). The *S. australe* aqueous, *T. lanceolata* aqueous, *T. ferdinandiana* ethyl acetate leaf and *A. acidula* methanolic fruit extract IC_{50} values: 283 µg/ mL, 230 µg/mL, 487 µg/mL and 480 µg/mL, also categorised these extracts as part of the same class (potent antiproliferative agents). The *T. ferdinandiana* ethyl acetate fruit and aqueous leaf extract IC_{50} values of 800 µg/mL and 606 µg/mL were classed as good antiproliferative agents (< 1000 µg/mL). Similarly *T. lanceolata* methanolic leaf and *E. angustifolium* methanolic fruit (IC_{50} values: 810 µg/mL, 859 µg/mL) were also considered good antiproliferative agents. The antiproliferative potencies against HeLa were in the order of *S. australe* (fruit and leaf) > *S. leuhmannii* (fruit and leaf) > *D. pruriens* (fruit and leaf) > *T. ferdinandiana* (fruit and leaf) > *T. lanceolata* (fruit and leaf) > *A. acidula* (fruit) > *E. angustifolium*.

Forty-six of the 56 plant extracts significantly inhibited ($p<0.01$) Caco-2 carcinoma proliferation (Fig 3.1- 3.9). Contrary to the trends observed with HeLa carcinoma proliferation, the *D. pruriens* (fruit), *T. ferdinandiana* (fruit and leaf) and *T. lanceolata* (fruit and leaf) ethyl acetate extracts were as potent antiproliferative agents against Caco-2 proliferation, with IC_{50} values: 372 µg/mL, 600 µg/mL, 102 µg/mL, 146 µg/mL, 225 µg/mL

respectively. Of the 46 extracts tested, Caco-2 values antiproliferative IC_{50} values ranged from 27 µg/mL (*S. australe* aqueous fruit extract) to a high of 10244 µg/mL (*T. ferdinandiana* methanolic fruit extract). Only 21 out of the 46 extracts inhibited Caco-2 proliferation by 50%. Incidentally, a larger number of extracts inhibited Caco-2 proliferation compared to HeLa. The *S. leuhmannii* aqueous fruit and leaf, and *S. australe* aqueous fruit extracts IC_{50} values of 124 µg/mL, 43 µg/mL and 27 µg/mL were very potent antiproliferative agents against Caco-2 proliferation (IC_{50} < 200 µg/mL). The *S. australe* aqueous fruit extract was the most potent antiproliferative agent with an IC_{50} of 27 µg/mL. The next most potent extract was the *S. leuhmannii* aqueous leaf extract, with an IC_{50} of 43 µg/mL. In addition, *T. lanceolata* ethyl acetate fruit, *T. lanceolata* aqueous leaf and *T. ferdinandiana* ethyl acetate extracts were also very potent antiproliferative agents with IC_{50} values of 146 µg/mL, 150 µg/mL and 102 µg/mL respectively. A further 10 extracts were classed as potent antiproliferative agents, with IC_{50} values < 500 µg/mL. Most *D. pruriens* fruit and leaf were potent antiproliferative agents against Caco-2 proliferation. Indeed, *D. pruriens* methanolic fruit, methanolic leaf, aqueous fruit, aqueous leaf and ethyl acetate fruit extracts, with IC_{50} values of 169 µg/mL, 212 µg/mL, 354 µg/mL, 295 µg/mL and 372 µg/mL were invariably potent at inhibiting Caco-2 proliferation. The *S. australe* methanolic fruit and *S. leuhmannii* methanolic leaf extracts (IC_{50} values of 279 µg/mL and 387 µg/mL respectively) were also considered potent Caco-2 inhibitors. In addition, the *T. ferdinandiana* methanolic fruit and *T. lanceolata* ethyl acetate leaf extracts were also potent inhibitory agents, with IC_{50} values of 438 µg/mL and 224.5 µg/mL respectively. Five extracts were good antiproliferative agents with IC_{50} < 1000 µg/mL. The *T. ferdinandiana* ethyl acetate fruit, *S. leuhmannii* methanolic fruit, *S. australe* methanolic leaf, *A. acidula* methanolic fruit and *A. acidula* aqueous fruit extracts (IC_{50} values of 600 µg/mL, 791 µg/mL, 653 µg/mL, 769

µg/mL, 885 µg/mL respectively), were classed as good antiproliferative agents. The antiproliferative potencies against Caco-2 were in the order of *D. pruriens* (fruit and leaf) > *S. australe* (fruit and leaf) > *S. leuhmannii* (fruit and leaf) > *T. lanceolata* (fruit and leaf) > *T ferdinandiana* (fruit and leaf) > *A. acidula* (fruit).

Table 3.2 IC_{50} values of the Australian fruits and leaves against HeLa and Caco-2, and also 24h LC_{50} values against *Artemia franciscana* nauplii (expressed as µg/mL with a 95% confidence interval).

Extracts		M			A			E			C			H		
Species	part/cell line	HeLa	Caco-2	LC_{50}	HeLa	Caco-2	LC_{50}	HeLa	Caco-2	LC_{50}	HeLa	Caco-2	LC_{50}	HeLa	Caco-2	LC_{50}
S. leuhmannii	fruit	884	791	414	86	124	478	DNI	DNI	181	N	N	4149	N	N	DNI
	leaf	165	387	450	128	43	813	DNI	DNI	ND	N	N	N	N	N	N
S. australe	fruit	134	279	1879	172	27	3310	58	DNI	514	N	N	DNI	N	N	DNI
	leaf	187	653	294	283	325	244	DNI	DNI	DNI	N	N	N	N	N	N
D. pruriens	fruit	276	169	6443	316	354	2883	305	372	DNI	N	N	N	N	N	N
	leaf	376	212	DNI	DNI	295	DNI	DNI	DNI	DNI	N	N	N	N	N	N
E. angustifolium	fruit	859	DNI	5418	DNI	DNI	3762	DNI	DNI	DNI	N	N	N	N	N	N
K. pomifera	fruit	DNI	DNI	1965	DNI	DNI	ND	DNI	DNI	1515	DNI	DNI	7843	DNI	DNI	DNI
P. elatus	fruit	DNI	DNI	1664	DNI	DNI	1956	DNI	DNI	1293	DNI	DNI	5946	DNI	DNI	DNI
A. acidula	fruit	480	769	1500	DNI	885	1872	1560	1301	1609	DNI	DNI	1984	DNI	DNI	5294

Table 3.2 IC_{50} values of the Australian fruits and leaves against HeLa and Caco-2, and also 24h LC_{50} values against *Artemia franciscana* nauplii (expressed as µg extract/mL with a 95% confidence interval). (cont'd)

Extracts / Species	part/cell line	M			A			E			C			H		
		HeLa	Caco-2	LC_{50}	HeLa	Caco-2	LC_{50}	HeLa	Caco-2	LC_{50}	HeLa	Caco-2	LC_{50}	HeLa	Caco-2	LC_{50}
T.ferdinandiana	fruit	1250	437.5	860	1060	1520	1200	800	600	DNI	DNI	DNI	DNI	DNI	DNI	DNI
	leaf	2240	10244	1133	606	1230	1330	487	102	767	DNI	DNI	DNI	DNI	DNI	DNI
T. lanceolata	fruit	1910	3070	3573	1663	1330	2376	1755	146	ND	DNI	DNI	DNI	DNI	DNI	DNI
	leaf	810	1030	2665	230	150	3096	1800	224.5	ND	DNI	DNI	DNI	DNI	DNI	DNI
		HeLa									**Caco-2**					
Cisplatin IC_{50}		15710									8400					
potassium dichromate LC_{50}			224													
Mevinphos LC_{50}				1336												

Numbers indicate the mean IC_{50} or LC_{50} values of triplicate determinations. DNI indicates that IC_{50}/LC_{50} were not obtained as % mortality did not exceed 50% highest dose tested during initial screen. M= methanol, A = aqueous, E = ethyl acetate, C = chloroform, H = hexane. ND indicates IC_{50}/LC_{50} values could not be determined as they were below 50% mortality for all doses tested. DNI = Tests were not performed due to lack of availability of plant material.

3.4 *A. franciscana* nauplii toxicity assay

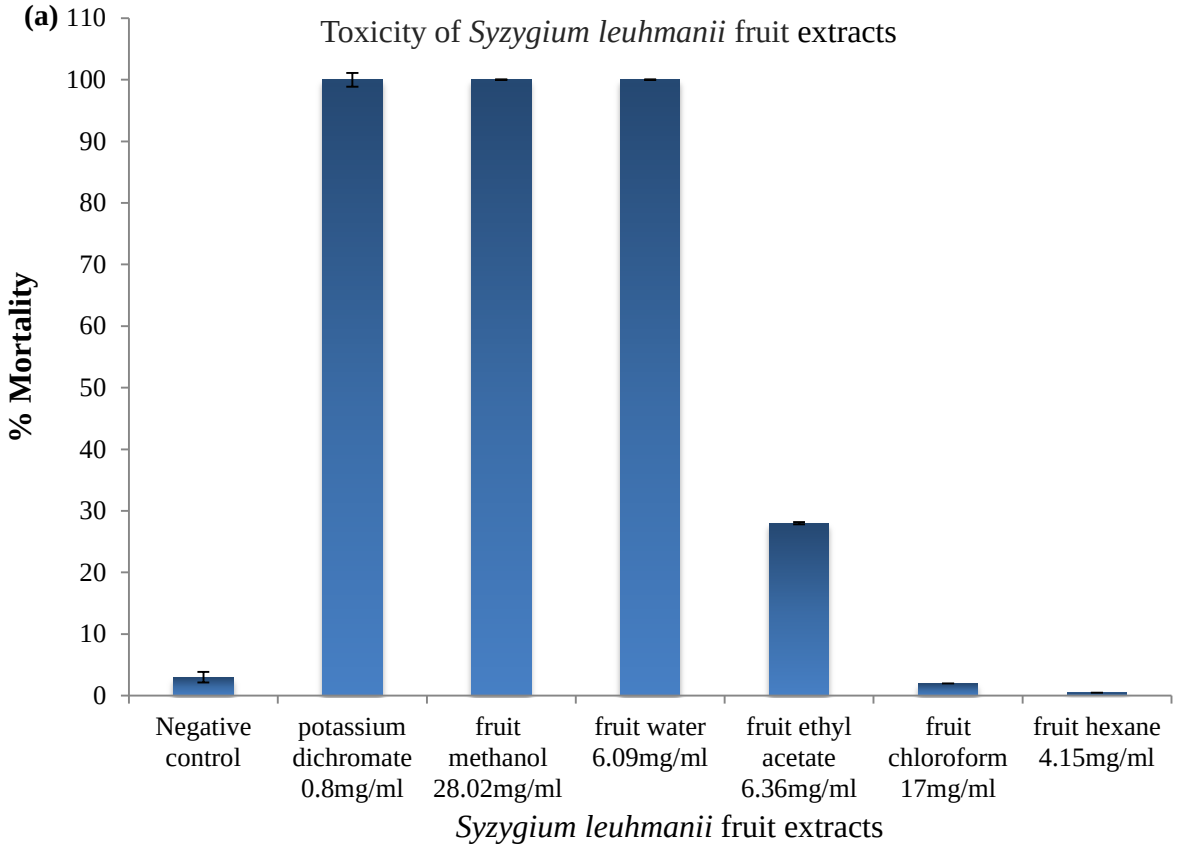
3.4.1 Toxicity of *Syzygium* species extracts.

The *S. leuhmannii* and *S. australe* methanolic and aqueous fruit extracts and leaf displayed > 50% mortality rates at 24h and were thus deemed to be toxic (Figure 3.10 a – 3.11 a). In contrast, the *S. leuhmannii* and *S. australe* mid-polarity ethyl acetate fruit and leaf induced < 50% mortality and hence were classed as non-toxic (Figure 3.10 – 3.11) (a – b). Similarly, the lower polarity (chloroform and hexane) fruit extracts induced < 50% mortality and hence deemed to be non-toxic.

The *S. leuhmannii* and *S. australe* (fruit and leaf) extracts were further tested across a range of concentrations to quantify the toxicity by determining the LC_{50} value (Table 3.2). Interestingly, the *S. leuhmannii* higher polarity (methanolic and aqueous) and mid polarity ethyl acetate fruit extracts had substantially lower of LC_{50} values < 500 µg/mL indicating high toxicity. Indeed, the mid-polarity ethyl acetate fruit extract was especially toxic (LC_{50} value 181 µg/mL).

The *S. leuhmannii* higher polarity methanolic and aqueous leaf extracts were toxic, with LC_{50} values of 450 µg/mL and 813 µg/mL. The *S. australe* methanolic and aqueous leaf extracts had substantially lower LC_{50} values < 500 µg/mL indicating high toxicity. In summary, *S.*

leuhmannii and *S. australe* (fruit and leaf) lower polarity extracts did not induce mortality > 50% mortality at 24h and hence were considered to be non-toxic. In contrast, *S. leuhmannii* methanolic, aqueous and ethyl acetate fruit extracts induced low LC_{50} values (< 500 µg/mL) indicating considerable toxicity. Likewise, *S. australe* methanolic, aqueous and ethyl acetate fruit extracts were deemed to be toxic for the same reason. *S. leuhmannii* and *S. australe* have previously been shown to have high ascorbic acid contents [179] which may significantly decrease the assay pH. The same study reported that similar mortality could be induced by decreasing the pH of the aqueous environment by a similar extent. This may account for these apparent toxicities.


(a)
Toxicity of Syzygium leuhmanii fruit extracts
% Mortality
110
100
90
80
70
60
50
40
30
20
10
0
Negative control
potassium dichromate 0.8mg/ml
fruit methanol 28.02mg/ml
fruit water 6.09mg/ml
fruit ethyl acetate 6.36mg/ml
fruit chloroform 17mg/ml
fruit hexane 4.15mg/ml
Syzygium leuhmanii fruit extracts

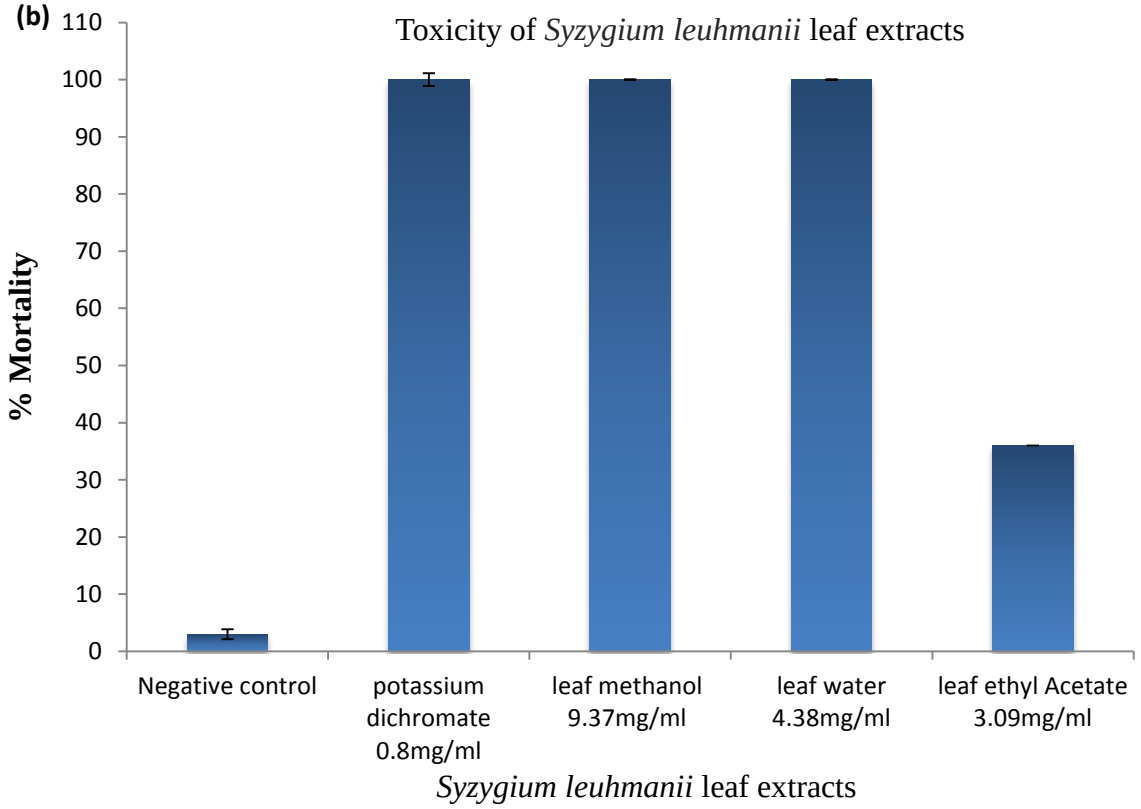
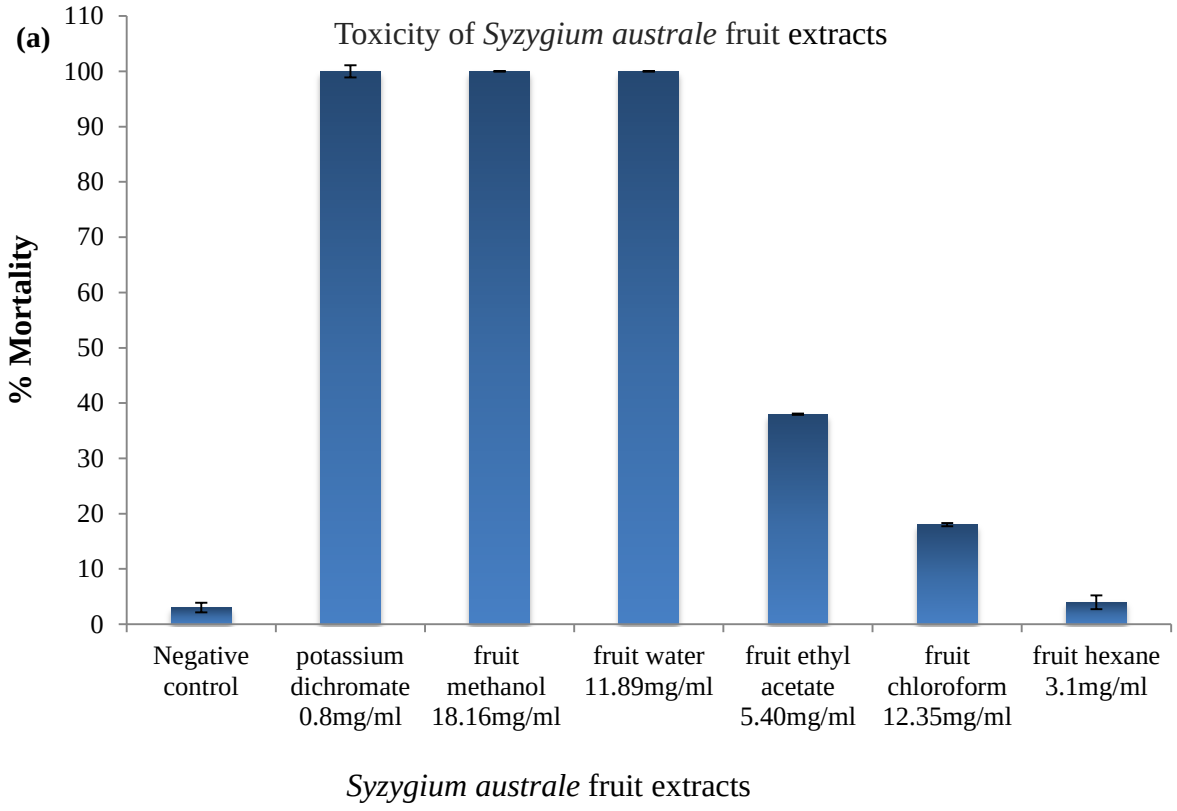

(b)
Toxicity of Syzygium leuhmanii leaf extracts
% Mortality
110
100
90
80
70
60
50
40
30
20
10
0
Negative control
potassium dichromate 0.8mg/ml
leaf methanol 9.37mg/ml
leaf water 4.38mg/ml
leaf ethyl Acetate 3.09mg/ml
Syzygium leuhmanii leaf extracts

Figure 3.10: The lethality of the *S. leuhmannii* (a) fruit and (b) leaf extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.


(a)
Toxicity of Syzygium australe fruit extracts
% Mortality
110
100
90
80
70
60
50
40
30
20
10
0
Negative control
potassium dichromate 0.8mg/ml
fruit methanol 18.16mg/ml
fruit water 11.89mg/ml
fruit ethyl acetate 5.40mg/ml
fruit chloroform 12.35mg/ml
fruit hexane 3.1mg/ml
Syzygium australe fruit extracts

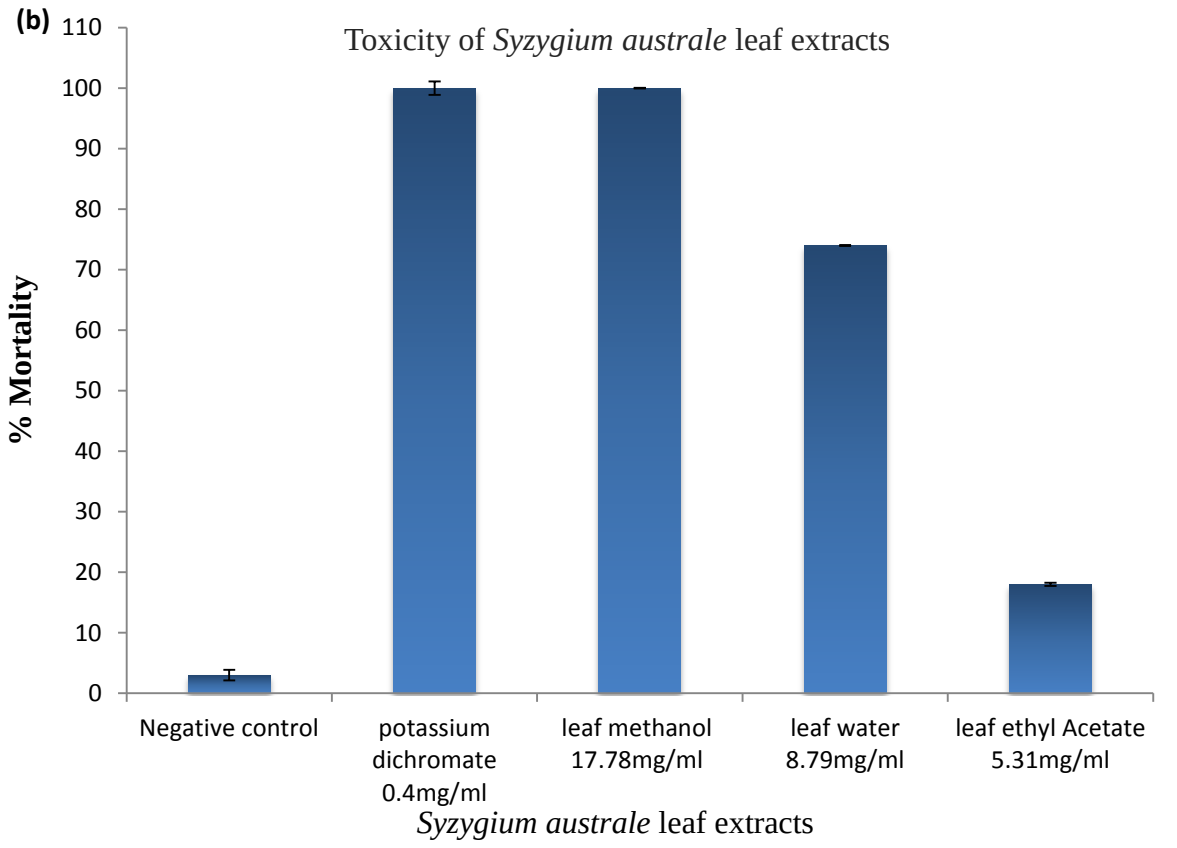
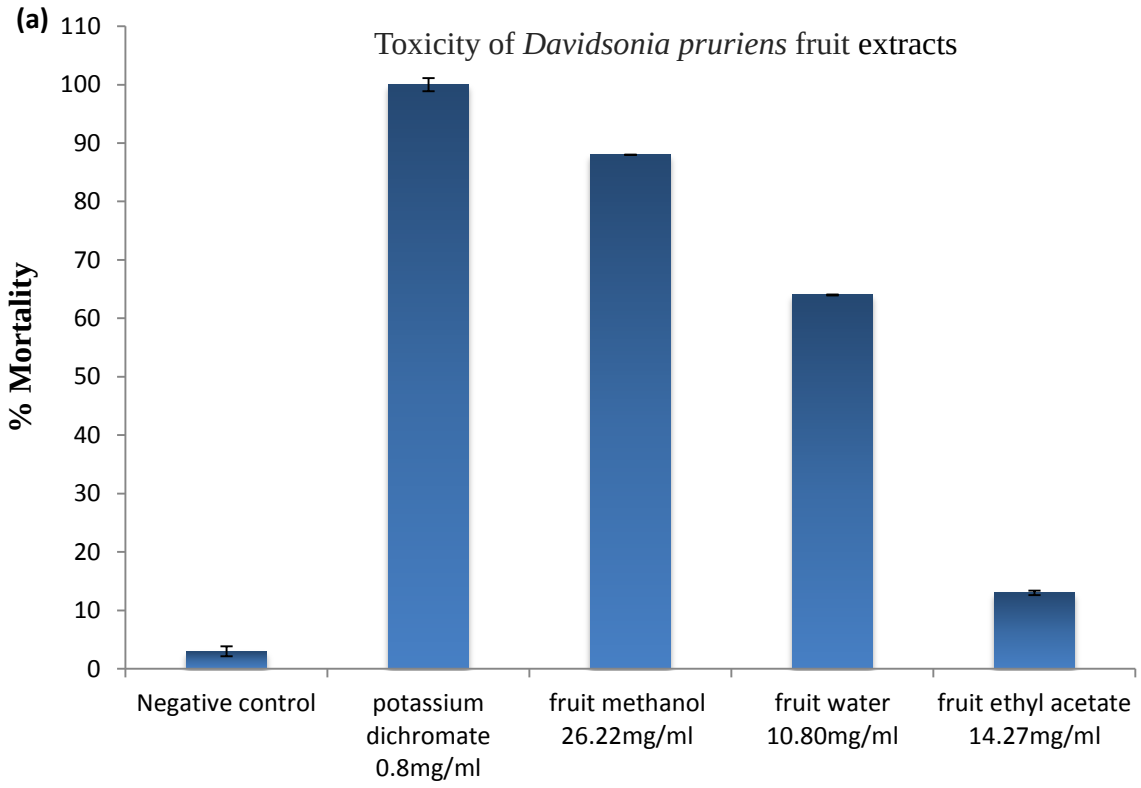

(b)
Toxicity of Syzygium australe leaf extracts
% Mortality
110
100
90
80
70
60
50
40
30
20
10
0
Negative control
potassium dichromate 0.4mg/ml
leaf methanol 17.78mg/ml
leaf water 8.79mg/ml
leaf ethyl Acetate 5.31mg/ml
Syzygium australe leaf extracts

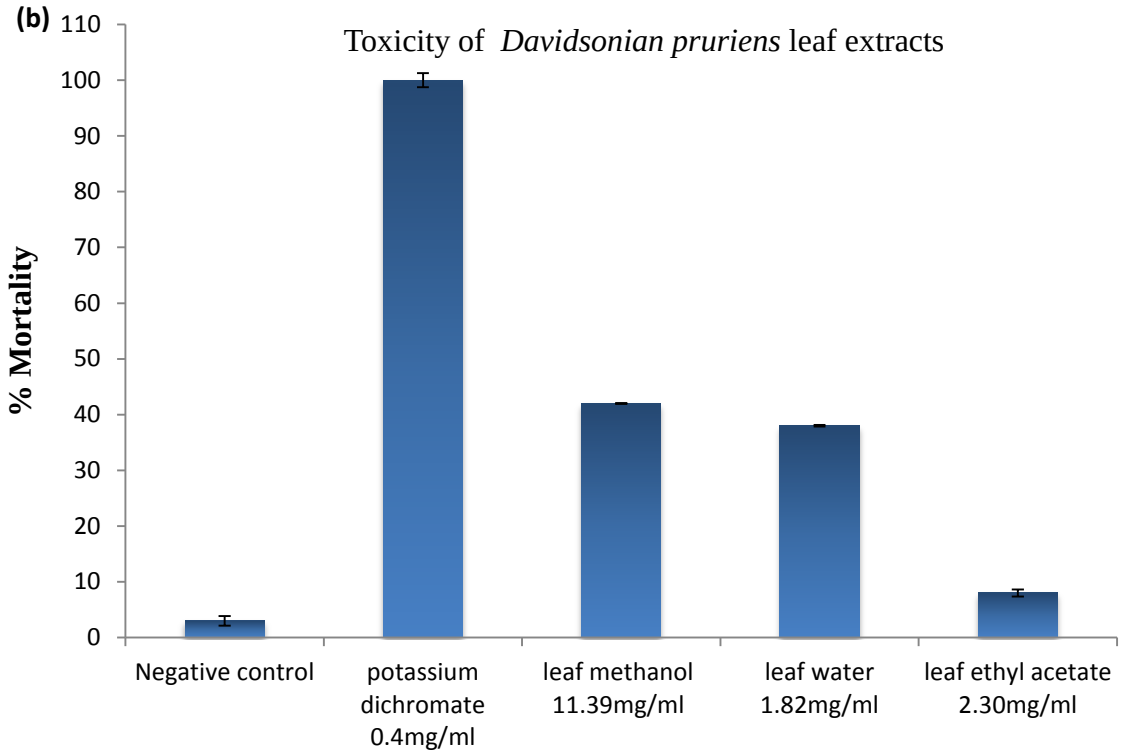
Figure 3.11: The lethality of the *S. australe* (a) fruit and (b) leaf extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

3.4.2 Toxicity of *Davidsonia pruriens* extracts

The *D. pruriens* methanolic and aqueous fruit extracts displayed > 50% mortality rates at 24h and were thus deemed to be toxic (Figure 3.12 a). The mid-polarity ethyl acetate fruit extracts induced < 50% mortality and hence considered non-toxic. Similarly, the *D. pruriens* methanolic, aqueous, mid polarity ethyl acetate leaf extracts showed < 50% mortality rates at 24h and were also considered to be non-toxic (Figure 3.12 b). The *D. pruriens* fruit and leaf extracts were further tested across a range of concentrations to quantify the toxicity by determining the LC_{50} value (Table 3.2). All *D. pruriens* fruit and leaf extracts displayed low toxicity (LC_{50} > 1000 µg/mL) following 24 hours exposure (Table 3.2). No LC_{50} values are reported for the *D. pruriens* for any mid-polarity ethyl acetate extract as less than 50% mortality was seen for all concentrations tested. In summary, all *D. pruriens* (fruit and leaf) extracts were nontoxic (LC_{50} > 1000 µg/mL) following 24 hours exposure.



Davidsonian pruriens fruit extracts



Davidsonian pruriens leaf extracts

Figure 3.12: The lethality of the *D. pruriens* (a) fruit and (b) leaf extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

3.4.3 Toxicity of *Elaeocarpus angustifolius* extracts

The *E. angustifolius* methanolic and aqueous fruit extracts displayed > 50% mortality rates at 24h and were thus deemed to be toxic (Figure 3.13). In contrast, the mid-polarity ethyl acetate fruit extracts induced < 50% mortality and hence were therefore considered to be non-toxic. The *E. angustifolius* fruit extracts were further tested across a range of concentrations to quantify the toxicity by determining the LC_{50} value (Table 3.2). *E. angustifolius* methanolic and aqueous fruit extracts were determined to be nontoxic (LC_{50} > 1000 µg/mL) following 24 hours exposure (Table 3.2). No LC_{50} values are reported for the *E. angustifolius* ethyl acetate fruit extract as less than 50% mortality was seen for all concentrations tested. In summary, all *E. angustifolius* fruit extracts displayed nontoxic (LC_{50} > 1000 µg/mL).

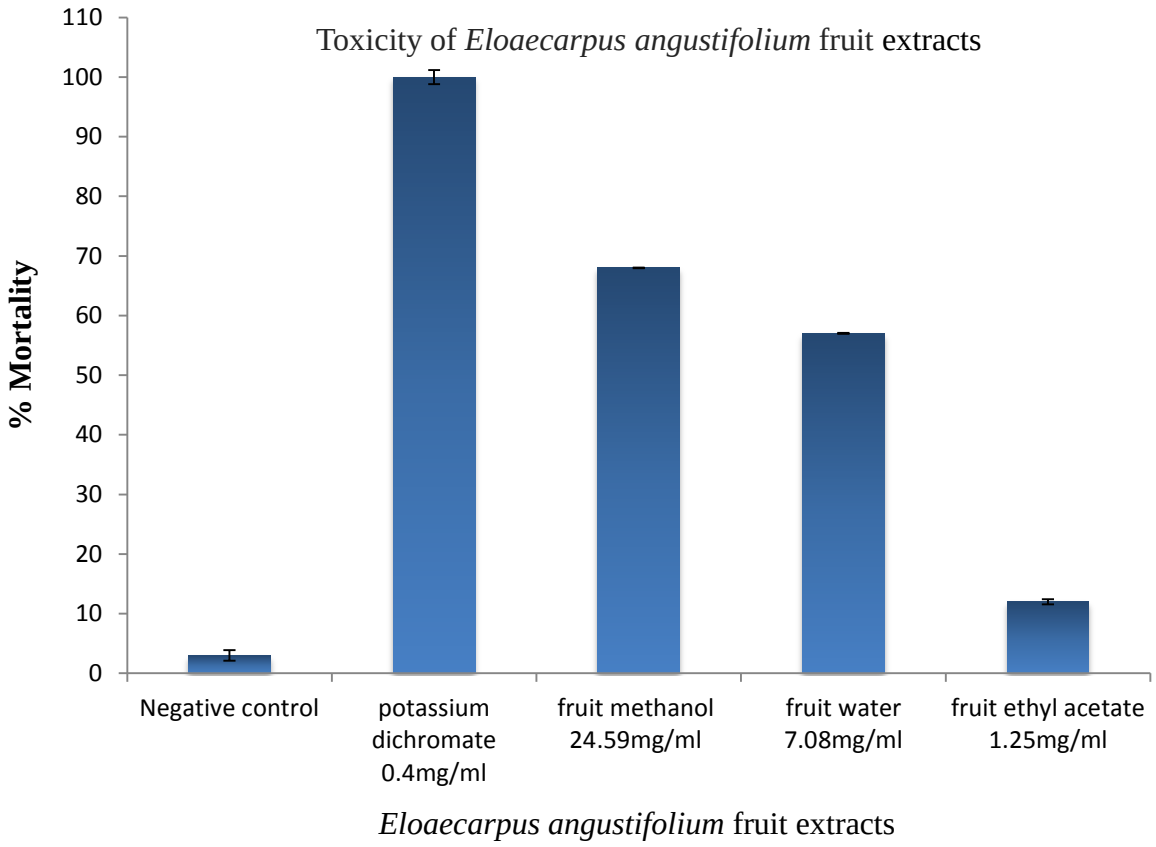


Figure 3.13: The lethality of the *E. angustifolius* fruit extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

3.4.4 Toxicity of *Kunzea pomifera* extracts

The *K. pomifera* methanolic, ethyl acetate and chloroform fruit extracts displayed > 50% mortality rates at 24h and were thus deemed to be toxic (Figure 3.14). In contrast, aqueous and hexane fruit extracts induced < 50% mortality and were therefore considered to be non-toxic. The *K. pomifera* fruit extracts were further tested across a range of concentrations to quantify the toxicity by determining the LC_{50} values (Table 3.2). *K. pomifera* methanolic, ethyl acetate, and chloroform fruit extracts were nontoxic (LC_{50} > 1000 µg/mL) following 24 hours exposure (Table 3.2). No LC_{50} values are reported for the *K. pomifera* for the high polarity methanolic and lower polarity hexane fruit extract as less than 50% mortality was seen for all concentrations tested. Therefore, all *K. pomifera* fruit extracts displayed low toxicity (LC_{50} > 1000 µg/mL) following 24 hours exposure at all concentrations, and hence were considered to be non-toxic.

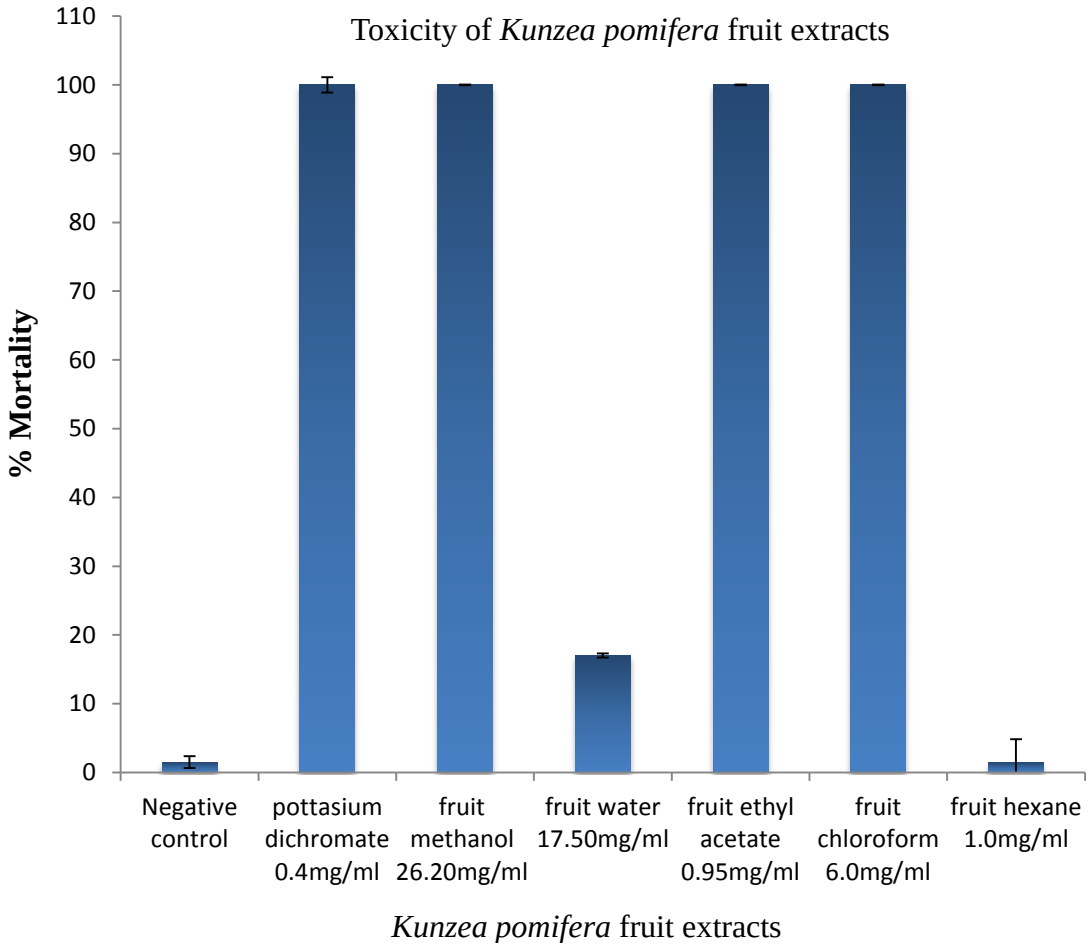


Figure 3.14: The lethality of the *K. pomifera* fruit extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

3.4.5 Toxicity of *Podocarpus elatus* extracts

The *P. elatus* methanolic, aqueous, ethyl acetate and chloroform fruit extracts all displayed > 50% mortality rates in the *Artemia* nauplii bioassay following 24h exposure and were thus deemed to be toxic (Figure 3.15). In contrast, the lower polarity hexane fruit extract on the other hand, induced < 50% mortality and was therefore hence considered to be non-toxic. The *P. elatus* fruit extracts were further tested across a range of concentrations to quantify the toxicity by determining the LC_{50} values (Table 3.2). *P. elatus* methanolic, aqueous, ethyl acetate, and chloroform fruit extracts were nontoxic displayed low toxicity (LC_{50} > 1000 µg/mL) following 24 hours exposure (Table 3.2). No LC_{50} values are reported for the *P. elatus* for hexane fruit extract as less than 50% mortality was seen for all concentrations tested. In summary, all *P. elatus* fruit extracts were nontoxic in the *Artemia* nauplii bioassay (LC_{50} > 1000 µg/mL)

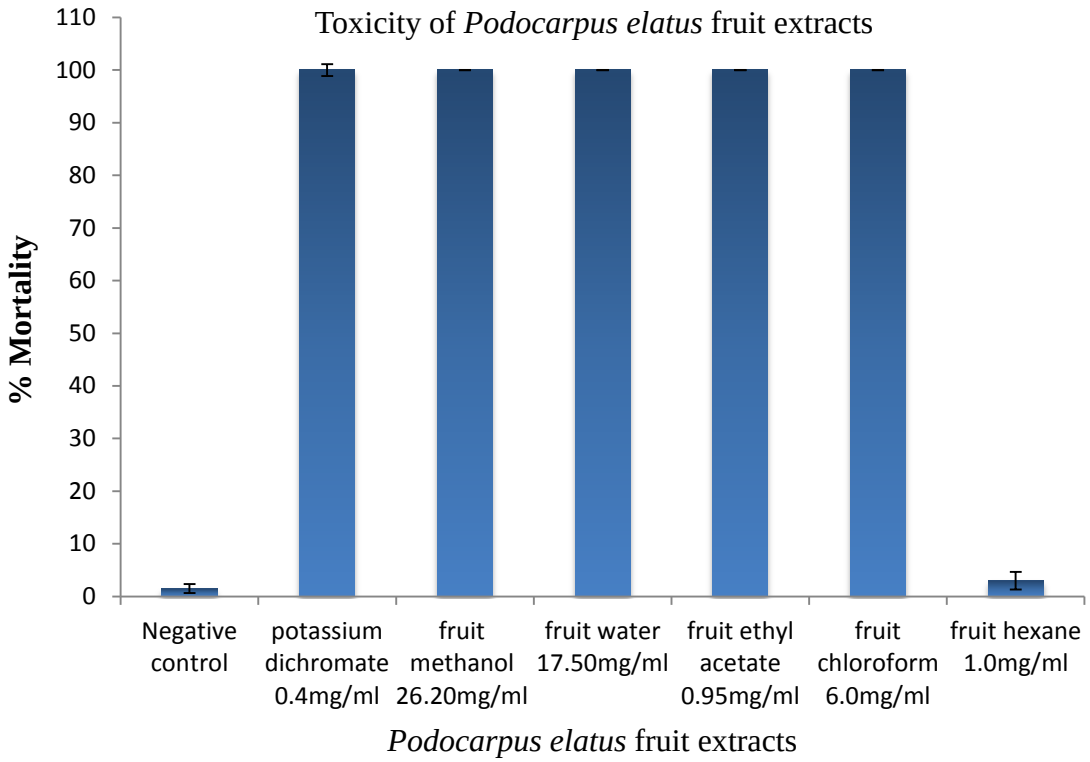


Figure 3.15: The lethality of the *P. elatus* fruit extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

3.4.6 Toxicity of *Acronychia acidula* extracts

All *A. acidula* fruit extracts displayed > 50% mortality rates at 24h and were thus deemed to be toxic (Figure 3.16). The *A. acidula* fruit extracts were further tested across a range of concentrations to quantify the toxicity by determining the LC_{50} values (Table 3.2). All *A. acidula* fruit extracts displayed low toxicity LC_{50} > 1000 µg/mL following 24 hours exposure (Table 3.2).

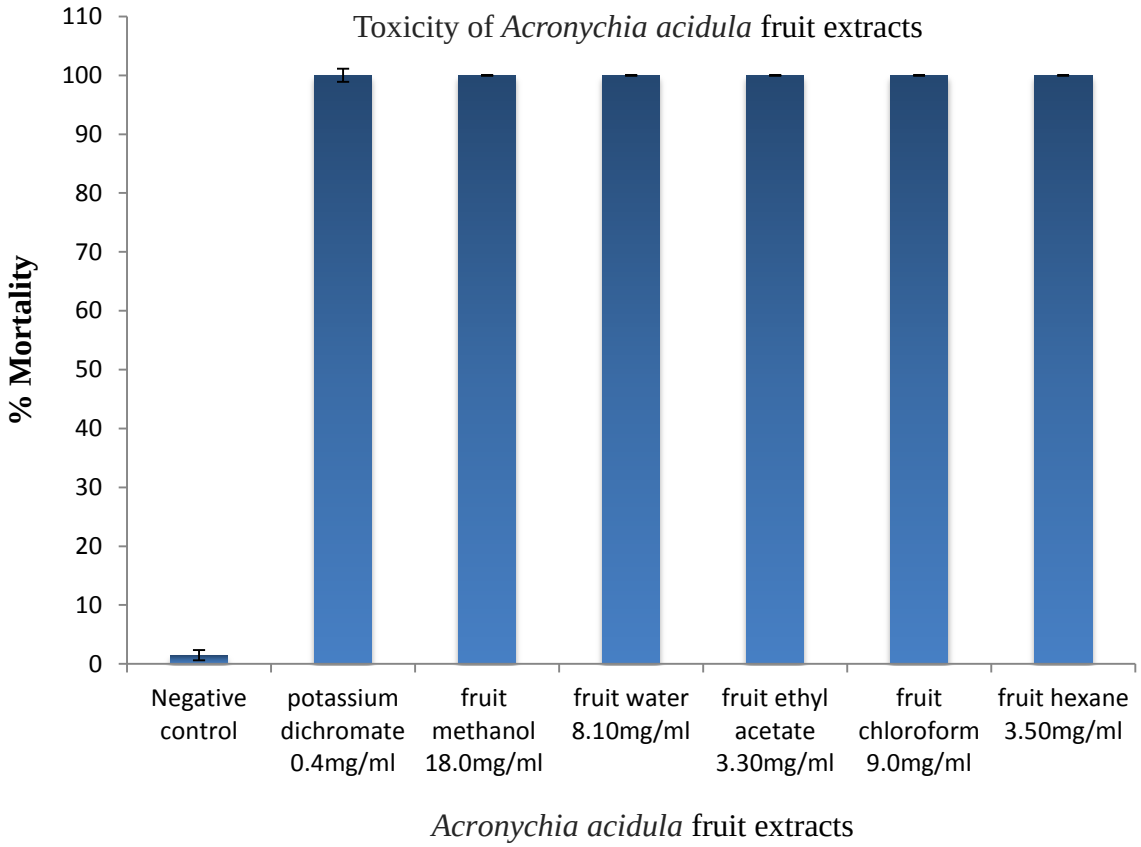
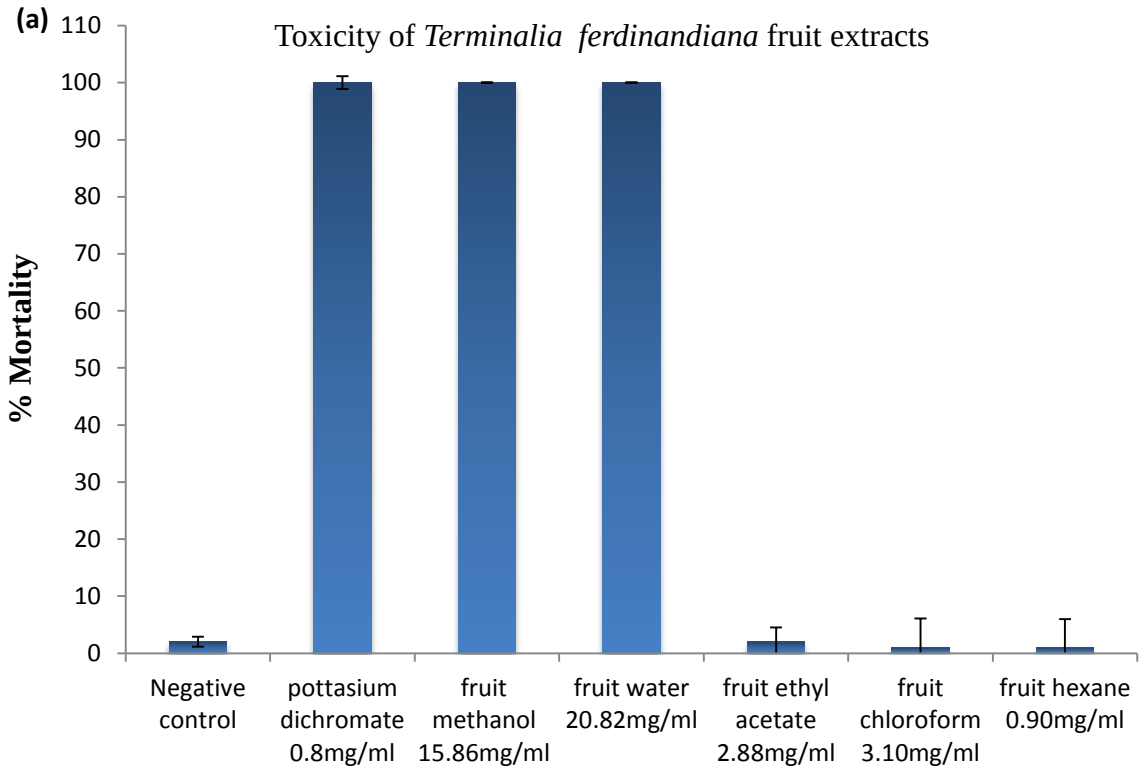


Figure 3.16: The lethality of the *A. acidula* fruit extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

3.4.7 Toxicity of *Terminalia ferdinandiana* extracts

The *T. ferdinandiana* methanolic and aqueous fruit extracts displayed > 50% mortality rates at 24h and were thus deemed to be toxic (Figure 3.17 a). The mid-polarity ethyl acetate and lower polarity (chloroform and hexane) fruit extracts induced < 50% mortality and were thus considered to be non-toxic. In contrast, the *T. ferdinandiana* methanolic, aqueous and ethyl acetate leaf extracts showed > 50% mortality rates at 24h and were therefore considered to be toxic (Figure 3.17 b). The chloroform and hexane leaf extracts induced < 50% mortality and were therefore considered to be non-toxic.

The *T. ferdinandiana* fruit and leaf extracts were further tested across a range of concentrations to quantify the toxicity by determining the LC_{50} value (Table 3.2). All *T. ferdinandiana* fruit and leaf extracts were non-toxic (LC_{50} > 1000 µg/mL) following 24 hours exposure, with some notable exceptions (Table 3.2). The *T. ferdinandiana* methanolic fruit and ethyl acetate leaf extracts displayed moderate toxicity (LC_{50} values: 860 µg/mL, 767 µg/mL) following 24 hours exposure. No LC_{50} values are reported for *T. ferdinandiana* fruit and leaf for any chloroform or hexane extracts as less than 50% mortality was seen for all concentrations tested. In summary, the *T. ferdinandiana* methanolic fruit and ethyl acetate leaf extracts were the only extracts which were deemed to be toxic. All other *T. ferdinandiana* fruit and leaf extracts were non-toxic, with LC_{50} values > 1000 µg/mL following 24 hours exposure.


(a)
Toxicity of *Terminalia ferdinandiana* fruit extracts
% Mortality
110
100
90
80
70
60
50
40
30
20
10
0
Negative control
pottasium dichromate 0.8mg/ml
fruit methanol 15.86mg/ml
fruit water 20.82mg/ml
fruit ethyl acetate 2.88mg/ml
fruit chloroform 3.10mg/ml
fruit hexane 0.90mg/ml
Terminalia ferdinandiana fruit extracts

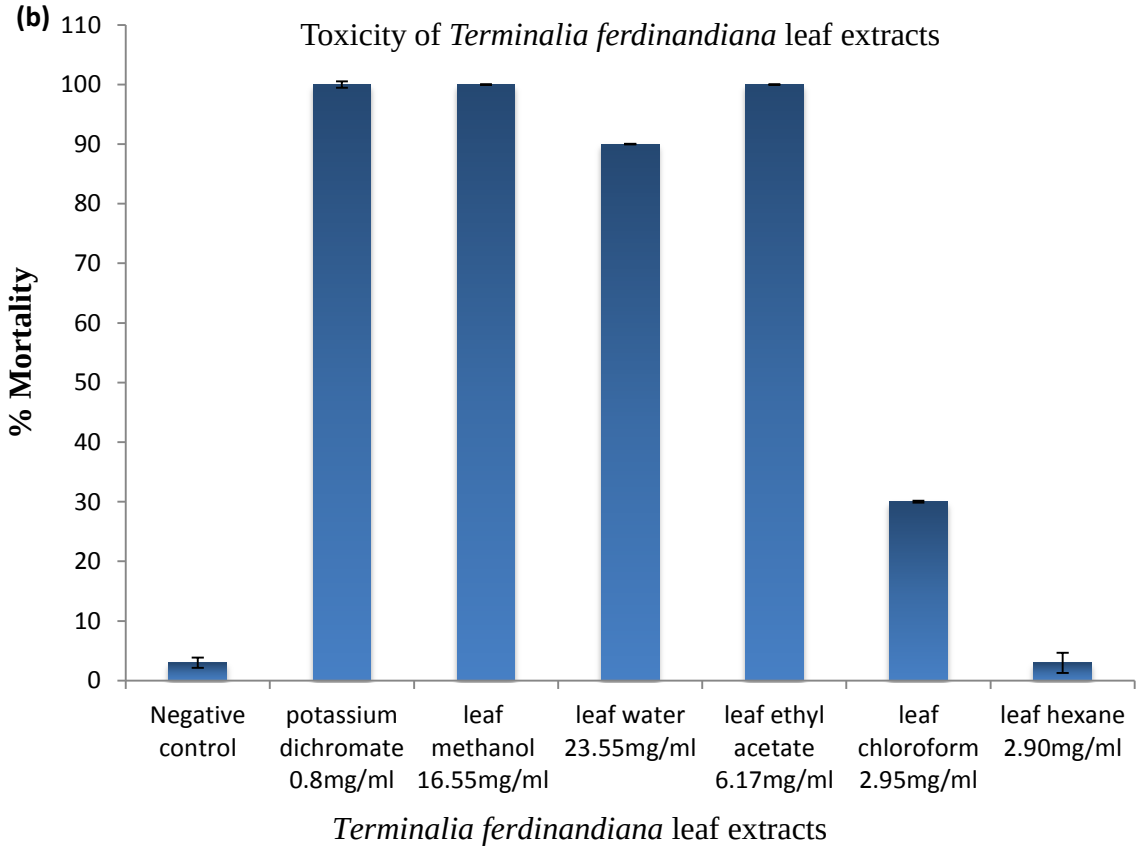
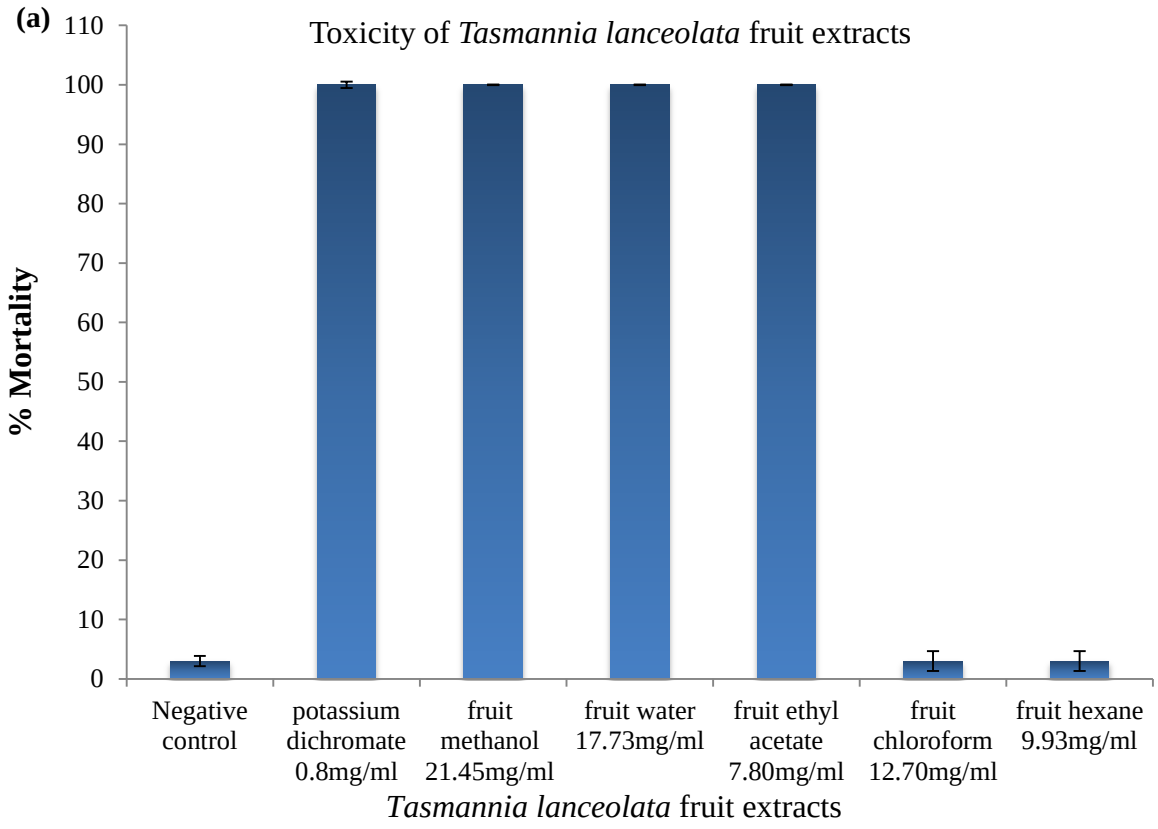

(b)
Toxicity of *Terminalia ferdinandiana* leaf extracts
% Mortality
110
100
90
80
70
60
50
40
30
20
10
0
Negative control
potassium dichromate 0.8mg/ml
leaf methanol 16.55mg/ml
leaf water 23.55mg/ml
leaf ethyl acetate 6.17mg/ml
leaf chloroform 2.95mg/ml
leaf hexane 2.90mg/ml
Terminalia ferdinandiana leaf extracts

Figure 3.17: The lethality of the *T. ferdinandiana* (a) fruit and (b) leaf extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

3.4.8 Toxicity of *Tasmannia lanceolata* extracts

The *T. lanceolata* methanolic, aqueous and ethyl acetate, fruit extracts displayed > 50% mortality rates at 24h and were thus deemed to be toxic (Figure 3.18 a). The chloroform and hexane fruit extracts induced < 50% mortality and were therefore considered to be non-toxic. Similarly, the *T. lanceolata* methanolic, aqueous and ethyl acetate leaf extracts showed > 50% mortality rates at 24h and were thus, considered to be toxic (Figure 3.18 b). The chloroform and hexane leaf extracts induced < 50% mortality and were therefore considered to be non-toxic.

The *T. lanceolata* fruit and leaf extracts were further tested across a range of concentrations to quantify the toxicity by determining the LC_{50} value (Table 3.2). All *T. lanceolata* fruit and leaf methanolic, aqueous and ethyl acetate extracts were non-toxic (LC_{50} > 1000 µg/mL) following 24 hours exposure (Table 3.2). No LC_{50} values are reported for the *T. ferdinandiana* fruit and leaf for any lower polarity chloroform and hexane extract as less than 50% mortality was seen for all concentrations tested. In summary, all *T. lanceolata* extracts were non-toxic in the Artemia nauplii toxicity assay.


(a)
Toxicity of Tasmannia lanceolata fruit extracts
% Mortality
110
100
90
80
70
60
50
40
30
20
10
0
Negative control
potassium dichromate 0.8mg/ml
fruit methanol 21.45mg/ml
fruit water 17.73mg/ml
fruit ethyl acetate 7.80mg/ml
fruit chloroform 12.70mg/ml
fruit hexane 9.93mg/ml
Tasmannia lanceolata fruit extracts

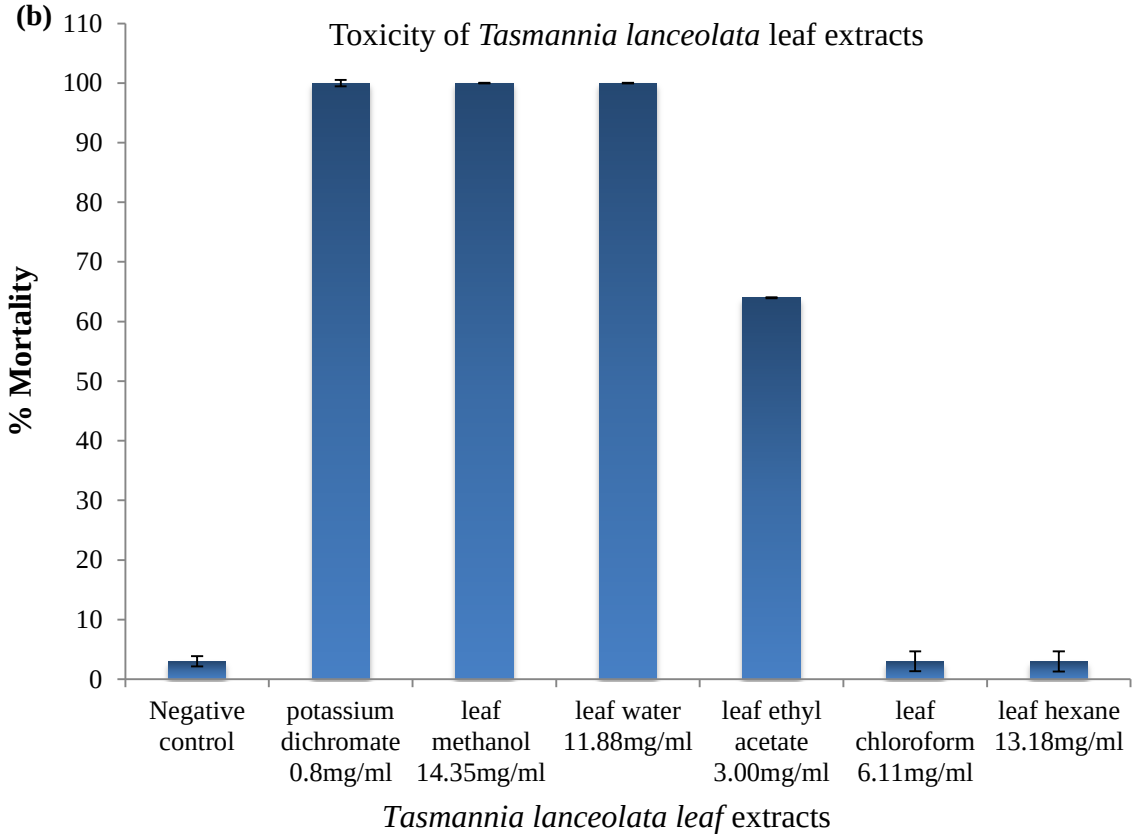

(b)
Toxicity of Tasmannia lanceolata leaf extracts
% Mortality
110
100
90
80
70
60
50
40
30
20
10
0
Negative control
potassium dichromate 0.8mg/ml
leaf methanol 14.35mg/ml
leaf water 11.88mg/ml
leaf ethyl acetate 3.00mg/ml
leaf chloroform 6.11mg/ml
leaf hexane 13.18mg/ml
Tasmannia lanceolata leaf extracts

Figure 3.18: The lethality of the *T. lanceolata* (a) fruit and (b) leaf extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *A. franciscana* nauplii following 24 hours exposure. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

3.5 Discussion

Diets consisting of fruits and vegetables rich in polyphenolic compounds (flavonoids, tannins and phenolic acids), have been linked with reduced morbidity and mortality brought about by chronic diseases [123, 133, 137-139, 141-150]. As mentioned in chapter 1, these polyphenolics are potent antioxidants that are well correlated with their ability to inhibit scavenge free radicals, prevent inflammation, retard progression of carcinogenesis and mutagenesis among many others. Many chronic diseases are caused by the inability to regulate the redox state of the cell due to injury both internally and externally via free radical damage. Hence, it has been postulated that by altering redox state of cell, it may shift the cell back to its normal state or induce apoptosis in aberrant carcinoma cells.

The studies reported in this chapter have attempted to verify the hypothesis that an alteration to the redox state of a cell may induce cytostatic and/or cytotoxic effects in carcinoma cell lines. In order to achieve this, the study attempted to establish a direct correlation between the levels of antioxidant content present in the selected plants and their corresponding efficacies as antiproliferative agents against well characterised carcinomas *in vitro*. A second aim was to determine the toxicities, if any, of these extracts to determine their relative safety.

All plant species tested were determined to be active and subsequently good inhibitors of HeLa and Caco-2 proliferation with some notable exceptions, where proliferation was noted instead. Several species displayed very good inhibitions against HeLa and Caco-2. The *T.*

lanceolata, *S. australe*, *S. leuhmannii*, *D. pruriens* and *T. ferdinandiana* extracts were particularly potent with IC_{50} values as low as 27 µg/mL determined (*S. australe* leaf versus Caco-2 cells).

A general trend was noted: the degree of inhibition decreased with decreasing polarity across the extracts, even within the same plant species. Similarly, the extract yields also differed with polarity as did the phytochemical classes. The methanolic and aqueous extracts were of higher yields and richer in phytochemicals than those of the ethyl acetate, chloroform and hexane extracts. The antioxidant capacities in the methanolic and aqueous extracts were also notably higher. In summary, the antioxidant capacities were in the order of *Terminalia ferdinandiana* (fruit and leaf) > *Tasmannia lanceolata* (fruit and leaf) > *Syzygium leuhmannii* (fruit and leaf) > *Syzygium australe* (fruit and leaf) > *Davidsonia pruriens* (fruit and leaf) > *Elaeocarpus angustifolius* (fruit) > *Acronychia acidula* (fruit) > *Kunzea pomifera* (fruit) > *Podocarpus elatus* (fruit).

The trends generally indicated that this inhibitory efficacy positively correlated with the antioxidant capacity present in the extract. Hence, the higher the antioxidant content, the greater the inhibition of proliferation. This was a generally noted trend amongst the methanolic and aqueous extracts. Conversely, very low antioxidant capacity often induced proliferation of the carcinomas cells *in vitro*, which was often observable with the ethyl acetate extracts. Indeed, the *S .leuhmannii* fruit and leaf ethyl acetate extracts induced proliferation in Caco-2 and to a lesser extent in HeLa. Likewise such activity was also evident for the *D. pruriens* leaf ethyl acetate and *E. angustifolium* ethyl acetate fruit extracts.

The lower polarity chloroform and hexane extracts also displayed similar activity, as generally observed for the *K. pomifera* and *T. lanceolata* fruit extracts.

The *T. ferdinandiana* methanolic fruit extract had the highest antioxidant content recorded at 660 mg AA equivalents per g. The antioxidant capacity correlated well with inhibition of proliferation. Indeed, a decrease in cell proliferation was observed with HeLa cells, indicative of cytotoxicity. The same extract also strongly inhibited Caco-2 proliferation (IC_{50} value < 500 µg/mL). Moderate toxicity observed in the *Artemia* assay was reported with LC_{50} value > 500 µg/mL.

Alternatively, the *S. leuhmannii* aqueous fruit extract (moderate antioxidant capacity) was quantified at 59 mg AA equivalents per g. This high antioxidant capacity correlated with the strong inhibition of HeLa (IC_{50} value: 86 µg/mL), whilst also inhibiting Caco-2 proliferation (IC_{50} value: 124 µg/mL). However, despite the great efficacy at inhibiting HeLa and Caco-2 proliferation, the *S. leuhmannii* aqueous fruit extract displayed high toxicity (LC_{50} value < 500 µg/mL). It may be postulated that there could be other components in the extract which may have contributed to toxicity in *Artemia* assay and subsequent potent inhibition in the cell proliferation assay, and not necessarily be due to the redox potential of the extract.

Other trends were also observed. The *D. pruriens* methanolic and aqueous fruit extracts were relatively low in antioxidant content, however potent antiproliferative agents against HeLa and Caco-2 (IC_{50} values < 500 µg/mL) and with low toxicities LC_{50} > 1000 µg/mL).

Likewise the *A. acidula* were also relatively low in antioxidant capacity, of low toxicity, and good antiproliferative agents. Therefore, these plant species did not fulfil the selection criteria and were not selected.

The *T. ferdinandiana* was chosen for further study because of its extremely high ascorbic acid and antioxidant capacity. In addition, *T. ferdinandiana* extracts were good antiproliferative agents against HeLa and Caco-2, and were low in toxicity. The *T. ferdinandiana* methanolic fruit and the ethyl acetate leaf extract were considered low toxicity LC_{50} < 1000 µg/mL. Of particular note, the *T. ferdinandiana* ethyl acetate fruit extract and were good antiproliferative agents against the HeLa and Caco-2 IC_{50} < 1000 µg/mL. The *T. ferdinandiana* ethyl acetate leaf extract was classed as a potent antiproliferative agent against HeLa IC_{50} < 500 µg/mL, whilst a very potent antiproliferative agent against Caco-2 IC_{50} < 200 µg/mL.

Similarly the *T. lanceolata* was also chosen as it had high antioxidant capacity but low ascorbic acid content. It was a good antiproliferative agent against both HeLa and Caco-2 and yet it was low in toxicity. The *T. lanceolata* ethyl acetate fruit extract was also a very potent antiproliferative agent against Caco-2 IC_{50} < 200 µg/mL and considered non-toxic. Conversely, the *T. lanceolata* aqueous leaf was a potent antiproliferative agent against HeLa IC_{50} < 500 µg/mL and a very potent antiproliferative agent against Caco-2 IC_{50} < 200 µg/mL carcinomas, and yet non-toxic (LC_{50} > 1000 µg/mL). The *T. lanceolata* methanolic leaf was a good antiproliferative agent against HeLa IC_{50} < 1000 µg/mL, whilst the *T.lanceolata* ethyl acetate leaf extract was a potent antiproliferative against Caco-2 IC_{50} < 500 µg/mL and yet non-toxic LC_{50} > 1000 µg/mL.

In summary the *T.* ferdinandiana and *T.lanceolata* (fruit and leaf) were chosen for further investigations into their mechanisms of action because of their high antioxidant content, low toxicity and high potency as antiproliferative agents against HeLa and Caco-2 carcinomas. Both species had high antioxidant capacity > 100 mg AA equivalents per g, and were non-toxic LC_{50} > 1000 µg/mL with exceptions. However as noted, several other plant extracts were potent antiproliferative agents, and should not be neglected in future studies. In particular, the *Syzygium* spp. and the *D. pruriens* extracts appear promising. However no single project can adequately examine all of the extracts. Instead, a major aim of my project was to narrow the focus for further studies. In summary, *T. ferdinandiana* and *T. lanceolata* were chosen as these were amongst the most potent inhibitory agents, had high antioxidant capacities, and were non-toxic. None of the other plant species met all of these criteria.

However these studies highlight the need for further examination of the *T. ferdinandiana* and *T. lanceolata* extracts. Only 2 cell lines were examined in this screening process. Therefore these studies need to be extended using a broader panel of carcinoma cell lines. Furthermore, the 2 carcinomas tested gave no indication as to the antiproliferative pathways used, or whether the mechanisms involved were cytostatic/cytotoxic, or both. Thus, the subsequent chapter screens *T. ferdinandiana* and *T. lanceolata* extracts against an extended panel of carcinoma cells, and examines the possible pathways and mechanisms involved using specialised cell lines.

CHAPTER 4: CELL LINE STUDIES – EXTENDED AND DIAGNOSTIC SCREENING OF SELECTED HIGH ANTIOXIDANT PLANTS.

Two Australian species plant screened for antiproliferative activity in the previous chapter (*Terminalia ferdinandiana* and *Tasmannia lanceolata*) were chosen for further investigations based because on their high antioxidant content, inhibitions at stock concentrations and significant IC_{50} values measured against both (HeLa and Caco-2) carcinomas. As the response against different cancer cells and cells from different tissues to antiproliferative drugs may vary, it is vital to test cells from different tissues when developing new cancer chemotherapeutic treatments. Therefore, the most promising extracts were chosen from the previous studies (Chapter 3) and screened against an extended panel of cell lines. Whilst the HeLa and Caco-2 cell lines used in the initial screening studies were ideal preliminary screening tools, they are also significant causes of mortality and morbidity in Western populations. These cell lines enhance the prospect of comparative studies with other cancer chemotherapeutic studies undertaken in other labs worldwide. However, for a greater understanding of the chemotherapeutic potential of the *T. ferdinandiana* and *T. lanceolata* extracts, there is a need to screen them against a larger and varied panel of cancer cell lines. Furthermore, testing against other cell lines may provide mechanistic detail into the antiproliferative activity of these extracts. Antiproliferative studies presented in the previous chapter indicated that *Terminalia ferdinandiana* extracts may exhibit both cytostatic activity and also cytotoxic potential against HeLa and Caco-2. Indeed, negative proliferation on

exposure to *T. ferdinandiana* fruit (methanolic and aqueous) and leaf (methanolic and ethyl acetate) extracts at stock concentrations were likely to be at least in part due to cytotoxic mechanisms. Therefore, these extracts were classed as very potent antiproliferative agents against these carcinomas. The studies presented in this chapter extend upon those reported in chapter 3, by examining the effect on proliferation of an extended panel of cells. Furthermore, the cell lines tested were targeted due to their ability to provide information about the antiproliferative mechanisms.

4.1 Extended cell line studies: Testing of *T. ferdinandiana* and *T. lanceolata* extracts against diagnostic cell lines.

Further investigation into the antiproliferative activity of *T. ferdinandiana* and *T. lanceolata* utilised two pairs of cell lines (JEG-3/JAR and MC3T3-E1/MG-63). These cell lines were chosen for their differential production/secretion of proteins associated with cellular redox state and apoptotic mechanisms. They are thus ideally suited to provide further insight into the antiproliferative pathways involved. Whilst being similar in most ways, the JEG-3 and JAR cell pair varies in the relative levels of thioredoxin expressed. JEG-3 (placental choriocarcinoma) cell line secretes a substantially higher level of thioredoxin in comparison to JAR cells [304]. In addition, JAR cells produce an intracellular macrophage-like tumour necrosis factor (TNF) in actively proliferating cells. In contrast, JEG-3 cells do not increase their secretion of TNF during proliferation [305]. These differences may provide mechanistic details of the pathways affected by the extracts tested. Hence, TNF produced by these choriocarcinomas (especially the JAR cells) may function as an autocrine growth factor rather than as an inducer of apoptotic cell death [305].

The second panel of cell lines for screening were MC3T3-E1/MG-63 cell lines. The MC3T3-E1 cells are normal murine calvarial osteoblasts which have the ability to proliferate, express collagen and mineralise in the presence of ascorbic acid [306]. Hence, the increased levels of ascorbic acid present in the methanolic, aqueous and ethyl acetate extracts in *T. ferdinandiana* fruit would assist in increased proliferation and formation of collagen and calcium matrix by MC3T3-E1 cells. In contrast, the *T. lanceolata* extracts do not have detectable levels of ascorbic acid [179], and thus a different response would be expected of the extracts from both plants which may use similar mechanisms. The MC3T3-E1, a normal murine cell line, has a known susceptibility to tumour necrosis factor (TNF) [307]. It is speculated that if TNF analogues are present in the extracts, they may stimulate apoptosis and cause increased inhibition of cellular proliferation. Furthermore, a previous study also reported that dexamethasone induced apoptosis of MC3T3-E1 can also occur through the mitochondrial pathway via activation of caspases 1, 3 6, 8, 9, 11 and 12 [308]. Thus, screening the extracts against these cells may provide detail about apoptotic mechanisms.

Conversely, the MG-63 is an osteosarcoma cell line that has no known susceptibility to TNF and may not follow the extrinsic apoptotic pathway via TNF induction. Several studies have indicated that MG-63 cells follow a Fas-activated apoptotic pathway when treated with IFN-γ, which induces Fas antigen expression leading to apoptosis [307]. Other studies have reported induction of apoptosis in MG-63 cells through the intrinsic mitochondrial pathway [309]. Curcumin was able to induce activation of caspase-3 and similarly quercetin (flavonoid) induced caspase-3 and caspase-9 leading to apoptosis in MG-63 cells [309, 310]. Hence, examination of the responses of these cells to the extracts may provide mechanistic detail

about the anti-proliferative activity of the extracts through their anti-proliferative/proliferative responses to the bioactive phytochemicals present in these plants.

4.1.1 Antiproliferative potential of *T. ferdinandiana* extracts against JEG-3 and JAR cells

Fruit

Methanolic and aqueous *Terminalia ferdinandiana* fruit extracts were particularly potent inhibitors of JEG-3 and JAR cell proliferation (Figure 4.1 a). Indeed, negative JEG-3 proliferation was noted for the *T. ferdinandiana* methanolic fruit extract, suggesting that the antiproliferative activity was at least in part due to cytotoxic mechanisms (Figure 4.1 a). The *T. ferdinandiana* methanolic fruit extract was also potent, inhibiting 95% JAR cell proliferation compared with the untreated control. The *T. ferdinandiana* aqueous fruit extract strongly inhibited JEG-3 proliferation by 99% compared to the negative control. A similar but more potent inhibition was noted with the *T. ferdinandiana* aqueous fruit extract which induced negative proliferation with JAR cells, indicating cytotoxicity being involved. The *T. ferdinandiana* ethyl acetate, chloroform and hexane fruit extracts inhibited JEG-3 cell proliferation by 64%, 37% and 14% respectively. A similar trend was observed with the ethyl acetate, chloroform and hexane fruit extracts which inhibited JAR cellular proliferation by (60%, 36% and 21%) compared with the untreated control (Figure 4.1a). As expected, the *T. ferdinandiana* chloroform and hexane fruit extracts were lesser in potency inhibiting JAR cellular proliferation.

Leaf

A similar trend was apparent with *T. ferdinandiana* leaf extracts (Figure 4.1 b). All extracts were effective inhibitors of JEG and JAR cell proliferation. The *T. ferdinandiana* methanolic, aqueous and ethyl acetate leaf extracts strongly inhibited JEG cell proliferation. As with corresponding fruit extracts, negative JEG-3 proliferation was noted for the *T. ferdinandiana* methanolic and ethyl acetate leaf extract, suggesting that the antiproliferative activity was possibly due to cytotoxic pathways (Figure 4.1 b). Likewise, negative proliferation was also observed in JAR cells on exposure to *T. ferdinandiana* aqueous and ethyl acetate leaf extracts. The *T. ferdinandiana* aqueous leaf extract strongly inhibited JEG-3 proliferation by 85%, but was less potent against JAR cells by 76% compared to the negative control. The *T. ferdinandiana* chloroform and hexane leaf extracts inhibited JEG-3 cell proliferation by 60% and 34%, whilst JAR were inhibited by 43% and 29% respectively (Figure 4.1 b). In summary, The *T. ferdinandiana* (fruit and leaf) extracts were potent inhibitors of JEG-3 and JAR (Figure 4.1 a - b). The *T. ferdinandiana* leaf extracts seemed more potent than the *T. ferdinandiana* fruit extracts against both carcinomas.

IC_{50}s

The antiproliferative efficacies of the plant extracts were further quantified by IC_{50} determination (Table 4.1). The inhibition of JEG-3/JAR proliferation for all plant extracts was dose dependant, with the level of inhibitory activity decreasing at lower concentrations

(Table 4.1). The IC_{50} values were evaluated by the same selection criteria as used in chapter 3 to grade the effectiveness of the plant extracts. Only two out the five *T. ferdinandiana* fruit extracts had IC_{50} values < 1000 µg/mL. The *T. ferdinandiana* methanolic fruit extract was the most potent antiproliferative agent against JEG-3 cells, with an IC_{50} value of 525 µg/mL. Hence, the *T. ferdinandiana* methanolic fruit extract was classed as a good antiproliferative agent (IC_{50}< 1000 µg/mL). In contrast, the *T. ferdinandiana* fruit extracts were categorised as weak antiproliferative agents against JAR cells with the methanolic, aqueous and ethyl acetate fruit extracts IC_{50} values > 1000 µg/ mL. The lower polarity extracts were not able to inhibit > 50% cell proliferation for all concentrations tested and thus, an IC_{50} could not be determined for these extracts.

The *T. ferdinandiana* leaf extracts were significantly more potent than the fruit extracts. All *T. ferdinandiana* leaf extracts tested displayed significant ($p < 0.01$) anti-proliferative effects against JEG-3 carcinoma cells. Four of the five leaf extracts had IC_{50} values in a range between 147 – 733.3 µg/mL (substantially < 1000 µg/mL). Amongst these leaf extracts, the methanolic leaf extract was classed as a very potent antiproliferative agent against JEG-3, with an IC_{50} value of 146.7 µg/mL (< 200 µg/mL) (Table 4.1). The *T. ferdinandiana* methanolic leaf extract was less potent against JAR cells in comparison to JEG cells with an IC_{50} 601.7 µg/mL, and was therefore classed as good antiproliferative agent against JAR (< 1000 µg/mL).

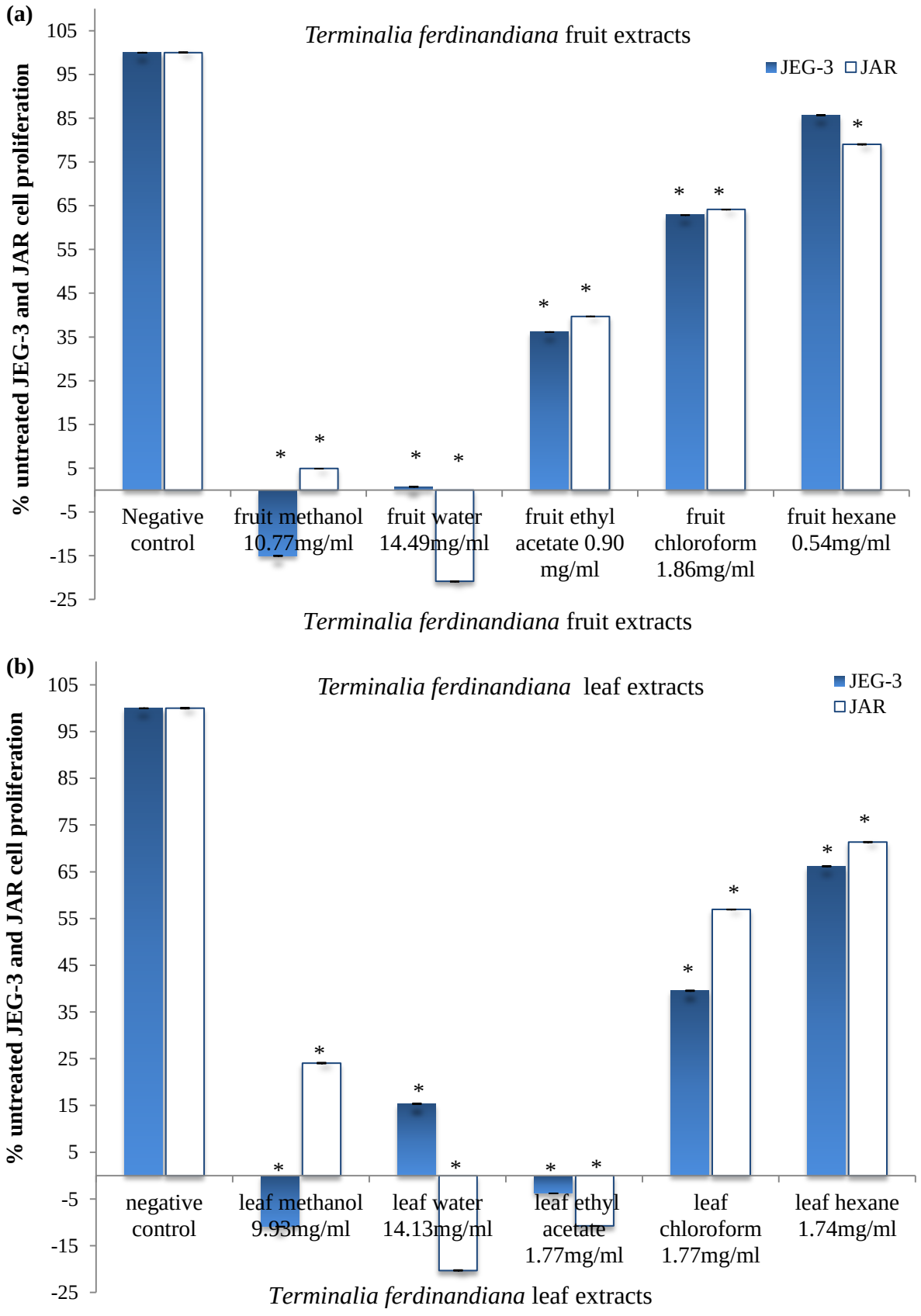

(a)
% untreated JEG-3 and JAR cell proliferation
105
95
85
75
65
55
45
35
25
15
5
-5
-15
-25
Terminalia ferdinandiana fruit extracts
JEG-3
JAR
Negative control
fruit methanol 10.77mg/ml
fruit water 14.49mg/ml
fruit ethyl acetate 0.90 mg/ml
fruit chloroform 1.86mg/ml
fruit hexane 0.54mg/ml
Terminalia ferdinandiana fruit extracts
(b)
% untreated JEG-3 and JAR cell proliferation
105
95
85
75
65
55
45
35
25
15
5
-5
-15
-25
Terminalia ferdinandiana leaf extracts
JEG-3
JAR
negative control
leaf methanol 9.93mg/ml
leaf water 14.13mg/ml
leaf ethyl acetate 1.77mg/ml
leaf chloroform 1.77mg/ml
leaf hexane 1.74mg/ml
Terminalia ferdinandiana leaf extracts

Figure 4.1 Cell proliferation (MTS) assays of *T. ferdinandiana* (a) fruit and (b) leaf extracts and untreated controls against JEG-3 (human placental choriocarcinoma cancer cell line) ATCC ® HTB-36™ (solid bars) and JAR (human placental choriocarcinoma cancer cell line) ATCC ® HTB-144™ (clear bars). Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed at least three times.

4.1.2 Antiproliferative potential of *T. ferdinandiana* extracts against MC3T3-E1 and MG-63 cells

Fruit

The *T. ferdinandiana* methanolic, aqueous and ethyl acetate fruit extracts were potent inhibitors of MC3T3-E1 cell proliferation. Indeed, negative MC3T3-E1 proliferation was noted for the *T. ferdinandiana* methanolic and aqueous fruit extracts, suggesting that the antiproliferative activity may have been due to cytotoxic activity (Figure 4.2 a). The *T. ferdinandiana* ethyl acetate was also a potent antiproliferative agent, inhibiting 79% MC3T3-E1 proliferation compared to the untreated control. In contrast, the lower polarity chloroform and hexane fruit extracts inhibited cellular proliferation to a lesser extent (46% and 33% respectively) (Figure 4.2 a). In summary, the *Terminalia ferdinandiana* methanolic and aqueous fruit extracts were the most potent antiproliferative agents, exhibiting negative proliferation in MC3T3-E1, implying possible cytotoxic activity.

A consistent trend was apparent with MG-63 cells on exposure to *T. ferdinandiana* fruit extracts (Figure 4.2 a). All *T. ferdinandiana* fruit extracts were effective inhibitors of MG-63 cell proliferation. The higher polar methanolic, aqueous and mid polarity ethyl acetate *T. ferdinandiana* fruit extracts strongly inhibited MG-63 cell proliferation. Indeed negative MG-63 proliferation was noted for the *T. ferdinandiana* methanolic and ethyl acetate fruit extracts, suggesting that the antiproliferative activity was not only cytostatic but possibly cytotoxic (Figure 4.2 a). The *T. ferdinandiana* aqueous fruit extract strongly inhibited MG-63 proliferation by 83%, compared to the negative control. The lower polarity *T. ferdinandiana* chloroform and hexane fruit extracts also inhibited MG-63 cell proliferation, albeit by a lesser degree, (56% and 26% respectively) (Figure 4.1 b). In summary, the *T. ferdinandiana* methanolic and ethyl acetate fruit extracts were the most potent antiproliferative agents, exhibiting negative proliferation against MG-63, indicating possible cytotoxic activity.

Leaf

An alternative trend was apparent for the *T. ferdinandiana* leaf extracts (Figure 4.2 b). All extracts were effective inhibitors of MC3T3-E1 cell proliferation. Indeed, negative MC3T3-E1 proliferation was noted for the *T. ferdinandiana* methanolic and ethyl acetate fruit extracts, suggesting that they may possess cytotoxic activity (Figure 4.2 a). The higher polarity *T. ferdinandiana* aqueous leaf extract was not as potent, although moderate inhibition (66%) was achieved. In contrast, the lower polarity chloroform and hexane leaf extracts inhibited cellular proliferation at higher than expected levels by (80% and 60%) respectively (Figure 4.2 a). This strong inhibition by the lower polarity extracts was unexpected as

opposing trends were noted prior in HeLa or Caco-2 cell studies. In summary, the *T. ferdinandiana* methanolic and ethyl acetate leaf extracts were the most potent antiproliferative agents, exhibiting negative proliferation in MC3T3-E1, implying possible cytotoxic activity. The *T. ferdinandiana* methanolic, ethyl acetate and chloroform leaf extracts were strong antiproliferative agents against MC3T3-E1 proliferation.

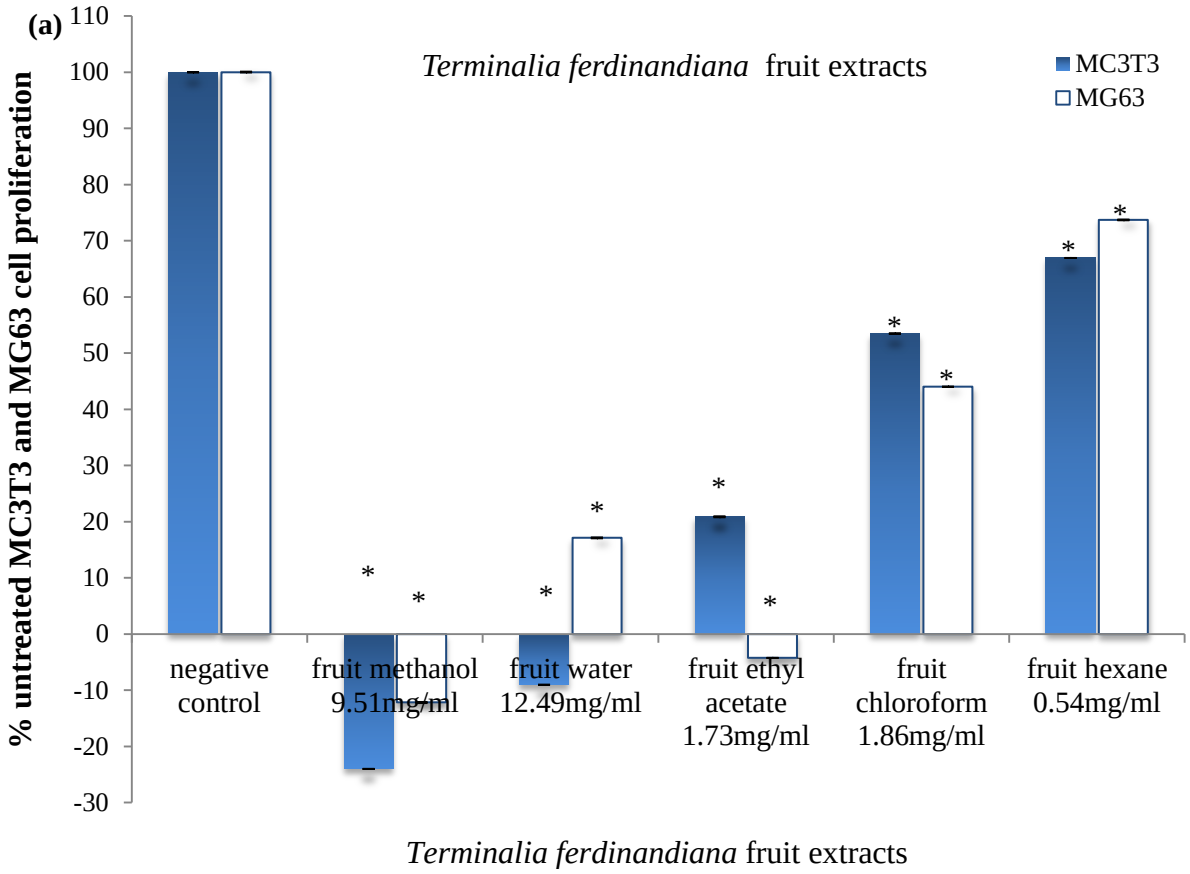
All *T. ferdinandiana* leaf extracts were effective inhibitors of MG-63 cell proliferation with some notable exceptions. The *T. ferdinandiana* methanolic and aqueous leaf extracts were potent inhibitors of MG-63 cell proliferation. Indeed, negative MG-63 proliferation was noted for *T. ferdinandiana* aqueous leaf extract, suggesting that the antiproliferative activity was possibly due to cytotoxic mechanisms (Figure 4.2 b). The *T. ferdinandiana* ethyl acetate, chloroform and hexane fruit extracts also inhibited Caco-2 cell proliferation by 69%, 35% and 1% (non-significant) respectively. In summary, the *T. ferdinandiana* aqueous leaf extract was the most potent antiproliferative agent against MG-63, exhibiting negative proliferation implying possible cytotoxic activity. Similarly, the *T. ferdinandiana* methanolic leaf extract was also a potent antiproliferative agent against MG-63.

IC_{50}s

The antiproliferative efficacies of the *T. ferdinandiana* extracts were further quantified by IC_{50} determination. The inhibition of MC3T3-E1/MG-63 proliferation for all plant extracts was dose dependant, with the level of inhibitory activity decreasing at lower concentrations

(Table 4.1). The IC_{50} values were evaluated by the same selection criteria used previously to grade the effectiveness of the plant extracts. Three of the five *T. ferdinandiana* fruit extracts had IC_{50} values substantially < 1000 µg/mL. The *T. ferdinandiana* methanolic fruit extract was the most potent antiproliferative agent against MC3T3-E1 cells with IC_{50} values of 443 µg/mL (< 500 µg/mL). Hence this extract was classed as a potent antiproliferative agent.

The *T. ferdinandiana* leaf extracts were significantly more potent than the fruit extracts. Although all *T. ferdinandiana* leaf extracts tested displayed significant ($p < 0.01$) antiproliferative effects against MC3T3-E1 cells, three of the five had IC_{50} values substantially < 1000 µg/mL. The *T. ferdinandiana* methanolic and ethyl acetate leaf extracts were very effective antiproliferative agents against MC3T3-E1 with, IC_{50} value of 40 µg/mL and 6 µg/mL respectively (< 500 µg/mL), and were thus classed as very potent antiproliferative agents (Table 4.1). The *T. ferdinandiana* ethyl acetate leaf extract with its very low IC_{50} values justifies its potency to inhibit 50% of the murine cell population with just 6 µg/mL of the extract. It is possible that the mechanisms involved could also be cytotoxic in nature. Similar is true with the methanolic extract with IC_{50} value of 40 µg/mL. Two of the five *T. ferdinandiana* fruit extracts also had IC_{50} values substantially < 1000 µg/mL against MG-63 cells. The *T. ferdinandiana* ethyl acetate fruit extract was the most effective antiproliferative agent against MG-63 cells with an IC_{50} values of 357 µg/mL. Hence, this extract was classed as a potent antiproliferative agent (< 500 µg/mL). Similarly, two of the five *T. ferdinandiana* leaf extracts had IC_{50} values substantially < 1000 µg/mL. The *T. ferdinandiana* ethyl acetate leaf extract was the most effective antiproliferative agent against MG-63 cells with IC_{50} values of 384 µg/mL, and therefore was classed as a potent antiproliferative agent (< 500 µg/mL).


(a)
% untreated MC3T3 and MG63 cell proliferation
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
Terminalia ferdinandiana fruit extracts
MC3T3
MG63
negative control
fruit methanol 9.51mg/ml
fruit water 12.49mg/ml
fruit ethyl acetate 1.73mg/ml
fruit chloroform 1.86mg/ml
fruit hexane 0.54mg/ml
Terminalia ferdinandiana fruit extracts

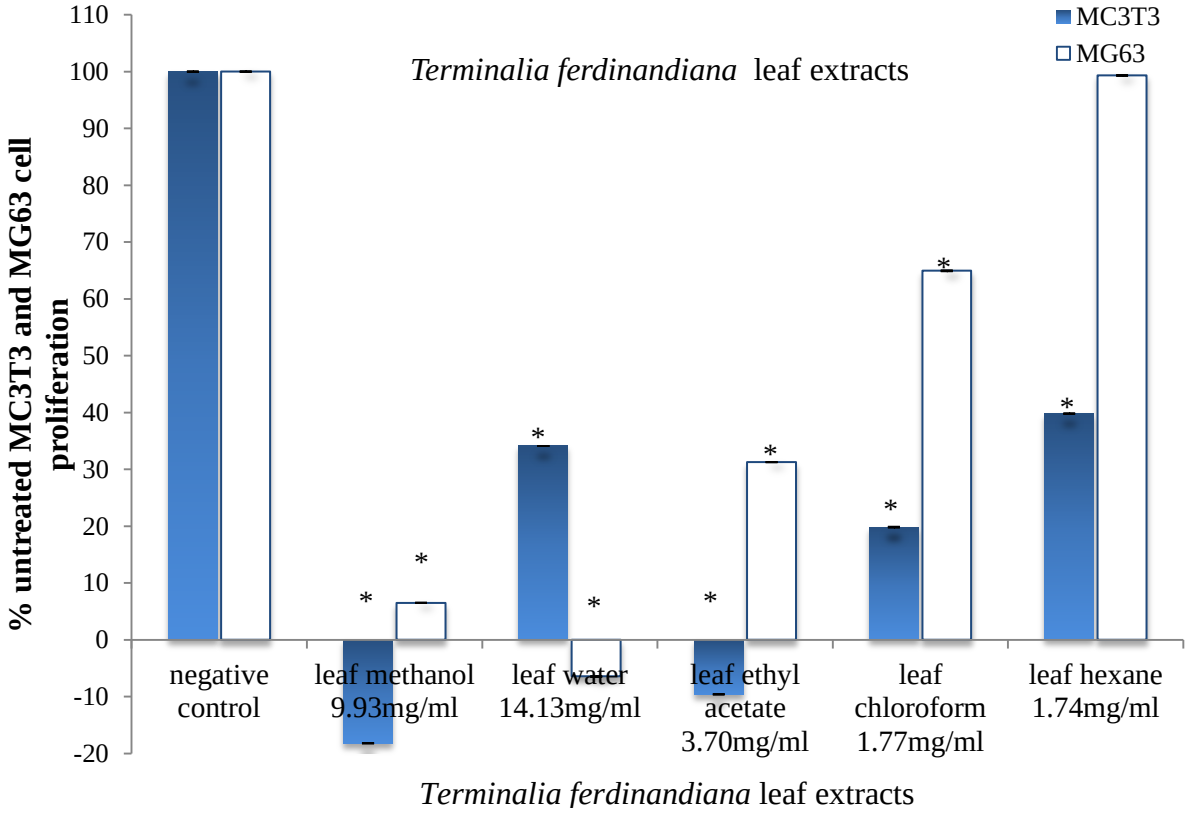

% untreated MC3T3 and MG63 cell proliferation
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
Terminalia ferdinandiana leaf extracts
MC3T3
MG63
negative control
leaf methanol 9.93mg/ml
leaf water 14.13mg/ml
leaf ethyl acetate 3.70mg/ml
leaf chloroform 1.77mg/ml
leaf hexane 1.74mg/ml
Terminalia ferdinandiana leaf extracts

Figure 4.2 Cell proliferation (MTS) assays of *T. ferdinandiana* (a) fruit and (b) leaf extracts and untreated controls against MC3T3-E1 Sub-clone 4 (murine osteoblast calvarial cell line) (ATCC® CRL-2593™) (solid bars) and MG-63 (human osteosarcoma cancer cell line) (ATCC® CRL-1427™) (clear bars). Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

4.1.3 Antiproliferative potential of *T. lanceolata* extracts against JEG-3 and JAR.

Fruit

Methanolic and aqueous *T. lanceolata* fruit extracts strongly inhibited JEG-3 proliferation (Figure 4.3 a). Indeed, negative JEG-3 proliferation was noted for the *T. lanceolata* methanolic and aqueous fruit extracts, suggesting that the antiproliferative activity but was possibly cytotoxic (Figure 4.3 a). *T. lanceolata* ethyl acetate and chloroform fruit extracts also inhibited JEG-3 cell proliferation (by 57% and 39% respectively) (Figure 4.3 a). The *T. lanceolata* hexane fruit extract also induced minor inhibition of JEG-3 cells, although this inhibition was not significant (7% inhibition compared to the negative control cell proliferation).

Consistent with the trends observed with JEG-3 cell proliferation for *T. lanceolata* fruit extracts, JAR cells proliferation were similarly inhibited by the higher polarity *T. lanceolata* fruit extracts, and to a lesser extent by the mid polarity and lower polarity extracts. The

higher polarity *T. lanceolata* methanolic fruit extract blocked 96% JAR cell proliferation. Indeed, negative JAR proliferation was noted for the *T. lanceolata* aqueous fruit extracts, suggesting possible cytotoxic activity (Figure 4.3 a). The mid polarity ethyl acetate, as well as the lower polarity chloroform and hexane fruit extracts, inhibited JAR proliferation by 61%, 55% and 39% respectively compared to the untreated control. In summary, the *T. lanceolata* methanolic and aqueous fruit extracts were the most potent inhibitors of JEG-3 and JAR proliferation, with notable negative proliferation.

Leaf

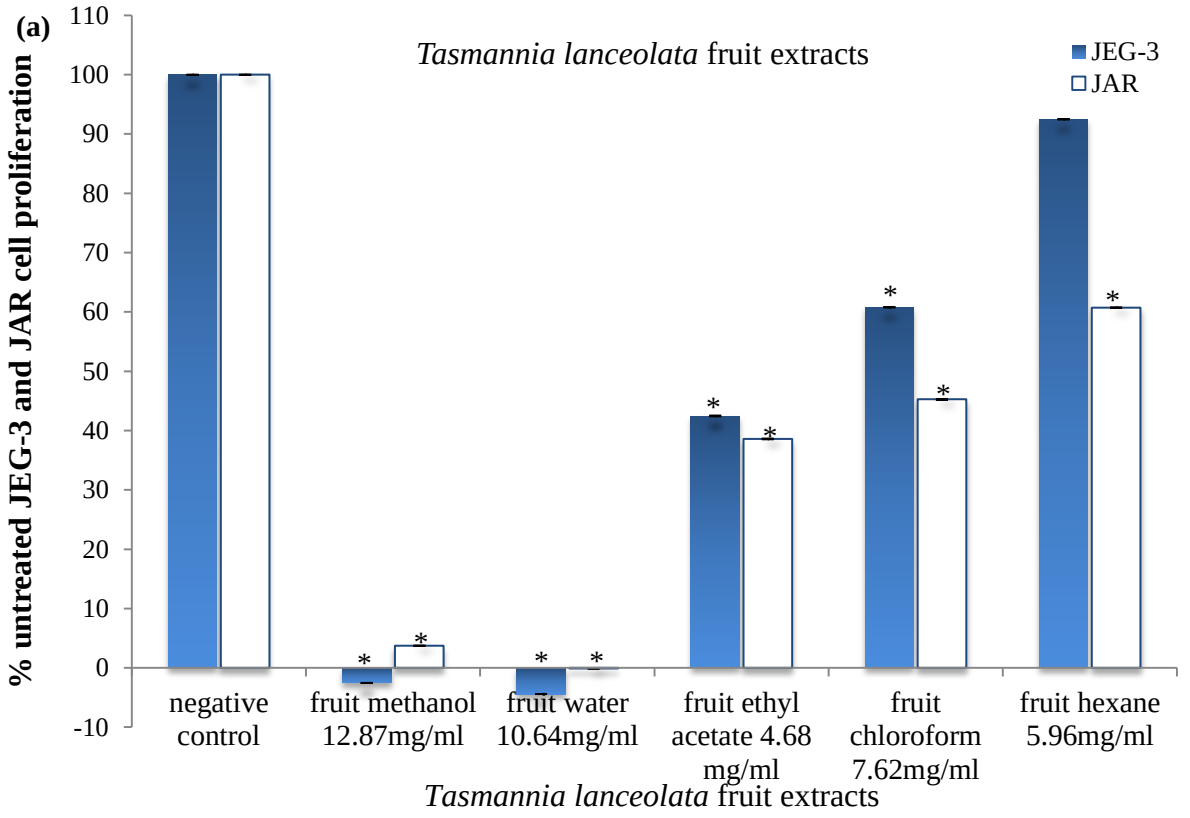
The highly polar and mid polarity *T. lanceolata* leaf extracts followed a similar trend to the *T. lanceolata* fruit extracts. However, the activity of lower polarity extracts were contrary previous trends (Figure 4.3 b). As expected, the methanolic and aqueous *T. lanceolata* leaf extracts strongly inhibited JEG-3 proliferation (Figure 4.3 b). Indeed, negative JEG-3 proliferation was noted for the *T. lanceolata* methanolic and aqueous leaf extracts, suggesting that the antiproliferative activity may be at least in part cytotoxic (Figure 4.3 b). Similarly, the ethyl acetate leaf extracts inhibited JEG-3 proliferation by 48% compared to the negative control. The lower polarity chloroform and hexane *T. lanceolata* leaf extracts seemingly displayed a trend of increased antiproliferative efficacy with increased hydrophobicity. Indeed, the *T. lanceolata* chloroform leaf extract inhibited JEG-3 proliferation by 66%, whilst the *T. lanceolata* hexane leaf inhibited JEG-3 proliferation by 92% (Figure 4.3 b).

The antiproliferative activity observed with JAR cells followed similar trends to the JEG-3, although the trend was less pronounced. The methanolic and aqueous *T. lanceolata* leaf extracts strongly inhibited JAR proliferation (Figure 4.3 b). Indeed, negative JAR proliferation was noted for the *T. lanceolata* methanolic and aqueous leaf extracts, indicating that inhibition of proliferation may be cytotoxic (Figure 4.3 b). The *T. lanceolata* ethyl acetate and the lower polarity chloroform and hexane leaf extracts also inhibited JAR proliferation by 32%, 20% and 60% respectively compared to the negative control (Figure 4.3 b). In summary, *T. lanceolata* methanolic and aqueous leaf extracts were the most potent inhibitors of JEG-3 and JAR proliferation, notably with negative proliferation.

IC_{50}s

The antiproliferative efficacies of the plant extracts were further quantified by IC_{50} determination. The inhibition of JEG-3/JAR proliferation for all plant extracts was dose dependant, with the level of inhibitory activity decreasing at lower concentrations (Table 4.1). The *T. lanceolata* fruit extracts were categorised as relatively weak antiproliferative agents against JEG and JAR cells. The *T. lanceolata* methanolic, aqueous and ethyl acetate (fruit and leaf) extracts had IC_{50} values determined > 1000 µg/mL. The lower polarity chloroform and hexane extracts, were not able to inhibit > 50% cell proliferation at any concentrations tested. Thus, an IC_{50} could not be determined for these extracts.

Two of the five *T. lanceolata* leaf extracts had IC_{50} values substantially < 1000 µg/mL against JEG-3 cells. The *T. lanceolata* aqueous leaf extract was the most potent antiproliferative agent against JEG-3 cells, with an IC_{50} value of 488 µg/mL and was therefore classed as a potent antiproliferative agent (< 500 µg/mL). Similarly, two out of the five *T. lanceolata* leaf extracts had IC_{50} values substantially < 1000 µg/mL against JAR cells. The *T. lanceolata* methanolic and aqueous leaf extracts were the most potent antiproliferative agents against JAR cells, both with IC_{50} value of 450 µg/mL and hence were classed as potent antiproliferative agents (< 500 µg/mL).


(a)
% untreated JEG-3 and JAR cell proliferation
110
100
90
80
70
60
50
40
30
20
10
0
-10
Tasmannia lanceolata fruit extracts
JEG-3
JAR
negative control
fruit methanol 12.87mg/ml
fruit water 10.64mg/ml
fruit ethyl acetate 4.68 mg/ml
fruit chloroform 7.62mg/ml
fruit hexane 5.96mg/ml
Tasmannia lanceolata fruit extracts

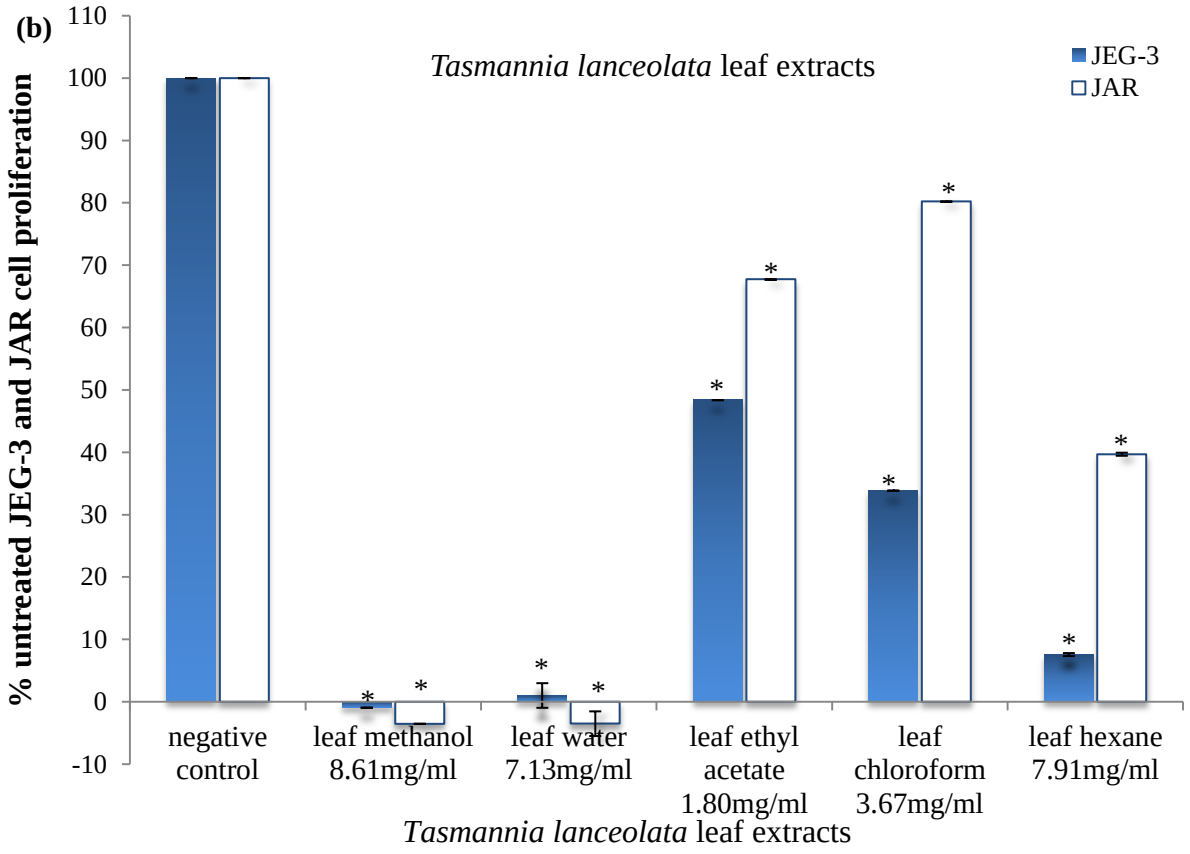

(b)
% untreated JEG-3 and JAR cell proliferation
110
100
90
80
70
60
50
40
30
20
10
0
-10
Tasmannia lanceolata leaf extracts
JEG-3
JAR
negative control
leaf methanol 8.61mg/ml
leaf water 7.13mg/ml
leaf ethyl acetate 1.80mg/ml
leaf chloroform 3.67mg/ml
leaf hexane 7.91mg/ml
Tasmannia lanceolata leaf extracts

Figure 4.3 Cell proliferation (MTS) assays of *T. lanceolata* (a) fruit and (b) leaf extracts and untreated controls against JEG-3 (human placental choriocarcinoma cancer cell line) ATCC ® HTB-36™ (solid bars) and JAR (human placental choriocarcinoma cancer cell line) ATCC ® HTB-144™ (clear bars). Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

4.1.4 Antiproliferative potential of *T. lanceolata* extracts against MC3T3-E1 and MG-63 cells

Fruit

The methanolic and aqueous *T. lanceolata* fruit extracts were potent inhibitors of MC3T3-E1 cell proliferation (Figure 4.4 a). Indeed, negative MC3T3-E1 proliferation was noted for *T. lanceolata* methanolic, aqueous extracts and ethyl acetate fruit extracts, suggesting that the antiproliferative activity may have been due to cytotoxic mechanisms (Figure 4.4 a). Interestingly, the lower polarity *T. lanceolata* chloroform fruit extract was also a potent inhibitor of MC3T3-E1 proliferation (77% inhibition). The *T. lanceolata* hexane fruit extract inhibited MC3T3-E1 by a lesser degree (52% respectively compared to the negative control).

Similar trends observed with MC3T3-E1 cell proliferation were also notably present with MG-63 cells (Figure 4.4 a). The methanolic, aqueous and ethyl acetate *T. lanceolata* fruit extracts strongly inhibited MG-63 cellular proliferation with negative MG-63 proliferation noted for the *T. lanceolata* methanolic and aqueous fruit extracts. This suggests that the

antiproliferative activity may have been due to cytotoxicity (Figure 4.4 a). The *T. lanceolata* ethyl acetate, chloroform and hexane fruit extracts inhibited MG-63 cellular proliferation by 92%, 72% and 48% respectively compared to the untreated control. In summary, the *T. lanceolata* methanolic, aqueous and ethyl acetate fruit extracts were very potent inhibitors of both MC3T3-E1 and MG-63 cellular proliferation.

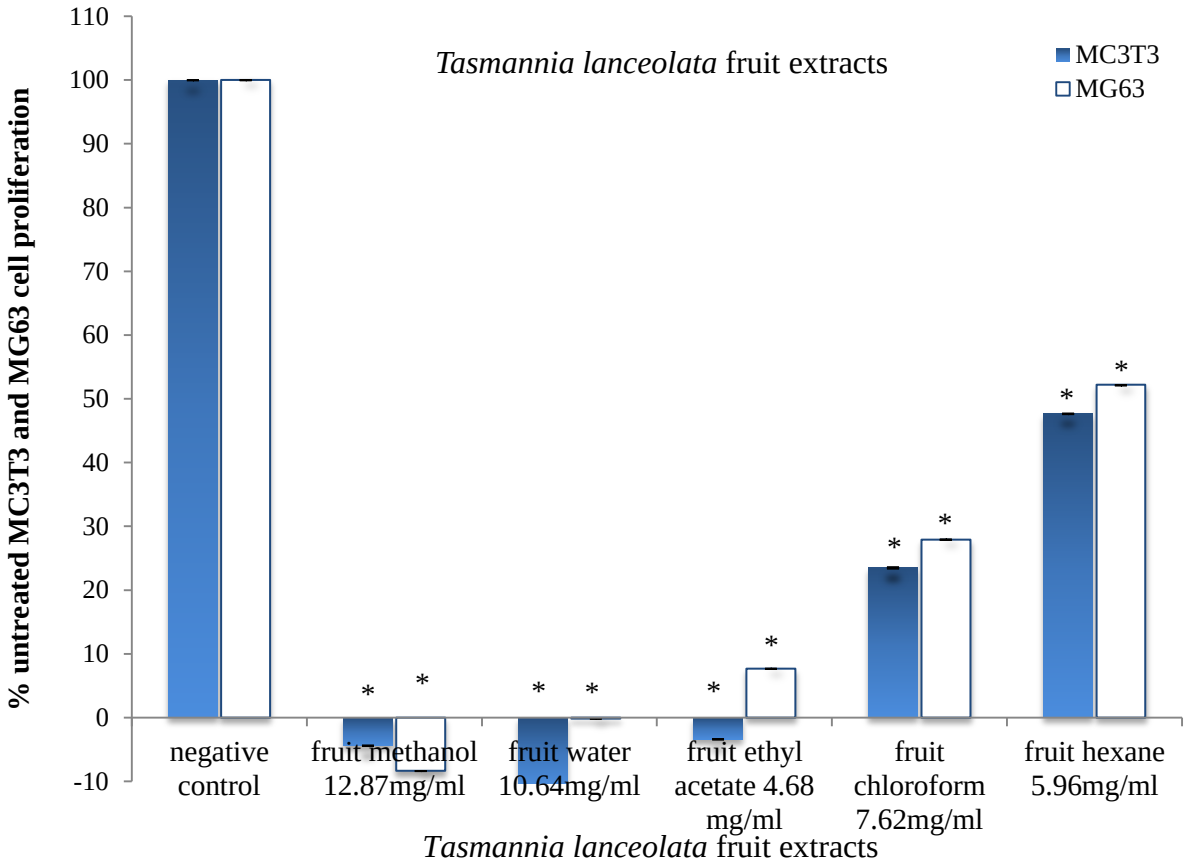
Leaf

T. lanceolata leaf extracts were substantially more potent than the fruit against both MC3T3-E1 and MG-63 cell lines. The methanolic *T. lanceolata* leaf extract potently inhibited MC3T3-E1 cellular proliferation by 89% (Figure 4.4 b). Both the *T. lanceolata* methanolic and ethyl acetate leaf extracts were also potent inhibitors of MC3T3-E1 proliferation with negative proliferation observed, suggest cytotoxicity (Figure 4.4 b). The *T. lanceolata* chloroform and hexane leaf extracts also strongly inhibited MC3T3-E1 proliferation by 89% and 92% respectively.

Similarly, the *T. lanceolata* methanolic leaf extract blocked 96% MG-63 cell proliferation, whilst the *T. lanceolata* aqueous leaf extract induced negative cellular proliferation in MG-63 cells (Figure 4.4 b). The ethyl acetate, chloroform and hexane *T. lanceolata* leaf extracts inhibited MG-63 proliferation by 82%, 85% and 99% respectively compared to the negative control. In summary, the *T. lanceolata* aqueous and ethyl acetate leaf extract were the most potent against both MC3T3-E1 and MG-63 proliferation. In addition, the *T. lanceolata* leaf extracts were generally more potent antiproliferative agents against both MC3T3-E1 and MG-63 cells than were the fruit.

IC_{50}s

The antiproliferative efficacies of the plant extracts were further quantified by IC_{50} determination. The inhibition of MC3T3-E1/MG-63 proliferation for all plant extracts was dose dependant, with the level of inhibitory activity decreasing at lower concentrations (Table 4.1). The IC_{50} values were evaluated by the same selection criteria to grade the effectiveness of the plant extracts as previously used. The *T. lanceolata* aqueous fruit extract, with an IC_{50} value of 536.3 µg/mL was classed as a good antiproliferative agent against MC3T3-E1 cells (< 1000 µg/mL). The other *T. lanceolata* fruit extracts were determined to have values > 1000 µg/mL and were subsequently categorised as relatively moderate antiproliferative agents. Two of the five *T. lanceolata* fruit extracts had IC_{50} values substantially < 1000 µg/mL against MG-63 cells. The *T. lanceolata* methanolic fruit extract was the most potent antiproliferative agent against MG-63 cells, with IC_{50} values of 200 µg/mL. Hence, this extract was classed as a potent antiproliferative agent (< 500 µg/mL). Similarly the *T. lanceolata* ethyl acetate fruit extract had an IC_{50} value of 275 µg/mL and was therefore classified as a potent antiproliferative agent against MG-63 cells. Four of the five *T. lanceolata* leaf extracts had IC_{50} values in the range of 195 µg/mL to 857.5 µg/mL (substantially < 1000 µg/mL against MC3T3-E1 cells). The *T. lanceolata* aqueous leaf extract was the most potent antiproliferative agent against MC3T3-E1 cells with IC_{50} values of 195 µg/mL and was therefore classed as a very potent antiproliferative agent (< 200 µg/mL). All *T. lanceolata* leaf extracts had IC_{50} values in the range of 53 µg/mL to 990 µg/mL (substantially < 1000 µg/mL) against the MG-63 cells. The *T. lanceolata* methanolic leaf extract was the most potent antiproliferative agent against MG-63 with IC_{50} value of 53 µg/mL and was hence classed as a very potent antiproliferative agent (< 200 µg/mL).


Tasmannia lanceolata fruit extracts
MC3T3
MG63
% untreated MC3T3 and MG63 cell proliferation
110
100
90
80
70
60
50
40
30
20
10
0
-10
negative control
fruit methanol 12.87mg/ml
fruit water 10.64mg/ml
fruit ethyl acetate 4.68 mg/ml
fruit chloroform 7.62mg/ml
fruit hexane 5.96mg/ml
Tasmannia lanceolata fruit extracts

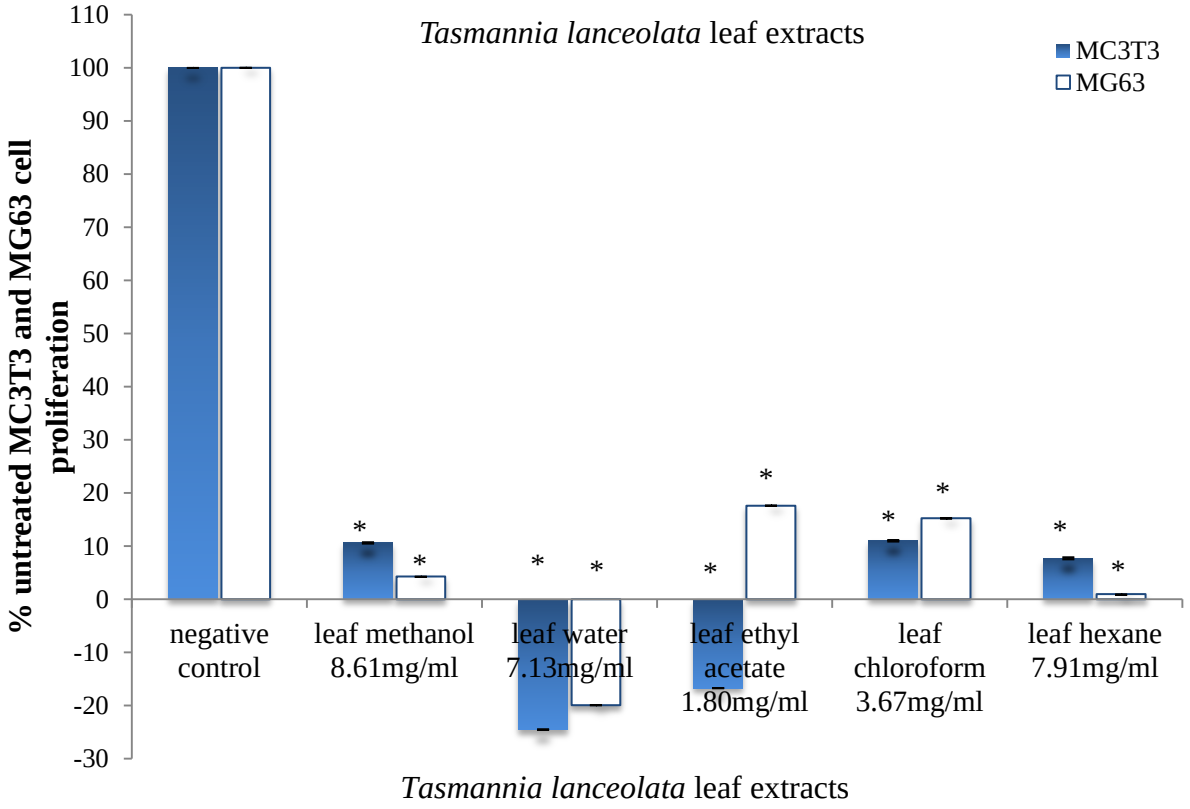

Tasmannia lanceolata leaf extracts
MC3T3
MG63
% untreated MC3T3 and MG63 cell proliferation
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
negative control
leaf methanol 8.61mg/ml
leaf water 7.13mg/ml
leaf ethyl acetate 1.80mg/ml
leaf chloroform 3.67mg/ml
leaf hexane 7.91mg/ml
Tasmannia lanceolata leaf extracts

Figure 4.4 Cell proliferation (MTS) assays of *T. lanceolata* (a) fruit and (b) leaf extracts and untreated controls against MC3T3-E1 Sub-clone 4 (murine osteoblast calvarial cell line) (ATCC® CRL-2593™) (solid bars) and MG-63 (human osteosarcoma cancer cell line) (ATCC® CRL-1427™) (clear bars). Results are expressed as mean percentages ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$). All bioassays were performed in at least triplicate.

4.1.5 Quantification of antiproliferative efficacy by IC_{50} determination

Table 4.1 *T. ferdinandiana* and *T. lanceolata* fruit and leaf extract IC_{50} values (expressed as µg/ml) with a 95% confidence interval against JEG-3/JAR and MC3T3-E1/MG-63 cell lines.

Species	Part	M		A		E		C		H	
		JEG-3	JAR	JEG-3	JAR	JEG-3	JAR	JEG-3	JAR	JEG-3	JAR
KP	fruit	525	1050	1040	1170	645	1290	ND	ND	ND	ND
	leaf	146.7	601.7	330	751.7	440.8	690	733.3	ND	ND	ND
TP	fruit	2951.7	4293	2881.7	1950	4680	4290	ND	ND	ND	ND
	leaf	900	450	487.5	450	1800	ND	3202.5	ND	3465	1980

Species	Part	M		A		E		C		H	
		MC3T3	MG-63	MC3T3	MG-63	MC3T3	MG-63	MC3T3	MG-63	MC3T3	MG-63
KP	fruit	443	1046	667	780	780	357	ND	3720	ND	ND
	leaf	40	845.5	740	1500	6	384	3540	3540	ND	ND
TP	fruit	1400	200	536.3	1191.7	1365	275	3810	3810	ND	5960
	leaf	245	52.5	195	315	200	525	1188.3	843.3	857.5	990

Values indicate the mean IC_{50} for triplicate determinations. KP = *Terminalia ferdinandiana*, TP = *Tasmannia lanceolata*, M = methanol, W = aqueous, E = ethyl acetate, C = chloroform, H = hexane. ND = indicates that IC_{50} value was not achieved as inhibition of proliferation did not exceed 50% at any concentration tested.

4.2 Cell Imaging Studies

The antiproliferative studies presented in this and the previous chapter highlight that these extracts have profound effects on cellular proliferation. In all cases, cellular proliferation was blocked and in some this effect was quite dramatic. Indeed, Figures (3.1 - 3.9, 4.1 - 4.4) show that not only was cell growth blocked, but in some cases (generally the methanolic, aqueous and ethyl acetate extracts), negative cellular proliferation was recorded. This decrease in relative cell proliferation compared to the untreated control cells indicated that these extracts may not just inhibit growth, but act via cytotoxic/apoptotic mechanisms. However, whilst this is indicated by these results, further studies are required to test this hypothesis and cell imaging studies were undertaken to confirm this finding. The Caco-2 cell line was chosen for cell imaging studies as it is a commonly used cell line (allowing for comparative studies), has well defined cell morphology, has a reasonable doubling time compared with the other cell lines studied, and was responsive to the *T. ferdinandiana* and *T. lanceolata* extracts. Extracts were carefully selected to accurately represent, the morphological changes that occurred in the antiproliferative assays, thereby giving a better understanding of the results reported.

The key organelles for comparison between treated and untreated cells in these studies were the nucleus, cytoplasm, vacuoles and cell membrane, all of which were clearly visible at 20X optical magnification of the cell in the untreated control cell image (Figure 4.5 A). The outline of the cell is clear, distinct and compact without abnormalities in size and density. Stress granules were not visible at this magnification. These are normally indicators of cellular stress, if present. In Figures 4.5 (B - E), typical apoptotic phenomena were noted in

the morphology of all cells tested against the *T. ferdinandiana* ethyl acetate (fruit and leaf), *T. lanceolata* aqueous fruit and methanolic leaf extracts which were tested at sub-lethal concentrations. Dosages of the extracts tested were at 70% of determined IC_{50} values for the respective cell line and listed as follow: *T. ferdinandiana* ethyl acetate fruit (420 µg/mL) IC_{50} 600 µg/mL, *T. ferdinandiana* ethyl acetate leaf (71.4 µg/mL) IC_{50} 102 µg/mL, *T. lanceolata* fruit ethyl acetate (102.2 µg/mL) IC_{50} 146 µg/mL and *T. lanceolata* aqueous leaf (105 µg/mL) IC_{50} 150 µg/mL extracts.

Images of Caco-2 cells exposed to the *T. ferdinandiana* ethyl acetate fruit extract (Figure 4.5 B), and *T. ferdinandiana* ethyl acetate leaf (Figure 4.5 C) confirm the antiproliferative studies described in the previous chapter: the *T. ferdinandiana* ethyl acetate fruit extract inhibited Caco-2 proliferation by 26%, whilst the *T. ferdinandiana* ethyl acetate leaf extract inhibited proliferation to below the level at the onset of the experiment. This correlates with the morphological phenomena noted. Apoptosis is generally characterised by presence of an intact membrane, marginalisation of cytoplasm and chromatin condensation [311]. These characteristics were visible in the Caco-2 cells treated with *T. ferdinandiana* fruit ethyl acetate extract Figures 4.5 (B - C). Marginalisation of the cytoplasm of cells undergoing apoptosis also produces an enormously large centrally placed vacuole which is clearly visible in Figure 4.5 (B - C). This characteristic is present in the cell in the centre-left of the image and in cells above it (top centre) (Figure 4.5 B). The same characteristic is also apparent in Figure 4.5 C (bottom left extending to top right across the field of view). The presence of granules in the visible field, are (stress granules) are indicators that cell is under stress Figure 4.5 (B - C). More acute morphological changes were evident in the cells treated with *T. lanceolata* compared to those treated with the *T. ferdinandiana* extracts

‘Blebbing’ is the appearance of ‘spherical bubbles’ on the surface of the membrane and is a typical characteristic generally apparent in apoptotic cells. ‘Blebbing’ is also seen in cells tested against *T. ferdinandiana* leaf ethyl acetate and *T. lanceolata* ethyl acetate fruit extract Figure 4.5 (C – D), this also correlates to the results presented in chapter 3 which reported that *T. lanceolata* aqueous leaf extract inhibited Caco-2 proliferation by 98%. Cells treated the same way had clearly evident morphological changes observed that are indicative of the induction of apoptosis.

Extensive ‘blebbing’ was more apparent for cells treated with the *T. lanceolata* ethyl acetate fruit extract Figure 4.5 (D - E), with apoptotic bodies (sizeable dense bodies) observed amongst the cells treated with *T. lanceolata* aqueous leaf extract (Figure 4.5 E). Furthermore, necrosis (uncontrolled destruction of cell) was visible in the cells that were present in the centre and right in Figure 4.5 E. These cells displayed a loss in membrane integrity with no clear outline of the cell visible, extensive chromatin condensation and the presence of numerous vacuoles (Figure 4.5 E). Also visible in the bottom of Figure 4.5 E were the ‘ghosts’ of cell where cellular content are no longer present within cell. These are clear indications of necrosis.

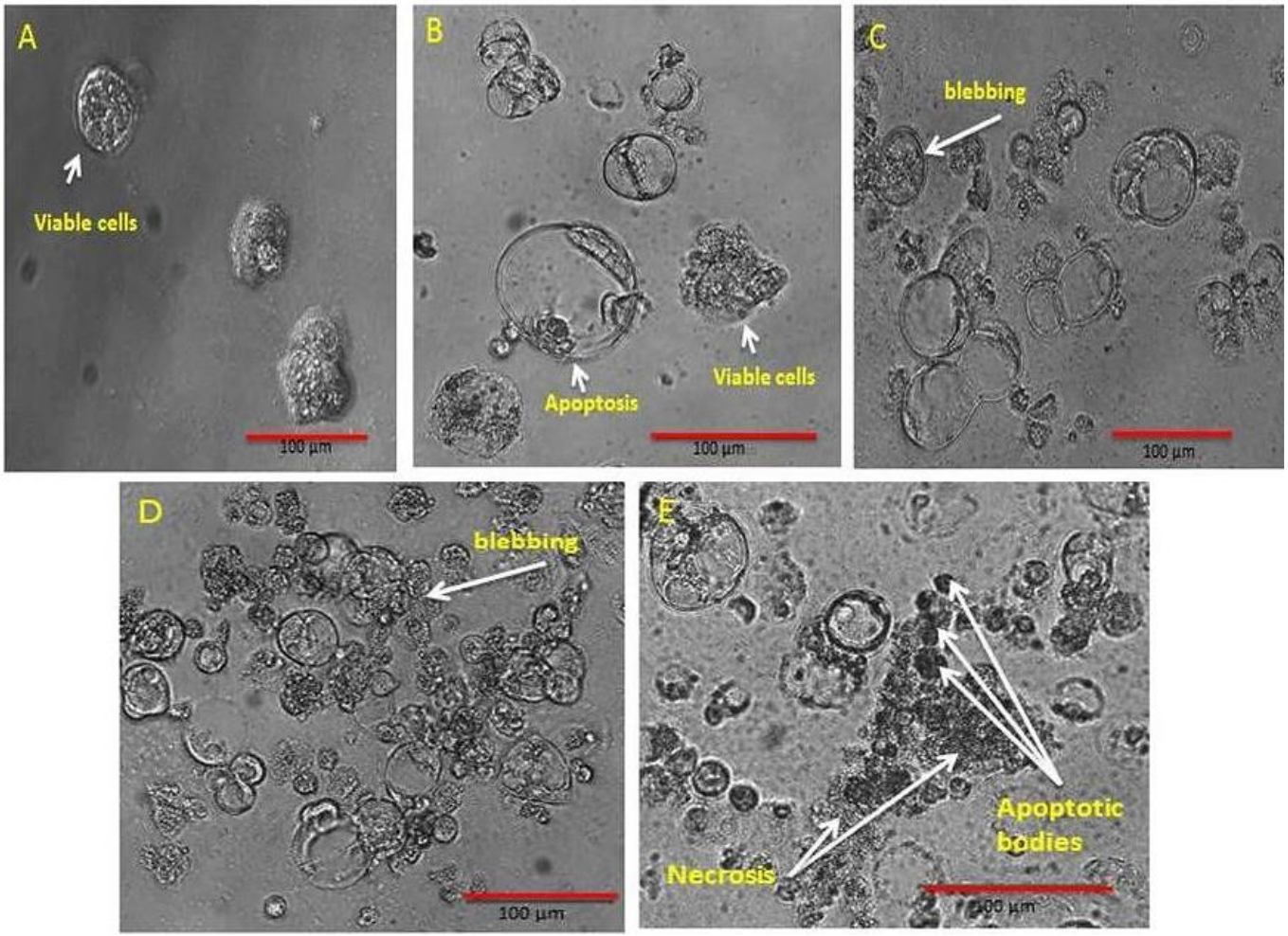


Figure 4.5 Morphological study of the effect of plant extracts (*T. ferdinandiana* fruit and leaf) and *T. lanceolata* (fruit and leaf) against Caco-2 (human colorectal carcinoma cancer cell line Caco-2 ATCC ® HTB-37™) cells. Images of the following treatments:

(a) Viable cells without treatment,

(b) *T. ferdinandiana* fruit ethyl acetate,

(c) *T. ferdinandiana* leaf ethyl acetate,

(d) *T.lanceolata* fruit ethyl acetate,

(e) *T.lanceolata* leaf aqueous extracts.

4.3 Discussion

The *T. ferdinandiana* methanolic, aqueous and ethyl acetate fruit and leaf extracts were good at inhibiting JEG-3 and JAR proliferation (IC_{50} values < 1000 µg/mL) with some exceptions. This trend was prevalent among the higher and mid polarity extracts of both the fruit and leaf extracts, but was absent among the lower polarity chloroform and hexane extracts. In addition, the *T. ferdinandiana* methanolic fruit and leaf extracts induced negative proliferation in JEG-3 cells. Similarly, negative proliferation was also visible in JAR cells by the induction of the *T. ferdinandiana* aqueous fruit, aqueous leaf and ethyl acetate leaf extracts.

Generally, greater inhibition was noted against JEG-3 than JAR cells. Indeed, in most cases the inhibition against JEG-3 was twice that of the JAR cells. For example, the *T. ferdinandiana* methanolic fruit extract had an IC_{50} value of 525 µg/mL for JEG-3, but an IC_{50} value of 1050 µg/mL for JAR (Table 4.1). This trend was apparent in all the higher polarity and mid polarity extracts of both *T. ferdinandiana* fruit and leaf. Although the increased levels of thioredoxin produced by JEG-3 cells were anticipated to have had a protective function, this was not the case. Instead, JEG-3 proliferation was more inhibited than JAR. Alternatively the increased levels of TNF produced by JAR cells may have functioned as a growth promoter and served a protective role instead. Similar outcomes were seen in a previous study, where JAR cells proliferated more rapidly than JEG-3 due to increased levels of intracellular TNF [305]. The *T. ferdinandiana* methanolic fruit extract has the highest antioxidant content recorded at 660 mg AA equivalents per g, as previously mentioned in

(chap 3). It can also be postulated that increased levels of thioredoxin produced by JEG-3 cells may not have played a significant part in protection as expected, as the extract in itself is of extremely high antioxidant capacity. Nonetheless, ascorbic acid has been known to function as a reducing agent and may have been instrumental in maintaining an already reduced cellular redox state, negating all effects of thioredoxin. It is also likely that the involvement of TNF in this manner as a promoter of cellular proliferation instead of cellular inhibition may also indicate that an extrinsic pathway may not be involved in apoptosis. Although evidences of cytostatic mechanisms seem to be implicated, and negative proliferation suggests that cytotoxicity maybe involved, further testing and morphological observation are needed to verify this. This is purely speculative at this juncture without ascertaining which caspases are involved. This would warrant further investigation as to the precise pathways involved.

Conversely, the *T.lanceolata* fruit and leaf extracts followed a different trend contrary to that observed with the *T. ferdinandiana* extracts. Indeed, the *T.lanceolata* fruit and leaf extracts inhibited JAR proliferation more than JEG-3, which was a total reversal compared with *T. ferdinandiana* extracts. Interestingly, several instances of cytotoxicity were also observed with the *T. lanceolata* extracts. The *T. lanceolata* methanolic, aqueous fruit extracts and aqueous leaf induced negative proliferation, indicating possible cytotoxic potential in JEG-3 cells. Similarly, the *T. lanceolata* aqueous fruit extract, methanolic and aqueous leaf extract also induced negative proliferation in JAR cells. However, despite this, the *T. lanceolata* fruit extracts were classed as weak antiproliferative agents (IC_{50} value > 1000 µg/mL).

Interestingly, a similar phenomenon noted with JEG-3 and JAR IC_{50} values were also noted with *T. lanceolata* extracts. Indeed, the determined JEG-3 IC_{50} values were doubled that of JAR. The *T. lanceolata* methanolic leaf extract was a good antiproliferative agent against JEG (IC_{50} value < 1000 µg/mL) but was an even more potent antiproliferative agent against JAR (IC_{50} value < 500 µg/mL). This trend was notable with the *T. lanceolata* aqueous leaf extract, albeit to a lesser degree (IC_{50} value < 500 µg/mL). In chapter 3, the *T. lanceolata* methanolic leaf extract was determined to have a moderate antioxidant capacity compared to the *T. ferdinandiana* methanolic fruit extract, and subsequently much lower ascorbic acid content. It can be postulated that the higher levels of thioredoxin present in JEG-3 cells may have had a protective role in this instance without the presence of high levels of ascorbic acid in the extract. It is also possible that TNF may be functioning as a promoter of apoptosis in the JAR cells under the influence of *T. lanceolata* extracts in contrast with what was observed with *T. ferdinandiana* extracts. This may require a metabolomic study which would indeed provide a clearer understanding through the identification of the compounds present in these extracts. In addition further studies to ascertain apoptosis through morphological determination and caspase determination may be required.

The *T. ferdinandiana* leaf and fruit extracts were also good antiproliferative agents against the next panel of cell lines MC3T3-E1/MG-63. Although a similar trend observed with JEG-3 and JAR was also noted, albeit with exceptions. However, the mechanisms of action may be fundamentally different. The MC3T3-E1 is a normal murine osteoblastic cell line which has increased proliferative capacity in the presence of ascorbic acid. The increased levels of ascorbic acid present in *T. ferdinandiana* methanolic, aqueous and to a much lesser degree the ethyl acetate fruit extracts, ought to have normally favoured the increased proliferation of

this cell line. However, the reverse was apparent. Indeed, the *T. ferdinandiana* methanolic fruit, aqueous fruit, methanolic leaf, aqueous leaf and the ethyl acetate leaf extracts inhibited MC3T3-E1 proliferation to a much greater extent than MG-63 cells, and instead induced negative proliferation in MC3T3-E1, possibly by a cytotoxic mechanism. Past studies have indeed documented that MC3T3-E1 cells have a known susceptibility to TNF. It is possible that TNF analogues present in the *T. ferdinandiana* fruit extracts may have triggered apoptosis and caused increased inhibition of cellular proliferation, via an extrinsic pathway. Alternatively, extract compounds may have induced expression of higher TNF levels, thereby inducing apoptosis. Furthermore, the MG-63 cells have no known susceptibility to TNF, and may even follow an intrinsic mitochondrial pathway or an extrinsic Fas-activated pathway. Hence, MG-63 cells are not inhibited by the presence of any such compounds if present. Thus, it may be postulated that the increased inhibition of MC3T3-E1 proliferation in comparison to MG-63 may suggest a TNF induced extrinsic apoptotic pathway. Likewise, the *T. ferdinandiana* leaf extracts followed a similar trend observed with the methanolic and aqueous fruit extracts. Negative proliferation was also notable with the *T. ferdinandiana* methanolic and ethyl acetate leaf extracts, suggesting involvement of cytotoxic pathways. Although the aqueous leaf extracts induced significant negative proliferation in MG-63 cells, the IC_{50} value seemingly indicated the *T. ferdinandiana* aqueous leaf extract was a moderate antiproliferative agent (IC_{50} value > 1000 µg/mL). Another notable phenomenon was the extremely potent inhibition of MC3T3-E1 cells by the *T. ferdinandiana* methanolic and ethyl acetate leaf extracts. The methanolic leaf registered an IC_{50} value of 40 µg/mL which is 20X less potent compared to MG-63 IC_{50} value. Similarly the ethyl acetate leaf IC_{50} value of 6 µg/mL was 64X lesser than its MG-63 IC_{50} value. They were among the lowest recorded for any cell line tested. It clearly indicates the extremely potent ability of these leaf extracts in

inhibiting the MC3T3-E1 cell line, which was further evidenced by the negative proliferation observed in these studies. Metabolomic investigation of the extracts is needed to elucidate the composition and caspase studies are required to be undertaken to confirm if the TNF induced extrinsic pathway was involved.

Although this may explain the trend observed with the inhibition of cell proliferation in both cell lines on exposure with *T. ferdinandiana* methanolic, aqueous fruit and leaf extracts, it does not account for results observed with *T. ferdinandiana* ethyl acetate fruit extract. The *T. ferdinandiana* ethyl acetate fruit induced negative proliferation in MG-63 cells, possibly via a cytotoxic mechanism. It also acted contrary to the trend observed with the *T. ferdinandiana* methanolic and aqueous fruit extracts. The inhibition of MG-63 cellular proliferation was much stronger in comparison to the MC3T3-E1 cells. Indeed, the *T. ferdinandiana* ethyl acetate fruit extract IC_{50} values indicate that the MG-63 cells were inhibited twice as much as that of MC3T3-E1 cells. The antioxidant content of the *T. ferdinandiana* ethyl acetate fruit extract is between 7- to 16-fold lower compared to the aqueous and methanolic fruit extract. The lack of high levels of ascorbic acid content present in the ethyl acetate extract in comparison to both the methanolic and aqueous may have been a contributed to this anomaly. Interestingly, most of the *T. ferdinandiana* high polarity and mid polarity fruit and leaf extracts also possess moderate to high levels of tannins, with the exception of the ethyl acetate fruit extract (chapter 3). The absence of tannins as a phytochemical class in the extract may have also been responsible for its activity in the cell lines. Tannins are a known high antioxidant polyphenolic class of compounds with an ability to affect redox potential [16], and subsequently may have influenced the inhibition of proliferation in both MC3T3-E1 and MG-63 cell lines.

The *T. lanceolata* extracts were also good inhibitors of both MC3T3-E1 and MG-63 cell proliferation. However, it was harder to spot trends which were less obvious as the extracts gave varied results. There were greater incidences of negative proliferation, notably among the MCT3-E1 cells induced by *T. lanceolata* methanolic, aqueous, ethyl acetate fruit and aqueous, ethyl acetate leaf extracts compared to MG-63 cells. However, the *T. lanceolata* methanolic fruit and ethyl acetate leaf extracts were classed as moderate antiproliferative agents against MC3T3-E1 proliferation (IC_{50} > 1000 µg/mL). Likewise, negative proliferation was also noted among the MG-63 cells induced by *T. lanceolata* methanolic, aqueous fruit and aqueous leaf extracts. However, the *T. lanceolata* ethyl acetate leaf extract was classed as a moderate antiproliferative against MG-63 proliferation (IC_{50} > 1000 µg/mL). Greater inhibition of MG-63 proliferation was noted with the *T. lanceolata* methanolic and ethyl acetate fruit extracts, although the converse was true with the aqueous fruit extract. MG-63 proliferation may have been inhibited possibly through an intrinsic mitochondrial pathway or through a fas-induced extrinsic pathway. The verification of the pathways would require a metabolomic analysis of the extracts and the determination of apoptotic proteins involved in this pathway. Similarly, the *T. lanceolata* methanolic leaf extract may have also possibly followed either the intrinsic mitochondrial pathway or the Fas-induced extrinsic pathway, and similar investigations are required to further elucidate this mechanism. Alternatively, the *T. lanceolata* aqueous fruit, aqueous leaf and ethyl acetate leaf extract inhibited MC3T3-E1 proliferation more than MG-63. It is possible that these cells may either follow a mitochondrial mediated pathway or a TNF-induced extrinsic pathway, leading to apoptosis. Further elucidation would require metabolomic profiling of the extracts and determination of apoptotic proteins involved to confirm the mechanisms of action and pathway involved.

Cell images of Caco-2 cells exposed to the selected extracts at sub lethal concentrations revealed that both apoptosis and necrosis were involved. Furthermore, these images were not able to reveal cytostatic behaviour, but clearly displayed all morphological characteristics associated with cytotoxicity. Indeed, the cell imaging studies correlated well with the observations reported in MTS screening assays (Chapter 3).

These extracts tested against the selected cell lines are essentially crude mixtures, very complex and may illicit multiple mechanisms that may be occurring simultaneously within cells. Thus further studies testing for cell cycle arrest are also warranted. The findings in this chapter did confirm that apoptosis and necrosis were both caused by the *T. ferdinandiana* and *T. lanceolata* extracts to varying degrees depending on which extract was used. However, more information needs to be collected as to the identity of compounds present in each extract tested and if these compounds work synergistically or otherwise. The following chapters offer a detailed exploration of the identity of the compounds present in the *T. ferdinandiana* and *T. lanceolata* extracts tested.

CHAPTER 5: COMPARATIVE METABOLOMIC PROFILE ANALYSIS OF *TERMINALIA FERDINANDIANA* FRUIT EXTRACTS

5.1 Introduction

The cell growth studies presented in chapters 3 and 4 have established the *T. ferdinandiana* fruit extracts as potent anti-proliferative agents. Further studies were undertaken to determine the composition of the extracts. The most common methodologies for doing this include bioactivity driven isolations and phytochemical fingerprint analysis. Whilst these techniques have been used quite successfully in the determination of bioactive components in bioactive plant extracts in the past, both also have drawbacks and difficulties. Bioactivity driven separations are particularly useful when the bioactivity is due to a single, major component within the extract. However, the analysis can be complicated when a very complex mixture is separated and analysed as fractionation may lead to the isolation of a large number of compounds or semi-purified fractions which need to be reassessed for bioactivity. This can be a daunting task when complex mixtures of hundreds to thousands of phytochemicals are evaluated. Instead, the usual methodology involves partial fractionation, re-assaying, followed by further fractionation etc. However, as many components with similar activities but different physical and chemical properties may be present in a crude extract, fractionating

may result in multiple bioactive fractions, further complicating elucidation of bioactive components.

Furthermore, phytochemical components often do not have the same bioactivities when separated, or have much less potent effects. This has frustrated research into the therapeutic properties of many plant species. For example, *Aloe vera* juice and extracts have been reported to be good therapeutic agents for the treatment of a wide variety of diseases and medicinal complaints [312]. Many of the same studies have also been frustrated by fractionation studies as many of *Aloe vera*'s bioactive components have variable concentration dependent effects. Furthermore, many require the presence of other components and it has been postulated that synergy between components is required for bioactivity. Thus, whilst bioactivity driven separations are often a good means of determining active components, it may be a long laborious process that often fails to determine the molecular basis for the therapeutic properties of a mixture.

Similarly, phytochemical fingerprinting may be useful in determining bioactive components within a mixture and has successfully been used to examine potential therapeutic phytochemical components for a variety of medicinal plants. For example, recent studies have reported anti-inflammatory activities for several South African plant species [313, 314]. These same studies also screened the extracts for the presence of the well characterised phytoalexin stilbene compound 3, 5, 4'-trihydroxy-trans-stilbene (resveratrol). This compound has well established therapeutic properties for the treatment of inflammation and affects several phases of the inflammatory process, although its effects on cytokine

production have been most extensively reported [244]. As resveratrol is a relatively common phytochemical (particularly in plants with anti-inflammatory activity), these earlier studies screened for its presence and reported it to be present in a high percentage of the most promising plant species. However, this technique relies on an understanding of the likely phytochemistry of the plant and therefore may be most useful for the analysis of taxonomically related species. Furthermore, targeting a particular compound or class of compound often will overlook other compounds which may have a greater contribution to the therapeutic properties of the extract.

A less targeted metabolomics approach may be useful in narrowing the focus of a phytochemical analysis. This methodology has been used in several recent studies with great effect. The West Australian plant *Scaevola spinescens* R. Br. has a variety of therapeutic properties including antiviral activity [315]. A non-biased phytochemical analysis and comparison of the antiviral extracts not only putatively identified several hundred phytochemical components, but also determined that only 2 of these compounds were present in all of the active extracts, but not in the inactive extracts [301]. Interestingly, one of these compounds had already been reported as having good antiviral activity, thus validating this non-biased metabolomics comparison approach in narrowing the focus for further studies. A similar non-biased metabolomics approach has been utilised in my studies to not only determine as many of the compounds present in the anti-proliferative extracts as possible, but also to compare the metabolomics profiles of the extracts to narrow the phytochemical focus.

The aim of such plant metabolomics studies is to identify the complete complement of metabolites in a plant sample as rapidly as possible and without bias [316]. This can be a difficult, time consuming task requiring a range of analytical methodologies to achieve an

extensive examination of the plants metabolome. The diversity of chemical properties and the wide concentration ranges of these compounds makes this difficult as the method used not only needs to identify a vast amount of compounds in a single sample, but also needs to be robust and reproducible enough to allow samples to be reliably compared [302].

Early metabolomic studies have focused mainly on gas chromatography - mass spectrometry (GC-MS) based approaches. Such approaches allow plant extracts to be rapidly screened for metabolites of the major metabolic pathways including amino acids, organic acids, sugars, sugar alcohols and glycosides [317]. However, this method is limited in the types of compounds which may be separated and detected. GC-MS is generally used for the detection of low polarity compounds. Thus, a large amount of compounds of medium polarity or high polarity are often not detected by GC-MS metabolomic analysis without additional derivatisation steps of the target analytes, making non-targeted analysis difficult. Liquid chromatography – mass spectroscopy (LC-MS) is better suited for the analysis of compounds of medium and high polarity. Coupling LC with high mass accuracy spectrometry techniques using mild ionisation techniques such as electrospray ionisation (ESI) can generate large amounts of useful information for compound identification and metabolomic analysis. Using these methods, molecular ions can be detected and their empirical formulas accurately determined and compared to databases. Furthermore, coupling this with collision induced fragmentation analysis allows for structural characterisation of unknown compounds, lending weight to database identifications using accurate mass data.

To detect the greatest amount of compounds with the greatest variety of solubility properties, both LC-MS analysis and GC-MS headspace analysis were used in my studies. As the 2 techniques should separate and detect compounds of overlapping polarity, a combination of the 2 techniques will provide the greatest chance of detecting all compounds present in each extract and allow for the most accurate non-biased metabolomics comparison analysis.

5.2 Materials and Methods

5.2.1 Preparation of samples for metabolomic analysis

A 0.5 ml volume of each *T. ferdinandiana* fruit extract was dried at room temperature under vacuum. The resultant dried extracts were resuspended in 5% acetonitrile and filtered through a Sarstedt 0.22 µm filter cartridge.

5.2.2 Non-targeted HPLC-MS QTOF analysis

A 2 µl aliquot of each extract was injected onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C18 column (2.1 x 100 mm, 1.8 µm particle size) and analysed as described in chapter 2.

5.2.3 Non-targeted GC-MS head space analysis

Aliquots (0.5 µL) of each extract were injected onto a Shimadzu GC-2010 Plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass spectrometer. Chromatographic separation was accomplished using a 5% phenyl, 95% dimethylpolysiloxane (30m x0.25 mm id x 0.25um) capillary column (Restek USA) as described in chapter 2. Helium (99.999%) was employed as a carrier gas at a flow rate of 0.79 ml/min.

5.3 Results

5.3.1 Qualitative HPLC-MS analysis

A major aim of this study was to establish a HPLC-MS/MS method for the metabolic profiling of the compounds in the bioactive extracts and to use this method to qualitatively differentiate and identify a significant number of these compounds. Optimised HPLC-MS parameters were developed and used to profile and compare the compound profiles from different extractions of *T. ferdinandiana* fruit. The resultant total compound chromatograms (TCC) for the positive ion and negative ion chromatograms of the methanolic fruit extracts are presented in Figure 5.1 (a, b) respectively. The positive ion chromatogram had a significantly greater number of mass signal peaks detected. However, the negative ion chromatogram had a higher base peak signal to noise ratio in the total ion chromatograms which may have hidden some peaks in the negative ionisation mode.

The positive ion TCC for the methanolic *T. ferdinandiana* fruit extract revealed numerous peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. Nearly all of the methanol extract compounds had eluted by 10 minutes (corresponding to approximately 20% acetonitrile). Indeed, multiple over lapping peaks eluted in the first 2 minutes with 5% acetonitrile. However, the presence of several peaks eluting later in the chromatogram (particularly evident in the positive ionisation mode) (Figure 5.1 a) indicates the broad spread of polarities of the compounds in the extract.

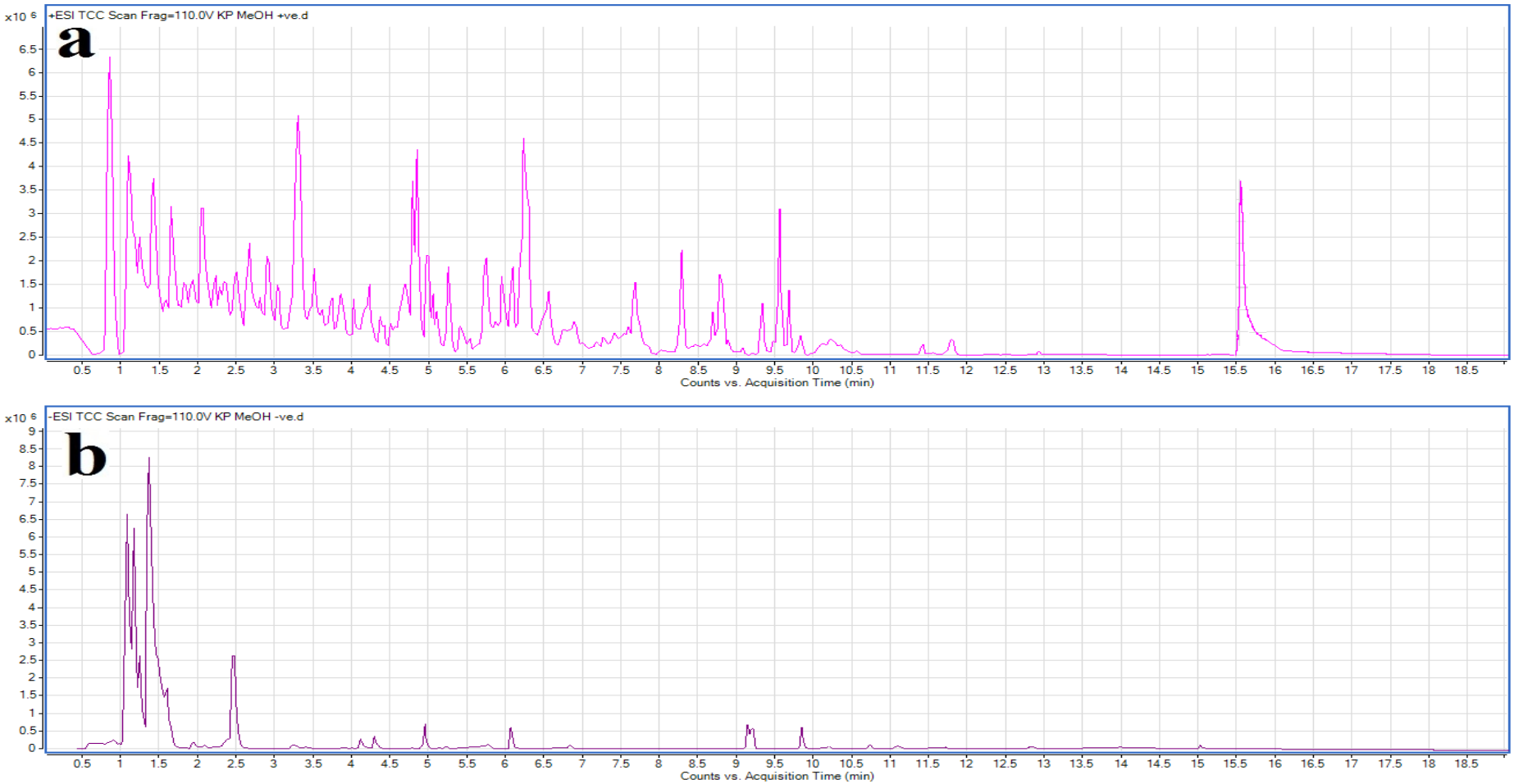


Figure 5.1: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µl injections of methanolic *T. ferdinandiana* fruit extract.

Multiple peaks were also evident in the aqueous *T. ferdinandiana* fruit extract in both the positive (Figure 5.2 a) and negative ion (Figure 5.2 b) TCC chromatograms, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. As for the methanolic extract, nearly all of the aqueous extract compounds had eluted by 10 minutes (approximately 20% acetonitrile).

The *T. ferdinandiana* fruit extract ethyl acetate extract (Figure 5.3) had substantially fewer peaks, with a greater spread in polarity. Whilst a high percentage of the compounds eluted in the isocratic phase of the chromatograms, several large peaks were also evident later in the chromatograms (10 - 20 min) corresponding to mid polarity compounds at approximately 25-60% acetonitrile. This is particularly evident for the positive ion (Figure 5.3 a) TCC chromatogram. Fewer peaks were evident in the later phase of the negative ion chromatograms (Figure 5.3 b).

The *T. ferdinandiana* fruit chloroform (Figure 5.4) and hexane extracts (Figure 5.5) both contained a relative abundance of peaks in the middle stages of the chromatogram. In contrast with the methanolic, aqueous and ethyl acetate extracts, both of these extracts displayed a greater abundance of peaks in the negative ion chromatograms than in the positive ion chromatogram. Many of these peaks were coincident with peaks in the methanolic, aqueous and ethyl acetate extracts, indicating the presence of similar compounds between the extracts.

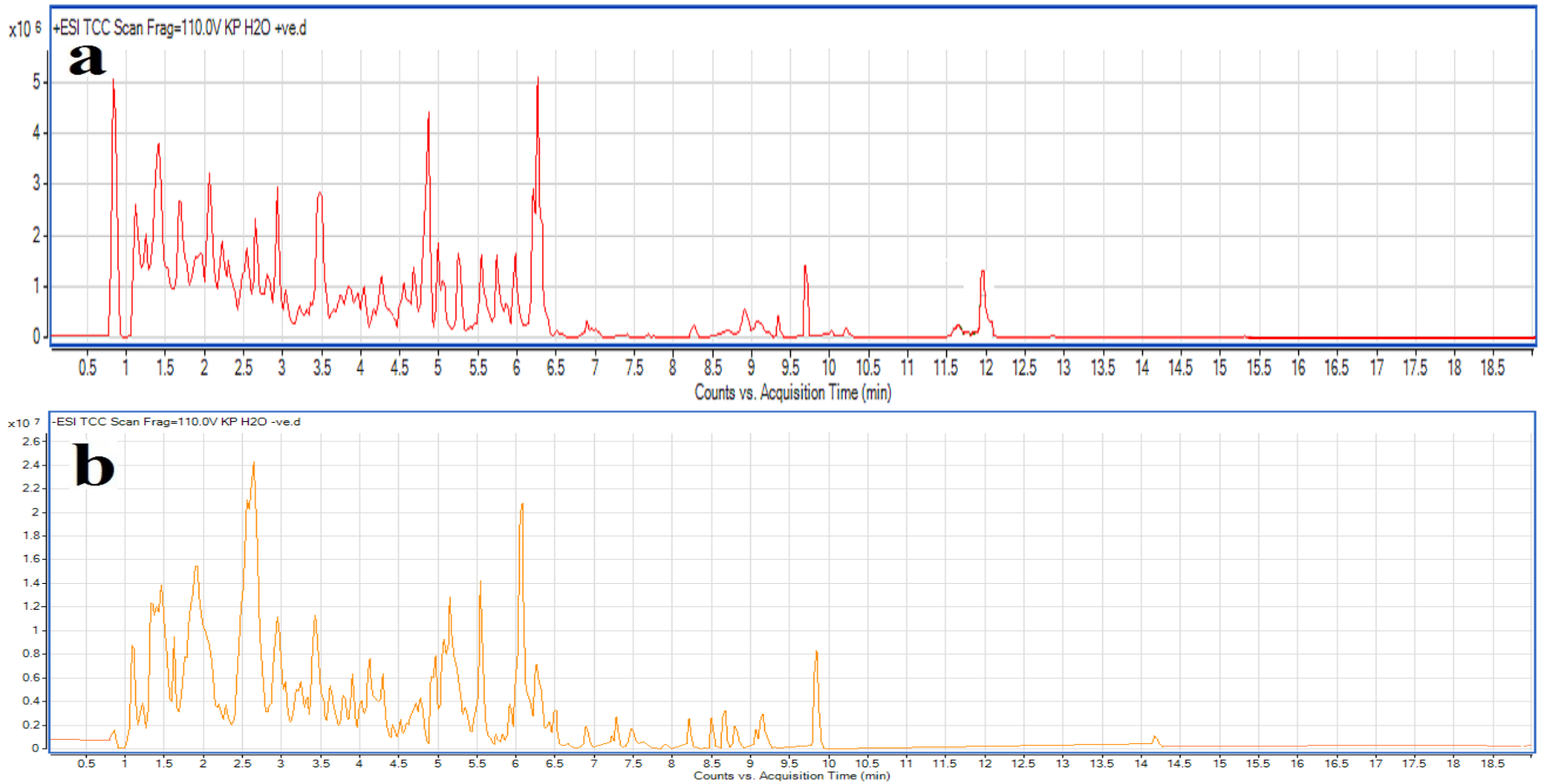


Figure 5.2: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µl injections of aqueous *T. ferdinandiana* fruit extract.

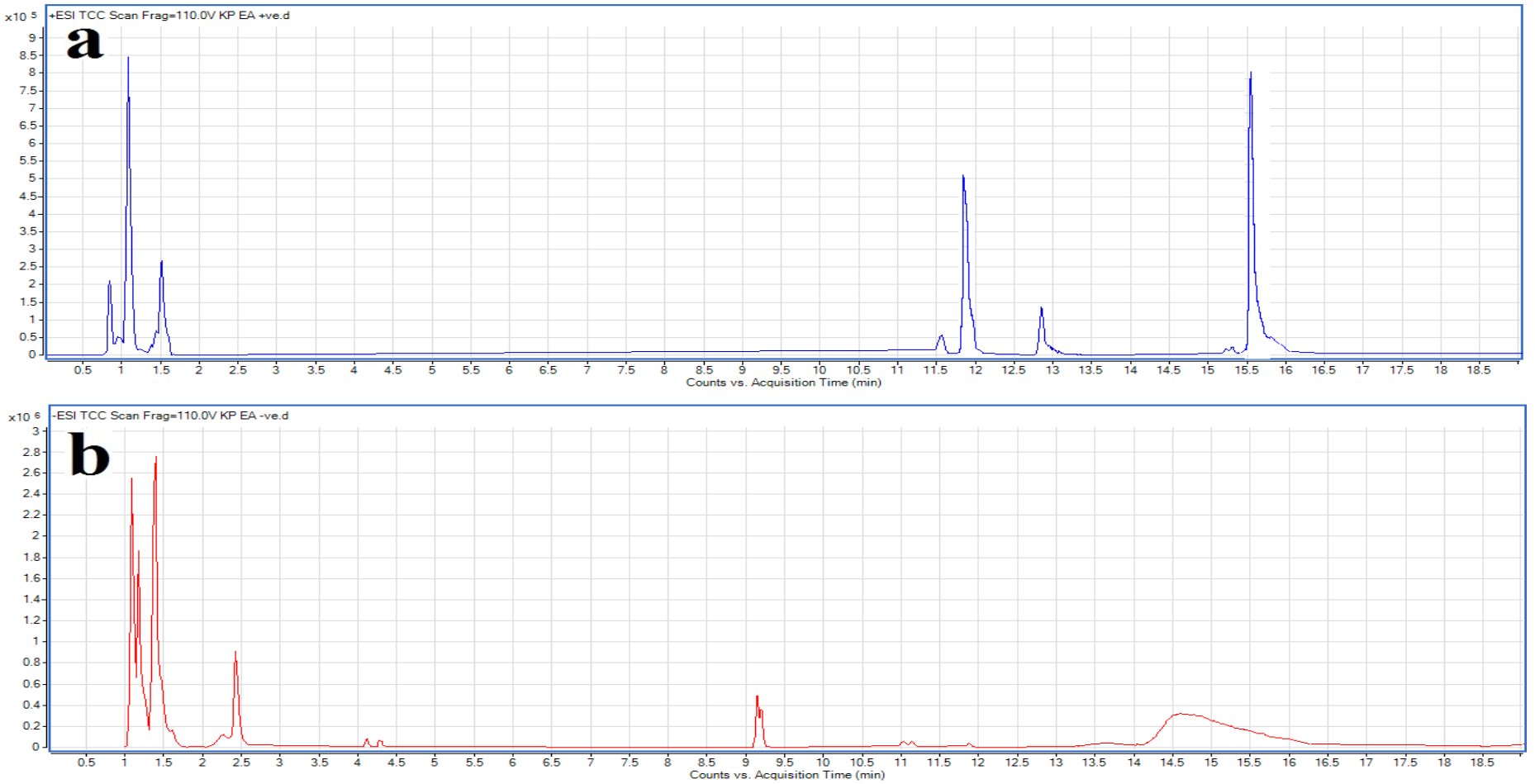


Figure 5.3: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µl injections of *T. ferdinandiana* fruit ethyl acetate extract.

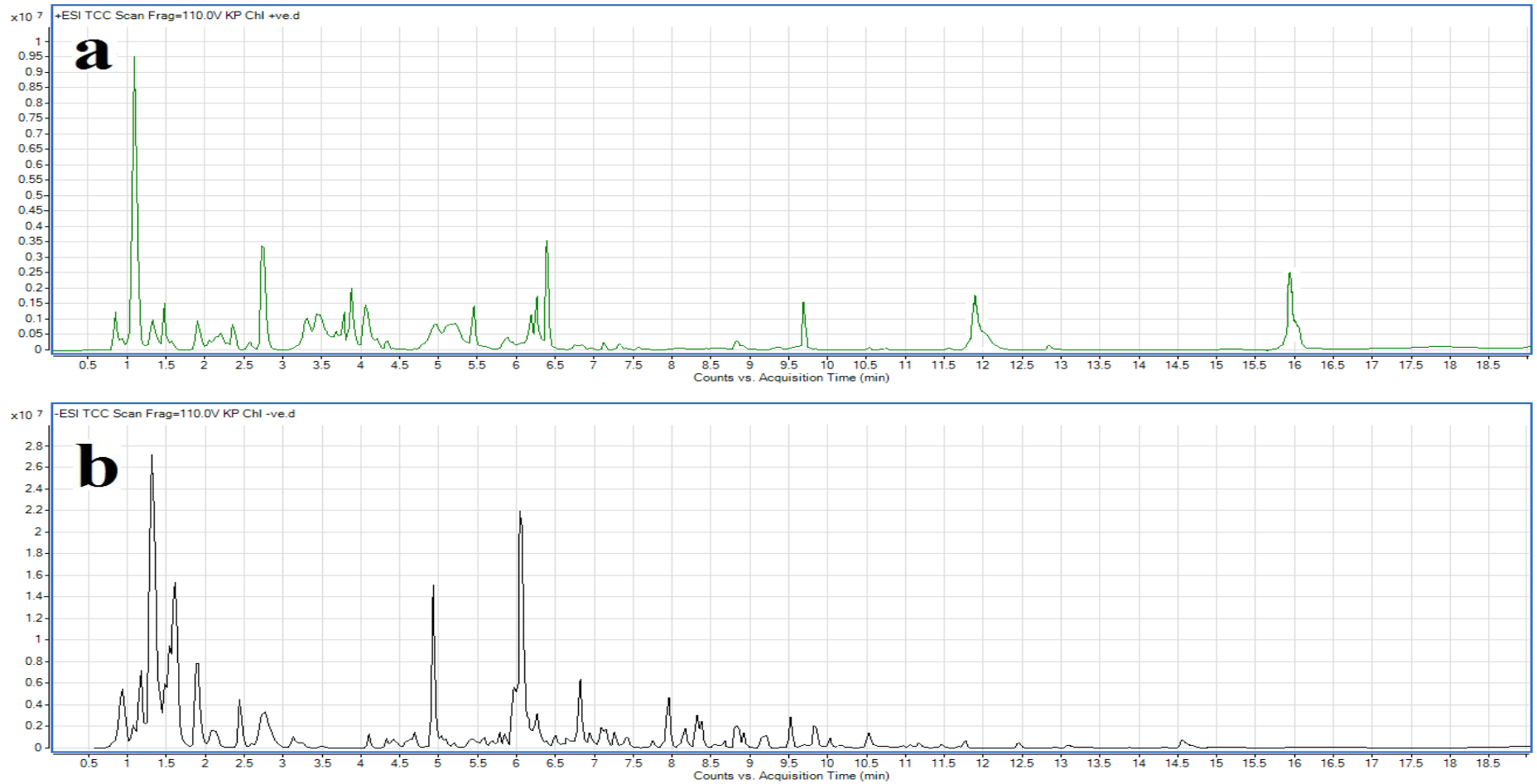


Figure 5.4: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µl injections of *T. ferdinandiana* fruit chloroform extract.

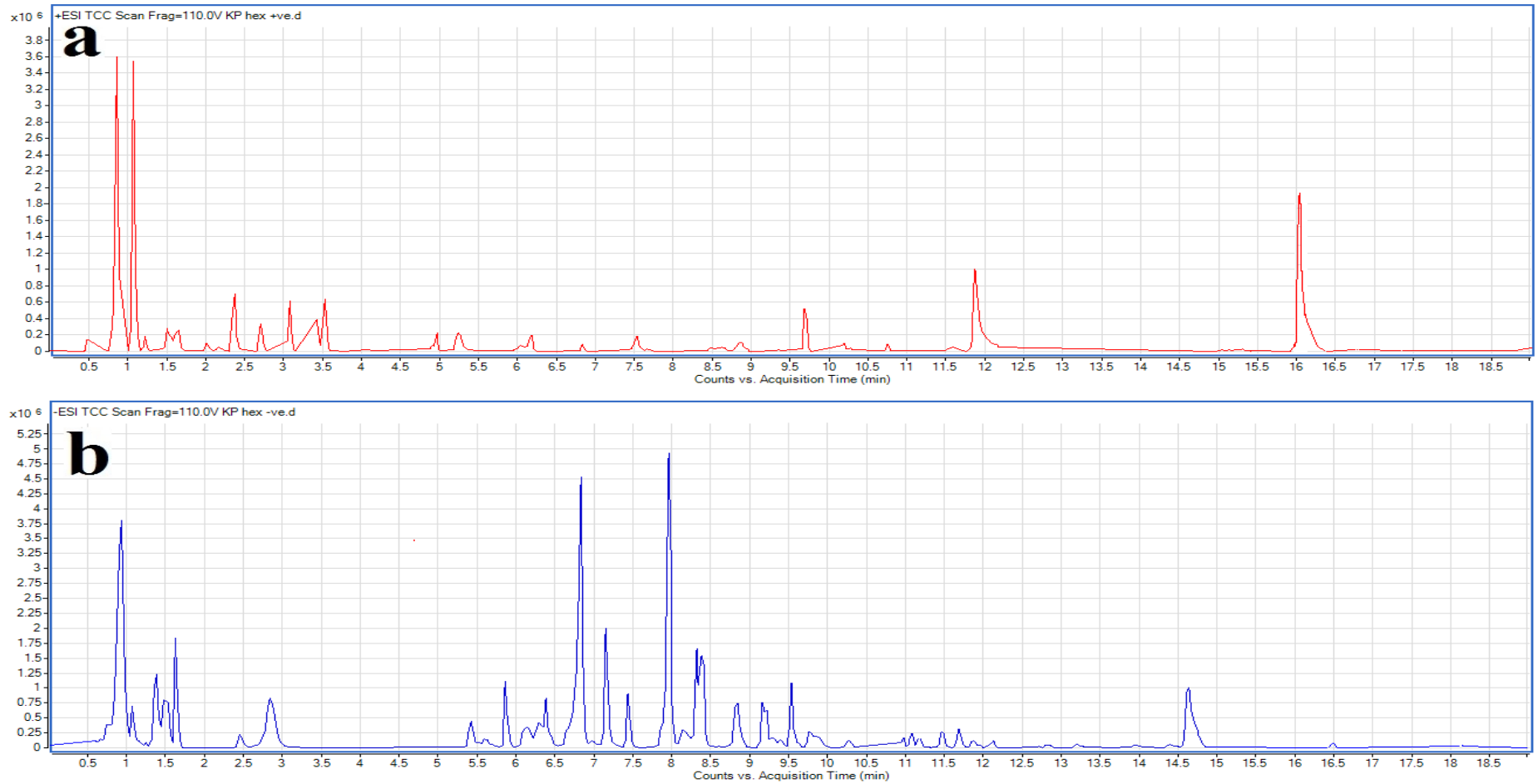


Figure 5.5: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µl injections of *T. ferdinandiana* fruit hexane extract.

5.3.1.1 Qualitative mass spectral analysis of the *T. ferdinandiana* fruit extracts

In total, 1115 unique polar to mid polarity mass signals were noted for the *ferdinandiana* fruit extracts by LC-MS analysis (Table 5.1). Putative empirical formulas were achieved for all of these compounds. Of the 1115 unique molecular mass signals detected, 785 compounds (70.4%) were putatively identified by comparison against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (800 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds). As all of the *T. ferdinandiana* fruit extracts displayed anti-proliferative activity (Chapters 3 and 4), a comparison of the metabolomics profiles across these extracts was used to narrow the focus of phytochemicals which may contribute to this bioactivity. Of the 1115 unique mass signals detected in the *T. ferdinandiana* fruit extracts, only 8 were present in all of the anti-proliferative extracts. These are shown in order of increasing molecular mass in Figure 5.6. The tannin compounds gallic acid (Figure 5.6 a) and chebulic acid (Figure 5.6 h) as well as the antioxidant ascorbic acid (Figure 5.6 d) were observed in all extracts. The presence of these 3 compounds is noteworthy as tannins and ascorbic acid have been reported to be present in high concentrations in extracts from many *Terminalia* spp. [8]. Indeed, tannins and high antioxidant capacity (often attributed to ascorbic acid) are defining phytochemical features of the genus *Terminalia*. Also putatively identified across all *T. ferdinandiana* fruit extracts were methoxy carbonyl oxy-methyl methyl carbonate (Figure 5.6 b), apionic acid (Figure 5.6 c), glucuronic acid (Figure 5.6 e), 5-(4-hydroxy-2, 5-dimethylphenoxy)-2, 2-dimethyl pentanoic acid (Figure 5.6 f) and C17 sphingosine (Figure 5.6 g).

Table 5.1: Qualitative HPLC-MS QTOF analysis of the *T. ferdinandiana* fruit extract, elucidation of empirical formulas and putative identification (where possible) of the compounds.

Name	Formula	Mass	RT	M	W	E	C	H
Acetoacetic acid	C4 H6 O3	102.032	1.081	-			-	
Hydroxypyruvic acid	C3 H4 O4	104.012	1.36				-	
3-furanoic acid	C5 H4 O3	112.015	2.057	+	+		-	
3-oxo-4-pentenoic acid	C5 H6 O3	114.032	1.082	-			-	
Proline	C5 H9 N O2	115.063	1.12	+	+			
maleic acid	C4 H4 O4	116.011	1.614				-	-
isoamyl nitrite	C5 H11 N O2	117.079	1.094	+	+			
succinic acid	C4 H6 O4	118.027	1.091	-	-		-	
Purine	C5 H4 N4	120.044	1.501			+		+
	C3 H9 N S2	123.017	0.833		+	+	+	+
2-Amino-5-chloropyridine	C5 H5 Cl N2	128.012	1.597				-	
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	C6 H8 O3	128.045	1.505	+				
Octodrine	C8 H19 N	129.152	3.43				+	+
Pyroglutamic acid	C5 H7 N O3	129.042	1.609	+/-	+			
Vigabatrin	C6 H11 N O2	129.08	1.244	+	+			

2-Methylenesuccinic acid (itaconic acid)	C5 H6 O4	130.027	2.041	+/-			-	
Leucine	C6 H13 N O2	131.094	1.643	+	+			
glutaric acid	C5 H8 O4	132.043	1.091	-	-		-	
2,3-Dihydroxyvaleric acid	C5 H10 O4	134.058	1.871	+				
trihhdroxybutanoic acid	C4 H8 O5	136.037	1.372	-	-	-	-	-
	C3 H11 N3 O3	137.08	2.955	+				
salicyclic acid	C7 H6 O3	138.032	4.681				-	
6-Hydroxynicotinic acid	C6 H5 N O3	139.027	1.521		+			
Metipirox	C7 H9 N O2	139.061	1.077			+		
(1S,5R)-4-hydroxy-6,7-dioxabicyclo[3.2.1]oct-2-en-8-one	C6 H6 O4	142.026	2.049	+	+			-
Triparanol	C7 H13 N O2	143.095	1.132	+	+			
(E)-2-Methylglutaconic acid	C6 H8 O4	144.042	4.135	+				
	C3 H3 N3 O4	145.012	0.999				+	
Propionylglycine methyl ester	C6 H11 N O3	145.074	1.091	+/-				
2R-aminoheptanoic acid	C7 H15 N O2	145.109	1.177	+				
3S)-5-Oxotetrahydro-3-furancarboperoxoic acid	C5 H6 O5	146.022	1.134	-			-	
Isosorbide	C6 H10 O4	146.058	2.458				-	

4-hydroxy enanthoic acid	C7 H14 O3	146.094	7.156					-	-
Ribonolactone	C5 H8 O5	148.037	1.425	-		-		-	
2,3-Dihydroxy-3-methylvaleric acid	C6 H12 O4	148.075	2.297		-			-	
	C8 H6 O3	150.032	5.078		+/-			-	
D-Ribose	C5 H10 O5	150.053	1.098	-				-	
piperitone	C10 H16 O	152.12	6.246	+	+			+	
Valdetamide	C9 H17 N O	155.131	1.481					+	
Furan 2,5-dicarboxylic acid	C6 H4 O5	156.007	2.631		-				
IRETOL	C7 H8 O4	156.04	1.849	+					
5-oxo-7-octenoic acid	C8 H12 O3	156.078	8.121					+	-
	C9 H19 N O	157.147	1.048					+	
2-methylene-4-oxo-pentanedioic acid	C6 H6 O5	158.023	1.582					-	
4,6-dioxoheptanoic acid	C7 H10 O4	158.057	4.023		-				
3-Oxovalproic acid	C8 H14 O3	158.094	6.335					-	-
2-Methylbutyrylglycine	C7 H13 N O3	159.09	1.206	+					
2-Propylbenzimidazole	C10 H12 N2	160.103	5.938					-	
2-Oxoadipic acid	C6 H8 O5	160.037	1.148	-					
2-hydroxy-4-oxoGlutaric acid	C5 H6 O6	162.019	1.335					+/-	

ethyl (ethylperoxy) (oxo) acetate	C6 H10 O5	162.053	1.07	-	-		-	
methoxycarbonyloxymethyl methyl carbonate	C5 H8 O6	164.032	1.45	-	-	-	-	-
	C5 H12 N2 S2	164.044	0.841	+	+	+	+	+
	C4 H11 N3 O4	165.075	2.9	+				
2-amino-4'-hydroxy-Propiophenone	C9 H11 N O2	165.079	2.612	+	+			
1,3-Benzodioxol-5-ylformiat	C8 H6 O4	166.026	6.12		-		-	-
Apionic acid	C5 H10 O6	166.048	1.368	-	-	-	-	-
3-Methylxanthine	C6 H6 N4 O2	166.052	1.073		-			
Ethionamide	C8 H10 N2 S	166.059	3.42		-			
Atrolactic acid	C9 H10 O3	166.061	5.559		-		-	
D-4-Hydroxyphenylglycine	C8 H9 N O3	167.058	1.42	+				
	C7 H4 O5	168.006	2.455					
vanillic acid	C8 H8 O4	168.042	5.021				-	
ACECLIDINE	C9 H15 N O2	169.11	5.405	+			+	
(1S,5R)-4-Oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2-carboxylic acid	C7 H6 O5	170.021	2.454	+/-	+/-		-	-
2-decylenic acid	C10 H18 O2	170.131	6.242	+				
Gabapentin	C9 H17 N O2	171.126	6.171				+	

(1S,5R)-4-Oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2-carboxylic acid	C7 H6 O5	170.022	2.612		+	-			
2-Heptenedioic acid, 4-oxo-	C7 H8 O5	172.037	1.439	+					
DL-3,4-Dihydroxyphenylglycol	C8 H10 O4	170.056	3.114				-		
(1r,2r)-cyclohexane-1,2-dicarboxylic acid	C8 H12 O4	172.072	2.343	+			-	+/-	
2-decylenic acid	C10 H18 O2	170.131	6.248		+				
9-hydroxy-5Z-nonenoic acid	C9 H16 O3	172.11	8.377				-	-	
Castelamarin	C9 H14 O3	170.094	9.801				-	-	
2-Heptenedioic acid, 4-oxo-	C7 H8 O5	172.035	1.339		+		+		
(1r,2r)-cyclohexane-1,2-dicarboxylic acid	C8 H12 O4	172.072	2.356				+		
swainsoniine	C8 H15 N O3	173.105	1.697	+	+		-		
9-amino-nonanoic acid	C9 H19 N O2	173.142	2.568				+		
shikimic acid	C7 H10 O5	174.053	1.334	-			-		
2-Propylglutaric acid	C8 H14 O4	174.089	5.858				-	-	
4-hydroxy pelargonic acid	C9 H18 O3	174.125	6.929				-		
ascorbic acid	C6 H8 O6	176.03	1.372	-	-	-	-	-	
6,8-dihydroxy-octanoic acid	C8 H16 O4	176.105	6.677				-	-	
2-Isopropylmalic acid	C7 H12 O5	176.066	3.608		-		-		

ESCULETIN	C9 H6 O4	178.027	5.189				-		
gluconolactone	C6 H10 O6	178.048	1.158	-		-	-		
Methyl-p-coumarate	C10 H10 O3	178.063	8.551				-		
methyl eugenol	C11 H14 O2	178.099	11.171			-	-	-	
Phenacetine	C10 H13 N O2	179.092	2.886	+			+		
	C12 H4 O2	180.021	0.84					+	
caffeic acid	C9 H8 O4	180.042	5.077				-		
glucose	C6 H12 O6	180.063	1.071	-		-	-	-	
	C10 H16 N2 O	180.126	2.536	+	+				
Tyrosine	C9 H11 N O3	181.074	2.642		+				
	C10 H2 N2 O2	182.012	5.983				-		
1,2-Epoxy-3,4-butanediol 4-methanesulfonate	C5 H10 O5 S	182.022	4.921				-		
Mannitol	C6 H14 O6	182.079	1.054				-		
AZOBENZENE	C12 H10 N2	182.084	4.823	+	+				
	C10 H18 N2 O	182.142	2.66	+	+				
Oxisuran	C8 H9 N O2 S	183.039	0.839	+	+		+/-	+	
Undecenamide 10-	C11 H21 N O	183.163	2.002				+	+	
2,4-DINITROPHENOL	C6 H4 N2 O5	184.014	1.186	-		-		-	
(2-Methyl-4-oxo-4H-pyran-3-	C8 H8 O5	184.037	4.943	-	-		-		

yloxy)-acetic acid								
4-Hydroxybicyclo[2.2.2]oct-1-yl acetate	C10 H16 O3	184.109	8.159				-	
Valdipromide	C11 H23 N O	185.178	2.159				+	+
	C9 H2 N2 O S	185.986	1.625					+
2,3,4,5-Tetrahydroxybenzoic acid	C7 H6 O6	186.014	1.496	-	-			
Endothal	C8 H10 O5	186.051	1.753	+			+/-	
2-oxo capric acid	C10 H18 O3	186.124	7.158				-	
Capuride	C9 H18 N2 O2	186.137	2.793	+				
8-Amino-7-oxononanoate	C9 H17 N O3	187.121	5.411		+			
Fluoresone	C8 H9 F O2 S	188.031	1.39	-	-		+/-	
3-Methylsuberic acid	C9 H16 O4	188.105	6.827	-			-	-
Val Ala	C8 H16 N2 O3	188.116	2.329	+	+			
	C12 H2 N2 O	190.016	1.117				+	
Dimethyl acetoxymalonate	C7 H10 O6	190.049	1.08	-			-	
citric acid	C6 H8 O7	192.029	1.431	-	-		-	
quinic acid	C7 H12 O6	192.063	1.37	-		-	-	
N-Hydroxymethylnorcotinine	C10 H12 N2 O2	192.092	7.519					+
p-Butylaminobenzoic acid	C11 H15 N O2	193.109	6.175				+	+
	C8 H19 N O4	193.132	2.748		+			

Hydroxy-7-methyl-4H,5H-pyrano[4,3-b]pyran-dione	C9 H6 O5	194.021	4.011		-		-	
glucuronic acid	C6 H10 O7	194.043	1.34	-	-	-	-	-
ferulic acid	C10 H10 O4	194.058	6.254				-	
Feroxidin	C11 H14 O3	194.092	7.954					-
2-Phenyl-1,3-propanediyl monocarbamate	C10 H13 N O3	195.089	12.135					-
	C15 O	195.994	0.473					+
HAEMATOMMIC ACID	C9 H8 O5	196.036	4.089		-		-	
(3S,4S,5R)-2-(Dihydroxymethyl)tetrahydro-2H-pyran-2,3,4,5-tetrol	C6 H12 O7	196.059	1.336	-			-	
ORSELLINIC ACID, ETHYL ESTER	C10 H12 O4	196.074	2.541		+			
	C8 H6 O6	198.017	3.893		-			
O,O,O-Triethylphosphorothioate	C6 H15 O3 P S	198.046	6.051				-	
ethyl gallate isomer 1	C9 H10 O5	198.053	2.383	+				
ethyl gallate isomer 2	C9 H10 O5	198.053	4.906	+			-	
ethyl gallate isomer 3	C9 H10 O5	198.053	6.065	-	-		-	
	C11 H18 O3	198.125	8.382				-	-
	C5 H5 N5 O2 S	199.016	0.84					+

	C14 H17 N	199.136	11.795	+	+		+	
	C11 H21 N O2	199.158	1.556				+	
1h-pyrido[3,2-b][1,4]benzothiazine (Prothipendyl-M (ring))	C11 H8 N2 S	200.044	1.478				-	
ethylgallic acid	C9 H12 O5	200.066	2.002	+				
gelsemiol	C10 H16 O4	200.105	7.429				-	-
	C9 H16 N2 O3	200.116	2.665		+			
2S-hydroxy-10-undecenoic acid	C11 H20 O3	200.141	8.319				-	
11-amino-undecanoic acid	C11 H23 N O2	201.173	1.458	+			+	+
	C9 H6 N4 S	202.033	0.847		-		-	-
	C8 H10 O6	202.048	2.207	+	+		-	
sebacic acid	C10 H18 O4	202.12	6.376				-	-
Ile Ala	C9 H18 N2 O3	202.132	2.636	+	+			
	C9 H9 N5 O	203.08	1.105	+	+			
Isopentenyladenine	C10 H13 N5	203.117	1.776		+			
	C8 H12 O6	204.063	2.039				-	
	C10 H6 O5	206.02	2.835		+			
	C14 H6 S	206.022	0.853	+	+			
2,3-O-	C7 H10 O7	206.045	1.191		-			

(Oxomethylene)hexopyranose							
	C10 H22 O4	206.152	6.236	+			
CARYLOPHYLLENE OXIDE	C14 H22 O	206.167	15.035	-			
Phenylacetylglycine methyl ester	C11 H13 N O3	207.09	5.695	+			-
FRAXETIN	C10 H8 O5	208.037	3.69	+			
Glucoheptonic acid-1,4-lactone	C7 H12 O7	208.058	1.242	-		-	-
	C8 H16 O6	208.094	1.451	+			
PROPOXUR	C11 H15 N O3	209.103	5.404	+			+
D-Saccharic acid	C6 H10 O8	210.038	1.422	-	-		
vanilpyruvic acid	C10 H10 O5	210.054	4.366	+			-
Sedoheptulose	C7 H14 O7	210.074	1.141				-
3-(2,5-dimethoxyphenyl)propanoic acid	C11 H14 O4	210.087	6.827				-
3-Hydroxyphenylglycine	C9 H9 N O5	211.049	1.227	+			
(2-Hydroxyphenoxy)malonic acid	C9 H8 O6	212.035	2.634		+		
danielone	C10 H12 O5	212.068	7.218		-		-
12-oxo-10Z-dodecenoic acid	C12 H20 O3	212.141	8.789	+			-
3E,5E-tridecadienoic acid	C13 H22 O2	210.162	6.347		+		-
CHLOROXINE	C9 H5 Cl2 N O	212.973	1.901				+
Droxidopa	C9 H11 N O5	213.064	1.515		+		-

	C10 H2 N2 O2 S	213.982	1.628					+
Deoxyribose 5-phosphate	C5 H11 O7 P	214.025	1.159	-	-			
Monolinuron (Phenylurea)	C9 H11 Cl N2 O2	214.048	4.123	+				
	C7 H14 N6 O2	214.118	4.899				+	+
	C11 H21 N O3	215.152	7.137				-	
Nithiazid	C6 H8 N4 O3 S	216.028	1.173	+			-	
xanthotoxin	C12 H8 O4	216.04	1.076			-		
Terbacil	C9 H13 Cl N2 O2	216.064	1.557	+	+			
desethyletomidate	C12 H12 N2 O2	216.09	4.654	+	+			
Undecanedioic acid	C11 H20 O4	216.136	8.599				-	
Val Val	C10 H20 N2 O3	216.147	1.66	+				
Pymetrozine	C10 H11 N5 O	217.095	1.142		+			
	C13 H2 N2 O2	218.01	0.875				-	-
	C8 H10 O7	218.043	1.219	+				
TRIACETIN	C9 H14 O6	218.076	1.32				+	
4-[(6-Hydroxyhexyl)oxy]-4-oxobutanoic acid	C10 H18 O5	218.115	6.287				-	-
Thr Val	C9 H18 N2 O4	218.127	1.482	+	+			
1) 1-(3-Chloropropyl)-4-(2-methoxyethyl)piperidine	C11 H22 Cl N O	219.14	3.064					+

	C8 H12 O7	220.058	1.129			-	
Gladiolic acid	C11 H10 O5	222.054	5.885	+			
KOBUSONE	C14 H22 O2	222.162	13.984	-			
gardenine	C11 H13 N O4	223.085	2.616	+	+		
Rimiterol	C12 H17 N O3	223.121	3.702		+		
Cys Cys	C6 H12 N2 O3 S2	224.027	0.922			-	-
Aspidinol	C12 H16 O4	224.103	4.711			+	
Isomoxole	C12 H20 N2 O2	224.153	3.277	+			
	C8 H3 N O5 S	224.973	2.045	+		-	
	C16 H2 O2	226.004	0.845	+			
chorismic acid	C10 H10 O6	226.046	2.245	+/-	-	-	
3-Nitrotyrosine	C9 H10 N2 O5	226.055	3.643	+			
Methallatal	C10 H14 N2 O2 S	226.075	4.36	+	+		
genipin	C11 H14 O5	226.084	8.209		-		
Allixin	C12 H18 O4	226.12	8.311			-	-
	C6 H18 N4 O5	226.127	2.986	+	+		
Cyclohexyl(1-hydroxycyclopentyl)acetic acid	C13 H22 O3	226.154	10.901			-	
Hydroxypyridin tartrate	C9 H9 N O6	227.042	3.321		-		
(2R,3S)-3-(3-	C9 H8 O7	228.024	3.47		-	-	

Carboxylatopropanoyl)-5-oxotetrahydro-2-furancarboxylate						
Ozagrel	C13 H12 N2 O2	228.09	4.783	+	+	
Octahydro-4a,9a(2H,5aH)-oxanthrenediol	C12 H20 O4	228.134	6.235	+		-
Pro Leu	C11 H20 N2 O3	228.148	1.693	+		
Pro Leu	C11 H20 N2 O3	228.148	2.668	+	+	
	C11 H11 N5 O	229.096	1.224	+		
	C6 H4 N2 O2 P2 S	229.948	1.031			-
Clidafidine	C9 H8 Cl2 N2 O	230.006	4.195		-	
D-Ribose 1-phosphate	C5 H11 O8 P	230.02	3.671	+		
	C9 H10 O7	230.043	2.657	+	-	-
	C10 H6 N4 O3	230.044	2.281	+		
	C8 H10 N2 O6	230.052	1.58			+
BENZANTHRONE	C17 H10 O	230.073	2.941	+	+	+
2,4-Dihydroxytacrine	C13 H14 N2 O2	230.106	4.964	+	+	
Leu Val	C11 H22 N2 O3	230.163	2.669	+	+	
5H-Oxireno[4,5]furo[3,2-g][1]benzopyran-5-one, 1a,8b-dihydro-3-methoxy-	C12 H8 O5	232.035	4.371	+		
Nifurprazine	C10 H8 N4 O3	232.06	1.601		+	

N2-Succinyl-L-ornithine	C9 H16 N2 O5	232.108	7.595	+			
	C6 H15 N7 O3	233.124	1.345		+		
Lomustine	C9 H16 Cl N3 O2	233.09	2.276	+			
2,4-DICHLOROPHENOXYACETIC ACID, METHYL ESTER	C9 H8 Cl2 O3	233.982	0.808	-			
	C9 H6 N4 O4	234.04	1.331				-
	C15 H10 N2 O	234.08	10.204		+		
	C12 H13 N O4	235.085	5.523	+	+		
TCPY/1-[1-(2-Thienyl)cyclohexyl]pyrrolidine	C14 H21 N S	235.142	1.125	+	+		
	C8 H12 O8	236.053	1.347				-
Methoxsalen Metabolite	C12 H12 O5	236.067	5.295				-
Phe Ala	C12 H16 N2 O3	236.117	3.219	+			
COTARNINE	C12 H15 N O4	237.101	2.804	+	+		
2-Keto-3-deoxyoctonate (KDO)	C8 H14 O8	238.069	1.158	-			
Protheobromine	C10 H14 N4 O3	238.105	2.134				-
3-[(1S,2S,4aR,6S,8aS)-6-Hydroxy-2-methyl-1,2,4a,5,6,7,8,8a-octahydro-1-naphthalenyl]propanoic acid	C14 H22 O3	238.157	11.095	-		-	-

	C12 H9 N5 O	239.08	1.553		+		
Methyldopate	C12 H17 N O4	239.115	2.088	+			
Tebatizole	C12 H21 N3 S	239.145	3.848	+			
	C11 H21 N5 O	239.174	5.226		+	+	
Cystine	C6 H12 N2 O4 S2	240.028	3.981		-		
Fluindione	C15 H9 F O2	240.062	1.866	+	-	-	
	C17 H8 N2	240.067	10.211	-			
Cyanazine (Fortrol)	C9 H13 Cl N6	240.084	1.057	-			
2-Hydroxy-3-(4-methoxyethylphenoxy)-propanoic acid	C12 H16 O5	240.099	9.141		-	-	
Budralazine	C14 H16 N4	240.136	11.858			-	-
Ethyl-5-hexylbarbituric acid	C12 H20 N2 O3	240.148	1.342	+	+		
Ethyl-5-hexylbarbituric acid	C12 H20 N2 O3	240.148	1.928	+			
Tranid	C10 H12 Cl N3 O2	241.059	2.62	+		-	
	C5 H10 N2 O9	242.039	2.883	+	-	+	
	C10 H10 O7	242.043	2.22	+	+		
3,3'-(2-Oxo-1,1-cyclohexanediyl)dipropanoic acid	C12 H18 O5	242.115	8.179			-	-
Hydroxyamobarbital	C11 H18 N2 O4	242.126	5.183			-	

	C13 H22 O4	242.151	10.196				-	
Mono-N-depropylprobenecid	C10 H13 N O4 S	243.06	1.07	-		-		
Pro Ala Gly	C10 H17 N3 O4	243.122	2.551	+				
2) 1-oxa-4-azaspiro[4.5]decane-3,3-dimethanol, 8-propyl-	C13 H25 N O3	243.184	1.88				+	
Diethyl 3,4-dihydroxy-2,5-furandicarboxylate	C10 H12 O7	244.059	1.897	+	+		-	
Diethyl 3,4-dihydroxy-2,5-furandicarboxylate	C10 H12 O7	244.059	2.283	+	-			
4-(3-Methoxy-3-oxopropyl)-2,2-dimethyltetrahydro-2H-pyran-4-carboxylic acid	C12 H20 O5	244.129	5.237				-	+/-
6E,8E,12E-Hexadecatrien-10-ynoic acid	C16 H20 O2	244.142	2.661	+				
Leu Ile	C12 H24 N2 O3	244.179	4.219	+	+			
TOLPERISONE	C16 H23 N O	245.181	1.098				+	
1,2,3,4-Cyclopentanetetracarboxylic acid	C9 H10 O8	246.035	0.842	+/-			-	
	C6 H10 N6 O5	246.072	1.588		+			
Mephobarbital	C13 H14 N2 O3	246.101	4.546	+	+			
3,3'-diindolylmethane	C17 H14 N2	246.117	2.991	+	+			
Santonin	C15 H18 O3	246.124	8.272		+			

Oxydi-4,1-butanediyl diacetate	C12 H22 O5	246.146	5.698			-
1,2,3,4-Cyclopentanetetracarboxylic acid	C9 H10 O8	246.04	5.984			-
SEMUSTINE	C10 H18 Cl N3 O2	247.106	1.201	+	+	
Oxomethylphenidate	C14 H17 N O3	247.119	6.736			+
7-DESHYDROXYPYROGALLIN-4-CARBOXYLIC ACID	C12 H8 O6	248.03	3.679	+	+	
Nitrefazole	C10 H8 N4 O4	248.051	1.389	-	-	-
Pyridoxamine Phosphate	C8 H13 N2 O5 P	248.054	1.573			-
Glu Thr	C9 H16 N2 O6	248.103	8.282	+		
6-Hydroxymelatonin	C13 H16 N2 O3	248.116	5.974			-
Zoliprofene	C12 H11 N O3 S	249.05	2.21	+		
Diflunisal	C13 H8 F2 O3	250.045	2.828	+		
2H-1-Benzopyran-6-acetic acid, 7-hydroxy-8-methoxy-2-oxo-	C12 H10 O6	250.046	3.131	+		
2H-1-Benzopyran-6-acetic acid, 7-hydroxy-8-methoxy-2-oxo-	C12 H10 O6	250.046	3.679		+	
Ubiquinone	C14 H18 O4	250.119	6.446			+
3-(1-adamantyl)-2-oxopropyl acetate	C15 H22 O3	250.157	11.714	-		

Muramic acid	C9 H17 N O7	251.1	1.696		+		
Thiacloprid	C10 H9 Cl N4 S	252.027	1.761	+	+/-		
(2,3-Dihydro-1,4-benzodioxin-2-ylmethyl)malonic acid	C12 H12 O6	252.063	4.317			-	
Ala Tyr	C12 H16 N2 O4	252.112	2.475		+		
Cys Met	C8 H16 N2 O3 S2	252.058	9.172			-	-
Pro His	C11 H16 N4 O3	252.121	2.035	+			
Pro His	C11 H16 N4 O3	252.121	3.547	+	+		
Carbantel	C12 H16 Cl N3 O	253.095	1.608	+	+		
4-(2-hydroxy-3-isopropyl-aminopropyl)benzoic acid	C13 H19 N O4	253.131	3.026	+			
ANABASAMINE	C16 H19 N3	253.153	2.689		+		
	C10 H10 N2 S3	254	1.902			+	
methyl 4-[2-(2-formyl-vinyl)-3-hydroxy-5-oxo-cyclopentyl]-butanoate	C13 H18 O5	254.113	7.955			-	-
	C4 H8 N4 O S4	255.958	0.84				+
	C10 H8 O8	256.023	5.387		-		
Clorindione	C15 H9 Cl O2	256.027	0.847	+			
PISCIDIC ACID	C11 H12 O7	256.057	1.689		+		
Timoprazole	C13 H11 N3 O S	257.059	2.142			+	

	C14 H27 N O3	257.2	3.69				+	
Aceglatone	C10 H10 O8	258.034	3.374		-		-	
Bis(ethylthio)methyl-2-methoxyphenol	C12 H18 O2 S2	258.07	1.319				+	
His Cys	C9 H14 N4 O3 S	258.074	3.113	+	+			
Hydroxythiopental	C11 H18 N2 O3 S	258.108	4.712	+				
	C13 H22 O5	258.146	6.76				+/-	-
Glucosamine 6-sulfate	C6 H13 N O8 S	259.038	1.069			+		
L-Glutamic acid dibutyl ester	C13 H25 N O4	259.179	1.213					+
1,4-Dimethyl-3,6-dinitrotetrahydroimidazo[4,5-d]imidazole-2,5(1H,3H)-dione	C6 H8 N6 O6	260.053	1.276	+	+			
penta hydroxystilbene	C14 H12 O5	260.068	8.66		-		-	
Diaveridin	C13 H16 N4 O2	260.124	3.853		+		+/-	
Gamma-Glu-Leu	C11 H20 N2 O5	260.137	1.651	+	+			
	C11 H19 N O6	261.122	2.891		+			
Pinacidil-N-Oxide	C13 H19 N5 O	261.157	2.135	+	+			
Alphaprodine	C16 H23 N O2	261.171	9.81				+	
2,4-DICHLOROPHENOXYBUTYRIC ACID, METHYL ESTER	C11 H12 Cl2 O3	262.013	0.853	+				

D-Mannitol 1-phosphate	C6 H15 O9 P	262.046	5.866	+	-		
4,4'-[1,2-Ethanediylbis(oxy)]bis(4-oxobutanoic acid)	C10 H14 O8	262.069	1.408	+/-	+/-		
Parthenin	C15 H18 O4	262.119	8.714	+			
Phe Pro	C14 H18 N2 O3	262.132	3.525	+			
Miloxacin	C12 H9 N O6	263.041	2.493		+		
	C11 H21 N O6	263.137	2.098		+		
	C7 H21 N9 O2	263.182	4.482			+	
O-Desmethylvenlafaxine	C16 H25 N O2	263.187	9.35			+	
6-Chlorothymolsulfonic acid	C10 H13 Cl O4 S	264.025	1.81	+			
(3-Methoxy-4-hydroxyphenyl)ethylene glycol sulfate	C9 H12 O7 S	264.025	2.217	+	+		
frutinone A	C16 H8 O4	264.045	1.572			-	
Mono-N-hydroxydapsone	C12 H12 N2 O3 S	264.055	2.787		+	-	
Diftalone	C16 H12 N2 O2	264.09	8.229	+	+		
Thialbarbital	C13 H16 N2 O2 S	264.098	10.748			+	+
absinic acid	C15 H20 O4	264.136	7.744			-	
2H-Indol-2-one, 1,3-dihydro-4-[2-hydroxy-3-[(1-	C14 H20 N2 O3	264.148	3.999	+	+		

methylethyl)amino]propoxy]-									
	C6 H3 N O11	264.968	2.605	+					
Mesulfamide	C7 H10 N2 O5 S2	266.006	6.005		-				
	C8 H6 N6 O5	266.041	2.778	+					
typharin	C17 H14 O3	266.095	5.443				+		
Nevirapine	C15 H14 N4 O	266.117	7.088				-		
5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-Pentanoic acid (Gemfibrozil M1)	C15 H22 O4	266.152	9.171	-	-	-	-	-	
Adenosine	C10 H13 N5 O4	267.097	3.219	+					
	C8 H4 N4 O5 S	267.992	0.918				+		
Metoprine	C11 H10 Cl2 N4	268.026	1.923		-				
dimethyl 2-(1,3-benzodioxol-5-yloxy)propanedioate	C12 H12 O7	268.053	7.144		-		-	-	
3-METHYLCHOLANTHRENE	C21 H16	268.129	7.12				+		
Dihydroxymelphalan	C13 H20 N2 O4	268.143	3.578	+					
(1R,2R)-3-oxo-2-pentyl-cyclopentanehexanoic acid	C16 H28 O3	268.204	10.228	+					
Bromamide	C11 H15 Br N2 O	270.037	2.295	+	-				
Phenyl glucuronide	C12 H14 O7	270.073	3.612	+					
Idebenone Metabolite	C13 H18 O6	270.11	4.913				-		

(Benzenebutanoic acid, 2,5-dihydroxy-3,4-dimethoxy-6-methyl-)								
N-Demethylpromethazine	C16 H18 N2 S	270.122	2.443		+			
Dihydroisolysergic acid II	C16 H18 N2 O2	270.138	14.585				-	
	C14 H22 O5	270.146	13.226				-	-
Pirogliride	C16 H22 N4	270.181	10.238	+	+			
	C14 H25 N O4	271.179	3.868				+	
	C12 H8 N4 O4	272.056	2.89	+	-		+	
5-S-Cysteinyldopamine	C11 H16 N2 O4 S	272.085	2.042	+	+			
Sotalol	C12 H20 N2 O3 S	272.117	4.689	+				
	C10 H20 N6 O3	272.16	6.027	+	+		+	+
Amidithion	C7 H16 N O4 P S2	273.031	2.139				+	
CARPROFEN	C15 H12 Cl N O2	273.053	2.225			-		
Furcloprofene	C15 H11 Cl O3	274.035	1.586				-	
SUPROFEN METHYL ESTER	C15 H14 O3 S	274.067	1.831	+	-			
umtatin	C15 H14 O5	274.084	9.062		-			
Diprogulicacid	C12 H18 O7	274.103	2.382	+	+			
Mexafylline	C14 H18 N4 O2	274.142	6.797				-	
Ethenodeoxyadenosine	C12 H13 N5 O3	275.098	3.214		-			

Aditeren	C13 H17 N5 O2	275.137	1.813	+			
1,3,6,7,8-Pentahyroxyxanthen-9-one	C13 H8 O7	276.026	5.538		-		
p-Hydroxytiaprofenic acid	C14 H12 O4 S	276.047	3.11		-	-	-
Ser Asn Gly	C9 H16 N4 O6	276.112	4.388	+	+		
Saccharopine	C11 H20 N2 O6	276.132	1.671	+			
Saccharopine	C11 H20 N2 O6	276.137	9.555	+			
Queuine	C12 H15 N5 O3	277.116	1.17		+		
	C12 H23 N O6	277.153	1.973	+			
	C6 H6 N4 O S4	277.945	0.912	+			
3-Methoxymandelic acid-4-O-sulfate	C9 H10 O8 S	278.006	6.255		-		
Exifone	C13 H10 O7	278.042	6.491		-		
	C10 H14 O9	278.064	1.542				-
ulopterol	C15 H18 O5	278.114	6.871				+
Tyr Pro	C14 H18 N2 O4	278.127	2.366		+		
	C11 H13 N5 O4	279.096	1.121	+	+		
7-Morpholinomethyltheophylline	C12 H17 N5 O3	279.131	1.868	+			
7-Morpholinomethyltheophylline	C12 H17 N5 O3	279.132	1.441	+	+		
	C11 H12 N4 O5	280.08	1.104	-			-

3-[(4-Carboxy-4-methylpentyl)oxy]-4-methylbenzoic acid (Gemfibrozil M3)	C15 H20 O5	280.131	8.684	+	-			
N-HISTIDYL-2-AMINONAPHTHALENE (betaNA)	C16 H16 N4 O	280.132	7.675	+				
Tyr Val	C14 H20 N2 O4	280.142	2.617	+				
	C13 H3 N3 O5	281.007	0.85	+				
	C7 H6 O10 S	281.97	14.76			-		
	C16 H26 O4	282.184	11.546	+		+		
	C16 H5 N5 O	283.051	6.827				+	+
	C16 H33 N3 O	283.259	13.697			-		
Rhein	C15 H8 O6	284.035	0.87				-	-
Tiopinac	C16 H12 O3 S	284.052	1.775	+	+/-			
6-Formylindolo [3,2-B] carbazole	C19 H12 N2 O	284.09	5.167				-	
His Glu	C11 H16 N4 O5	284.117	4.837	+				
His Glu	C11 H16 N4 O5	284.118	6.458	+				
Zomebazam	C15 H16 N4 O2	284.127	7.677	+				
6,7-Dimethyltetrahydropterin	C15 H19 N5 O	285.157	7.169				-	
Pro Leu Gly	C13 H23 N3 O4	285.169	2.262		+			

Pyrocatechol glucuronide	C12 H14 O8	286.067	1.943	+	+			
	C12 H6 N4 O5	286.034	2.273		-		-	
5-(3,4-Dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin	C15 H14 N2 O4	286.091	1.311				-	
Gly Asn Pro	C11 H18 N4 O5	286.132	4.66	+				
Difenoxuron	C16 H18 N2 O3	286.133	12.926				-	
Hexadecanedioic acid	C16 H30 O4	286.214	12.813	-				-
	C14 H25 N O5	287.174	6.324				+	
Leu Gly Val	C13 H25 N3 O4	287.185	3.282	+				
Leu Gly Val	C13 H25 N3 O4	287.185	2.38	+	+			
C17 Sphinganine	C17 H37 N O2	287.283	8.789	+	+	+	+	+
3-[[2-(3,4-dihydroxy-5-oxo-2H-furan-2-yl)-2-hydroxy-ethoxy]methyl]tetrahydrofuran-2,5-dione	C11 H12 O9	288.049	1.505	+	+/-		-	
dihydrokaempferol	C15 H12 O6	288.064	7.253				-	
	C8 H12 N6 O6	288.081	1.999		-			
Soterenol	C12 H20 N2 O4 S	288.111	4.327		+			
Arg Gly Gly	C10 H20 N6 O4	288.154	5.541	+			+	
17-Epiestriol	C18 H24 O3	288.169	3.807		+			

4,12-dihydroxy-hexadecanoic acid	C16 H32 O4	288.23	9.186				-	
	C13 H6 O8	290.005	5.542		-			
Ser Gln Gly	C10 H18 N4 O6	290.127	4.705		+			
clavirin I	C17 H22 O4	290.148	1.796		+			
	C13 H8 O8	292.022	5.075		-			
Tiamiprine	C9 H8 N8 O2 S	292.046	1.562				-	
edetate	C10 H16 N2 O8	292.092	5.382	+				
PICROTOXININ	C15 H16 O6	292.092	4.9	+				
His His	C12 H16 N6 O3	292.129	9.518				-	
N-Acetylmuramic acid	C11 H19 N O8	293.112	1.524	+	+			
B-Octylglucoside	C14 H28 O6	292.185	4.076				+	+
Ondansetron	C18 H19 N3 O	293.147	2.305	+	+			
	C14 H2 N2 O6	293.992	5.885		-			
Pyrifenox	C14 H12 Cl2 N2 O	294.03	1.319				+/-	
2-Butanone, 4-[6-(sulfooxy)-2-naphthalenyl]-	C14 H14 O5 S	294.056	1.376	-			-	
Sulfacytine	C12 H14 N4 O3 S	294.074	4.569	+				
(2-Chlorophenyl)(4-hydroxyphenyl)phenylmethane	C19 H15 Cl O	294.078	1.095				-	

	C17 H18 N4 O	294.148	9.555	+				
gingerol	C17 H26 O4	294.179	6.807	+			-	-
12-oxo-9-octadecynoic acid	C18 H30 O3	294.219	8.786	+				
Indican	C14 H17 N O6	295.106	4.974	+	+		-	
	C12 H8 O9	296.017	5.155		-			
Disulfiram	C10 H20 N2 S4	296.051	2.075	+	+			
flunixin	C14 H11 F3 N2 O2	296.075	1.557				-	
	C9 H7 N5 O S3	296.981	0.833	+	+			
5'-Methylthioadenosine	C11 H15 N5 O3 S	297.086	2.645		+			
	C11 H6 O10	297.994	6.001		-			
	C12 H10 O9	298.031	3.668		-			
	C9 H10 N6 O6	298.067	3.504	+	+			
SAPPANONE A 7-METHYL ETHER	C17 H14 O5	298.088	1.358	-		-		
Metochlopramide	C14 H22 Cl N3 O2	299.137	2.247		+			
Norethindrone	C20 H26 O2	298.19	1.964		+			
	C16 H29 N O4	299.21	4.32				+	
	C12 H4 N4 O6	300.013	0.903				-	-
L-Homocysteine sulfonic acid	C8 H16 N2 O6 S2	300.048	3.512		-			
Tazobactam	C10 H12 N4 O5 S	300.051	2.714	+	+			

5,8,11-Eicosatriynoic acid	C20 H28 O2	300.213	4.027					+	
	C17 H36 N2 O2	300.278	9.06		+				
TEGASEROD	C16 H23 N5 O	301.186	2.919	+	+			+/-	+
Val Ala Leu	C14 H27 N3 O4	301.2	3.898		+				
quercetin	C15 H10 O7	302.043	1.584					-	
2,3-Dihydroxyphenyl ?-D-glucopyranosiduronic acid	C12 H14 O9	302.063	3.243	-	-	+		+	
Cambendazole	C14 H14 N4 O2 S	302.086	1.299					-	
MUCRONULATOL((+/-))	C17 H18 O5	302.114	8.686	+					
benzeneacetic acid, ?-phenyl-, 4-methylphenyl ester	C21 H18 O2	302.127	4.404	+	+				
benzeneacetic acid, ?-phenyl-, 4-methylphenyl ester	C21 H18 O2	302.137	7.676	+					
6-Ketoestriol	C18 H22 O4	302.152	6.937					-	
Formestane	C19 H26 O3	302.185	4.545		+				
Chlorambucil	C14 H19 Cl2 N O2	303.078	2.498		+				
Thr Ile Ala	C13 H25 N3 O5	303.177	3.012	+					
Val Ser Val	C13 H25 N3 O5	303.179	2.647	+	+				
taxifolin	C15 H12 O7	304.06	1.149					-	
Acetylhelenalin	C17 H20 O5	304.13	9.082	+	+				

Acetylhelenalin	C17 H20 O5	304.13	9.454	+			
Enpiprazole	C16 H21 Cl N4	304.151	4.211	+			
2',3'-Cyclic UMP	C9 H11 N2 O8 P	306.03	0.922				-
	C11 H14 O10	306.057	2.414	-	-	-	-
gallocatechin	C15 H14 O7	306.077	6.142		-		
Inproquone	C16 H22 N2 O4	306.162	3.058	+	+		
Anitrazafen	C18 H17 N3 O2	307.127	1.644	+	+		
Phe Ala Ala	C15 H21 N3 O4	307.153	3.747		+		
	C13 H8 O9	308.018	5.258		-		
	C14 H4 N4 O5	308.018	4.09		-		
2,2'-[Methylenebis(oxy)]bis(ethylmalonic acid	C11 H16 O10	308.075	1.668		-		
4-Methyl-2-oxo-2H-chromen-7-yl ?-D-xylopyranoside	C15 H16 O7	308.093	6.265		-		
3beta-CHLOROANDROSTANONE	C19 H29 Cl O	308.197	10.209	+			
Pro His Gly	C13 H19 N5 O4	309.139	3.485	+			
Ribulose 1,5-diphosphate	C5 H12 O11 P2	309.989	5.962		-		
Brophebarbital	C12 H11 Br N2 O3	309.997	6.266		-		
Diflubenzuron	C14 H9 Cl F2 N2 O2	310.03	3.239		-		

1-O-Acetyl-2,3-O-isopropylidene-5-O-(methylsulfonyl)-?-D-ribofuranose	C11 H18 O8 S	310.072	4.588	+		-	
PICROTIN	C15 H18 O7	310.104	6.839	+			
Trepibutone	C16 H22 O6	310.138	6.523	+	+		
methyl 8-[2-(2-formyl-vinyl)-3-hydroxy-5-oxo-cyclopentyl]-octanoate	C17 H26 O5	310.179	11.714				-
Mestranol	C21 H26 O2	310.189	3.309	+			
16-hydroperoxy-9Z,12,14E-octadecatrienoic acid	C18 H30 O4	310.214	10.969			-	-
Sulfaclomid	C12 H13 Cl N4 O2 S	312.05	2.678		-	-	
	C14 H16 O8	312.084	4.317			-	
8,13-dihydroxy-9,11-octadecadienoic acid	C18 H32 O4	312.23	11.921			-	-
Flubendazole	C16 H12 F N3 O3	313.085	2.936		+		
Norcisapride	C14 H20 Cl N3 O3	313.117	2.482	+	+		
Val Pro Val	C15 H27 N3 O4	313.2	3.499	+			
Salicyl phenolic glucuronide	C13 H14 O9	314.063	4.21		-		
eucomnalin	C17 H14 O6	314.085	12.863	-			-
chicoric acid	C22 H18 O2	314.126	5.985			-	

	C16 H29 N O5	315.205	3.522				+
Leu Leu Ala	C15 H29 N3 O4	315.211	3.873	+	+		
Pazoxide	C12 H10 Cl2 N2 O2 S	315.986	0.923			-	-
Bromoallylmethylbutylbarbituric acid (Sigmodal)	C12 H17 Br N2 O3	316.047	2.467		-		
	C15 H12 N2 O6	316.071	10.273			-	-
	C9 H12 N6 O7	316.077	3.046	+			
Bornyl bromoisovalerate	C15 H25 Br O2	316.107	5.026	+	+		
	C11 H12 N10 O2	316.113	3.999	+	+		
	C11 H12 N10 O2	316.114	3.853	+			
EPIAFZELECHIN TRIMETHYL ETHER	C18 H20 O5	316.129	9.556	+			
Timolol	C13 H24 N4 O3 S	316.152	8.793			-	
Ala Lys Val	C14 H28 N4 O4	316.209	3.774			+	
Glu Val Ala	C13 H23 N3 O6	317.159	2.693		+		
Leu Trp	C17 H23 N3 O3	317.173	2.7	+			
	C17 H35 N O4	317.257	4.782		+		
	C10 H6 O12	317.987	5.911		-		
myricetin	C15 H10 O8	318.038	1.171		+		
1-(2,3-Dihydro-1,4-benzodioxin-	C16 H14 O5 S	318.054	6.061		-	+/-	

6-yl)-2-(phenylsulfonyl)ethanone							
PHENOLPHTHALEIN	C20 H14 O4	318.093	2.134	+			
Gly Trp Gly	C15 H18 N4 O4	318.13	4.027	+	+		
Zearalenone	C18 H22 O5	318.143	1.941		+		
	C14 H25 N O7	319.163	7.673	+			
Asn Ser Thr	C11 H20 N4 O7	320.131	7.793	+			
1-({4-[(Cyclopropylmethyl)amino]-6-(isopropylamino)-1,3,5-triazin-2-yl}oxy)-2,5-pyrrolidinedione	C14 H20 N6 O3	320.158	2.815		+		
	C13 H6 O10	321.998	4.589		-		
	C19 H2 N2 O2 S	321.981	0.891	+			
Glyhexamide	C16 H22 N2 O3 S	322.137	1.643		+		
Sterigmatocystin	C18 H12 O6	324.067	1.37	-			
	C24 H2 O2	322.004	0.925			-	-
Methoxy-pyridinyl-suIfaniI,1-acetamide	C13 H14 N4 O4 S	322.068	4.048	+			
	C13 H9 N9 O2	323.087	1.496	+			
Ala Tyr Ala	C15 H21 N3 O5	323.147	2.64		+		
4-Hydroxyalprazolam	C17 H13 Cl N4 O	324.076	1.301			-	
acetohexamide	C15 H20 N2 O4 S	324.12	7.676	+		-	

Dihydroxycarteolol M1	C16 H24 N2 O5	324.168	3.332	+				
Dihydroxycarteolol M1	C16 H24 N2 O5	324.169	2.732	+				
Dihydroxycarteolol M1	C16 H24 N2 O5	324.169	3.497	+				
Dihydroxycarteolol M1	C16 H24 N2 O5	324.169	3.853	+	+			
quinine	C20 H24 N2 O2	324.187	7.816	+				
	C18 H2 N2 O5	325.998	1.322				-	
	C16 H6 O8	326.007	7.479		-			
	C19 H6 N2 O4	326.031	2.217		-			
Naphtho[2",3":4',5']imidazo[2',1':2,3][1,3]thiazolo[4,5-b]quinoxaline	C19 H10 N4 S	326.062	3.181		-			
CUNEATIN METHYL ETHER	C18 H14 O6	326.085	1.352	-				
tetranor-PGEM	C16 H22 O7	326.136	8.498		-		-	
2R-hydroperoxy-9Z,12Z,15Z-octadecatrienoic acid	C18 H30 O5	326.208	11.515				-	-
Leu Pro Val	C16 H29 N3 O4	327.216	4.187	+	+			
	C18 H33 N O4	327.241	11.555	+	+	+	+	+
	C12 H8 O11	328.007	5.156		-			
	C14 H8 N4 O6	328.045	2.11	+				
9,15-dioxo-11R-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioic acid	C16 H24 O7	328.15	4.995		+			

9,12,13-trihydroxy-10,15-octadecadienoic acid	C18 H32 O5	328.224	8.437				-
His Ala Cys	C12 H19 N5 O4 S	329.112	4.311	+	+		
Thr Ile Pro	C15 H27 N3 O5	329.195	3.155	+	+		
Ile Val Val	C16 H31 N3 O4	329.232	4.219	+	+		
Furosemide	C12 H11 Cl N2 O5 S	330.001	7.628		-		
3',5'-Cyclic Inosine monophosphate (cIMP)	C10 H11 N4 O7 P	330.038	8.314			-	
9,12,13-trihydroxy-10-octadecenoic acid	C18 H34 O5	330.24	8.824			-	
	C13 H6 N4 O7	330.025	1.617		-		
9,12,13-trihydroxy-10-octadecenoic acid	C18 H34 O5	330.241	8.796		-	-	
D-Ribose 1-phosphate	C5 H11 O8 P	230.018	3.315		+		
Nitrosonifedipine	C17 H18 N2 O5	330.123	5.019	+	+	-	
9,12,13-trihydroxy-10-octadecenoic acid	C18 H34 O5	330.239	9.3				-
9,12,13-trihydroxy-10-octadecenoic acid	C18 H34 O5	330.241	8.824				-
MY-5445	C20 H14 Cl N3	331.091	3.754		+		
Val Val Asp	C14 H25 N3 O6	331.175	2.806		+		

Leu Thr Val	C15 H29 N3 O5	331.213	9.987	+	+	
	C18 H8 N2 O5	332.046	1.872		-	
2-[3-(Dimethylamino)-2,4,6-trinitrophenyl]-1-hydroxy-2-methylhydrazine 1-oxide	C9 H12 N6 O8	332.072	2.132	+	-	
3-(2-Methyl-3-furyl)-6-(2-naphthyl)[1,2,4]triazolo[3,4-b][1,3,4]thiadiazole	C18 H12 N4 O S	332.074	1.94	+/-	-	
3,5-Pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-, monomethyl ester	C16 H16 N2 O6	332.102	4.64	+	+	
3,5-Pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-, monomethyl ester	C16 H16 N2 O6	332.102	9.556	+		
3,5-Pyridinedicarboxylic acid, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-, monomethyl ester	C16 H16 N2 O6	332.105	2.985	+		
	C10 H16 N6 O7	332.108	2.291	+	+	
combretastastin A-1	C18 H20 O6	332.125	6.94	+		
Spectinomycin	C14 H24 N2 O7	332.156	1.944	+		
Trp Lys	C17 H24 N4 O3	332.184	6.241	+	+	-
hautriwaic acid	C20 H28 O4	332.197	11.764	+		
Ciglitazone	C18 H23 N O3 S	333.144	5.342	+		

Phe Pro Ala	C17 H23 N3 O4	333.169	3.932	+	+		
CARBETAPENTANE	C20 H31 N O3	333.228	8.41				+
Lormetazepam	C16 H12 Cl2 N2 O2	334.023	1.166				+
nicotinamide mononucleotide	C11 H15 N2 O8 P	334.056	1.278		-		
combretastastin	C18 H22 O6	334.14	7.274	+			
Ala Val Phe	C17 H25 N3 O4	335.184	4.582	+	+		
Gangletene	C20 H33 N O3	335.244	11.412	+			+
	C11 H8 N6 O7	336.047	3.75		+		
(7-Phenoxy-2,3-dihydro-1,4-benzodioxin-2-yl)methyl methanesulfonate	C16 H16 O6 S	336.071	1.225		-		-
CYCLOPIAZONIC ACID	C20 H20 N2 O3	336.15	2.969	+	+		
Nuvenzepine	C19 H20 N4 O2	336.155	10.518				-
Platyphylline	C18 H27 N O5	337.187	3.523				+
ellagic acid dihydrate	C14 H10 O10	338.03	1.883		+/-		
1-(5'-Phosphoribosyl)-5-amino-4-imidazolecarboxamide (AICAR)	C9 H15 N4 O8 P	338.065	5.195				-
	C13 H10 N10 O2	338.099	3.294	+	+		
Moxnidazole	C13 H18 N6 O5	338.135	6.498	+			
Diethylpropion(metabolite X-glucuronide)	C17 H23 N8	339.205	9.556	+			-

Deloslamide	C9 H20 Cl3 N2 O3 P	340.029	1.313				+
AESCULIN	C15 H16 O9	340.081	4.215	+	+		
Diethylpropion(metabolite VIII-glucuronide)	C15 H19 N O8	341.112	2.447	+	+		
His Trp	C17 H19 N5 O3	341.148	5.126	+			
Leu Leu Pro	C17 H31 N3 O4	341.232	4.893	+			
Leu Leu Pro	C17 H31 N3 O4	341.232	4.727	+	+		
	C14 H6 N4 O7	342.026	2.043		-		
	C14 H14 O10	342.058	3.309	+			
Coumachlor	C19 H15 Cl O4	342.059	3.822	+			
ferulic acid dehydrodimer	C19 H18 O6	342.116	1.066	-		-	-
Coniferin	C16 H22 O8	342.132	6.065	+			
Acitretin Ro 23-4750	C21 H26 O4	342.179	3.292	+	+		
Acitretin Ro 23-4750	C21 H26 O4	342.18	3.737	+			
2,3-Dinor-TXB2	C18 H30 O6	342.204	6.962			-	-
b-D-Glucopyranosiduronic acid	C15 H21 N O8	343.127	4.162	+	+		
b-D-Glucopyranosiduronic acid	C15 H21 N O8	343.129	1.776	+			
Pro Val Glu	C15 H25 N3 O6	343.175	2.67	+	+		
Val Ile Leu	C17 H33 N3 O4	343.247	4.843	+			
Val Ile Leu	C17 H33 N3 O4	343.247	4.629	+	+		

	C15 H4 O10	343.982	0.899			+	
METHOXYCHLOR	C16 H15 Cl3 O2	344.016	1.401		+/-		
	C14 H8 N4 O7	344.039	4.675		-		
	C19 H8 N2 O5	344.044	0.838		-		
trimethyl ellagic acid	C17 H12 O8	344.054	9.825	+/-	-	-	
	C16 H8 N8 O2	344.077	3.7	+	-		
8-Epiiridotrial glucoside	C16 H24 O8	344.146	3.85	+	+	-	
16-Hydroxy-4-carboxyretinoic acid	C20 H24 O5	344.161	9.295	+			
Glu Val Val	C15 H27 N3 O6	345.189	1.731	+	+		
Coroxon	C14 H16 Cl O6 P	346.032	1.166		+	-	
Nifedipine	C17 H18 N2 O6	346.117	4.732	+			
Deutzioside	C15 H22 O9	346.123	3.711	+	+		
Pro Met Thr	C14 H25 N3 O5 S	347.149	5.701			-	
Indoramin	C22 H25 N3 O	347.196	8.463			-	
	C18 H37 N O5	347.268	8.79	+			+
	C12 H12 O12	348.031	2.63		-		
18-bromo-17E-octadecene	C18 H21 Br O2	348.066	3.336		-	-	
1-Naphthoic acid glucuronide	C17 H16 O8	348.083	4.161	+	+		
Diethylstilbestrol monosulfate	C18 H20 O5 S	348.104	1.805	+	+		

Gibberellin A34	C19 H24 O6	348.155	9.171	-		-		-
Ser Ser Arg	C12 H24 N6 O6	348.177	4.5	+	+			
Cetiedil	C20 H31 N O2 S	349.21	6.238	+	+			
strychnine N-oxide	C21 H22 N2 O3	350.169	2.63	+	+			
ballotenol	C20 H30 O5	350.207	9.826	+				+
Chlorpromazine sulfone	C17 H19 Cl N2 O2 S	350.085	1.572		-			
Pirenzepine	C19 H21 N5 O2	351.172	2.251		+			
Halazepam	C17 H12 Cl F3 N2 O	352.062	1.369	-				
Deacetylaloesin	C17 H20 O8	352.115	7.425	+				
	C14 H20 N6 O5	352.15	7.175	+				
	C15 H16 N10 O	352.15	6.847	+				
Thromboxane A2	C20 H32 O5	352.223	8.79	+	+		+/-	
Thr Ser Phe	C16 H23 N3 O6	353.154	2.607		+			
Urefibrate	C15 H12 Cl2 N2 O4	354.023	3.155		-			
	C20 H6 N2 O5	354.027	1.701		-			
5-Amino-6-(5'-phosphoribosylamino)uracil	C9 H15 N4 O9 P	354.058	2.435	+	+			
N-Desmethylketazolam	C19 H15 Cl N2 O3	354.079	1.283				-	
chlorogenic acid	C16 H18 O9	354.093	3.495	+				
Isofazolac	C23 H18 N2 O2	354.13	7.558	+				

Ala His Gln	C14 H22 N6 O5	354.166	6.241	+	+		+	
chebulic acid	C14 H12 O11	356.039	1.301	+/-	+/-	-	-	-
BISSALICYL FUMARATE	C18 H12 O8	356.047	1.888		+			
5-Amino-6-(5'-phosphoribitylamino)uracil	C9 H17 N4 O9 P	356.075	3.694		+/-			
Asn His Ser	C13 H20 N6 O6	356.148	6.735	+				
Etibendazole	C18 H16 F N3 O4	357.107	1.491		+			
Phenylephrine 3-O-glucuronide	C17 H25 O8	357.152	4.35		-			
N-Desmethyltamoxifen	C25 H27 N O	357.216	5.453				+	
Prednisone	C21 H26 O5	358.175	2.619	+				
Leu Ala Arg	C15 H30 N6 O4	358.234	12.849			+	+	
Asn Ile Asn	C14 H25 N5 O6	359.174	2.618		+			
	C10 H8 N4 O3 S4	359.949	0.916	+				
	C16 H8 O10	360.012	6.874		-			
Trisalicyclide	C21 H12 O6	360.062	1.585				-	
lariciresinol	C20 H24 O6	360.156	7.148	+				
Val Ser Arg	C14 H28 N6 O5	360.214	6.335					-
Octocrylene	C24 H27 N O2	361.21	2.833				+	
	C17 H6 N4 O6	362.028	5.308		-			
Proflazepam	C18 H16 Cl F N2 O3	362.082	3.332		-			

procumbide	C15 H22 O10	362.119	3.025	+	+	
Trp Ala Ser	C17 H22 N4 O5	362.155	4.416	+		
secoisolariciresinol	C20 H26 O6	362.173	8.155			-
18-bromo-octadecanoic acid	C18 H35 Br O2	362.184	8.929			-
20alpha-Dihydroprednisolone	C21 H30 O5	362.208	12.501	+		
	C11 H8 O14	363.991	0.917			-
Gibberellin A44 diacid	C20 H28 O6	364.185	2.272	+	+	
AM-2201-d5	C24 H17 D5 F N O	364.201	9.953			-
His Pro Asn	C15 H22 N6 O5	366.165	1.654	+	+	
Leu Cys Met	C14 H27 N3 O4 S2	365.148	6.94			-
Tifencillin	C16 H18 N2 O4 S2	366.071	1.578			-
	C16 H8 N4 O7	368.039	4.009		-	
2-Hydroxy-N'-(3-nitrobenzoyl)-4-oxo-1,4-dihydro-3-quinolinecarbohydrazide	C17 H12 N4 O6	368.075	5.403		-	-
Penicilloic acid V	C16 H20 N2 O6 S	368.108	1.994	+/-	+	
Tricresylphosphate	C21 H21 O4 P	368.113	1.075	-		
Tricresylphosphate	C21 H21 O4 P	368.114	7.468		-	
Fludoxopone	C21 H21 F N2 O3	368.147	6.465	+		-
Gly His Arg	C14 H24 N8 O4	368.193	8.798	+		+

Arg Pro Pro	C16 H28 N6 O4	368.218	7.519				+
	C15 H6 N4 O8	370.02	2.858		-		
	C17 H10 N2 O8	370.043	3.29		-		
	C15 H14 O11	370.053	3.688		-		
	C16 H10 N4 O7	370.056	2.346		-		
REBAMIPIDE	C19 H15 Cl N2 O4	370.074	1.616			-	
	C23 H18 N2 O3	370.131	6.25		+		
ESTRONE HEMISUCCINATE	C22 H26 O5	370.179	11.771		+		
Remoxipride	C16 H23 Br N2 O3	370.087	2.775	+			
Remoxipride	C16 H23 Br N2 O3	370.088	3.079	+	+		
Gln Ser His	C14 H22 N6 O6	370.16	5.119	+	+		
Ropizine	C24 H26 N4	370.221	3.544		+		
Tamoxifen	C26 H29 N O	371.231	6.775			+	
(2Z)-4-(2-hydroxyethoxy)-4-oxo-2-butenoic acid	C20 H12 N4 O2 S	372.071	3.348	+	+/-		
9-(5-O-Benzoylpentofuranosyl)-3,9-dihydro-6H-purin-6-one	C17 H16 N4 O6	372.107	5.594		+/-		
tangeritin	C20 H20 O7	372.119	5.712	+			
Trp Pro Ala	C19 H24 N4 O4	372.176	4.812	+			
Trp Pro Ala	C19 H24 N4 O4	372.176	4.663	+	+		

Ile Gln Asn	C15 H27 N5 O6	373.194	3.223	+	+		
Glu Ile Leu	C17 H31 N3 O6	373.221	4.288	+	+		
diospyrin	C22 H14 O6	374.08	2.415		-		
ESTRONE BENZOATE	C25 H26 O3	374.184	4.717	+			
Pro Leu Phe	C20 H29 N3 O4	375.216	5.469	+	+		
	C18 H4 N2 O8	375.999	0.867			-	-
Flutazolam	C19 H18 Cl F N2 O3	376.101	2.201	+			
Flutazolam	C19 H18 Cl F N2 O3	376.101	3.15	+	+		
Mesudipine	C19 H24 N2 O4 S	376.15	7.482	+			
Mesudipine	C19 H24 N2 O4 S	376.151	7.722	+			
Gln Lys Cys	C14 H27 N5 O5 S	377.169	6.071	+	+		
Val Leu Phe	C20 H31 N3 O4	377.231	5.389	+			
Famprofazone	C24 H31 N3 O	377.241	6.244	+	+		
	C16 H10 O11	378.022	2.269	+	+		
carbenicillin	C17 H18 N2 O6 S	378.094	5.105	+			
cnicin	C20 H26 O7	378.164	2.118	+	+		
Arg Gly Phe	C17 H26 N6 O4	378.2	3.823	+	+		
Doxapram	C24 H30 N2 O2	378.225	5.508			-	-
	C20 H8 N6 O3	380.065	1.34			-	

	C13 H12 N6 O8	380.072	1.061	+				
O-Acetylserine	C13 H21 N2 O7 P S	380.086	4.214	+	+			
Cys Met Gln	C13 H24 N4 O5 S2	380.121	4.318		+			
Gln His Pro	C16 H24 N6 O5	380.18	1.724		+			
Normeperidinic acid glucuronide	C18 H23 N O8	381.141	5.126	+				
Asp Thr Phe	C17 H23 N3 O7	381.154	2.65		+			
	C20 H39 N5 O2	381.31	12.853		+	+	+	
Olsalazine sulfate	C14 H10 N2 O9 S	382.016	0.911				-	-
Nerolidyl diphosphate	C15 H28 O7 P2	382.125	4.068	+	+			
Nerolidyl diphosphate	C15 H28 O7 P2	382.125	5.798	+				
Nerolidyl diphosphate	C15 H28 O7 P2	382.126	6.071	+				
Alpiropride	C17 H26 N4 O4 S	382.166	7.414	+				
	C12 H12 N6 O9	384.066	2.984	+	+/-			
	C29 H8 N2	384.069	3.529		-			
	C16 H16 O11	384.069	4.721		-			
Asn Asp His	C14 H20 N6 O7	384.142	6.8	+	+			
19-hydroxy-17-oxoandrost-5-en-3-beta-yl sulfate	C19 H28 O6 S	384.159	9.673	+			+	
JWH-398-d9	C24 H13 D9 Cl N O	384.196	8.426	+				
Pro Arg Ile	C17 H32 N6 O4	384.25	1.956	+	+			

Thioridazine 5-sulfoxide	C21 H26 N2 O S2	386.156	4.368	+	+		-
Clonitazene	C20 H23 Cl N4 O2	386.157	6.448	+			
Ile Arg Val	C17 H34 N6 O4	386.263	2.59		+		
Lys Gln Ile	C17 H33 N5 O5	387.247	4.731	+			
pamoate	C23 H16 O6	388.095	2.574	+			
pamoate	C23 H16 O6	388.096	2.883	+			
pamoate	C23 H16 O6	388.098	2.726	+			
Secologanin	C17 H24 O10	388.136	5.33		+		
Gln Asn Gln	C14 H24 N6 O7	388.172	4.214	+	+		
Trp Ser Pro	C19 H24 N4 O5	388.172	3.419	+			-
HETACILLIN	C19 H23 N3 O4 S	389.145	5.712	+	+		
	C17 H31 N11	389.277	7.717				-
resveratrol glucoside	C20 H22 O8	390.133	6.924				-
Ser Arg Glu	C14 H26 N6 O7	390.186	4.178	+			
20-trifluoro-LTB4	C20 H29 F3 O4	390.2	2.645	+			
	C14 H13 N7 O7	391.089	3.018	+	+		
	C19 H12 N4 O6	392.076	2.338	+			-
	C17 H12 N8 O4	392.098	1.919		-		
Ile Asn Phe	C19 H28 N4 O5	392.205	4.733	+	+/-		-

Ala Phe Arg	C18 H28 N6 O4	392.215	3.467	+	+	
Ala Phe Arg	C18 H28 N6 O4	392.216	4.542	+		
Phe Met Pro	C19 H27 N3 O4 S	393.173	2.666		+	
Phe Leu Asp	C19 H27 N3 O6	393.191	5.089		+	
Tyr Val Ile	C20 H31 N3 O5	393.224	4.268		+	
Diflufenican	C19 H11 F5 N2 O2	394.071	1.575			-
	C18 H18 O10	394.09	5.185	+	+	
	C8 H26 N8 S5	394.088	2.197		+	
	C11 H22 O15	394.095	5.719	+		
DEGUELIN(-)	C23 H22 O6	394.14	10.531			+
ROBUSTIC ACID METHYL ETHER	C23 H22 O6	394.144	4.031	+	+	
Mesuprine	C19 H26 N2 O5 S	394.163	1.699		+	
Tyr Gly Arg	C17 H26 N6 O5	394.197	6.365	+	+	
	C18 H12 N4 O7	396.07	2.548		-	
4-Hydroxylevamisole Glucuronide	C17 H20 N2 O7 S	396.102	3.812	+	+	-
Glibornuride M2 (p-carboxyglibornuride)	C18 H24 N2 O6 S	396.141	6.559	+		
Glibornuride M2 (p-carboxyglibornuride)	C18 H24 N2 O6 S	396.141	6.731	+		

Phe Gln Cys	C17 H24 N4 O5 S	396.143	6.407	+	+		
Phe Gln Cys	C17 H24 N4 O5 S	396.144	7.5	+			
Lys Phe Cys	C18 H28 N4 O4 S	396.177	7.674	+	+		
Lys His Ile	C18 H32 N6 O4	396.249	12.717			-	-
	C10 H10 N2 O13 S	397.988	0.949			-	
Clefamide	C17 H16 Cl2 N2 O5	398.048	3.206		-		
4-Dedimethyl-6-dehydro-anhydrotetracycline	C20 H18 N2 O7	398.119	2.099	+	+		
mitragynine	C23 H30 N2 O4	398.228	3.727		+		
	C19 H34 N4 O5	398.253	4.867		+		
	C18 H8 N8 O4	400.066	3.944	+	-		
N-AcetylCilastatin	C18 H28 N2 O6 S	400.171	5.113	+	+		
Enprostil	C23 H28 O6	400.189	9.336	+	+	+	
Ophiobolin A	C25 H36 O4	400.269	4.613		+		
nobiletin	C21 H22 O8	402.137	1.066	+/-	+		
Alepride	C22 H30 Cl N3 O2	403.196	2.108		+		
Zopiclone N-oxide	C17 H17 Cl N6 O4	404.096	2.532	+	+		
Mepitiostane	C25 H40 O2 S	404.276	8.153			-	
Gln Met Lys	C16 H31 N5 O5 S	405.2	7.404	+			
b-D-Glucopyranosiduronic acid,	C21 H26 O8	406.16	5.661	+			

3-(6-methoxy-2-naphthalenyl)-1-methylpropyl							
Phe Gln Ile	C20 H30 N4 O5	406.222	5.199	+	+		
Amantocillin	C20 H29 N3 O4 S	407.189	2.164	+			
3-(a-Naphthoxy)lactic acid glucuronide	C19 H20 O10	408.105	5.922	+	+		
Trp Gly Phe	C22 H24 N4 O4	408.175	1.876		+		
	C12 H15 N11 O6	409.122	1.907	+			
cis-3-(6-Hydroxy-7-methoxy-5-benzofuranyl)acrylic acid glucuronide	C18 H18 O11	410.083	6.068			-	
Aloesol	C20 H26 O9	410.156	4.549	+	+		
Aloesol isomer	C20 H26 O9	410.156	4.813	+			
Aloesol	C20 H26 O9	410.157	8.192	+	+		
2H-Indol-2-one, 1,3-dihydro-7-hydroxy-4-[2-(propylamino)ethyl]- glucuronide	C19 H26 N2 O8	410.171	10.185				+
COLFORSIN	C22 H34 O7	410.228	9.895			-	
Quinaprilat	C23 H26 N2 O5	410.187	5.008	+	+		
	C19 H8 N8 O4	412.067	2.408		-		
His Ala Trp	C20 H24 N6 O4	412.185	3.826	+			

Trandolapril lactam (RU 46178)	C24 H32 N2 O4	412.228	3.497	+		-	
GRAYANOTOXIN I	C22 H36 O7	412.243	8.83				-
Talniflumate	C21 H13 F3 N2 O4	414.079	1.906	+	+/-		
Flecainide	C17 H20 F6 N2 O3	414.137	2.855		+		
Phe Leu His	C21 H29 N5 O4	415.221	4.955			-	+
	C13 H16 N6 O10	416.092	1.866	+			
Asp Trp Pro	C20 H24 N4 O6	416.168	4.972			-	
Asn Trp Val	C20 H27 N5 O5	417.205	3.44	+			
Cloxestradiol	C20 H25 Cl3 O3	418.082	6.043			-	
Artelinic acid	C23 H30 O7	418.2	9.336	+			
Netobimin	C14 H20 N4 O7 S2	420.085	3.264		+		
	C16 H20 O13	420.091	2.502	+			
	C16 H20 O13	420.091	2.138	+	+		
	C17 H16 N4 O9	420.092	1.544	+	+		
Diketopiperazine derivative of Quinapril	C25 H28 N2 O4	420.208	4.657	+			
Testosteronephenylpropionate	C28 H36 O3	420.271	7.206			-	
His His Glu	C17 H23 N7 O6	421.163	1.648	+	+		
Phe Gln Gln	C19 H27 N5 O6	421.192	3.729	+	+		
Lys Gln Phe	C20 H31 N5 O5	421.23	4.511		+		

Asp Cys Trp	C18 H22 N4 O6 S	422.121	6.174		-		
Loxoprofen Metabolite (b-D-Glucopyranuronic acid, 1-[a-methyl-4-[(2-oxocyclopentyl)methyl]benzeneace	C21 H26 O9	422.156	4.615	+			
	C22 H18 N10	422.171	12.932	+			
	C15 H8 N2 O13	424.003	0.912			-	
salicortin	C20 H24 O10	424.135	4.87	+			
salicortin	C20 H24 O10	424.137	6.467	+			
Asp Tyr Gln	C18 H24 N4 O8	424.151	9.674	+	+	+	
Chitobiose	C16 H28 N2 O11	424.17	5.589			-	
Diflunisal acyl glucuronide	C19 H16 F2 O9	426.079	2.263	+			
Met Asn Tyr	C18 H26 N4 O6 S	426.152	6.459	+			
N-Desmethylclindamycin sulfoxide	C17 H31 Cl N2 O6 S	426.158	4.107	+	+		
Asn Phe Phe	C22 H26 N4 O5	426.188	4.529	+	+		
Asp His Arg	C16 H26 N8 O6	426.197	5.019	+	+		
	C17 H4 N2 O12	427.976	14.632		-	-	-
2H-1-Benzopyran-2-one, 6-(1,2-dihydroxyethyl)-7-hydroxy-8-methoxy- glucuronide	C18 H20 O12	428.093	1.952	+			

	C18 H16 N14	428.168	5.71		+	
Pro Trp Lys	C22 H31 N5 O4	429.237	6.384			+/-
	C19 H10 N8 O5	430.077	2.023		-	
	C22 H12 N2 O8	432.059	2.474		-	
Estradiol disulfate	C18 H24 O8 S2	432.087	3.318		-	
vitexin	C21 H20 O10	432.106	5.732	+/-	+/-	-
6b,11b,16a,17a,21-Pentahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide	C24 H32 O7	432.213	4.344		+	
Gln Trp Thr	C20 H27 N5 O6	433.195	5.453	+		
Dextrorphan glucuronide	C23 H31 N O7	433.205	3.158	+	+	
	C22 H14 N2 O8	434.074	2.333		-	
flurandrenolide	C24 H33 F O6	436.228	4.95	+		
N-FORMYLMETHIONYL-LEUCYLPHENYLALANINE	C21 H31 N3 O5 S	437.199	1.858		+	
	C26 H39 N5 O	437.315	7.549			+
	C32 H10 N2 O	438.079	1.573			-
Losartan Metabolite (1H-Imidazole-2-propanol, 4-chloro-5-(hydroxymethyl)-a-methyl-1-[[2'-(1H-tetrazo	C22 H23 Cl N6 O2	438.15	4.982	+	+	

Losartan Metabolite (1H-Imidazole-2-propanol, 4-chloro-5-(hydroxymethyl)-a-methyl-1-[[2'-(1H-tetrazo	C22 H23 Cl N6 O2	438.151	6.91	+			
Tyr Gln Glu	C19 H26 N4 O8	438.172	1.437		+		
Pro His Trp	C22 H26 N6 O4	438.201	3.717		+		
Arg Tyr Cys	C18 H28 N6 O5 S	440.182	9.688				+
Pro His Trp	C22 H26 N6 O4	438.201	3.614	+			
Met Gln Tyr	C19 H28 N4 O6 S	440.167	4.547		+		
Arg Tyr Cys	C18 H28 N6 O5 S	440.182	9.677	+	+	+	
1alpha,25-dihydroxy-26,27-dimethyl-22,22,23,23-tetradehydrovitamin D3/1alpha,25-dihydroxy-26,27-di	C29 H44 O3	440.326	3.554			+	
	C15 H10 N10 O7	442.074	2.277	+			
12-Dehydrotetracycline	C22 H22 N2 O8	442.146	4.079	+			
Citrodisalyl	C21 H16 O11	444.063	3.585		-		
	C15 H12 N10 O7	444.088	2.014	+	+/-		
Arg Asp Arg	C16 H31 N9 O6	445.247	12.932	+			
Glucosulfamide	C13 H22 N2 O11 S2	446.072	2.613		-		
Estrone 3-glucuronide	C24 H30 O8	446.195	9.823			-	

Asp Lys Trp	C21 H29 N5 O6	447.211	5.512		+		-	
Trp Gly Trp	C24 H25 N5 O4	447.195	2.653		+			
	C18 H16 N4 O10	448.086	2.378		-			
luteolin	C21 H20 O11	448.101	5.249	+	+/-			
	C21 H31 N5 O6	449.227	4.606	+			-	
Arg Phe Gln	C20 H31 N7 O5	449.236	3.574	+	+			
Asp Trp Met	C20 H26 N4 O6 S	450.157	13.964				-	-
Bentipimine	C27 H31 Cl N2 S	450.181	11.885			-		-
Arg Tyr Asn	C19 H29 N7 O6	451.219	6.384		+		+	
Met Phe Arg	C20 H32 N6 O4 S	452.224	4.446		+			
SAICAR	C13 H19 N4 O12 P	454.083	4.439		-			
resveratrol dihydro dimer	C28 H22 O6	454.142	4.545		+			
halcinonide	C24 H32 Cl F O5	454.195	5.222	+	+			
Octimibate	C29 H30 N2 O3	454.217	5.886		+			
10-Hydroxyprotriptyline glucuronide	C25 H29 N O7	455.199	1.884	+				
OBACUNOL	C26 H32 O7	456.211	2.767	+	+			
	C32 H27 N O2	457.203	5.663	+				
	C20 H10 O13	458.012	9.868		-			
	C19 H14 N4 O10	458.071	3.144		-			

	C31 H12 N2 O3	460.085	3.711		-		
Nisterime	C25 H33 Cl N2 O4	460.221	9.674	+			
trans-1,4-bis(2-Chlorobenzaminomethyl)cyclohexane dihydrochloride	C22 H30 Cl4 N2	462.123	1.375	-			
	C23 H29 N9 O2	463.245	5.171	+	+		
	C14 H12 N2 O16	464.019	0.911			-	-
	C22 H8 N8 O5	464.061	1.77		+		
isoquercitrin	C21 H20 O12	464.095	4.943		+		
	C23 H32 N2 O8	464.216	5.96			-	
Biotripyrrin-b	C25 H27 N3 O6	465.187	1.68		+		
	C33 H27 N3	465.221	3.033	+			
	C19 H27 N15	465.258	6.883		+		
	C22 H10 N8 O5	466.077	4.276	+			
	C22 H10 N8 O5	466.077	4.106	+			
Asp Trp Phe	C24 H26 N4 O6	466.182	4.074	+			
Phe Trp Asp	C24 H26 N4 O6	466.184	7.286	+			
a-Denopterin	C21 H24 N8 O5	468.184	3.68	+			
1alpha,25-dihydroxy-26,27-dimethyl-22,22,23,23-tetradehydro-24a,24b-	C31 H48 O3	468.358	3.825			+	

dihomovitamin D3/1alpha,25-dih							
His Trp Gln	C22 H27 N7 O5	469.206	3.717		+		
	C29 H18 N4 O3	470.138	3.407	+	-		
	C19 H22 N10 O5	470.177	4.759	+	+		
	C20 H26 N10 O4	470.213	6.883	+	+		
Gln Tyr Tyr	C23 H28 N4 O7	472.193	5.681	+	+		
Sulfochenodeoxycholic acid	C24 H40 O7 S	472.259	11.872				-
	C24 H17 N3 O8	475.102	1.49		-		
	C14 H20 O18	476.064	1.306			-	
	C19 H16 N4 O11	476.082	3.802		-		
	C25 H12 N6 O5	476.086	2.39		-		
	C23 H24 O11	476.132	7.477		-		
betamethasone valerate	C27 H37 F O6	476.253	9.685		+	+	
	C18 H31 N13 O3	477.268	4.943		+		
Chlortetracycline	C22 H23 Cl N2 O8	478.112	5.362		-		
1-O-Desmethyltetrabenazine glucuronide	C24 H33 N O9	479.213	2.64		+		
	C34 H29 N3	479.238	3.807		+		
	C17 H8 N2 O15	479.992	0.923			-	-
	C19 H32 N10 O5	480.255	5.132	+			

N-acetyl-LTE4	C25 H39 N O6 S	481.253	5.946	+	+			
	C26 H14 N2 O8	482.075	1.96		-			
cyanidin-3-glucoside chloride	C21 H21 Cl O11	484.085	4.113	-	-	-		
(22S)-1alpha,25-dihydroxy-22-ethoxy-26,27-dimethyl-23,24-tetradehydro-20-epivitamin D3/(22S)-1alph	C31 H48 O4	484.353	3.766	+			+	
	C19 H22 N10 O6	486.171	3.73	+				
Glucosylgalactosyl hydroxylysine	C18 H34 N2 O13	486.209	5.938	+	+		+	
	C25 H33 N11	487.292	11.879			+		+
	C21 H12 N8 O7	488.082	4.119		-			
Gln Arg Trp	C22 H32 N8 O5	488.251	4.598	+				
Trp Trp Val	C27 H31 N5 O4	489.238	4.204		+			
	C31 H59 N O9	589.419	15.311					
	C16 H14 N10 O9	490.095	2.884	+	-			
	C37 H14 O2	490.101	2.737	+	-			
	C24 H18 N4 O8	490.112	6.312	+				
	C38 H18 O	490.134	2.36		+			
	C21 H21 N11 O4	491.178	3.069	+	+			
	C19 H8 N8 O9	492.043	1.302				-	

	C24 H20 N4 O8	492.127	6.432		-	
	C25 H39 N3 O S3	493.226	2.632		+	
flumethasone pivalate	C27 H36 F2 O6	494.247	8.83			-
	C21 H20 O14	496.086	4.587		-	
Fenirofibrate glucuronide	C23 H25 Cl O10	496.118	2.809	+		
Mycophenolic acid glucuronide	C23 H28 O12	496.158	5.221		+	
	C22 H39 N7 O6	497.297	4.263	+	+	
Clobenoside	C25 H32 Cl2 O6	498.156	6.935		+	
Teclozan	C20 H28 Cl4 N2 O4	500.083	3.88		-	
punaglandin 6	C25 H37 Cl O8	500.224	6.02		+	
	C26 H48 N2 O7	500.346	5.595			+
D-Glucosaminide	C18 H35 N3 O13	501.222	6.956	+	+	
p-Hydroxycarvedilol sulfate	C24 H26 N2 O8 S	502.148	6.16	+	+	
arjungenin	C30 H48 O6	504.345	9.212		-	-
	C19 H10 N10 O8	506.069	4.106	+	+	
	C19 H10 N10 O8	506.069	4.283	+		
	C24 H10 N8 O6	506.073	3.95	+		
Maltotriitol	C18 H34 O16	506.178	6.963	+	+	
	C19 H25 N O15	507.123	2.735	+		

	C33 H13 N7	507.124	2.15	+	+	
	C21 H17 N9 O7	507.124	1.614	+		
	C39 H27 N	509.215	2.626		+	
	C20 H27 N15 O2	509.249	5.848		+	
	C38 H26 N2	510.209	6.01	+	+	
Flordipine	C26 H33 F3 N2 O5	510.243	4.205		+	
GPSer(8:0/8:0)	C22 H42 N O10 P	511.26	3.238	+	+	
	C20 H33 N17	511.31	4.944		+	
	C22 H8 N8 O8	512.046	5.117		-	
	C21 H20 O15	512.08	2.733	+	+	
	C31 H16 N2 O6	512.101	1.288			-
	C22 H16 N4 O11	512.081	2.159	+		
	C29 H20 O9	512.111	3.384		-	
	C34 H28 N2 O3	512.208	2.624		+	
	C32 H37 N O P2	513.236	4.322		+	
Tyr Trp Phe	C29 H30 N4 O5	514.219	4.162		+	
10S,11R-epoxy-punaglandin 4	C25 H35 Cl O9	514.203	5.848		+	
	C29 H34 N6 O3	514.27	11.899			+
	C22 H21 N5 O10	515.128	3.742	+		

	C17 H20 N6 O13	516.109	1.807	+		
	C21 H28 N10 O6	516.219	5.579	+	+	
Glucametacin	C25 H27 Cl N2 O8	518.142	5.128	+	+	
	C22 H29 N15 O	519.268	6.001		+	
	C22 H31 N15 O	521.284	5.972	+	+	
	C23 H26 N10 O5	522.209	5.688			-
	C20 H16 N10 O8	524.115	4.583		-	
	C23 H28 N10 O5	524.224	8.772	+		
	C23 H28 N10 O5	524.224	9.003	+		
26,26,26,27,27,27-hexafluoro-1alpha,24-dihydroxyvitamin D3/26,26,26,27,27,27-hexafluoro-1alpha,24-	C27 H38 F6 O3	524.281	5.299		+	
	C22 H18 N14 O3	526.169	5.366		+	
	C22 H34 N6 O9	526.239	7.355	+		
	C22 H34 N6 O9	526.24	5.97	+	+	
	C28 H42 N6 O4	526.326	9.207	+	+	
17beta-Estradiol 3-sulfate-17-(beta-D-glucuronide)	C24 H31 O11 S	527.167	2.932		+	
Imcarbofos	C17 H30 N4 O7 P2 S2	528.112	2.748	+		

	C28 H24 N4 O7	528.165	7.032	+	+		
Retinylphosphate mannose	C26 H41 O9 P	528.255	5.631	+	+		
	C39 H14 O3	530.094	2.922		-		
	C24 H22 N10 O5	530.177	7.461		-		
	C23 H33 N O13	531.196	2.913		+		
	C25 H29 N11 O3	531.244	3.117	+	+		
Sulindac glucuronide	C26 H25 F O9 S	532.12	4.107	+			
	C23 H14 N14 O3	534.138	6.653		-		
	C17 H42 N8 O S5	534.208	6.134		+		
	C17 H24 N6 O14	536.134	1.453	+	+		
Piretanide glucuronide	C23 H26 N2 O11 S	538.128	3.442	+			
	C24 H25 N15 O	539.238	6.144	+	+		
	C21 H8 N4 O14	540.005	0.891			-	-
IRIGENIN, DIBENZYL ETHER	C32 H28 O8	540.185	5.809		+		
Galbeta1-3GalNAcalpha-Thr	C21 H36 N2 O14	540.219	4.603	+	+		
(5b,7a,12a)-2-(3-methoxyphenyl)-2-oxoethyl ester-7,12-dihydroxy-cholan-24-oic acid	C33 H48 O6	540.342	4.132			+	
	C39 H30 N2 O	542.236	4.89		+		
p-Hydroxygliquidone	C27 H33 N3 O7 S	543.203	2.657		+		

	C23 H20 N4 O12	544.107	3.889		-	
	C22 H31 N15 O3	553.274	4.98		+	
Octotiamine	C23 H36 N4 O5 S3	544.188	5.704	+	+	
	C29 H55 N O8	545.393	15.388	+		
	C18 H14 N2 O18	546.022	0.916			-
	C25 H30 N12 O3	546.257	3.929		+	
	C24 H33 N15 O	547.3	5.218	+	+	
	C26 H16 O14	552.053	5.537		-	
	C37 H35 N3 O2	553.272	4.975	+		
	C31 H42 N10	554.359	5.262			+
	C32 H20 N4 O6	556.138	2.099	+	+	
	C22 H6 N2 O10	458.002	0.9			-
OLMESARTAN MEDOXOMIL	C29 H30 N6 O6	558.229	4.97	+	+	
	C21 H10 N2 O17	561.995	0.928			-
protoporphyrin IX	C34 H36 N4 O4	564.269	4.121		+	
Cicortonide	C29 H37 Cl F N O7	565.234	6.078	+		
	C27 H16 N14 O2	568.159	1.848	+		
Mioflazine	C29 H30 Cl2 F2 N4 O2	574.17	5.856		+	
	C24 H45 N15 O2	575.388	7.347			

Deserpidine	C32 H38 N2 O8	578.271	4.615	-			
	C30 H24 N14	580.23	6.837	+	+		
	C25 H22 N14 O4	582.196	6.31		-		
ENDECAPHYLLIN X	C18 H24 N4 O18	584.118	6.123	+	+/-		
	C32 H44 N10 O	584.37	4.056				+/-
	C30 H46 N6 O6	586.348	9.221				-
	C32 H55 N5 O5	589.42	15.311	+	+	+	+
	C29 H34 O3 S5	590.113	2.343		-		
	C15 H36 N4 O10 S5	592.104	2.947		-		
katecateroside	C28 H38 O14	598.225	6.878	+			
	C33 H46 N10 O	598.386	5.071				+
	C25 H25 N15 O4	599.222	6.309		+		
NORSTICTIC ACID PENTAACETATE	C28 H24 O15	600.112	5.818		-		
	C36 H40 N8 O	600.333	9.22				-
Dilazep	C31 H44 N2 O10	604.296	3.708	+	+		
cefpiramide	C25 H24 N8 O7 S2	612.121	1.758		-		
	C23 H14 N14 O8	614.113	1.25	+			
	C22 H30 O20	614.133	1.403		-		
	C45 H34 N2 O	618.27	3.341		+		

Amicetin	C29 H42 N6 O9	618.291	4.821		+			
	C27 H16 N4 O14	620.067	5.503		-			
CEFDITORIN PIVOXIL	C25 H28 N6 O7 S3	620.123	2.934		-			
	C25 H24 N20 O	620.245	6.88		+			
Hinderin	C23 H28 I2 O4	622.007	0.919				-	
	C29 H12 N2 O15	628.025	0.913				-	
	C30 H20 N4 O12	628.107	8.804		-			
	C31 H51 N15	633.446	15.209	+		+	+	+
corilagin	C27 H22 O18	634.082	4.943	-	-			
	C21 H18 N10 O14	634.1	1.421		+			
Diethyl 1,7-bis(2,4-dinitrophenyl)-1,7-dihydrodipyrazolo[3,4-b:4',3'-e]pyrazine-3,5-dicarboxylate	C24 H16 N10 O12	636.097	4.776	-	-			
	C31 H28 N10 O6	636.22	8.309	+				
	C25 H29 N21 O	639.286	4.318		+			
	C37 H54 O9	642.376	4.193				+	
	C42 H40 N6 O	644.326	4.666		+			
	C27 H22 O19	650.076	4.414		-			
	C28 H18 N4 O15	650.077	4.701		-			

	C28 H18 N4 O15	650.078	5.002		-		
	C29 H38 N18 O	654.351	4.188		+		
	C25 H16 N6 O16	656.062	4.92	+			
	C25 H28 N20 O3	656.266	7.585	+			
	C26 H22 N8 O12	638.136	1.447		-		
	C25 H24 N6 O15	648.13	5.533		-		
	C33 H26 N4 O11	654.16	2.904		-		
	C29 H12 N12 O8	656.09	3.238		-		
TENIPOSIDE	C32 H32 O13 S	656.154	3.433		-		
	C40 H37 N9 O	659.312	3.306		+		
	C20 H22 N8 O18	662.105	1.833		-		
	C26 H30 O20	662.132	3.309	+			
	C28 H22 N22	666.241	7.893	+			
	C27 H42 N2 O17	666.249	8.137	+			
	C26 H26 N20 O3	666.25	8.469	+			
	C27 H26 O20	670.101	3.644		-		
	C29 H14 N12 O9	674.102	2.01		-		
	C33 H55 N15 O	677.472	15.122	+		+	+
						-	

	C27 H24 N24	684.261	6.734	+				
	C22 H40 N26 O	684.386	4.782				+	
	C28 H42 N6 O14	686.276	7.544	+				
Neutramycin	C34 H54 O14	686.349	6.202		+			
	C29 H34 N4 O6 S5	694.109	4.338		-			
	C37 H50 N10 O4	698.402	5.868				+	
	C28 H32 N20 O3	696.296	4.781		+			
GPIns(12:0/13:0)	C34 H65 O13 P	712.416	8.101				+	
	C34 H59 N17 O	721.508	15.036				+	+
(Alginate)n	C24 H32 O26	736.13	6.671	+				
	C23 H24 N14 O15	736.154	2.228			-		
	C34 H6 N4 O17	741.974	0.926				-	
	C34 H46 O18	742.268	6.084	+				
	C32 H45 N19 O3	743.396	5.03		+			
	C30 H28 N14 O10	744.211	5.505		-			

+ and - indicates the mass spectral mode in which the molecule is detected. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract.

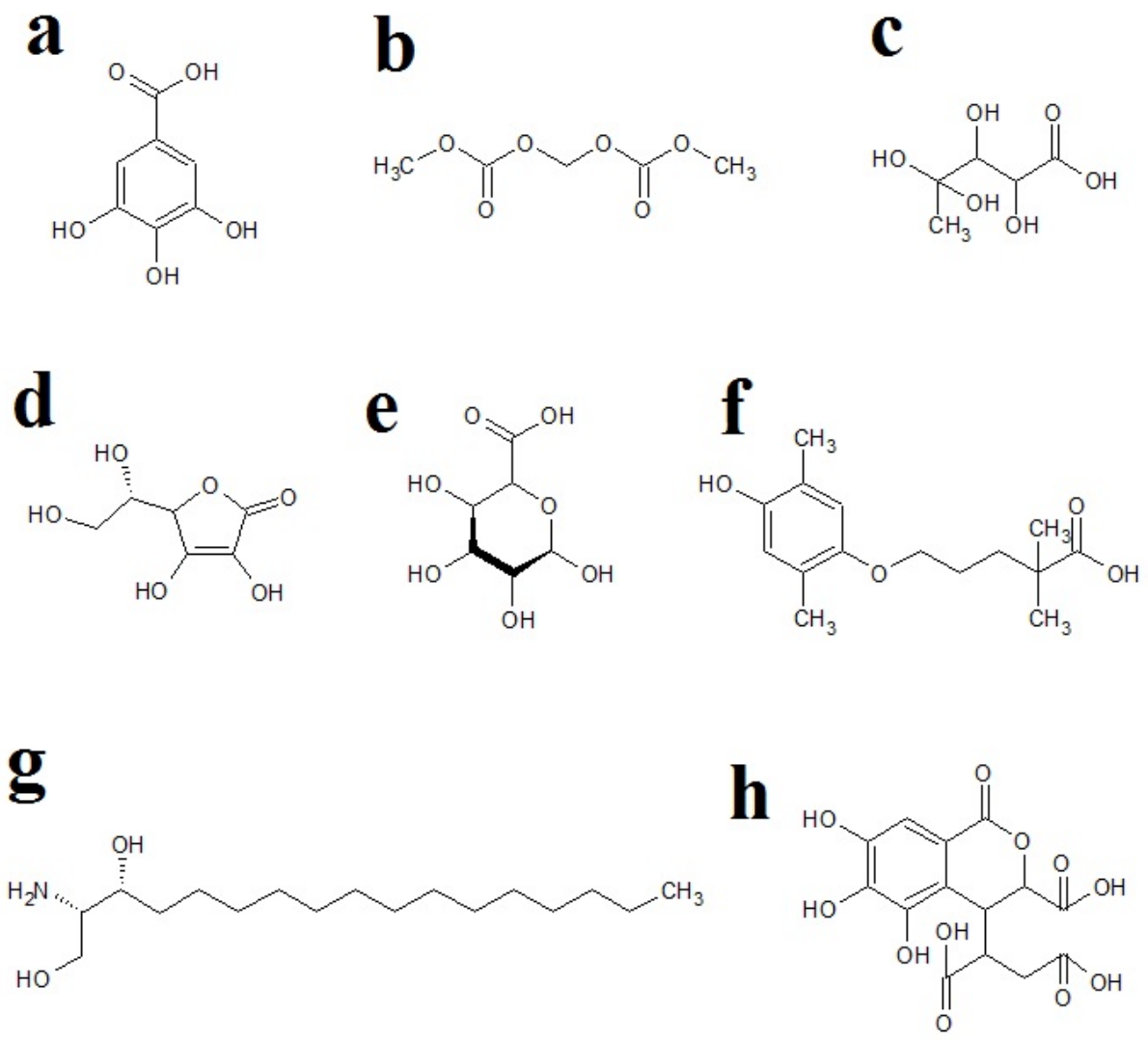


Figure 5.6: Chemical structures of *T. ferdinandiana* fruit compounds detected in all solvent extractions by HPLC-MS analysis: (a) gallic acid (trihydrobutanoic acid), (b) methoxycarbonyloxymethyl methyl carbonate, (c) apionic acid, (d) ascorbic acid, (e) glucuronic acid; (f) 5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl pentanoic acid, (g) C17 sphingosine, (h) chebulic acid.

5.3.1.2 GC-MS Head space analysis of the *T. ferdinandiana* fruit extracts

Optimised GC-MS parameters were developed and used to further examine the *T. ferdinandiana* fruit extract compound profiles. The resultant gas chromatograms are presented in Figures 5.7 - 5.11. The methanolic extract GC-MS chromatogram (Figure 5.7) displayed numerous overlapping peaks throughout the middle phases of the chromatogram. Indeed, a total of 108 peaks were detected in this chromatogram, with major peaks at approximately 10.2, 12.3, 14.1, 14.2, 16.0, 17.9, 20.9 and 28.9 min. Numerous overlapping peaks were also evident throughout the chromatogram, especially between 10-25 min. Many peaks were evident in the aqueous extract chromatogram (Figure 5.8) at elution times corresponding to peaks in the methanolic extract. Indeed, major peaks were also evident at 10.2 and 28.9 min. Many of the other major peaks that were present in the methanolic extract chromatogram were also present in the aqueous extract chromatogram, albeit with much reduced peak heights and areas. This indicates that methanol and water extract similar components, although many of these lower polarity compounds appear to be more effectively extracted into methanol than water. In addition to these peaks, a further major peak was present in the aqueous extract at 10.3. This peak was absent in the methanolic extract.

Much fewer peaks were evident in the ethyl acetate extract (Figure 5.9), chloroform extract (Figure 5.10) and hexane extract (Figure 5.11) chromatograms (59, 41 and 49 peaks respectively) than in the methanolic and aqueous extracts. Furthermore, many peaks in these extracts were at different elution times than seen for the more polar methanolic and aqueous extracts. The ethyl acetate, chloroform and hexane chromatograms all had major peaks

present at 19.7 min. In addition, the chloroform and hexane extracts had a further major peak present at approximately 15.6 - 15.7 min. This peak was lacking in the methanolic, aqueous, and ethyl acetate extracts.

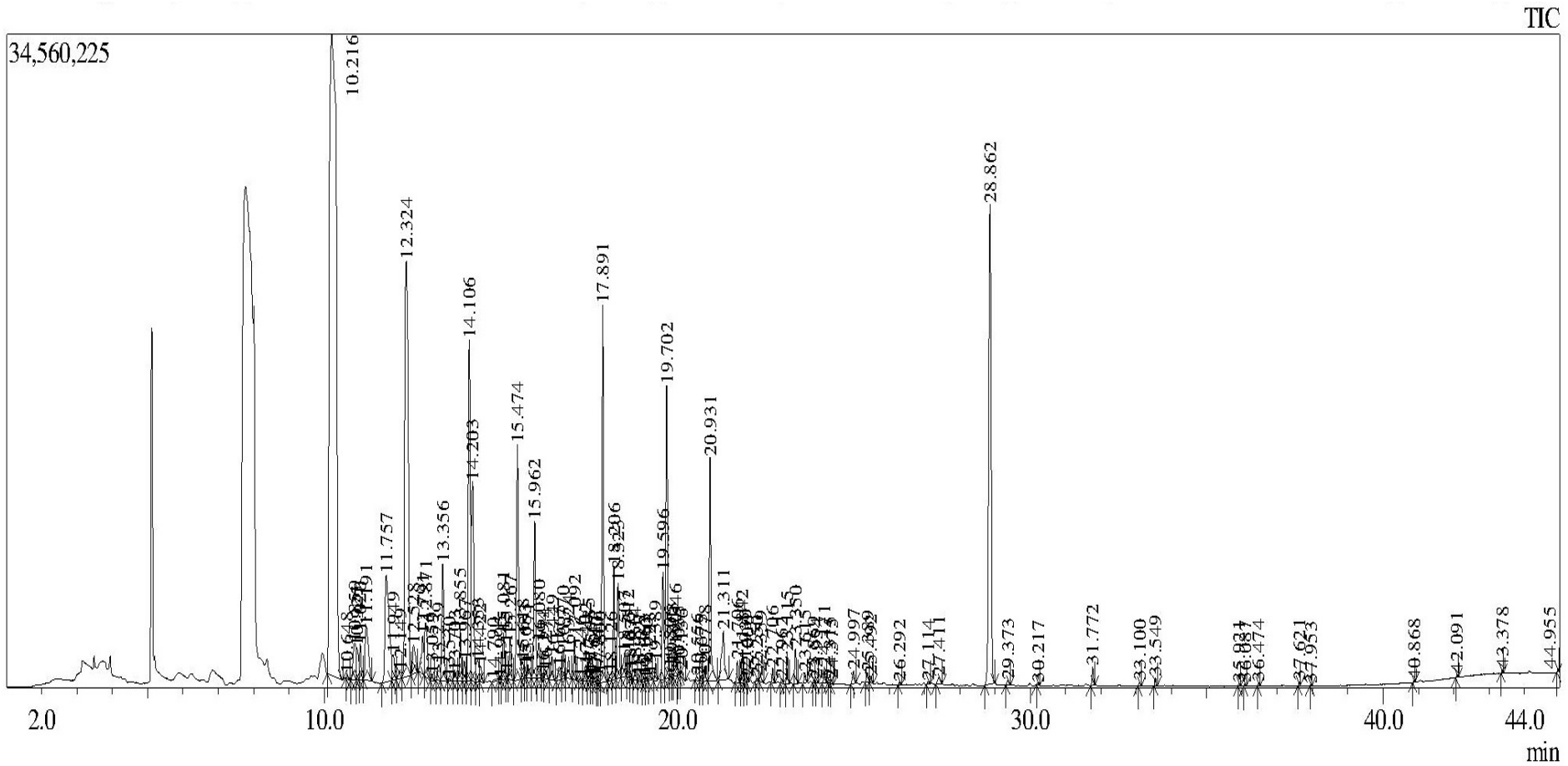


Figure 5.7: Head space gas chromatogram of 0.5 µL injections of *T. ferdinandiana* methanol fruit extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

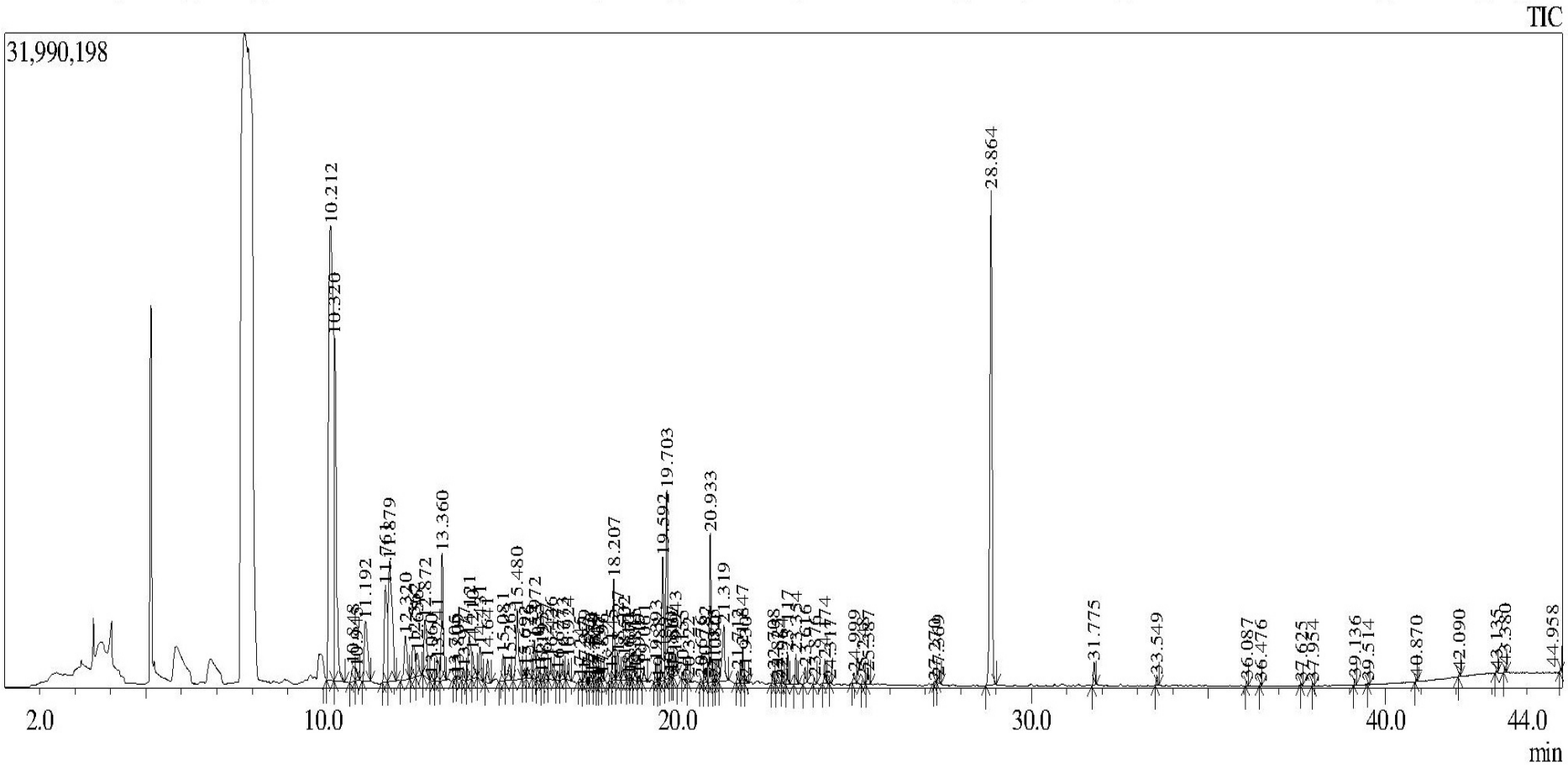


Figure 5.8: Head space gas chromatogram of 0.5 µL injections of aqueous *T. ferdinandiana* fruit extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

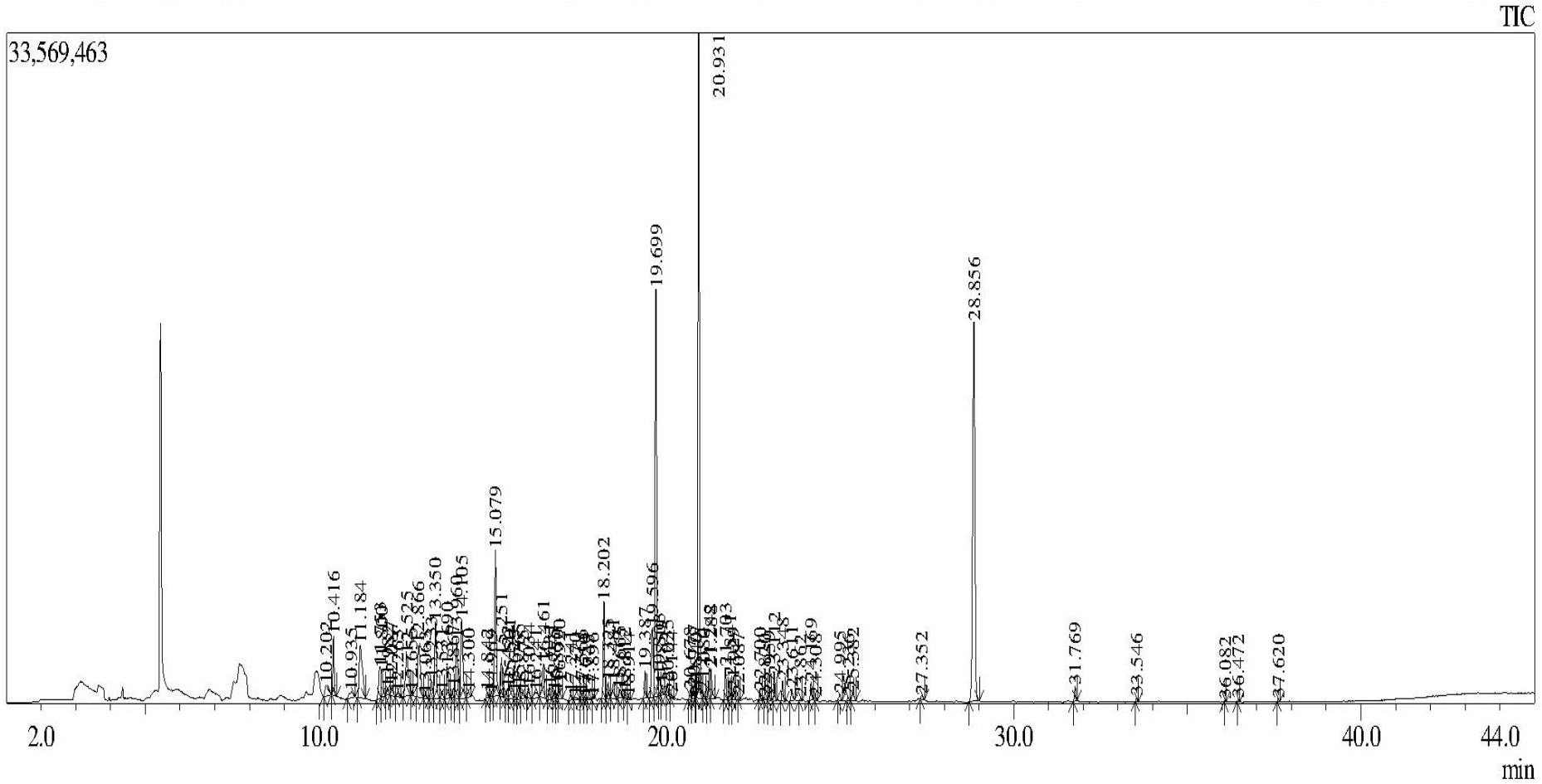


Figure 5.9: Head space gas chromatogram of 0.5 µL injections of *T. ferdinandiana* ethyl acetate fruit extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

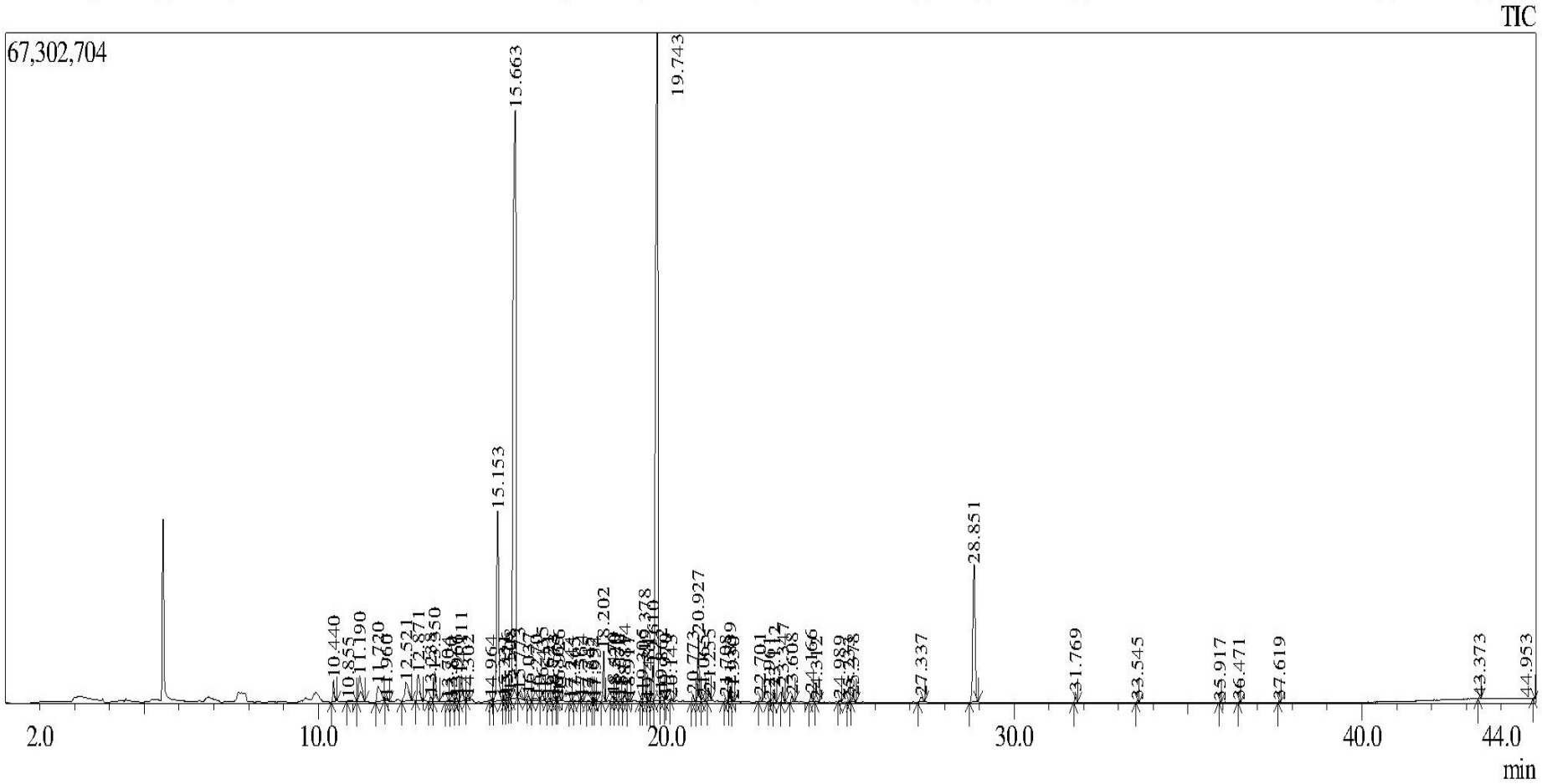


Figure 5.10: Head space gas chromatogram of 0.5 µL injections of *T. ferdinandiana* chloroform fruit extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

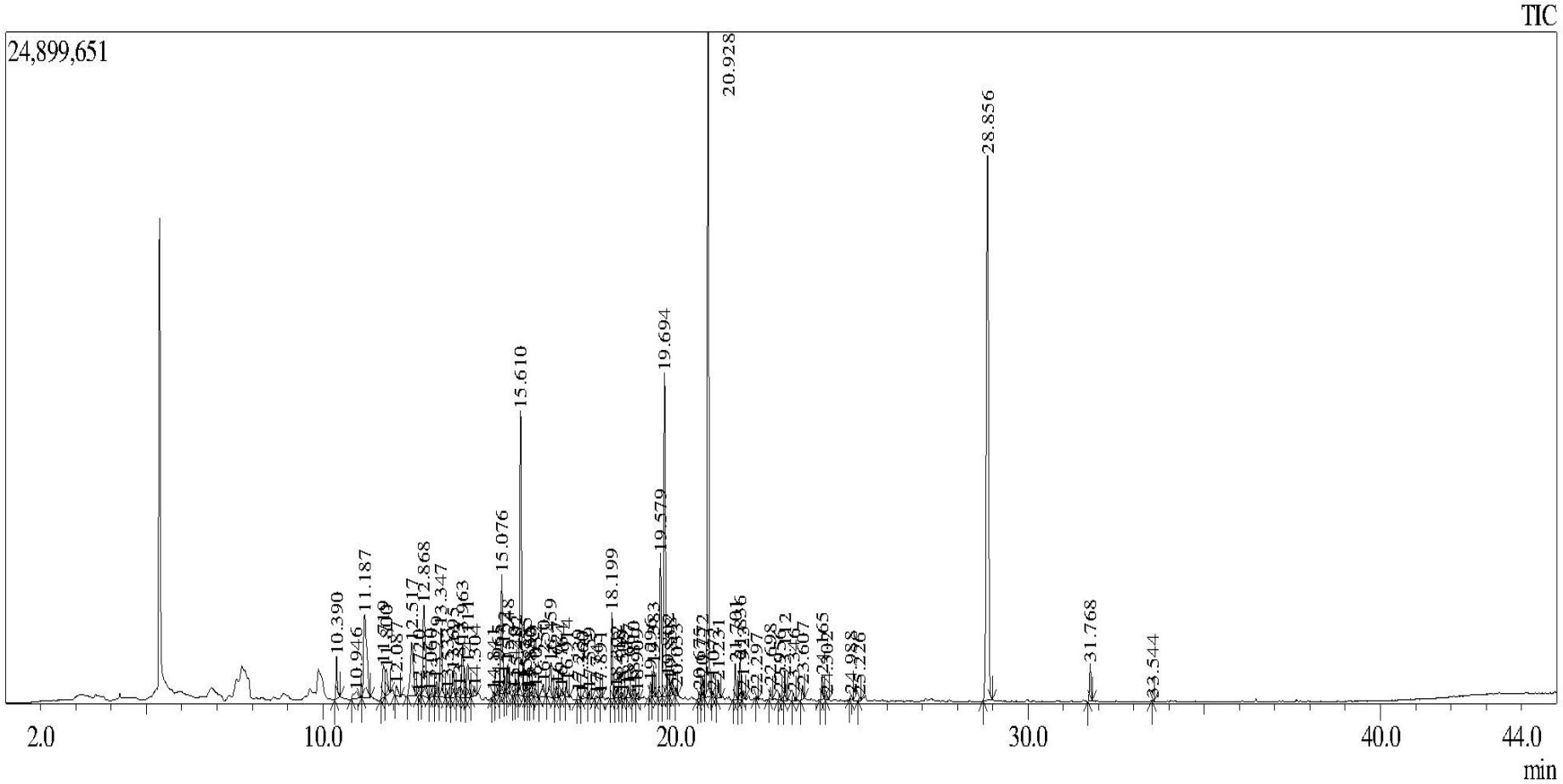


Figure 5.11: Head space gas chromatogram of 0.5 µL injections of *T. ferdinandiana* hexane fruit extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

5.3.1.2.1 Qualitative GC-MS headspace analysis of the *T. ferdinandiana* fruit extracts

In total, 128 unique mass signals were noted for the *ferdinandiana* fruit extracts by GC-MS headspace analysis (Table 5.2). Putative empirical formulas were achieved for all of these compounds. Of the 128 unique molecular mass signals detected, 97 compounds (75.8%) were putatively identified by comparison against the accurate mass GC-MS database. As for the LC-MS chromatogram analysis, a comparison of the metabolomics profiles across the GC-MS headspace chromatograms of these extracts was used to narrow the focus of phytochemicals which may contribute to this bioactivity. Of the 128 unique mass signals detected in the *T. ferdinandiana* fruit extracts, only 14 were present in all of the anti-proliferative extracts. In total, 13 of these compounds were putatively identified. These compounds are shown in order of increasing retention time in Figure 5.12. Of these, the imidazo compound 1 - (3H-imidazol-4-yl) - ethanone (Figure 5.12 a) was the only alkaloid detected in all of the extracts. A number of aliphatic keto- and aldo- compounds were also detected. Of these, 4-methyl-2-heptanone (Figure 5.12 b), 4, 6-dimethyl-2-heptanone (Figure 5.12 c), nonanal (Figure 5.12 d) and butyl 2-butoxyacetate (Figure 5.15 h) were present in all extracts. Similarly, the fatty acid octanoic acid (Figure 5.12 e), the alkane 3,3-dimethyl-hexane (Figure 5.12 i), as well as the aliphatic acid derivatives 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (Figure 5.12 j) and propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester (Figure 5.12 k) were also present in all anti-proliferative *T. ferdinandiana* extracts. The difurano derivative tetrahydro [2, 2'] bifuranyl-5-one (Figure 5.12 m) was also detected in all *T. ferdinandiana* extracts. All of the remaining compounds

present in all extracts (2-methyl-2-phenyl-oxirane, (Figure 5.12 f); m-di-tert-butylbenzene, (Figure 5.12 g); 3, 5-di-tert-butylphenol, (Figure 5.12 l)) were benzene derivatives.

Table 5.2: Qualitative GC-MS headspace analysis of the *T. ferdinandiana* extracts, elucidation of empirical formulas and putative identification (where possible) of each compound.

Molecular Mass	Molecular Formula	R.Time	Name	M	W	E	C	H
110	C5 H6 N2 O	10.52	1-(3H-Imidazol-4-yl)-ethanone	Y	Y	Y	Y	Y
118	C6 H14 O2	10.73	3-Methoxy-3-methylbutanol	Y				
96	C6 H8 O	10.845	2-Cyclohexen-1-one		Y			
132	C5 H8 O4	10.91	Dimethyl malonate	Y				
		10.99		Y	Y			
90	C3 H3 Cl O	11.105	2-chloroacrolein	Y				
128	C8 H16 O	11.345	4-methyl-2-heptanone	Y	Y	Y	Y	Y
112	C7 H12 O	11.765	2-heptenal					Y
144	C5 H8 N2 O3	11.875	(2E)-2-(Acetylhydrazono)propanoic acid			Y		
106	C7 H6 O	11.92	Benzaldehyde	Y	Y		Y	Y
110	C6 H6 O2	12.035	2-Formyl-5-methylfuran	Y	Y	Y		
		12.19		Y		Y		Y
158	C9 H18 O2	12.36	n-Heptyl acetate			Y		
126	C6 H6 O3	12.455	Methyl 2-furoate	Y	Y			
118	C6 H14 O2	12.595	Pinacol		Y			
232	C12 H24 O4	12.63	1-(1-Methoxypropan-2-yloxy)propan-2-y	Y		Y		Y
158	C9 H18 O2	12.69	Nonanoic acid				Y	
		12.825		Y	Y			

142	C9 H18 O	13.005	4,6-Dimethyl-2-heptanone	Y	Y	Y	Y	Y
130	C8 H18 O	13.12	2-Methyl-3-heptanol	Y	Y	Y		
128	C8 H16 O	13.295	Octanal	Y		Y	Y	Y
		13.48		Y	Y			
142	C10 H22	13.64	3,3,5-Trimethylheptane	Y		Y		Y
156	C11 H24	13.69	Undecane		Y			
278	C14 H30 O3 S	13.775	Sulfurous acid, 2-ethylhexyl hexyl ester	Y		Y		
120	C9 H12	13.918	Cumene				Y	Y
228	C15 H32 O	13.944	Decyl pentyl ether		Y	Y		
142	C10 H22	14.03	3,3,5-trimethyl-heptane	Y		Y	Y	Y
130	C8 H18 O	14.155	2-ethyl-1-hexanol	Y		Y		Y
146	C6 H10 O4	14.285	Butanedioic acid, dimethyl ester	Y	Y			
102	C5 H14 N2	14.395	N,N-Dimethyl-N-(3-aminopropyl)amine	Y	Y	Y	Y	
120	C8 H8 O	14.52	Benzeneacetaldehyde	Y	Y			
123	C7 H9 NO	14.735			Y			
298	C19 H38 O2	14.95	[(hexadecyloxy)methyl]-oxirane	Y		Y		Y
170	C12 H26	15.015	2,3-Dimethyldecane	Y	Y	Y		Y
168	C12 H24	15.14	4-methyl-1-undecene	Y				Y
114	C8 H18	15.205	3,3-Dimethylhexane	Y		Y		Y
120	C8 H8 O	15.29	o-Methylbenzaldehyde				Y	
160	C7 H12 O4	15.365	Dimethyl 2-methylsuccinate	Y	Y	Y		

112	C8 H16	15.425	n-Propylcyclopentane					Y	
130	C8 H18 O	15.448	1-Octanol			Y	Y		
170	C10 H18 O2	15.58	cis-Linalool oxide	Y		Y		Y	
120	C8 H8 O	15.66	3-Methylbenzaldehyde	Y		Y	Y	Y	
126	C6 H6 O3	15.73	Furyl hydroxymethyl ketone	Y	Y			Y	
		15.87		Y	Y			Y	
242	C13 H22 O4	16.03	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	Y	Y				
138	C8 H10 O2	16.008	2,4-Dihydroxy-1-ethylbenzene			Y			
136	C7 H8 N2 O	16.15	Benzoic acid, hydrazide	Y	Y		Y	Y	
		16.225		Y	Y				
		16.36		Y	Y	Y	Y		
142	C9 H18 O	16.54	Nonanal	Y	Y	Y	Y	Y	
168	C12 H24	16.715	4-Methyl-1-undecene	Y	Y	Y			
		16.83		Y	Y	Y			
		17.015		Y	Y	Y			
162	C6 H10 O5	17.16	Dimethyl dl-malate	Y					
174	C10 H22 O2	17.32	3,7-Dimethyl-1,7-octanediol	Y	Y			Y	
160	C7 H12 O4	17.445	Pentanedioic acid, dimethyl ester	Y	Y				
110	C6 H6 O2	17.53	2-Acetylfuran	Y	Y				
		17.61		Y	Y		Y	Y	
152	C10 H16 O	17.7	L-camphor	Y	Y				

		17.765		Y	Y			
		17.82		Y	Y			
111	C4 H5 N3 O	18.01	2-(Cyanoimino)oxazolidine	Y	Y			
		18.145		Y	Y			
168	C12 H24	18.385	4-Methyl-1-undecene					Y
154	C10 H18 O	18.405	endo-Borneol	Y	Y			
144	C8 H16 O2	18.56	Octanoic acid	Y	Y	Y	Y	Y
150	C9 H10 O2	18.655	Benzeneacetic acid, methyl ester	Y	Y			
314	C18 H34 O4	18.73	Oxalic acid, 6-ethyloct-3-yl isohexyl ester	Y	Y	Y		
		18.88		Y	Y	Y		Y
158	C9 H18 O2	18.99	3-Hexene, 1-(1-methoxyethoxy)-, (E)-	Y	Y		Y	Y
		19.09		Y				
154	C10 H18 O	19.16	Terpineol	Y	Y			
		19.295		Y				
150	C10 H14 O	19.34	2H-1b,4-Ethanopentaleno[1,2-b]oxirene, hexahydro-, (1a.alpha.,1b.beta.,4.beta.,4a.alpha.,5a.alpha.)-					Y
184	C13 H28	19.44	Nonane, 5-(2-methylpropyl)-	Y	Y	Y		Y
156	C10 H20 O	19.52	Decanal		Y		Y	
		19.645		Y	Y	Y		
134	C9 H10 O	19.775	Oxirane, 2-methyl-2-phenyl-	Y	Y	Y	Y	Y
156	C11 H24	19.83	2,6-Dimethylnonane	Y	Y	Y		Y

186	C10 H18 O3	19.895	2,2-Dimethylpropanoic anhydride	Y	Y		Y		
126	C6 H6 O3	20.01	5-Hydroxymethylfurfural	Y	Y				
184	C13 H28	20.08	4-methyl-dodecane	Y		Y		Y	
		20.225		Y	Y	Y	Y		
		20.63		Y					
268	C19 H40	20.725	5-Methyloctadecane	Y	Y	Y		Y	
154	C10 H18 O	20.84	1-Ethyl-4,4-dimethyl-cyclohex-2-en-1-ol				Y	Y	
140	C9 H16 O	20.865	2,3,4,5-Tetramethylcyclopent-2-en-1-ol	Y	Y	Y			
190	C14 H22	21.13	m-Di-tert-butylbenzene	Y	Y	Y	Y	Y	
158	C9 H18 O2	21.455	Nonanoic acid	Y	Y	Y	Y		
168	C12 H24	21.78	4-methyl-1-undecene	Y	Y	Y		Y	
188	C10 H20 O3	21.89	Butyl 2-butoxyacetate	Y	Y	Y	Y	Y	
		21.97		Y		Y	Y	Y	
		22.035		Y	Y				
		22.29		Y				Y	
		22.445		Y					
140	C9 H16 O	22.77	2,3,4,5-Tetramethylcyclopent-2-en-1-ol	Y	Y				
114	C8 H18	23.01	3,3-dimethyl-hexane	Y	Y	Y	Y	Y	
180	C13 H24	23.43	1,1,6,6-Tetramethylspiro[4.4]nonane	Y	Y	Y	Y		
286	C16 H30 O4	23.685	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	Y	Y	Y	Y	Y	
172	C10 H20 O2	23.91	n-Decanoic acid	Y	Y	Y			

		23.995		Y					
216	C12 H24 O3	24.24	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	Y	Y	Y	Y	Y	
		24.34		Y	Y	Y		Y	
		24.425		Y			Y		
		25.065		Y	Y	Y	Y	Y	
226	C14 H26 O2	25.445	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	Y	Y		Y		
152	C9 H16 N2	25.555	6-Butyl-5-methyl-1,6-dihydropyrimidine	Y					
202	C10 H18 O4	26.355	Octanedioic acid, dimethyl ester	Y					
		27.165		Y					
186	C12 H26 O	27.515	1-Dodecanol	Y	Y	Y	Y		
206	C14 H22 O	29.005	3,5-Di-tert-butylphenol	Y	Y	Y	Y	Y	
166	C9 H10 O3	29.445	Ethyl 3-hydroxybenzoate	Y					
216	C11 H20 O4	30.275	Nonanedioic acid, dimethyl ester	Y					
156	C8 H12 O3	31.835	Tetrahydro[2,2']bifuranyl-5-one	Y	Y	Y	Y	Y	
		33.15		Y					
		33.605		Y	Y	Y			
368	C24 H48 O2	35.96	Heneicosanoic acid, isopropyl ester	Y			Y		
		36.125		Y	Y	Y			
278	C16 H22 O4	36.515	Diisobutyl phthalate	Y	Y	Y			
330	C20 H26 O4	37.625	Phthalic acid, di-(1-hexen-5-yl) ester				Y		
178	C10 H10 O3	37.66	2-Propionylbenzoic acid	Y	Y	Y			

		37.985		Y			
286	C17 H34 O3	39.18	Carbonic acid, bis(2-ethylhexyl) ester		Y		
		40.9		Y	Y		
		42.13		Y	Y		
		43.41		Y	Y		Y
		44.975		Y	Y		Y

Y indicates the presence of that compound in the indicated extract.

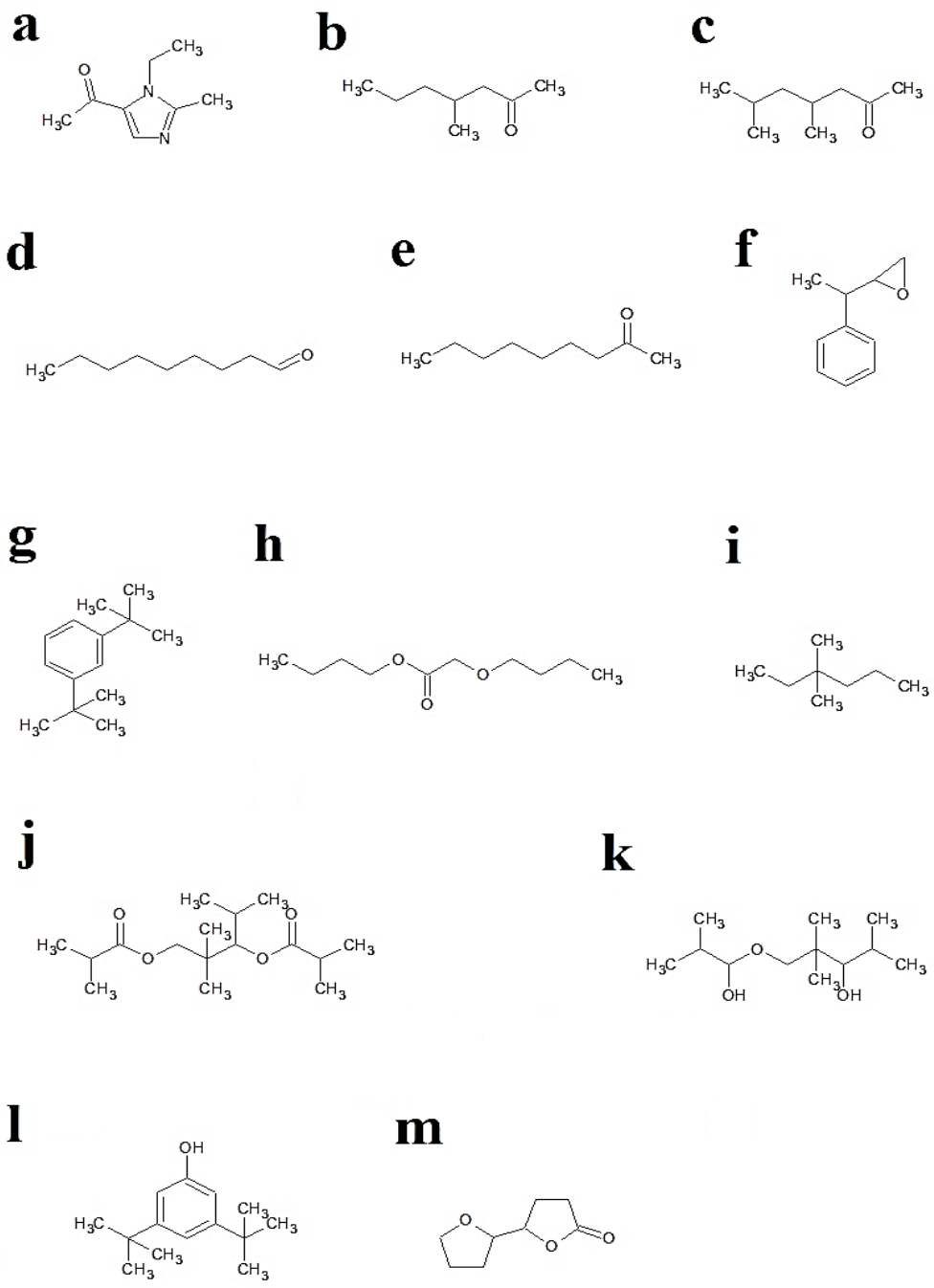


Figure 5.12: Chemical structures of *T. ferdinandiana* compounds detected in all solvent extractions using headspace GC-MS analysis: (a) 1-(3H-imidazol-4-yl)-ethanone, (b) 4-methyl-2-heptanone, (c) 4,6-dimethyl-2-heptanone, (d) nonanal, (e) octanoic acid; (f) 2-methyl-2-phenyl-oxirane, (g) m-di-tert-butylbenzene, (h) butyl 2-butoxyacetate, (i) 3,3-dimethyl-hexane, (j) 2,2,4-Trimethyl-1,3-pentanediol diisobutyrate, (k) propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester, (l) 3,5-di-tert-butylphenol, (m) tetrahydro[2,2']bifuranyl-5-one.

5.4 Discussion

The phytochemical compositions of all *T. ferdinandiana* extracts were determined and compared to identify compounds common between all of the extracts with anti-proliferative activity against the panel of carcinoma cell lines. Similar metabolomics comparison studies have previously been used very successfully to narrow the focus of phytochemicals and allow for the identification of bioactive components in extracts from other plant species. In a recent study examining the anti-viral activity of *Scaevola spinescens*, a comparison of the metabolomic profiles of solvents of varying polarities was able to highlight 2 compounds from 239 detected mass signals as possibly contributing to this activity [301]. Of these 2 compounds, one had been previously been reported to have anti-viral activity, validating this approach.

An important consideration of any metabolomic technique is that it is unable to detect all compounds in a complex mixture, but instead will only detect a portion of the compounds in the solvent. This is not necessarily a problem when a directed/biased study is undertaken to detect a particular compound or class of compounds and the separation and detection conditions can be optimised for the study. However, when the aim of the study is metabolomic profiling (as is required for comparative analyses) rather than metabolomic fingerprinting, the technique conditions must be chosen and optimised to separate and detect the largest amount of compounds, with the broadest possible physical and chemical characteristics. Generally, HPLC-MS/MS is a good choice for such metabolomic profiling studies as it detects a larger amount of compounds of varying polarities than the other commonly used techniques. However, this method is limited to studies of the mid to high

polar compounds and is not as useful for studies aimed at highly non-polar compounds. For these compounds, GC-MS analysis may provide further information. Both high accuracy QTOF HPLC-MS and GC-MS headspace analysis were used to examine the metabolomics profiles of the various *T. ferdinandiana* fruit extracts in this study to ensure that the greatest numbers of compounds were detected. A degree of overlap between the metabolomics profiles is not only expected, but is beneficial to narrow the focus of compounds for later studies.

A number of interesting compounds were identified by LC-MS in all extracts displaying anti-proliferative activity. The presence of the tannin compounds gallic acid (Figure 5.6 a) and chebulic acid (Figure 5.6 h), were particularly interesting as tannins have previously been reported to have anti-proliferative effects against a variety of carcinoma cell lines. Gallotannins have been reported to inhibit cellular growth through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins [318, 319], and by inhibiting glucosyltransferase enzymes [320]. Elligitannins are also highly potent inhibitors of cell growth and have also been reported to function via several mechanisms including interaction with cytoplasmic oxidoreductases [318, 321]. The ellagitannin arjunin extracted from a related *Terminalia* spp. *T. arjuna* has been reported to inhibit carcinoma proliferation and stimulate apoptosis [322]. Furthermore, a number of studies have reported high tannin content in a variety of plants used in traditional medicine to treat cancer [8, 323].

Also noteworthy was the detection on C17 sphingosine in all anti-proliferative *T. ferdinandiana* extracts. This is an interesting finding as two sphingosine kinase isozymes

(SphK1 and SphK2) are upregulated in many cancers, resulting in increased production of the potent bioactive compound sphingosine-1-phosphate (S1P) [324]. S1P induces a variety of responses including stimulation of TNF production [325-327], which in turn results in NF-κB activation and the subsequent down-regulation of apoptosis [328]. Thus, increased levels of S1P have tumour promoting effects and several recent studies have aimed at inhibiting SphK1 and SphK2 in order to inhibit cancer progression. Interestingly, whilst S1P is a potent mediator of cancer progression, its precursors and other related sphingolipids antagonise the activity of S1P and instead arrest carcinoma growth and induce apoptosis [329]. Indeed, a recent study demonstrated that several sphingosine analogues, including C17 sphingosine, are potent specific inhibitors of SphK1 [330]. This study also reported that the sphingosine analogues were highly specific and did not inhibit any of the other numerous protein kinases tested. As a result, exposure to the sphingosine analogues had dramatic effects on the growth and survival of human leukaemia U937 and Jurkat cells. The effects were manifested in several ways: the sphingosine analogues dramatically decreased cellular growth and proliferation and enhanced apoptosis and cleavage of Bcl-2. It appears that sphingosine analogues such as C17 sphingosine may be useful anticancer compounds via both cytostatic and cytotoxic mechanisms. Thus it is likely that C17 sphingosine may contribute to the anti-proliferative activity of the *T. ferdinandiana* extracts reported in chapters 3 and 4.

Two of the remaining compounds putatively identified in all inhibitory extracts by LC-MS metabolomics profiling analysis contain lactone moieties (ascorbic acid (Figure 5.6 d), glucuronic acid (Figure 5.6 e)). This is noteworthy as many lactone compounds have been reported to be useful mediators of apoptosis and several anti-proliferative natural compounds containing lactone moieties (e.g. costunolide and dehydrocostuslactone) have been described

[331]. Perhaps the most extensively studied of the natural lactone chemotherapeutics are sesquiterpene lactones such as eupatoriopicrin [332, 333] and parthenolide [334], which enhance apoptosis via multiple mechanisms. Furthermore, many clinically used cancer chemotherapeutics (e.g. camptothecin, testolactone, topotecan, belotecan, irinotecan, exatecan, gimatecan, silatecan, diflometecan) contain lactone moieties. In further studies, diacylglycerol (DAG)-lactones have been reported to be much more potent inducers of apoptosis in LNCaP cells than the positive control cytotoxins used in that study [335]. Moreover, the same study determined that the apoptotic induction of the DAG-lactones was mediated via protein kinase C activation. Enhancement of glutathione-S-transferase activity has also been reported for many lactones [336]. Glutathione-S-transferase has a regulatory role in the mitogen activated protein (MAP) kinase pathway that participates in cellular survival/apoptosis signals [337, 338]. As an increase in glutathione-S-transferase activity stimulates apoptosis, compounds which enhance this enzyme have emerged as promising new cancer therapy targets [339]. Thus, it is likely that lactone containing compounds may contribute to the anti-proliferative activity of the *T. ferdinandiana* extracts.

Also putatively identified across all *T. ferdinandiana* fruit extracts were methoxy carbonyl oxy-methyl methyl carbonate and 5-(4-hydroxy-2, 5-dimethylphenoxy)-2, 2-dimethyl pentanoic acid, which both contain ether moieties, and the aliphatic acid apionic acid. Whilst a literature search was unable to detect studies reporting anticancer activities for any of these compounds, they may still contribute to the anti-proliferative activity of these extracts, either directly, or through synergistic interactions with the bioactive components of the extracts and further studies are required to examine their therapeutic effects.

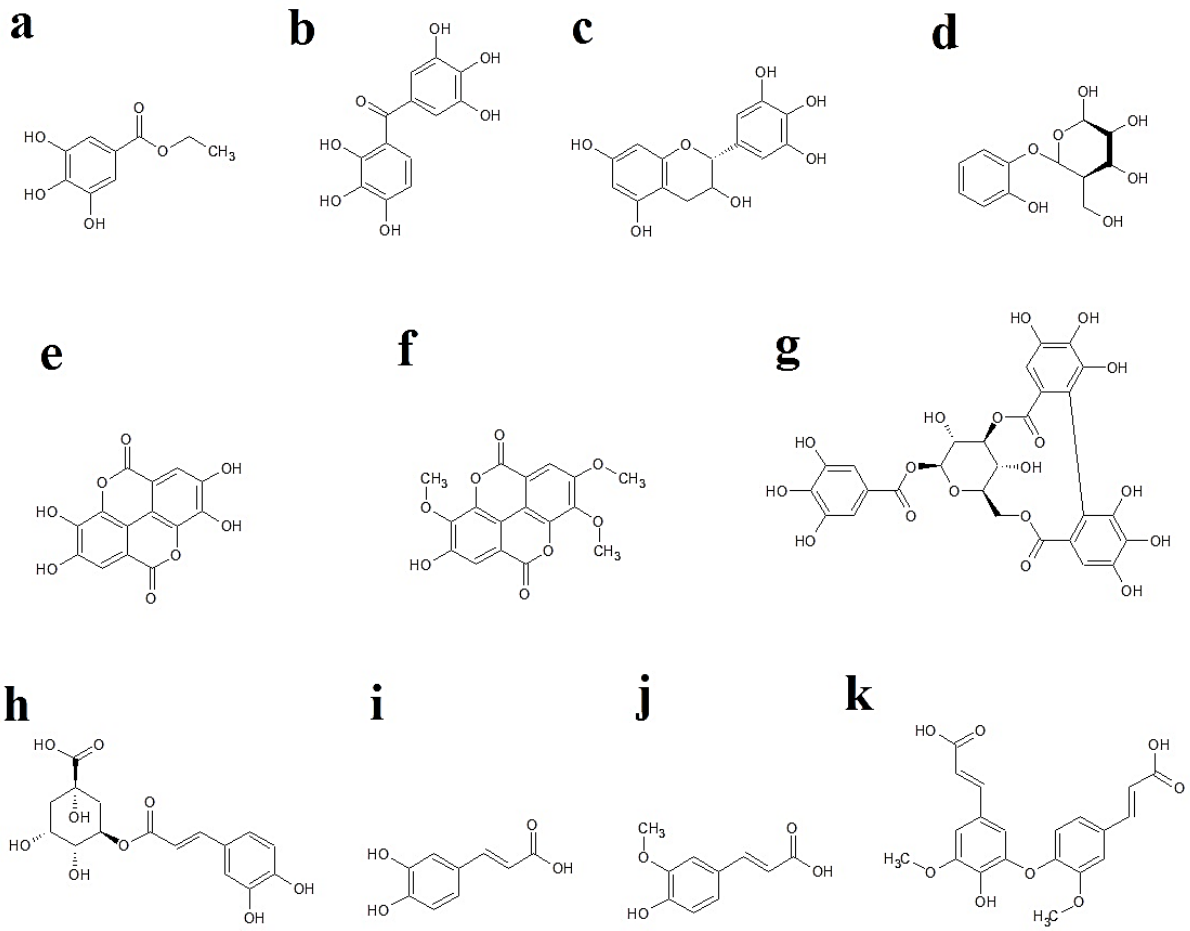


Figure 5.13: Chemical structures of *T. ferdinandiana* fruit tannin and pseudo-tannin compounds detected in some but not all solvent extractions: (a) ethyl gallic acid, (b) 4-galloyl pyrogalloyl, (c) gallocatechin, (d) pyrocatechol glucuronide, (e) ellagic acid, (f) ellagic acid dihydrate, (g) corilagen; (h) chlorogenic acid, (i) caffeic acid, (j) ferulic acid, (k) ferulic acid dihydrodimer.

Whilst metabolomic profile comparison analysis is a useful tool to narrow the phytochemical focus for future studies, a shortcoming is that some bioactive compounds which contribute to the activity of some, but not all extracts will be missed. An examination of the LC-MS metabolomics profiles of the *T. ferdinandiana* extracts illustrates this point. Some compounds with well documented anticancer activity were detected in some, but not all of the extracts

and thus were not highlighted in this analysis. This is particularly evident in the methanolic and aqueous extracts, which extracted the greatest number and diversity of phytochemicals. Particularly noteworthy in these extracts were the greater diversity of tannins and the presence of stilbenes. Indeed, seven tannins (ethyl gallic acid, exifone (4-galloylpyrogallol), gallocatechin, pyrocatechol diglucuronide, ellagic acid, trimethyl ellagic acid and corilagin) were detected only in the more polar extracts. Similarly, several pseudo-tannins including chlorogenic acid, caffeic acid, ferulic acid and ferulic acid dihydro dimer were detected in some, but not all extracts. In contrast to the tannins, these compounds, whilst often also detected in the methanolic extract, were most commonly detected in the less polar ethyl acetate and chloroform extracts. The potential of tannins in cancer therapy has already been described earlier in this chapter. It is likely that these compounds may have also contributed to the anticancer activities of the extracts in which they were detected.

Of further note, a number of stilbenes were detected in some (but not all) extracts. Stilbenes are phytoalexins produced by plants for protection against microbes but also have many other therapeutic properties. Resveratrol, which was identified in the methanolic *T. ferdinandiana* fruit extract in our study, has been particularly well studied and has been reported to be useful in the prevention and treatment of cancer [340, 341]. Furthermore, resveratrol is a potent specific inhibitor of NF-κB activation via its induction by TFN-α and IL-1β [244]. Thus, resveratrol treatment directly blocks cytokine production and inflammation via its inhibition of NF-κB activation. As inhibition of NF-κB potentiates apoptosis [342, 343], resveratrol is useful in the prevention and treatment of some cancers [340, 344, 345]. Qualitative HPLC-MS/MS analysis of the *T. ferdinandiana* fruit extract also detected the resveratrol glycoside piceid (2-[3-hydroxy-5[(E)-2-(4-hydroxyphenyl) ethenyl] phenoxy]-6-(hydroxymethyl) oxane-3, 4, 5-triol) in the

chloroform extract. Glycosylated stilbenes may be hydrolysed *in vivo* to remove glucose. Thus the presence of piceid in the extract is likely to result in the release of the resveratrol moiety *in vivo*. Piceid (and other glycosylated stilbenes) has also been shown to block IL-17 production in stimulated human mononuclear cells [346]. Other stilbenes (combretastatin, combretastatin A-1) were detected in the methanolic fruit extract. Combretastatins are well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin, thereby disrupting microtubule formation [246].

It is believed that they act by a similar mechanism to that of colchicine (N-[(7S)-1, 2, 3, 10-tetramethoxy-9-oxo-5, 6, 7, 9-tetrahydrobenzo[a]heptlen-7-yl] acetamide) by binding the colchicine binding site on the tubulin peptide and inhibiting polymerisation [247]. A number of other inflammatory and anticancer stilbenes have also previously been reported in other plant species. For example, 2, 3, 4, 5-tetrahydroxystilbene-2-O-β-D-glucoside (TSG) inhibits inflammation by suppressing the induction of pro-inflammatory mediators by reducing NF-κB binding to DNA and thus has potential as a cancer therapeutic [347]. The same study detected TSG in numerous herbs used to treat inflammation and cancer in Chinese traditional medicine. Whilst this compound was not detected in the compound databases used in our studies, it is possible that they may still be present in small quantities.

Qualitative GC-MS headspace analysis also identified a number of interesting compounds present in all of the anti-proliferative extracts which may contribute to the anti-proliferative activity of the *T. ferdinandiana* extracts. The presence of the imidazo-alkaloid compound 1-(3H-imidazol-4-yl)-ethanone was especially noteworthy. The imidazole moiety has a wide variety of biological activities [348]. Furthermore, imidazole derivatives are structural isomers of naturally occurring nucleotides, allowing them to be incorporated into new nucleic acids, resulting in inhibition of DNA and RNA synthesis [349]. This activity is responsible for many of the therapeutic properties of imidazole containing compounds, including their ability to block bacterial, fungal, protozoal and viral replication, as well as inhibiting carcinoma cell proliferation. For example, distamycin A is a natural imidazole antibiotic originally isolated from *Streptomyces distallicus* [350]. Distamycin A is a potent inhibitor of transcription and therefore is an efficient inhibitor of cellular proliferation [351]. A number of synthetic analogues of distamycin A have also been reported to have potent anticancer activity, with IC_{50} values as low as 1.53 µM [352]. Furthermore, many recent studies aimed at developing synthetic anticancer drugs have focussed on using the imidazole nucleus as a precursor. A novel imidazole analogue 2-b-D-ribofuranosylimidazole-4-carboxamide (imidazofurin) was synthesised and found to have potent anticancer activity [353]. A number of other promising anticancer drugs containing imidazole moieties including diaryl substituted imidazole analogues, pyrrole-imidazole polyamides, arylimidazonapthalimides and xantheno-imadazole derivatives have been developed [348].

The difurano derivative tetrahydro [2, 2'] bifuranyl-5-one was also detected in all *T. ferdinandiana* extracts. The anti-proliferative properties of lactones have already been discussed earlier in this chapter. Furanolactones are particularly promising and several recent studies have aimed at developing synthetic furanolactone compounds [354]. Similarly, the fatty acid octanoic acid may also have anti-proliferative activities. Short chain fatty acids similar to octanoic acid have been reported to inhibit the synthesis of further fatty acids and induce apoptosis in HL-60 [355]. These authors concluded that the induction of apoptosis was due to increased mitochondrial levels of oxidisable substrates following short chain fatty acid exposure. In contrast, other studies have reported differential effects, with some short chain fatty acids inhibiting carcinoma cell proliferation, whilst other fatty acids induced proliferation [356].

Whilst a literature review was unable to find reports of anticancer activity for the aliphatic acid derivatives 2, 2, 4-trimethyl-1, 3-pentanediol diisobutyrate and propanoic acid, 2-methyl-, 3-hydroxy-2, 2, 4-trimethylpentyl ester, they may also contribute to the anti-proliferative of the *T. ferdinandiana* extracts. Similar aliphatic acids (butyrate, phenyl butyrate and valproic acid) are weak inhibitors of histone deacetylase (HDAC) [357, 358]. Many types of cancer cells are sensitive to HDAC inhibitors, yet normal cells are relatively resistant making the development of HDAC inhibitors an attractive option in the development of new anticancer drugs [357].

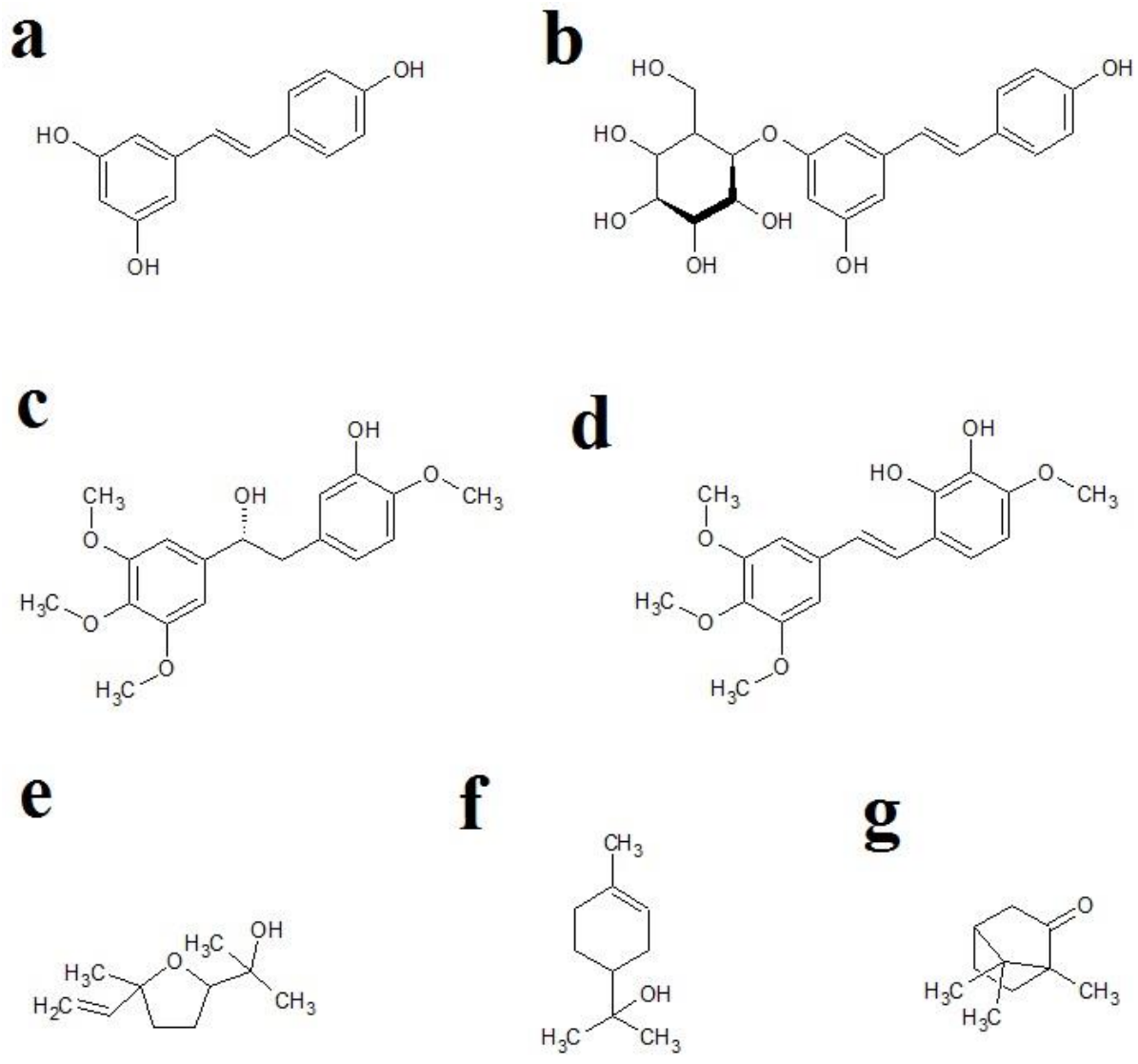


Figure 5.14: Chemical structures of *T. ferdinandiana* fruit compounds detected in some but not all solvent extractions: (a) resveratrol, (b) piceid, (c) combretastatin, (d) combretastatin A1, (e) linalool oxide; (f) terpineol, (g) camphor.

A number of other anti-proliferative compounds were present in one or more (but not all) extracts and thus were not highlighted by this study. Interestingly, several monoterpenoids (linalool oxide, terpineol, camphor) were detected in at least 2 of the extracts, but were not highlighted by the metabolomics comparison as they were not present in all of the *T.*

ferdinandiana extracts. Linalool has cytotoxic effects in SW620, T-47D, A549 and Hep G2 cells via a stimulation of TFN-α secretion [359]. Similarly, α-terpineol has been reported to inhibit RPMI 8226 myeloma, CCRF-CEM leukaemia, U937-GTB lymphoma, NCI-H69 lung carcinoma and ACHN renal adenocarcinoma cells [360]. The same study also reported that the antiproliferative activity of α-terpineol was via NF-κB inhibition. In contrast, in a study which screened multiple phytochemical components of Golden Feverfew against Hs605T, MCF-7 and SiHa carcinoma cells, camphor was found to be completely devoid of anti-proliferative activity [361]. Many other terpenes have documented anti-proliferative activity towards a variety of cancer cell lines. Lupeol is an effective growth inhibitor of human colorectal cancer cells [362]. Limonene and carvone were reported to be useful in the prevention and treatment of cancer in a murine test model, reducing stomach tumour formation by approximately 60% [363]. Furthermore, many extracts and essential oils from medicinal plants with traditional uses for the treatment of cancers have high terpenoid contents [364, 365]. Thus linalool oxide, terpineol and camphor may also contribute to the anti-proliferative activity of the polar extracts, but not to the activity of the other extracts.

In order to putatively identify as many compounds as possible in the extracts, the obtained spectra were compared with commercially available electron mass impact libraries. Furthermore, a library was prepared using approximately 800 compounds that are present in detectable amounts in a variety of plant species. When these libraries were screened against our extracts, a large number of the detected mass signals were identified. However, there were still a large number of compounds that were not identified by comparison to any of the libraries we used in our studies. These compounds will require significant further efforts before a structure and putative identification can be assigned.

It is noteworthy that the inability to detect all compounds by a single technique may mean that the bioactive compounds are missed. Furthermore, mass spectral techniques are generally not capable on their own to differentiate between structural isomers. Thus, comparison to the databases used in this study may have mistakenly identified an incorrect isomeric form of some compounds. Despite the promising results of our study, much further work is needed for a complete understanding of the anti-cancer properties of these extracts. Furthermore, it must also be noted that whilst this metabolomic profiling approach may provide us with promising leads, it is likely that these are not the only bioactive compounds in these extracts.

CHAPTER 6: COMPARATIVE METABOLOMIC PROFILE ANALYSIS OF *TASMANNIA LANCEOLATA* BERRY EXTRACTS

6.1 Material and Methods

6.1.1 Preparation of samples for metabolomic analysis

A 0.5 mL volume of each *T. lanceolata* berry extract was dried at room temperature under vacuum. The resultant dried extracts were resuspended in 5% acetonitrile and filtered through a Sarstedt 0.22 µm filter cartridge.

6.2 Non-targeted HPLC-MS QTOF analysis

A 2 µL aliquot of each *T. lanceolata* berry extract was injected onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C18 column (2.1 x 100 mm, 1.8 µm particle size) and analysed as described in Chapter 2.

6.3 Non-targeted GC-MS head space analysis

Aliquots (0.5 µL) of each extract was injected onto a Shimadzu GC-2010 Plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass spectrometer. Chromatographic separation was accomplished using a 5% phenyl, 95% dimethylpolysiloxane (30 m x0.25 mm id x 0.25 um) capillary column (Restek USA) as described in Chapter 2. Helium (99.999%) was employed as a carrier gas at a flow rate of 0.79 mL/min.

6.4 Results

6.4.1 HPLC-MS/MS analysis

The optimised HPLC-MS/MS parameters developed for the *T. ferdinandiana* fruit metabolomics study were also used to profile and compare the compound profiles from different extractions of *T. lanceolata* berry. The resultant positive ion and negative ion total compound chromatograms (TCC) of the methanolic fruit extracts are presented in Figure 6.1a and b respectively. Both the positive and negative ion TCC for the methanolic *T. lanceolata* berry extract chromatograms revealed numerous peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. Nearly all of the methanol extract compounds had eluted by 12 minutes (corresponding to approximately 32% acetonitrile). Indeed, multiple over lapping peaks eluted in the first 5 minutes with 5% acetonitrile. However, later eluting peaks were also evident at approximately 13.8 and 16

minutes in the chromatogram (particularly evident in the positive ionisation mode, Figure 6.1 a), indicating the broad spread of polarities of the compounds in that extract.

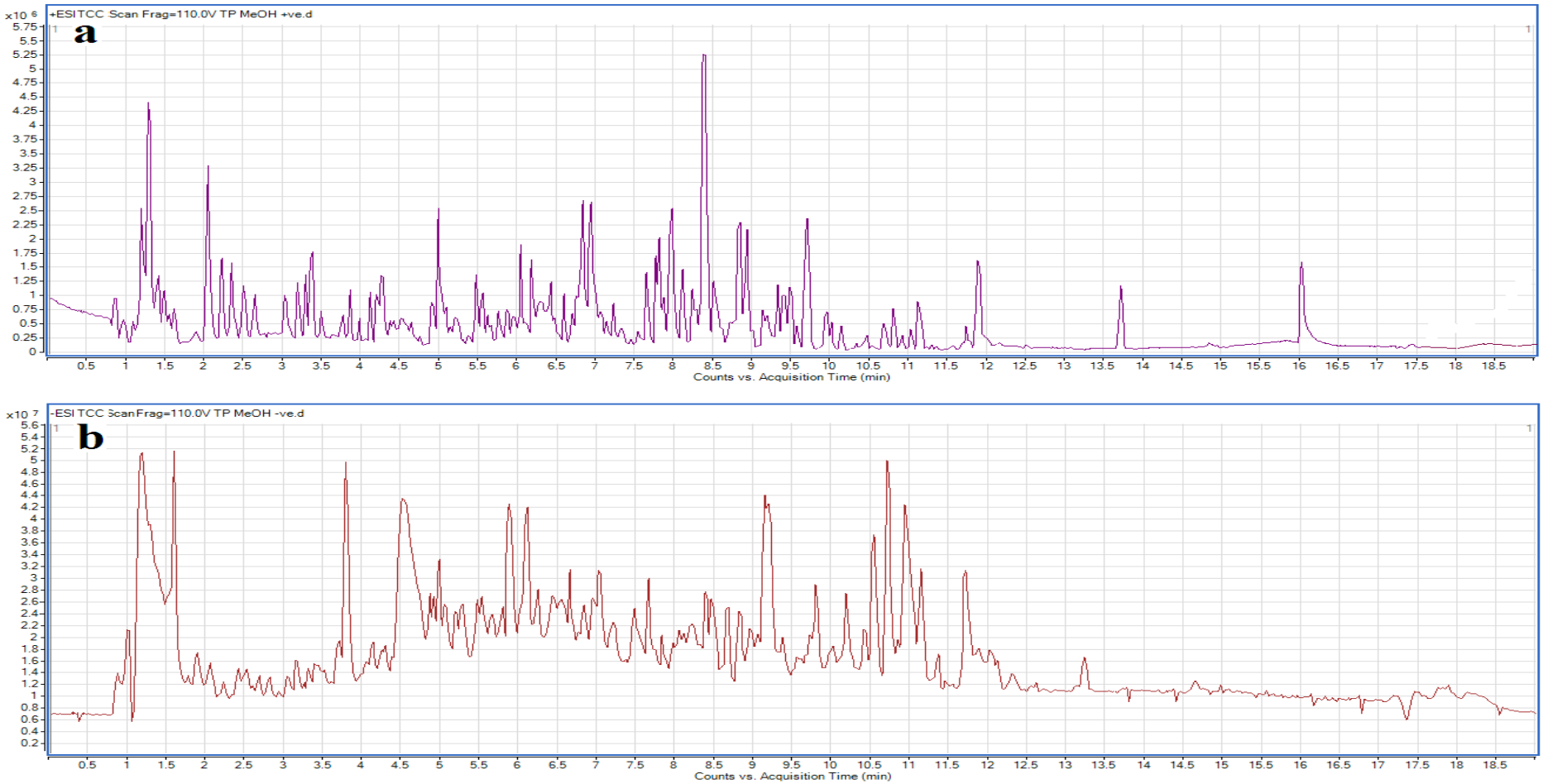


Figure 6.1: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µL injections of methanolic *T. lanceolata* berry extract.

Multiple peaks were also evident in the aqueous *T. lanceolata* berry extract in both the positive (Figure 6.2 a) and negative ion (Figure 6.2 b) TCC chromatograms, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. As for the methanolic extract, nearly all of the aqueous extract compounds had eluted by 12 minutes. (approximately 32% acetonitrile). The positive ion chromatogram had a greater number of mass signal peaks detected. However, the aqueous *T. lanceolata* berry extract negative ion chromatogram (Figure 6.2 b) had a substantially higher baseline signal that the positive ion chromatogram (Figure 6.2 a). Thus, some peaks may have been masked in the chromatogram.

The *T. lanceolata* berry ethyl acetate extract (Figure 6.3) had substantially fewer peaks, with a greater spread in polarity. Whilst numerous peaks were evident in the positive ion chromatogram (Figure 6.3 a), substantially fewer peaks were seen in the negative ion chromatogram (Figure 6.3 b). This is likely due to the high background signal of this chromatogram masking peaks in this mode. Whilst a substantial percentage of the detected compounds eluted in the isocratic phase of the positive ion chromatogram (the first 5 minutes), the majority of peaks were evident in the gradient phase of the positive ion chromatograms (5 - 30 min) corresponding to mid polarity compounds at approximately 5 - 84% acetonitrile. Fewer peaks were evident in the later phase of the chromatogram.

The *T. lanceolata* berry chloroform (Figure 6.4) and hexane extracts (Figure 6.5) both had a relative abundance of peaks in the middle stages of the chromatogram. In contrast with the methanolic, aqueous and ethyl acetate extracts, both of these extracts displayed a similar or

greater abundance of peaks in the negative ion chromatograms than in the positive ion chromatogram. Many of these peaks were coincident with peaks in the methanolic, aqueous and ethyl acetate extracts, indicating the presence of similar compounds between the extracts.

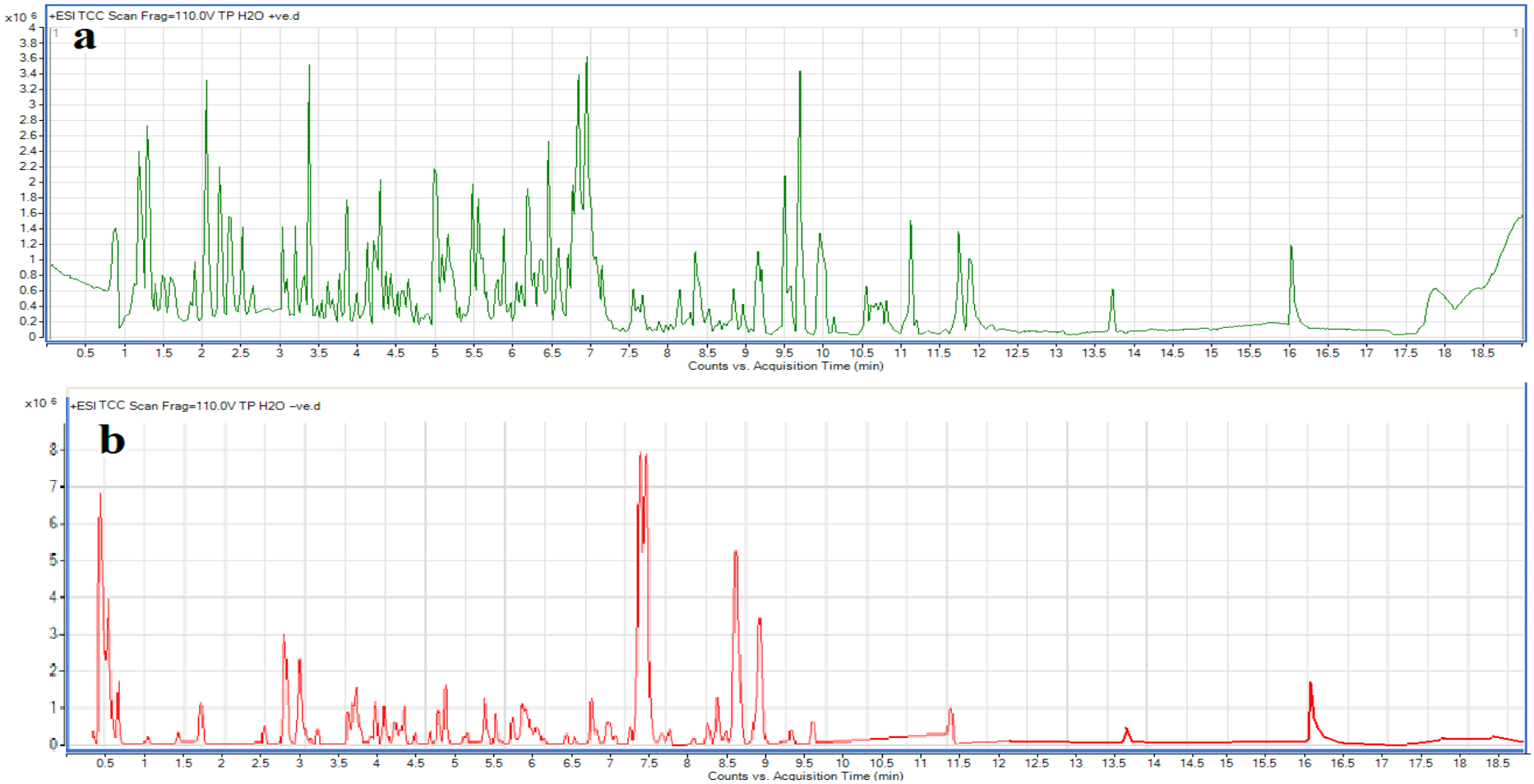


Figure 6.2: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µL injections of aqueous *T. lanceolata* berry extract.

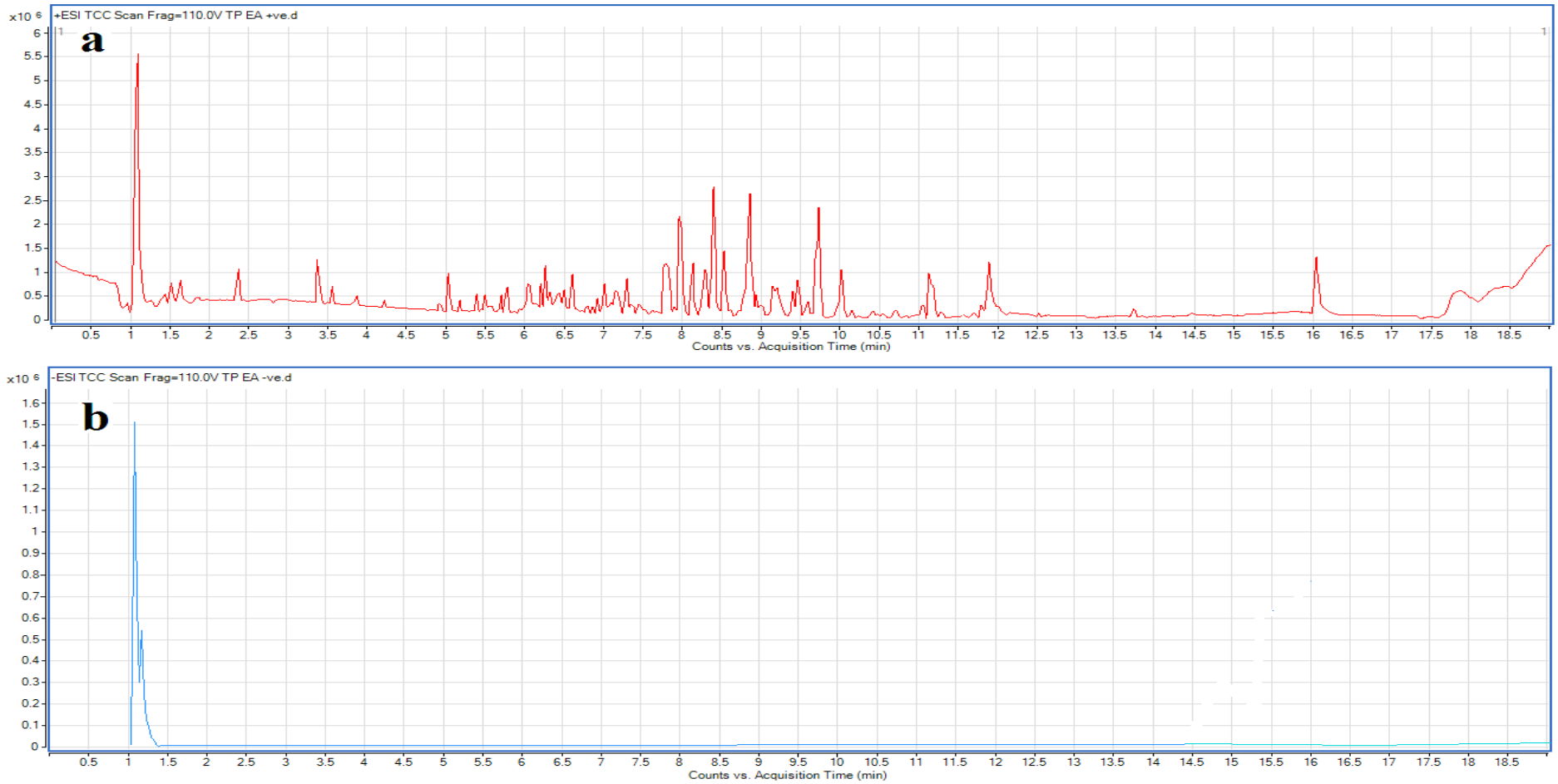


Figure 6.3: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µL injections of ethyl acetate *T. lanceolata* berry extract.

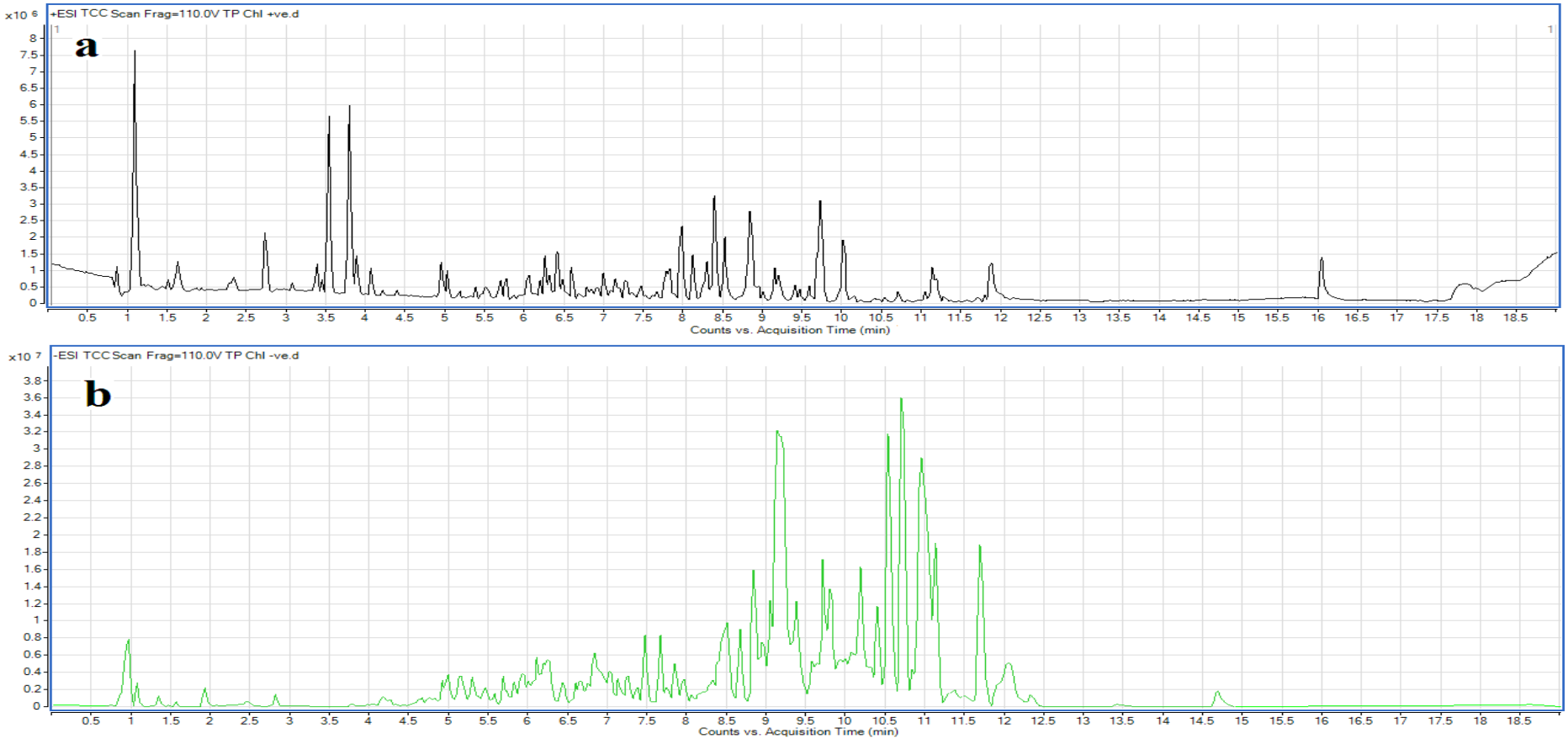


Figure 6.4: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µL injections of chloroform *T. lanceolata* berry extract.

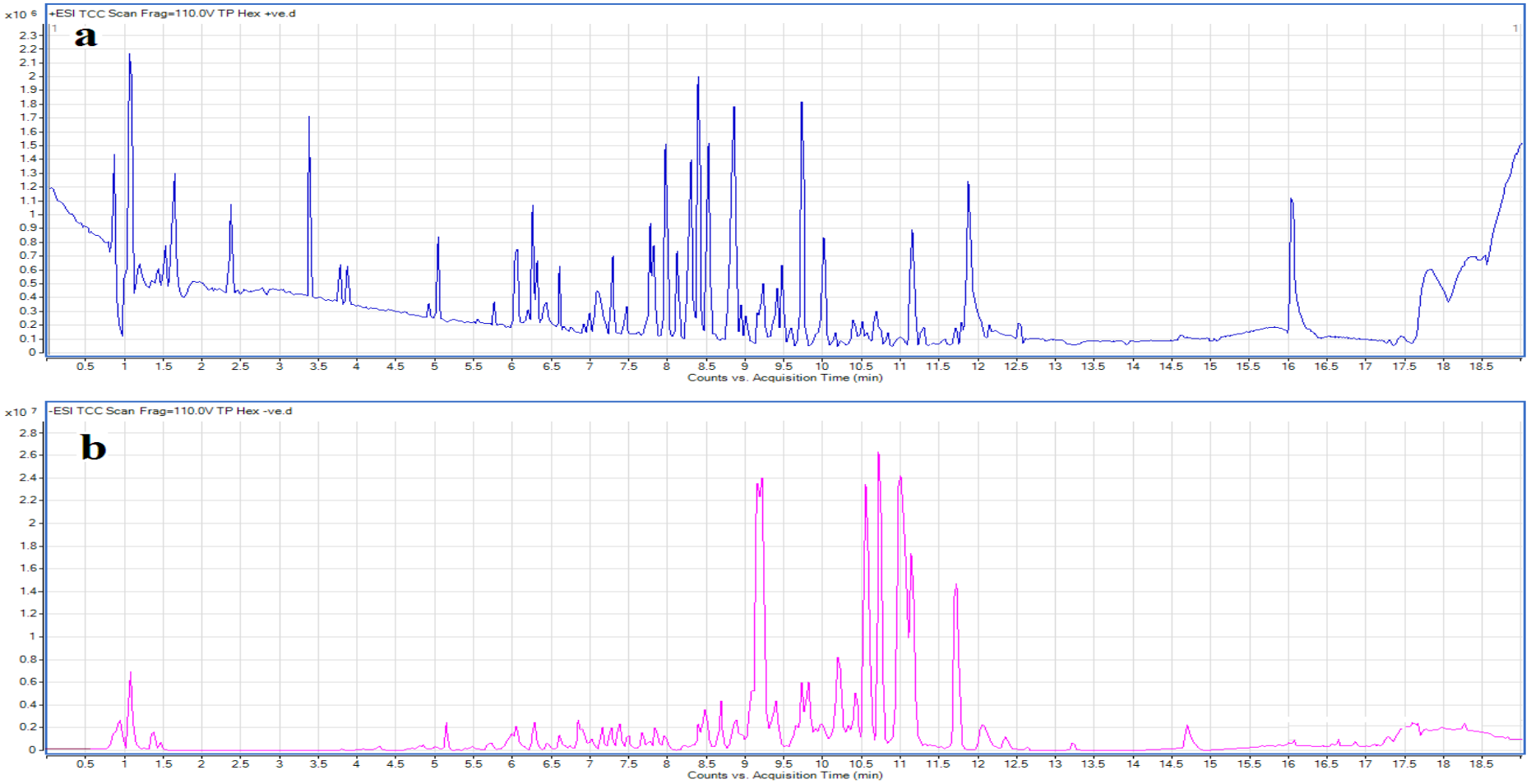


Figure 6.5: (a) Positive and (b) negative ion RP-HPLC total compound chromatograms (TCC) of 2 µL injections of hexane *T. lanceolata* berry extract.

6.4.1.1 Qualitative mass spectral analysis of the T. lanceolata berry extracts

The phytochemical compositions of the *T. lanceolata* berry extracts were examined by LC-MS QTOF analysis (Table 6.1). A total of 998 unique mass signals were detected from a comparison between the different extracts chromatographic profiles. Putative empirical formulas were achieved for all of these compounds. Of the 998 unique molecular mass signals detected, 879 compounds (88.1%) were putatively identified by comparison against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (800 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds).

Table 6.1: Qualitative HPLC-MS QTOF analysis of the *T. lanceolata* berry extracts, elucidation of empirical formulas and putative identification (where possible) of the compounds.

Name	Formula	Mass	Retention Time (mins)	M	W	E	C	H
Hydroxypyruvic acid	C3 H4 O4	104.012	1.176	-				
Quinone	C6 H4 O2	108.022	3.788	-				
Pyrocatechol	C6 H6 O2	110.037	3.808	-				
Hydroquinone	C6 H6 O2	110.038	0.662		-			
3-furanoic acid	C5 H4 O3	112.017	1.589	-				
3-oxo-4-pentenoic acid	C5 H6 O3	114.033	1.074					-
Proline	C5 H9 N O2	115.064	1.111	+				
maleic acid	C4 H4 O4	116.012	1.324	-				
2-Oxovaleric acid	C5 H8 O3	116.048	2.815	-			-	
succinic acid	C4 H6 O4	118.027	1.891	-			-	
3-Hydroxy-2-methyl-[R-(R,S)]-butanoic acid	C5 H10 O3	118.063	2.925	-				
Purine	C5 H4 N4	120.044	1.428	+				-
Benzoic acid	C7 H6 O2	122.037	5.849	-			-	-
	C3 H9 N S2	123.017	0.842	+	+	+		+
Guaiacol	C7 H8 O2	124.053	3.708			+		
5,7-octadienal	C8 H12 O	124.089	9.782	-			-	
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	C6 H8 O3	128.045	1.513			+		+
Octodrine	C8 H19 N	129.152	3.441		+		+	

3-Methyl-2-oxovaleric acid	C6 H10 O3	130.063	4.159				-	
glutaric acid	C5 H8 O4	132.043	1.95	-				
malic acid	C4 H6 O5	134.022	0.975	-	-			
trihhdroxybutanoic acid	C4 H8 O5	136.037	1.365					-
Allopurinol	C5 H4 N4 O	136.039	0.425		-			
salicyclic acid isomer 1	C7 H6 O3	138.032	7.281	-				
salicyclic acid isomer 2	C7 H6 O3	138.032	4.866	-				
3-AMINOPROPANESULPHONIC ACID	C3 H9 N O3 S	139.032	15.78	-				
(1S,5R)-4-hydroxy-6,7-dioxabicyclo[3.2.1]oct-2-en-8-one	C6 H6 O4	142.024	0.95	-			-	
7-hydroxy-5-heptynoic acid	C7 H10 O3	142.062	0.842	+	+	+		+
Valnoetamide	C8 H17 N O	143.131	1.664		+			
3-Hexenedioic acid	C6 H8 O4	144.042	2.415	+/-			-	
(E)-2-Methylglutaconic acid	C6 H8 O4	144.042	1.1					
Allysine	C6 H11 N O3	145.074	1.505		+			
Ribonolactone	C5 H8 O5	148.038	2.045	-		-		-
cinnamic acid	C9 H8 O2	148.05	6.092	-	-		-	
Mevalonic acid	C6 H12 O4	148.075	1.934	-				
	C7 H16 O3	148.11	4.809		+			
4-Ethylbenzoic acid	C9 H10 O2	150.068	8.415	-			-	
	C6 H14 O4	150.089	2.351	+	+	+	+	+
	C7 H4 O4	152.011	3.794	-				

Cystamine	C4 H12 N2 S2	152.042	3.702	-			
vanillin	C8 H8 O3	152.047	6.204	-		-	
Limonene-1,2-epoxide	C10 H16 O	152.12	9.357				-
Fosamine	C3 H8 N O4 P	153.02	2.039		+		
protocatechuic acid isomer 2	C7 H6 O4	154.026	6.248	-			
protocatechuic acid isomer 1	C7 H6 O4	154.026	3.806	-	-		
Acipimox	C6 H6 N2 O3	154.036	1.359	+			
vanillic alcohol	C8 H10 O3	154.062	3.704	-			
Furan 2,5-dicarboxylic acid	C6 H4 O5	156.007	1.59	-			
	C9 H19 N O	157.147	1.049			+	
2-methylene-4-oxo-pentanedioic acid	C6 H6 O5	158.022	2.033	-			
2-Isopropylmaleate	C7 H10 O4	158.058	5.121			-	
(+)-6-methyl caprylic acid	C9 H18 O2	158.13	12.047	-		-	-
2-Methylbutyrylglycine	C7 H13 N O3	159.092	1.256		+		
7-Hydroxyoctanoic acid	C8 H16 O3	160.11	4.045	+		+	
L-2-Aminoadipic acid	C6 H11 N O4	161.069	1.556	+			
ethyl (ethylperoxy) (oxo) acetate	C6 H10 O5	162.052	2.082	-	+		
coumaric acid	C9 H8 O3	164.047	5.934	-			
1,3-Benzodioxol-5-ylformiat	C8 H6 O4	166.027	5.874	-			
6-Methylmercaptopurine	C6 H6 N4 S	166.031	1.305		+		
D-Phenyllactic acid	C9 H10 O3	166.062	6.096	-		-	
Ethyl 4-hydroxybenzoate	C9 H10 O3	166.062	3.98		-		

	C6 H14 O5	166.085	1.149		+		
vanillic acid	C8 H8 O4	168.042	6.249	-			
3-Hydroxymandelic acid	C8 H8 O4	168.042	2.552	-			
vanillic acid	C8 H8 O4	168.042	3.703	-			
4-Hydroxy-3-methoxybenzoic acid	C8 H8 O4	168.043	1.613	-			
2Z,6E-decadienoic acid	C10 H16 O2	168.115	7.658	-		-	
(1S,5R)-4-Oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2-carboxylic acid	C7 H6 O5	170.022	3.793	-			
DL-3,4-Dihydroxyphenylglycol	C8 H10 O4	170.058	1.617	-			
Gabapentin	C9 H17 N O2	171.127	6.166		+	+	
Loprodiol	C5 H10 Cl2 O2	172.005	0.88	+/-		-	-
(1r,2r)-cyclohexane-1,2-dicarboxylic acid	C8 H12 O4	172.073	2.19	-			
9-oxo-nonanoic acid	C9 H16 O3	172.11	7.957	-		-	-
2,6-Piperidinedicarboxylic acid	C7 H11 N O4	173.069	1.197	-			
shikimic acid	C7 H10 O5	174.054	1.43	-			
6,7-dihydroxy-4-oxo-2-heptenoic acid	C7 H10 O5	174.054	1.223	-			
(+)-3-hydroxy pelargonic acid	C9 H18 O3	174.126	6.937	-			
2-Isopropylmalic acid	C7 H12 O5	176.067	1.463			-	
6,8-dihydroxy-octanoic acid	C8 H16 O4	176.105	6.589	-			
2-Cyclohexylphenol	C12 H16 O	176.121	8.147		-		
Aliflurane	C4 H3 Cl F4 O	177.978	0.846	-			
Methyl-p-coumarate	C10 H10 O3	178.063	7.48	-			

Amylmetacresol Pentylcresol	C12 H18 O	178.136	8.664		-				
caffeic acid	C9 H8 O4	180.043	5.102	+/-					
glucose	C6 H12 O6	180.063	1.713	-	+	+/-	+/-	-	
theophylline	C7 H8 N4 O2	180.065	1.201	-					
Acamprosate	C5 H11 N O4 S	181.041	5.407				-		
1,2-Epoxy-3,4-butanediol 4-methanesulfonate	C5 H10 O5 S	182.022	1.593	-					
Oxisuran	C8 H9 N O2 S	183.039	0.854		+				
Undecenamide 10-	C11 H21 N O	183.163	2.049				+		
(2-Methyl-4-oxo-4H-pyran-3-yloxy)-acetic acid	C8 H8 O5	184.037	5.879	-					
4-Hydroxybicyclo[2.2.2]oct-1-yl acetate	C10 H16 O3	184.11	8.166	-			-		
Valdipromide	C11 H23 N O	185.178	2.203				+		
10-oxo-decanoic acid	C10 H18 O3	186.126	8.477	-			-	-	
Undecylic acid	C11 H22 O2	186.165	9.462			+			
3-amino-2-naphthoic acid	C11 H9 N O2	187.062	6.037	-					
(3S,4R,5S)-5-(aminomethyl)-3,4-dihydroxycyclohex-1-ene-1-carboxylic acid	C8 H13 N O4	187.085	1.556	+					
N-n-Hexanoylglycine methyl ester	C9 H17 N O3	187.121	5.449	-			-		
5,8,11-dodecatriynoic acid	C12 H12 O2	188.084	5.924				-	-	
3-Methylsuberic acid	C9 H16 O4	188.105	6.819	-			-		
CLOPIDOL	C7 H7 Cl2 N O	190.992	1.896	+	+				
citric acid	C6 H8 O7	192.028	1.568	-	-				
quinic acid	C7 H12 O6	192.063	1.775	-	-		-	-	

2E,6E,8Z,10E-dodecatetraenoic acid	C12 H16 O2	192.116	6.825	-			+/-	-
	C9 H20 O4	192.136	4.911		+			
glucuronic acid	C6 H10 O7	194.044	1.174	-				
Azimexone	C9 H14 N4 O	194.116	3.361		+	+	+	
2,6-Diisopropylhydroquinone	C12 H18 O2	194.13	9.832	-			-	
2,6-Diisopropylhydroquinone isomer 2	C12 H18 O2	194.13	10.422	-			-	-
2,6-Diisopropylhydroquinone isomer 1	C12 H18 O2	194.13	9.573	-			-	
2,6-Diisopropylhydroquinone isomer 3	C12 H18 O2	194.132	6.797	-				
	C7 H4 N2 O3 S	195.994	0.351		+	+		
HAEMATOMMIC ACID	C9 H8 O5	196.04	1.233	+				
(3S,4S,5R)-2-(Dihydroxymethyl)tetrahydro-2H-pyran-2,3,4,5-tetrol	C6 H12 O7	196.059	1.155	-				
Alafosfalin	C5 H13 N2 O4 P	196.061	0.659		-			
3-dodecynoic acid	C12 H20 O2	196.145	11.124	-			-	-
	C9 H10 O S2	198.016	5.869	-				
	C8 H6 O6	198.017	3.77	-				
9-acetyl pelargonic acid	C11 H20 O3	200.141	8.321	-			-	-
11-amino-undecanoic acid	C11 H23 N O2	201.173	1.193		+		+	+
	C4 H6 N6 O4	202.046	1.756	+				
	C9 H6 N4 S	202.033	0.871	+/-			-	+/-
2-Naphthaleneacetic acid, 6-hydroxy-	C12 H10 O3	202.061	1.143	-				
sebacic acid	C10 H18 O4	202.12	7.736	-	-		-	

13-tetradecen-2,4-diyn-1-ol	C14 H20 O	204.151	8.339				-	
5-Methoxyindoleacetate	C11 H11 N O3	205.074	6.697	-				
eugenitol	C11 H10 O4	206.06	1.093					-
Ser Thr	C7 H14 N2 O5	206.094	4.19				-	
Furofenac	C12 H14 O3	206.098	6.899		+		+	
	C13 H18 O2	206.13	10.87	-	-			-
OXOTREMORINE	C12 H18 N2 O	206.138	10.331	-	-			
	C10 H22 O4	206.152	6.252	+	+			
CARYLOPHYLLENE OXIDE	C14 H22 O	206.167	11.15	-			-	-
Glucoheptonic acid-1,4-lactone	C7 H12 O7	208.059	1.202	-				
6-methoxymellein	C11 H12 O4	208.074	4.815				-	
5,8-tetradecadienal	C14 H24 O	208.18	7.607	-				
Sedoheptulose	C7 H14 O7	210.072	1.177	-	+			
Methoxyhydroxymethylhydrocinnamic acid	C11 H14 O4	210.089	5.712	-				
jasmonic acid	C12 H18 O3	210.126	9.736				-	-
(3S,7R)-epi-jasmonic acid	C12 H18 O3	210.126	11.746	-				-
altretamine	C9 H18 N6	210.159	8.575				-	
3E,5E-tridecadienoic acid	C13 H22 O2	210.161	9.573	-			-	-
danielone	C10 H12 O5	212.071	7.13				-	
Chrysoidine	C12 H12 N4	212.104	5.672					-
7-Oxo-11-dodecenoic acid	C12 H20 O3	212.141	9.833	-			-	-
Deoxyribose 5-phosphate	C5 H11 O7 P	214.025	1.211	-				

3-oxo-dodecanoic acid	C12 H22 O3	214.157	10.956	-			-	
xanthotoxin	C12 H8 O4	216.04	1.823	-				
10E,12E-tetradecadiene-4,6-diynoic acid	C14 H16 O2	216.115	10.097				-	
FOSFOSAL	C7 H7 O6 P	217.998	0.855	+	+			
5-amino-3,3,3-trihydroxy-3,4-dihydro-7H-3?5-[1,3]azaphospholo[4,5-d]pyrimidin-7-one	C5 H7 N4 O4 P	218.02	1.117		+			
EUPATORIOCHROMENE	C13 H14 O3	218.094	7.257				-	
4-[(6-Hydroxyhexyl)oxy]-4-oxobutanoic acid isomer 1	C10 H18 O5	218.115	6.24	-	+			
4-[(6-Hydroxyhexyl)oxy]-4-oxobutanoic acid isomer 2	C10 H18 O5	218.115	3.769	-				
5-hydroxycalamenene	C15 H22 O	218.166	6.944		+	+		
Pantothenic Acid	C9 H17 N O5	219.11	3.393	+/-	+			
1) 1-(3-Chloropropyl)-4-(2-methoxyethyl)piperidine	C11 H22 Cl N O	219.139	3.081	+	+			
DICAMBA	C8 H6 Cl2 O3	219.97	15.77			-		
3-(Imidazol-4-yl)-2-oxopropyl phosphate	C6 H9 N2 O5 P	220.023	0.873		+			
2,4-Diamino-6,7-dimethoxyquinazoline	C10 H12 N4 O2	220.095	2.83	-				
2-ISOPROPYL-3-METHOXYCINNAMIC ACID	C13 H16 O3	220.111	11.958	-			-	
Lidamidine	C11 H16 N4 O	220.131	3.981	-			-	
Butibufen	C14 H20 O2	220.146	10.933	-	-		-	-
Phenindione	C15 H10 O2	222.072	1.391	-				
Ethyl glucuronide	C8 H14 O7	222.074	1.491	-				
apiole	C12 H14 O4	222.089	4.956	-			-	-

Hydroxyibuprofen	C13 H18 O3	222.125	9.956	-			-	-
KOBUSONE	C14 H22 O2	222.162	9.203	-			-	
Cys Cys	C6 H12 N2 O3 S2	224.027	0.945				-	
7-HYDROXYETHYLTHEOPHYLLINE	C9 H12 N4 O3	224.089	1.192	+				
Aspidinol	C12 H16 O4	224.105	3.878	-			-	
Methyl jasmonate	C13 H20 O3	224.141	10.956	-	-		-	-
Febuprol	C13 H20 O3	224.142	8.013		-			
7-tetradecynoic acid	C14 H24 O2	224.176	7.287				-	-
Xanthene-9-carboxylic acid	C14 H10 O3	226.064	6.863	+	+			
Metyrapone	C14 H14 N2 O	226.115	10.702	-			-	-
Tamitinol	C11 H18 N2 O S	226.116	5.171				-	
Tuberonic acid	C12 H18 O4	226.119	11.715	-			-	
Allixin	C12 H18 O4	226.12	8.667				-	-
Cyclohexyl(1-hydroxycyclopentyl)acetic acid	C13 H22 O3	226.157	10.074	-		+	+/-	+/-
Tibenzate	C14 H12 O S	228.064	1.837	-				
Depdecin	C11 H16 O5	228.1	11.719	-				
12-HYDROXY-4,4-BISNOR-4,8,11,13-PODOCARPATETRAEN-3-ONE	C15 H16 O2	228.115	8.935	+				
Octahydro-4a,9a(2H,5aH)-oxanthrenediol isomer 1	C12 H20 O4	228.136	9.046	-				-
Octahydro-4a,9a(2H,5aH)-oxanthrenediol isomer 2	C12 H20 O4	228.136	5.812	-			-	+
Mifentidine	C13 H16 N4	228.137	7.049		-			

Visnagin	C13 H10 O4	230.058	4.864	-					
BENZANTHRONE	C17 H10 O	230.077	3.186		+				
Nabumetone alcohol	C15 H18 O2	230.131	11.749		+				
13-hydroxy-tridecanoic acid	C13 H26 O3	230.189	6.057	+					
Aloesone	C13 H12 O4	232.074	4.256				-		
d-Camphorsulfonate	C10 H16 O4 S	232.077	4.348	-				-	
Nalidixic acid	C12 H12 N2 O3	232.085	8.864		+				
	C12 H24 O4	232.167	8.206		+				
Lomustine	C9 H16 Cl N3 O2	233.093	1.18	+	+				
7H-Furo[3,2-g][1]benzopyran-7-one, 2,3-dihydro-2-hydroxy-9-methoxy-	C12 H10 O5	234.051	3.702	-					
alpha-Phenylcyclohexylglycolic acid	C14 H18 O3	234.126	5.812				-		
Azoxy-2-procarbazine	C12 H17 N3 O2	235.129	6.835				-		
drimenin	C15 H22 O2	234.163	7.768	+	+	+	+/-	+/-	
FRAXIDIN METHYL ETHER	C12 H12 O5	236.069	4.147				-		
Didanosine	C10 H12 N4 O3	236.091	2.246	-					
DIHYDRO-beta-TUBAIC ACID	C13 H16 O4	236.105	6.379	-					
4-hydroxyphenyl octanoate	C14 H20 O3	236.141	9.453	-			-	-	
capsidiol	C15 H24 O2	236.178	5.949	+	+	+	+	+	
2-Keto-3-deoxyoctonate (KDO)	C8 H14 O8	238.07	1.179	-					
kigelin	C12 H14 O5	238.082	6.562		+				
Isopropyl beta-D-ThiogalactoPyranoside	C9 H18 O5 S	238.088	8.741				-		

Secobarbital	C12 H18 N2 O3	238.132	6.725	-				
NOMIFENSIN	C16 H18 N2	238.142	3.858			+	+	
3-[(1S,2S,4aR,6S,8aS)-6-Hydroxy-2-methyl-1,2,4a,5,6,7,8,8a-octahydro-1-naphthalenyl]propanoic acid	C14 H22 O3	238.157	10.976	-			-	-
Thiram	C6 H12 N2 S4	239.989	0.906	-		+	-	-
Bentazone	C10 H12 N2 O3 S	240.058	7.234		-			
	C17 H8 N2	240.069	1.388		+			
2-Hydroxy-3-(4-methoxyethylphenoxy)-propanoic acid	C12 H16 O5	240.101	11.75				-	
Dinoterb	C10 H12 N2 O5	240.076	11.046		-			
(1R,2R)-3-oxo-2-pentyl-cyclopentanebutanoic acid	C14 H24 O3	240.172	8.493	-			-	-
flavandiol	C15 H14 O3	242.095	10.693					+
3,3'-(2-Oxo-1,1-cyclohexanediyl)dipropanoic acid	C12 H18 O5	242.114	1.917	+	+	+	+	
6E,8E,14E-Hexadecatriene-10,12-diynoic acid	C16 H18 O2	242.129	12.535					+
2) 1-oxa-4-azaspiro[4.5]decane-3,3-dimethanol, 8-propyl-	C13 H25 N O3	243.184	1.856		+		+	
Diethyl 3,4-dihydroxy-2,5-furandicarboxylate	C10 H12 O7	244.056	1.419	+				
Pro Glu	C10 H16 N2 O5	244.107	8.668	-				
OSTHOL	C15 H16 O3	244.111	8.474	-				
Etomidate	C14 H16 N2 O2	244.121	7.519	-			-	-
4-(3-Methoxy-3-oxopropyl)-2,2-dimethyltetrahydro-2H-pyran-4-carboxylic	C12 H20 O5	244.131	6.98	-				

acid								
OXDEMETONMETHYL	C6 H15 O4 P S2	246.017	3.794	-		+		
LEUCODIN	C15 H18 O3	246.126	10.57	+/-		+		
12-METHOXY-4,4-BISNOR-5alpha-8,11,13-PODOCARPATRIEN-3-OL	C16 H22 O2	246.161	9.9				-	
Propanoic acid, 2-hydroxy-3-[(4-hydroxy-1-naphthalenyl)oxy]-	C13 H12 O5	248.069	5.176				-	
Thr Glu	C9 H16 N2 O6	248.105	7.023	-			-	-
8-CYCLOPENTYLTHEOPHYLLINE	C12 H16 N4 O2	248.126	4.061	-				
amiloxate	C15 H20 O3	248.141	10.775	-			-	
salutarisolide	C15 H20 O3	248.142	7.969	+	+	+	+/-	+/-
	C13 H28 O4	248.199	10.247		+	+		
N,O-Didesmethylvenlafaxine	C15 H23 N O2	249.173	8.616		+			
CITRININ	C13 H14 O5	250.084	4.728	-	-		-	-
Desmethylmianserin	C17 H18 N2	250.15	8.396	-	-		-	-
3-(1-adamantyl)-2-oxopropyl acetate	C15 H22 O3	250.153	9.061	+/-	+	+	+/-	+
Trinexapac-ethyl	C13 H16 O5	252.1	5.121				-	
Cimetidine	C10 H16 N6 S	252.117	3.904	+				
Pro His	C11 H16 N4 O3	252.121	2.222	+/-	+			
	C14 H20 O4	252.136	5.4				+	
	C12 H20 N4 S	252.142	6.771	-				-
Didesmethylimipramine	C17 H20 N2	252.166	6.88	-				
PUNCTAPORIN B	C15 H24 O3	252.172	8.524	+/-	+	+	+/-	+

Dyphylline	C10 H14 N4 O4	254.102	3.02	-					
methyl 4-[2-(2-formyl-vinyl)-3-hydroxy-5-oxo-cyclopentyl]-butanoate	C13 H18 O5	254.113	6.953				-		
	C15 H26 O3	254.188	8.243	+					
Clorindione	C15 H9 Cl O2	256.028	0.866	-					
N1,N4-Diacetylsulfanilamide	C10 H12 N2 O4 S	256.05	10.243	-					
EDOXUDINE	C11 H16 N2 O5	256.106	3.045	-					
11-keto pentadecanoic acid	C15 H28 O3	256.2	10.022	-	+				
Mefenamic acid Metabolite (Anthranilic acid, N-(a3-hydroxy-2,3-xylyl)-)	C15 H15 N O3	257.103	1.123	+					
2-cyclohexylpiperidine oxalate	C13 H23 N O4	257.163	2.837				+		
Aceglatone	C10 H10 O8	258.041	1.891	-					
Glucosamine 6-sulfate	C6 H13 N O8 S	259.038	1.083					+	
L-Glutamic acid dibutyl ester	C13 H25 N O4	259.179	1.274	+	+		+		
Ifosfamide	C7 H15 Cl2 N2 O2 P	260.025	0.945				-		
Furafylline	C12 H12 N4 O3	260.088	1.471	+	+				
DIHYDROPTAEROXYLIN	C15 H16 O4	260.108	7.025				-	-	
Gamma-Glu-Leu	C11 H20 N2 O5	260.139	10.558	+	+	+	+	+	
Asn Lys	C10 H20 N4 O4	260.148	7.258				-	-	
Aminoparathion	C10 H16 N O3 P S	261.058	10.539	-				-	
	C11 H19 N O6	261.122	1.215	+	+				
METHYL 7-DESHYDROXYPYROGALLIN-4-	C13 H10 O6	262.048	3.803	-					

CARBOXYLATE								
EPOXY (4,5alpha)-4,5-DIHYDROSANTONIN	C15 H18 O4	262.121	6.865	-				
Parthenin	C15 H18 O4	262.121	8.512	+/-		+	+/-	-
Methohexital	C14 H18 N2 O3	262.132	5.927				-	
Etoglucid	C12 H22 O6	262.141	3.589	-	+			
SULFAMERAZINE	C11 H12 N4 O2 S	264.067	10.666	-				
1-(5-Ketohexyl)-3-methylxanthine	C12 H16 N4 O3	264.123	4.946	-				
absinic acid	C15 H20 O4	264.136	8.958	+/-	+/-	+	+/-	+/-
Stilbamidine	C16 H16 N4	264.138	8.594		-			
5-Hydroxysulfadiazine	C10 H10 N4 O3 S	266.043	3.523	-				
1-(3-Carboxypropyl)-3,7-dimethylxanthine	C11 H14 N4 O4	266.1	1.813	-				
Nevirapine	C15 H14 N4 O	266.117	5.472		-		-	
5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-Pentanoic acid (Gemfibrozil M1)	C15 H22 O4	266.15	9.192	+/-	+/-	+	+/-	+/-
5-[2-(hydroxymethyl)-5-methylphenoxy]-2,2-dimethyl-Pentanoic acid (Gemfibrozil M4)	C15 H22 O4	266.152	8.675	+/-			-	+/-
10,11-epoxy-3,7,11-trimethyl-2E,6E-tridecadienoic acid	C16 H26 O3	266.186	9.503	-			-	-
dimethyl 2-(1,3-benzodioxol-5-yloxy)propanedioate	C12 H12 O7	268.053	7.159	-			-	-
3-Deoxy-D-glycero-D-galacto-2-nonulosonic acid	C9 H16 O9	268.079	2.048	-				
Idebenone Metabolite (QS-4)	C13 H16 O6	268.095	4.564	-			-	
Moclobemide	C13 H17 Cl N2 O2	268.099	3.521	-			-	

Isomethiozin	C12 H20 N4 O S	268.136	9.954	-					
8-Butyl-5,6-dihydrobenzo[f]quinazoline-1,3-diamine	C16 H20 N4	268.167	8.663	+/-	+	+	+/-	+/-
(1R,2R)-3-oxo-2-pentyl-cyclopentanehexanoic acid	C16 H28 O3	268.203	8.65	-			-	-
	C16 H32 N2 O	268.251	1.526	-				
	C15 H27 N O3	269.2	8.51	+	+	+	+	+
Phenyl glucuronide	C12 H14 O7	270.073	3.44	-	-			
Idebenone Metabolite (Benzenebutanoic acid, 2,5-dihydroxy-3,4-dimethoxy-6-methyl-)	C13 H18 O6	270.111	4.918	-	-		-	
	C10 H22 O8	270.131	11.735	-				
	C12 H22 N4 O S	270.152	9.837	-				
Pirogliride	C16 H22 N4	270.18	7.213	+/-			+	
8-keto palmitic acid	C16 H30 O3	270.219	9.645	-			-	-
Arg Pro	C11 H21 N5 O3	271.166	1.586	+	+			
	C14 H25 N O4	271.179	3.878				+	
Lauroylsarcosine	C15 H29 N O3	271.215	7.396	+		+	+	+
QUINALIZARIN	C14 H8 O6	272.031	1.262	-				
Sotalol	C12 H20 N2 O3 S	272.118	6.84	-			-	
N-Despropylpergolide	C16 H20 N2 S	272.131	11.727	-	+			
3-methyl-tetradecanedioic acid	C15 H28 O4	272.197	8.327				-	
5-methyl-tetradecanedioic acid	C15 H28 O4	272.197	5.976	-			-	-
Carboxyprimaquine	C15 H18 N2 O3	274.13	6.955	-	+			

Flumazenil acid	C13 H10 F N3 O3	275.068	6.144	-					
Flutamide	C11 H11 F3 N2 O3	276.068	1.29	-					
Saccharopine	C11 H20 N2 O6	276.134	9.62					-	-
cyclandelate	C17 H24 O3	276.163	7.865	+	+	+	+	+	
Isovalerylglucuronide	C11 H18 O8	278.101	1.757	+				-	
ulopterol	C15 H18 O5	278.115	8.451	-					-
Pantetheine	C11 H22 N2 O4 S	278.133	2.406	-					
Pentoxifylline	C13 H18 N4 O3	278.136	3.327	-					
Phthalic acid Mono-2-ethylhexyl Ester	C16 H22 O4	278.152	6.734	-				-	-
Desmethylondansetron	C17 H17 N3 O	279.132	1.215	+	+				
Moxisylyt	C16 H25 N O3	279.184	9.11	+					
Met Met	C10 H20 N2 O3 S2	280.096	4.934	-					
6-hydroxy-7Z,9E-Octadecadiene-11,13,15,17-tetraynoic acid	C18 H16 O3	280.111	3.969	-					
3-[(4-Carboxy-4-methylpentyl)oxy]-4-methylbenzoic acid (Gemfibrozil M3)	C15 H20 O5	280.129	5.997	+/-				-	+/-
Hydroxystilbamidine	C16 H16 N4 O	280.133	7.841		-				
Pentoxifylline alcohol	C13 H20 N4 O3	280.152	1.918					-	
	C12 H20 N6 O2	280.166	12.134	-					
Benzenepropanoic acid, 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]- (9CI) ASL 8123	C15 H23 N O4	281.163	9.717	+	+	+	+	+	
	C17 H31 N O2	281.236	11.896	+					
Tyr Thr	C13 H18 N2 O5	282.121	10.562			+		+	

Dupracetam	C12 H18 N4 O4	282.132	1.475	+	+			
Gallic acid, 2-ethylhexyl ester isomer 1	C15 H22 O5	282.144	11.807	-				
Gallic acid, 2-ethylhexyl ester isomer 2	C15 H22 O5	282.146	7.459	-	-		-	-
Gallic acid, 2-ethylhexyl ester isomer 3	C15 H22 O5	282.146	5.305	-	-		-	-
Gallic acid, 2-ethylhexyl ester isomer 4	C15 H22 O5	282.146	9.013	-			-	-
Gallic acid, 2-ethylhexyl ester isomer 5	C15 H22 O5	282.146	10.025	-			-	
Octyl gallate	C15 H22 O5	282.148	6.758		-			
10-Hydroxydesipramine	C18 H22 N2 O	282.168	4.208	+	+			
Rhein	C15 H8 O6	284.036	0.893	-				
glyciteii	C16 H12 O5	284.074	0.874		+			
6-Formylindolo [3,2-B] carbazole	C19 H12 N2 O	284.094	1.405	+	+			
FLAVOKAWAIN B	C17 H16 O4	284.108	11.737	-				
Cimetidine sulfoxide	C10 H16 N6 O2 S	284.108	12.293	-				
His Glu	C11 H16 N4 O5	284.109	10.848	-				
Dihydro-tetrazapentacen	C18 H12 N4	284.11	5.904		-			
Zomebazam	C15 H16 N4 O2	284.126	4.321				-	
Dihydroartemisinin	C15 H24 O5	284.161	8.31	-	+	+	-	-
N-(2,3-Dimethylphenyl)-1,3,5-triazaspiro[5.5]undeca-1,4-diene-2,4-diamine	C16 H23 N5	285.195	8.842		+	+	+	+
Gummiferol isomer 1	C16 H14 O5	286.087	8.129					
Gummiferol isomer 2	C16 H14 O5	286.088	11.871	-				
Ethofumesate	C13 H18 O5 S	286.09	3.627		-			

Hydroxytolbutamide	C12 H18 N2 O4 S	286.098	5.678			-	
salicin	C13 H18 O7	286.105	2.688	+/-	+		
Gly Asn Pro	C11 H18 N4 O5	286.13	1.125	+			
Linogliride	C16 H22 N4 O	286.177	6.696			-	
10-hydroxy-16-oxo-hexadecanoic acid	C16 H30 O4	286.212	6.031	-		-	-
C17 Sphinganine	C17 H37 N O2	287.283	9.028		+		
Eriodictyol	C15 H12 O6	288.066	1.396	-			
Xantocilline	C18 H12 N2 O2	288.085	1.828		+		
PIROMIDIC ACID	C14 H16 N4 O3	288.119	3.035	+	+		
4-Methylumbelliferyl heptanoate	C17 H20 O4	288.134	9.186			+	+
N-(3-Indolylacetyl)-L-isoleucine	C16 H20 N2 O3	288.149	7.369				-
Isocarbophos	C11 H16 N O4 P S	289.053	10.725	-			
Sedoheptulose 7-phosphate	C7 H15 O10 P	290.042	1.177	-			
Brompheniramine (didemethylated)	C14 H15 Br N2	290.046	3.003	-			
Catechin	C15 H14 O6	290.08	5.324	-			
ROSOLIC ACID	C19 H14 O3	290.095	1.253	+/-	+		
5-O-Methylvisamminol	C16 H18 O5	290.116	10.017			-	
clavirin I	C17 H22 O4	290.152	11.747		+		
3-(2-chloro-10H-phenothiazin-10-yl)propan-1-amine	C15 H13 D2 Cl N2 S	292.071	3.308	-			
PICROTOXININ	C15 H16 O6	292.096	5.575	-		-	-
TETRAHYDROTRIMETHYLHISPIDIN	C16 H20 O5	292.131	6.517	-		-	-

Benzoylecgonine-d3	C16 H16 D3 N O4	292.15	4.221	-				
N-Acetylmuramic acid	C11 H19 N O8	293.112	10.715	+		+		+
7-Acetamidonitrazepam	C17 H15 N3 O2	293.114	9.184	+				
Dihydrolevobunolol	C17 H27 N O3	293.2	9.892	+				+
Cedrin	C15 H18 O6	294.11	6.426	-			-	-
Glu Phe	C14 H18 N2 O5	294.122	11.7	+	+	+	+	+
Triamiphos	C12 H19 N6 O P	294.132	4.106	+	+			
Tiadenol	C14 H30 O2 S2	294.166	4.292	-				
cinchonine	C19 H22 N2 O	294.168	4.305	+	+			
gingerol	C17 H26 O4	294.181	6.376	+	+	+	+/-	-
Glyconiazide	C12 H13 N3 O6	295.077	4.555	-				
	C17 H29 N O3	295.215	11.178	+		+	+	
Desmethylnaproxen-6-O-sulfate	C13 H12 O6 S	296.03	5.878	-				
Disulfiram	C10 H20 N2 S4	296.047	4.666	-				
flunixin	C14 H11 F3 N2 O2	296.076	1.215	-				
Penicillamine disulfide	C10 H20 N2 O4 S2	296.09	4.378	-				
Idebenone Metabolite (QS-6)	C15 H20 O6	296.125	5.997	-			-	-
Vomitoxin	C15 H20 O6	296.126	11.018	+/-		+	+/-	-
3'-Methoxy-E,E-dienoestrol	C19 H20 O3	296.146	2.872	-	+			
Lactone of PGF-MUM	C16 H24 O5	296.162	9.935	-			-	
	C13 H3 N3 O4 S	296.982	0.846	+	+			
	C17 H31 N O3	297.231	8.515	+		+	+	+

Cycliramine	C18 H19 Cl N2	298.127	1.285	+	+			
Idebenone Metabolite (Benzenehexanoic acid, 2,5-dihydroxy-3,4-dimethoxy-6-methyl-)	C15 H22 O6	298.141	5.997	-				
Trimeprazine	C18 H22 N2 S	298.15	6.742				-	
Alprostadil(15-keto-13,14-dihydro-PGE)	C16 H26 O5	298.176	7.054	-			-	-
Artemether	C16 H26 O5	298.178	8.306	-			-	-
EMODIC ACID	C15 H8 O7	300.028	8.356	-				
Lomevactone	C18 H17 Cl O2	300.088	2.536	-	-			
5-NITRO-2-PHENYLPROPYLAMINOBENZOIC ACID [NPPB]	C16 H16 N2 O4	300.113	9.714				-	
Demethylclomipramine	C18 H21 Cl N2	300.139	11.15	-			-	-
Azapropazone	C16 H20 N4 O2	300.157	7.689				-	
5a,7a-Dihydroxy-11-ketotetranorprostanoic acid	C16 H28 O5	300.193	5.462					-
TEGASEROD	C16 H23 N5 O	301.19	2.745			+	+	
quercetin	C15 H10 O7	302.043	6.707	-				
HAEMATOXYLIN	C16 H14 O6	302.078	1.135	-				
Cambendazole	C14 H14 N4 O2 S	302.085	2.918		-			
Terizidone	C14 H14 N4 O4	302.098	2.376	+	+			
MUCRONULATOL((+/-))	C17 H18 O5	302.114	11.349		+			
benzeneacetic acid, ?-phenyl-, 4-methylphenyl ester	C21 H18 O2	302.128	9.661	-	+		-	-
Ergoline-1,8-dimethanol, 10-methoxy-, (8b)-	C17 H22 N2 O3	302.167	5.086				-	

Ala Arg Gly	C11 H22 N6 O4	302.17	6.876				-		
DIHYDROROBINETIN	C15 H12 O7	304.057	6.116	-					
Melphalan	C13 H18 Cl2 N2 O2	304.076	11.695				-		
METHYL 7-DESOXYPURPUROGALLIN-7-CARBOXYLATE TRIMETHYL ETHER	C16 H16 O6	304.095	6.465				-	-	
gallocatechin	C15 H14 O7	306.074	4.776	-					
Gln Gly Thr	C11 H20 N4 O6	304.138	7.519				-		
Gly Gln Cys	C10 H18 N4 O5 S	306.096	1.239	-					
Matricin	C17 H22 O5	306.146	7.97	+/-	+	+	+/-	+/-	
dihydrocapsaicin	C18 H29 N O3	307.215	12.609	+					
4-Methyl-2-oxo-2H-chromen-7-yl ?-D-xylopyranoside	C15 H16 O7	308.09	4.719	-			-		
indicaxanthin	C14 H16 N2 O6	308.107	6.196				-		
TYRPHOSTIN B44 (-)	C18 H16 N2 O3	308.114	6.136	-					
2-[(1E)-1-(1-Cyclohexyl-2,4,6-trioxotetrahydro-5(2H)-pyrimidinylidene)ethyl]hydrazinecarboximidamide	C13 H20 N6 O3	308.162	9.382	-			-	-	
2-Pyrrolidinone, 4-(2-aminoethyl)-1-ethyl-3,3-diphenyl- (AHR 5904)	C20 H24 N2 O	308.184	3.709	-					
His Gly Pro	C13 H19 N5 O4	309.146	1.478	+	+				
Nadolol	C17 H27 N O4	309.195	9.716	+	+	+	+	+	
1-O-Acetyl-2,3-O-isopropylidene-5-O-(methylsulfonyl)-?-D-ribofuranose	C11 H18 O8 S	310.067	3.188	+	+				
Sulfadoxine	C12 H14 N4 O4 S	310.069	2.82	-					

2-Naphthalenepropanol, 6-methoxy-a-methyl-, hydrogen sulfate	C15 H18 O5 S	310.091	1.225	-					
PICROTIN	C15 H18 O7	310.107	6.665					-	-
Glu Tyr	C14 H18 N2 O6	310.117	10.717	+	+	+	+	+	
BIFONAZOLE	C22 H18 N2	310.145	10.263	+/-			-		
Demethylcitalopram	C19 H19 F N2 O	310.148	7.591					-	
methyl 8-[2-(2-formyl-vinyl)-3-hydroxy-5-oxo-cyclopentyl]-octanoate	C17 H26 O5	310.176	8.483	-			-	-	
Staurosporine aglycone	C20 H13 N3 O	311.101	4.36	-					
	C18 H25 N5	311.211	7.82				+	+	
Nivalenol	C15 H20 O7	312.119	4.365	-			-		
2-Methyl-2-propanyl (2S,3R,4R,5S,6S,7R)-2,3,4,5,6,7,8-heptahydroxyoctanoate	C12 H24 O9	312.142	2.222	-					
Granisetron	C18 H24 N4 O	312.192	5.398	+	+				
	C18 H27 N5	313.226	9.246		+	+	+		
Salicyl acyl glucuronide	C13 H14 O9	314.064	4.039	-					
Dantrolene	C14 H10 N4 O5	314.065	4.284	-					
S,S,S,-TRIBUTYLPHOSPHOROTRITHIOATE	C12 H27 O P S3	314.101	4.559	+			-	-	
chicoric acid	C22 H18 O2	314.136	4.124	+/-	+		-		
Granisetron metabolite 4	C17 H22 N4 O2	314.176	8.396	-			-		
2,4,5-Trichlorophenyl carbanilate	C13 H8 Cl3 N O2	314.96	3.802	-					
Gly His Cys	C11 H17 N5 O4 S	315.099	5.872	+					
1,8-Diamino-5-cyano-3-methoxy-6-(4-methylpiperazin-4-ium-1-yl)-2,7-	C15 H21 N7 O	315.182	6.745			+	+	+	

naphthyridin-2-ium							
isohamnetin	C16 H12 O7	316.061	1.261	-			
	C13 H16 O9	316.08	2.413	-			
	C11 H20 N6 O S2	316.114	3.518	+			
EPIAFZELECHIN TRIMETHYL ETHER	C18 H20 O5	316.129	7.865	+	+	+	
Timolol	C13 H24 N4 O3 S	316.152	3.143	-			
Ala Lys Val	C14 H28 N4 O4	316.209	3.787			+	
Combretastatin A-4	C18 H20 O5	316.129	9.942	+	+	+	
	C9 H3 N O8 S2	316.929	1.165	-			
myricetin	C15 H10 O8	318.038	5.899	-			
Gly Trp Gly	C15 H18 N4 O4	318.129	2.867	+	+		
Zearalenone	C18 H22 O5	318.15	10.491	-		-	
Fluvoxamine	C15 H21 F3 N2 O2	318.155	3.986	-			
	C13 H5 N O S4	318.926	1.164	-			
Dihydrotetrabenazine	C19 H29 N O3	319.215	10.361	-			
Dihydromyricetin	C15 H12 O8	320.053	5.878	+/-			
MYCOPHENOLIC ACID	C17 H20 O6	320.124	5.135				-
ENOXACIN	C15 H17 F N4 O3	320.13	7.175	-			-
Ser Thr Asn	C11 H20 N4 O7	320.134	7.429	-		-	
Asn Thr Ser	C11 H20 N4 O7	320.135	7.402				-
Thr Ser Asn	C11 H20 N4 O7	320.14	5.583	-			
1-({4-[(Cyclopropylmethyl)amino]-6-	C14 H20 N6 O3	320.159	10.519	-		-	-

(isopropylamino)-1,3,5-triazin-2-yl}oxy)-2,5-pyrrolidinedione									
Cys Cys Pro	C11 H19 N3 O4 S2	321.079	10.987	-			-	-
phaseolin	C20 H18 O4	322.122	8.386	+		+	+	
Pumitepa	C12 H19 N8 O P	322.141	7.665	-			-	
7-hydroxy Tetranor Iloprost	C18 H26 O5	322.178	9.081	-			-	-
Betaxolol(hydroxylation)	C18 H29 N O4	323.21	9.614	-				
Phe Ala Ser	C15 H21 N3 O5	323.146	6.738	+				
N-(2-Furylmethyl)-2-(4-methyl-1-piperazinyl)-4-quinazolinamine	C18 H21 N5 O	323.174	8.386	+	+	+	+	+
Bromadolin	C15 H21 Br N2 O	324.086	4.912	-				
	C17 H16 N4 O3	324.124	5.282	-			-	
Galactosylhydroxylysine	C12 H24 N2 O8	324.154	7.238	-			-	
Idebenone Metabolite (QS-8)	C17 H24 O6	324.158	6.332	+/-	+	+	+/-	+/-
Citalopram (propionic acid derivative)	C19 H16 F N O3	325.117	3.396	-				
Acetylaminodantrolene	C16 H14 N4 O4	326.101	4.305	-				
(1s, 4R, 5S, 8S)-4,8-dimethyl-4[(phenylsulfonyl)methyl]-2,3-dioxabicyclo[3.3.1] nonan-8-ol	C16 H22 O5 S	326.122	1.152	+				-
Tetranor-PGEM	C16 H22 O7	326.137	4.007	-		+	+/-	-
Pro Pro Asn	C14 H22 N4 O5	326.157	3.041	-			-	
azaperone	C19 H22 F N3 O	327.176	8.674	-				
BERGENIN	C14 H16 O9	328.082	1.183		+			
Eclanamin	C16 H22 Cl2 N2 O	328.117	4.831					-

DEOXYSAPPANONE B TRIMETHYL ETHER	C19 H20 O5	328.131	7.047				-	
9,15-dioxo-11R-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioic acid	C16 H24 O7	328.152	11.155	-			+	
Gly Arg Pro	C13 H24 N6 O4	328.187	6.035	-			-	-
endo-1-methyl-N-(9-methyl-9-azabicyclo[3.3.1]non-3-yl)-N-oxide	C18 H24 N4 O2	328.188	7.837	-			-	-
11-hydroperoxy-12,13-epoxy-9-octadecenoic acid	C18 H32 O5	328.224	8.359	-			-	-
Paroxetine	C19 H20 F N O3	329.147	9.171	-			-	
Prenylamine	C24 H27 N	329.221	8.295	+		+		
9S,10S,11R-trihydroxy-12Z-octadecenoic acid	C18 H34 O5	330.242	7.441		-			
3',5'-Cyclic Inosine monophosphate (cIMP)	C10 H11 N4 O7 P	330.036	3.812	-				
	C14 H18 O9	330.095	3.157	-				
8-Epiiridodial glucoside	C16 H26 O7	330.167	5.389	-	+			
Val Arg Gly	C13 H26 N6 O4	330.204	6.827	-			-	-
9S,10S,11R-trihydroxy-12Z-octadecenoic acid	C18 H34 O5	330.241	8.811	-	+		-	-
2,4-DICHLOROPHENOXYACETIC ACID, ISOOCTYL ESTER	C16 H22 Cl2 O3	332.094	5.32	-				
combretastastin A-1	C18 H20 O6	332.132	11.166	+/-	-	+/-	+/-	-
Trp Lys	C17 H24 N4 O3	332.183	5.479	+/-				
4-Oxo-norfloxacin	C16 H16 F N3 O4	333.106	3.07	-				
Tuclazepam	C17 H16 Cl2 N2 O	334.07	6.19	+				
plicadin	C20 H14 O5	334.087	6.678	+	+			

1-Naphthylmethanol glucuronide	C17 H18 O7	334.107	1.118	-			-	-
combretastastin	C18 H22 O6	334.141	9.934	-			-	-
	C20 H25 N5	335.211	11.752		+			
Clonixeril	C16 H17 Cl N2 O4	336.085	4.991				-	
berberine	C20 H18 N O4	336.122	6.946	-			-	-
Phe Asn Gly	C15 H20 N4 O5	336.142	3.214	-				
fluoxymesterone	C20 H29 F O3	336.212	5.546	-				
Decimemide	C19 H31 N O4	337.226	1.195	+				
7-O-Methylsterigmatocystin	C19 H14 O6	338.08	10.973	-			-	-
Moxnidazole	C13 H18 N6 O5	338.137	7.259			+	+	+
	C18 H21 N5 O2	339.169	10.336	+				
Perazine	C20 H25 N3 S	339.17	6.746				+	
Diethylpropion(metabolite X-glucuronide)	C17 H23 N8	339.205	6.73	+			+	
AESCULIN	C15 H16 O9	340.08	3.456	-				
p,gamma-Dihydroxyphenylbutazone	C19 H20 N2 O4	340.137	2.655	-			-	
Asn His Ala	C13 H20 N6 O5	340.151	5.89			+	-	
Diethylpropion(metabolite VIII-glucuronide)	C15 H19 N O8	341.112	7.866	+				
Fenetylline	C18 H23 N5 O2	341.185	3.301	+				
ferulic acid dehydrodimer	C19 H18 O6	342.117	1.184	+/-		+		
Megestrol	C22 H30 O3	342.219	1.133	+		+	+	
Dolichyl diphosphate	C12 H26 O7 P2	344.111	1.153		+			
Deoxyelephantopin	C19 H20 O6	344.124	8.39	+		+	+	

Iridotrial glucoside	C16 H24 O8	344.147	5.668	-			
Clopamide	C14 H20 Cl N3 O3 S	345.092	4.84	-			
S 26191	C15 H17 F3 N2 O4	346.11	9.019	-		-	
Deutzioside	C15 H22 O9	346.124	6.199	+			
Aucubin	C15 H22 O9	346.126	2.687	-	+		
CATECHIN TETRAMETHYLETHER	C19 H22 O6	346.139	9.742			-	-
Ala Ala Trp	C17 H22 N4 O4	346.163	5.496	+/-	+		
Pergolide sulfone	C19 H26 N2 O2 S	346.173	4.865	-			
Ala Thr Arg	C13 H26 N6 O5	346.198	5.792	-		-	
9-hydroperoxy-12,13-dihydroxy-10-octadecenoic acid	C18 H34 O6	346.236	7.328		+		
1-Naphthoic acid glucuronide	C17 H16 O8	348.086	2.799	-			
Torasemide	C16 H20 N4 O3 S	348.126	8.748	-			
Asn Asp Thr	C12 H20 N4 O8	348.127	10.156	-			
Gibberellin A29	C19 H24 O6	348.155	12.332	-		-	-
Cys Ala Arg	C12 H24 N6 O4 S	348.161	8.511			-	
Naltaiofos	C16 H16 N O6 P	349.074	10.712			-	
His Pro Pro	C16 H23 N5 O4	349.174	5.293	-			
Cetiedil	C20 H31 N O2 S	349.211	5.549		+		
Asp Asn Cys	C11 H18 N4 O7 S	350.091	1.205	-			
FELAMIDIN	C21 H18 O5	350.117	1.164		+		
Estrone sulfate	C18 H22 O5 S	350.122	3.138	-			

Phe Ala Asn	C16 H22 N4 O5	350.157	5.583	-				
Phe Lys Gly	C17 H26 N4 O4	350.193	4.653	-	+			
dipivefrin	C19 H29 N O5	351.204	10.921	+/-	+	+	+	+
4-Methylumbelliferyl beta-D-glucuronide	C16 H16 O9	352.079	3.442	-				
Cys Gln Cys	C11 H20 N4 O5 S2	352.092	3.07	-				
Deacetylaloesin	C17 H20 O8	352.116	5.854	-			-	-
Estradiol-17beta 3-sulfate	C18 H24 O5 S	352.135	3.681	-				
Gly Tyr Asn	C15 H20 N4 O6	352.137	2.5	-				
ajmalicine	C21 H24 N2 O3	352.173	4.217	-				
chlorogenic acid	C16 H18 O9	354.1	5.509	+/-				
Isofazolac	C23 H18 N2 O2	354.132	6.441		-		-	
Propofol glucuronide	C18 H26 O7	354.167	7.904	-				
Minocromil	C18 H16 N2 O6	356.104	0.527		-			
Gentiopicrin	C16 H20 O9	356.11	3.498	-			-	
Asn His Ser	C13 H20 N6 O6	356.149	4.222	+/-	+			
	C22 H28 O2 S	356.181	11.734	-				
PSOROMIC ACID	C18 H14 O8	358.068	6.215	-				
Mebeverine metabolite (Veratric acid glucuronide)	C15 H18 O10	358.091	5.229	+	+			
sweroside	C16 H22 O9	358.119	7.214	-			-	
2-Phenylaminoadenosine	C16 H18 N6 O4	358.14	7.667	-			-	
matairesinol	C20 H22 O6	358.142	8.242	-				

Canrenoate	C22 H30 O4	358.217	6.946	-			
Chlorthiophos	C11 H15 Cl2 O3 P S2	359.957	5.872	-			
	C27 H4 O2	360.022	0.875	-			
rosmarinic acid	C18 H16 O8	360.088	7.741	-			
3-Methoxy-4-hydroxyphenylglycol glucuronide	C15 H20 O10	360.106	3.479	-			
Elephantopin	C19 H20 O7	360.124	8.945			-	
NITRENDIPINE	C18 H20 N2 O6	360.134	6.593			-	
Asn Asn Asn	C12 H20 N6 O7	360.141	10.544	+/-	+	-	
lariciresinol	C20 H24 O6	360.156	5.071	+	+		
Thr Glu Leu	C15 H27 N3 O7	361.189	10.811		+		
Piretanide	C17 H18 N2 O5 S	362.092	2.654		+		
Asp Asp Asn	C12 H18 N4 O9	362.111	1.063			+	-
Gibberellin A8-catabolite	C19 H22 O7	362.134	11.881	-		-	
Gibberellin A8	C19 H24 O7	364.15	8.834	-			-
Ala Ser Trp	C17 H22 N4 O5	362.159	8.335	+			
secoisolariciresinol	C20 H26 O6	362.173	9.867			-	
Phe Ala Gln	C17 H24 N4 O5	364.173	4.115	+/-	+		
Ibuterol	C20 H31 N O5	365.221	4.116	+			
Tifencillin	C16 H18 N2 O4 S2	366.075	10.705			-	
DERRUBONE	C21 H18 O6	366.114	1.207	-			
Cyclovalone	C22 H22 O5	366.146	5.388	-			

Tyr Gly Lys	C17 H26 N4 O5	366.19	10.387	+		
Asp Pro His	C15 H21 N5 O6	367.148	4.45	+		
2-Hydroxy-N'-(3-nitrobenzoyl)-4-oxo-1,4-dihydro-3-quinolinecarbohydrazide	C17 H12 N4 O6	368.075	4.042	-		
Fludoxopone	C21 H21 F N2 O3	368.147	6.413	-		
GPA(6:0/6:0)	C15 H29 O8 P	368.163	5.772	-		
Testosterone sulfate	C19 H28 O5 S	368.169	4.112	-		
Asn Val His	C15 H24 N6 O5	368.181	1.329	+	+	
N-[(diphenylmethoxy)acetyl]-Glutamine	C20 H22 N2 O5	370.152	5.538			-
Lys His Ser	C15 H26 N6 O5	370.199	10.075	-	+	
Asn His Thr	C14 H22 N6 O6	370.159	5.467			+
Sulindac sulfone	C20 H17 F O4 S	372.082	3.701	-		
tangeritin	C20 H20 O7	372.128	1.146	-		
Hydroxyprogesterone acetate	C23 H32 O4	372.228	6.947	+		
13,14-dihydro-19(R)-hydroxyPGE1	C20 H36 O6	372.251	7.151	-		
6-O-Demethylterazosin	C18 H23 N5 O4	373.174	11.724	+		
Deoxyloganin	C17 H26 O9	374.158	4.48		+	
Leu Met Ile	C17 H33 N3 O4 S	375.227	10.739		+	
Picolinafen	C19 H12 F4 N2 O2	376.078	4.699	-		
Asp Asn Glu	C13 H20 N4 O9	376.116	6.837		+	-
Vitamin B2 (riboflavin)	C17 H20 N4 O6	376.136	3.307	-		
Arg Thr Thr	C14 H28 N6 O6	376.209	5.23			

carbenicillin	C17 H18 N2 O6 S	378.093	1.148	-		
Phe Val Asn	C18 H26 N4 O5	378.189	4.494	-	-	
	C18 H5 N O9	378.996	1.383	-		
Nicolinamidomethylaminopyrazolone	C21 H25 N5 O2	379.2	9.489		+	
Nicolinamidomethylaminopyrazolone	C21 H25 N5 O2	379.201	6.86		-	
	C21 H33 N O5	379.236	11.23	+		
fludrocortisone	C21 H29 F O5	380.199	3.476	-		
Azelastine	C22 H24 Cl N3 O	381.163	3.257	-		
Pro Glu His	C16 H23 N5 O6	381.165	3.471	-		
Olsalazine sulfate	C14 H10 N2 O9 S	382.013	0.9	-		-
3-iodo-hexadecanoic acid	C16 H31 I O2	382.138	6.146	-		
Glibornuride M4	C18 H26 N2 O5 S	382.164	7.086			-
Leu Asn His	C16 H26 N6 O5	382.192	7.019	-		
S-Adenosylhomocysteine	C14 H20 N6 O5 S	384.127	1.386	-		
His Asn Asp	C14 H20 N6 O7	384.14	3.976		+	
19-hydroxy-17-oxoandrost-5-en-3-beta-yl sulfate	C19 H28 O6 S	384.155	4.728	-		
3b,16a-Dihydroxyandrostenone sulfate	C19 H28 O6 S	384.163	4.276	-		
Thr His Gln	C15 H24 N6 O6	384.177	5.203	+	+	
Lys Thr His	C16 H28 N6 O5	384.214	5.479	-		
Pro Arg Asp	C15 H26 N6 O6	386.194	4.99	-	+/-	+
Oleandolide	C20 H34 O7	386.23	6.352	-		

DIHYDROSAMIDIN	C21 H24 O7	388.152	8.057	+/-				
Nisoldipine	C20 H24 N2 O6	388.165	6.887	-				
Gln Asn Gln	C14 H24 N6 O7	388.171	8.4	+	+			
methyl 9,12,13,15-bisepidioxy-16-hydroperoxy-10-octadecenoate	C19 H32 O8	388.21	5.522		+			
Arg Leu Thr	C16 H32 N6 O5	388.24	5.177	-				
Cefaloram	C18 H18 N2 O6 S	390.095	5.971	-				
resveratrol glucoside	C20 H22 O8	390.132	7.979				-	-
loganin	C17 H26 O10	390.152	11.153	-				
Trp Trp	C22 H22 N4 O3	390.167	6.386	+/-		+	+	
Val Trp Ser	C19 H26 N4 O5	390.189	5.103	-				
Ser Trp Val	C19 H26 N4 O5	390.19	9.945	+	+			
4-Formyl-2-methoxyphenyl 4,6-O-(2-furylmethylene)hexopyranoside	C19 H20 O9	392.114	1.173	-				
b-D-Glucopyranosiduronic acid, 3-(6-hydroxy-2-naphthalenyl)-1-methylpropyl	C20 H24 O8	392.145	7.572	-				
Asn Tyr Pro	C18 H24 N4 O6	392.171	6.052	-				
Met Arg Ser	C14 H28 N6 O5 S	392.182	5.094					
Dexamethasone	C22 H29 F O5	392.199	6.186	-				
Phe Asn Ile	C19 H28 N4 O5	392.204	4.991	-			-	
GPA(16:0/0:0)[cyclic]	C19 H37 O6 P	392.233	5.052	-				
aloesin	C19 H22 O9	394.125	6.348	+				
Val Tyr Asn	C18 H26 N4 O6	394.184	6.653	+/-			-	

10,11-epoxy-chlorovulone I	C21 H29 Cl O5	396.171	4.719	-				
Quinine ethylcarbonate	C23 H28 N2 O4	396.202	3.866		-			
Ile His Gln	C17 H28 N6 O5	396.211	6.975	-				
Sulfasalazine	C18 H14 N4 O5 S	398.074	1.073			+		+
Deoxypodophyllotoxin	C22 H22 O7	398.135	3.982	-				
Trp His Gly	C19 H22 N6 O4	398.175	5.063	-			-	
Lys Asp His	C16 H26 N6 O6	398.191	5.71	-				
Arg Pro Glu	C16 H28 N6 O6	400.206	6.02	-				
Ethinylestradiol benzoate	C27 H28 O3	400.211	4.745		-			
6,6'-(1-Oxo-2,3-dihydro-1H-indene-2,2-diyl)bis(3-amino-5H-imidazo[2,1-c][1,2,4]triazol-5-one)	C17 H10 N10 O3	402.095	6.785	-				
Thioridazine 5-sulfone	C21 H26 N2 O2 S2	402.145	7.029				-	
Delmadinone acetate	C23 H27 Cl O4	402.154	5.317	-				
Thr Pro Trp	C20 H26 N4 O5	402.189	4.247	+/-	+			
Cinitapride	C21 H30 N4 O4	402.227	5.774		-			
Uridine diphosphate (UDP)	C9 H14 N2 O12 P2	403.997	0.913				-	-
PICEID	C21 H24 O8	404.147	7.017	-	+			
SERICETIN	C25 H24 O5	404.163	5.124	-				
Trp Ser Leu	C20 H28 N4 O5	404.204	10.273	-				
Tyr Pro Gln	C19 H26 N4 O6	406.184	5.494	-				
Lincomycin	C18 H34 N2 O6 S	406.212	5.489	-				

Phe Gln Leu	C20 H30 N4 O5	406.22	4.399	+/-	+	
3-(a-Naphthoxy)lactic acid glucuronide	C19 H20 O10	408.104	5.778	+	+	
Phe Gly Trp	C22 H24 N4 O4	408.177	6.075	+	+	
Met Lys Met	C16 H32 N4 O4 S2	408.187	6.354	-		
Leu Asn Tyr	C19 H28 N4 O6	408.199	7.742	-		
Lys Phe Asp	C19 H28 N4 O6	408.2	4.732	-		
butoconazole	C19 H17 Cl3 N2 S	410.015	0.944			-
Aloesol	C20 H26 O9	410.156	4.984	+	+	
Thr Tyr Lys	C19 H30 N4 O6	410.215	6.544	-	-	
His Lys Glu	C17 H28 N6 O6	412.209	6.013	-	-	
narcotine	C22 H23 N O7	413.155	9.686		+	
Phe Thr Phe	C22 H27 N3 O5	413.192	5.198	-		
Podophyllotoxin (Podophyllum)	C22 H22 O8	414.134	3.966	-		
Diltiazem	C22 H26 N2 O4 S	414.159	4.35	-		
methyl 9,11-epidioxy-12,15-dihydroperoxy-5,7,13-eicosatrienoate	C21 H34 O8	414.223	6.109	-		-
methyl 6,8-epidioxy-5,15-dihydroperoxy-9,11,13-eicosatrienoate	C21 H34 O8	414.226	6.947	-		-
Trp Pro Leu	C22 H30 N4 O4	414.226	6.6	+/-	+	
Zuclopenthixol sulfoxide	C22 H25 Cl N2 O2 S	416.132	3.576	-		
Asp Trp Pro	C20 H24 N4 O6	416.169	4.98	-	-	
Glu Arg Leu	C17 H32 N6 O6	416.238	5.192	-	+	

Val Trp Ile	C22 H32 N4 O4	416.242	6.1	+			-	
(24S,25R)-25,26-epoxy-1alpha,24-dihydroxy-27-norvitamin D3/(24S,25R)-25,26-epoxy-1alpha,24-dihydro	C26 H40 O4	416.291	10.224	-				
Asp His Phe	C19 H23 N5 O6	417.167	7.724					+
Arg Arg Ser	C15 H31 N9 O5	417.245	7.587	+				
Thioridazine-2-sulfone-5-sulfoxide	C21 H26 N2 O3 S2	418.141	5.317	-				
	C23 H22 N4 O4	418.164	10		+			
Artelinic acid	C23 H30 O7	418.202	10.681		+			
Leu Thr Trp	C21 H30 N4 O5	418.22	10.554	-	+		+	
Met Arg Asp	C15 H28 N6 O6 S	420.18	6.697	+	+			
7alpha-(Thiomethyl)spironolactone sulfone	C23 H32 O5 S	420.196	11.143	-				
Isoflupredone acetate	C23 H29 F O6	420.196	10.437	-				
alpha,alpha'-Trehalose 6-phosphate	C12 H23 O14 P	422.075	1.211	-				
Asp Cys Trp	C18 H22 N4 O6 S	422.122	7.723			+	+	+/-
Phe Gln Glu	C19 H26 N4 O7	422.179	3.982	-				
Valtratum	C22 H30 O8	422.195	7.111	-				
fludrocortisone acetate	C23 H31 F O6	422.21	4.337	-				
Glu Lys Phe	C20 H30 N4 O6	422.215	8.274	-				
	C28 H38 N6 O4	522.296	11.143			+		
Tyr Lys Asn	C19 H29 N5 O6	423.214	6.925		+			
salicortin	C20 H24 O10	424.143	4.907		+			

Tyr Lys Asp	C19 H28 N4 O7	424.193	4.38	-			
Lys Tyr Asp	C19 H28 N4 O7	424.194	4.115	-			
GPA(8:0/8:0)	C19 H37 O8 P	424.221	5.158	-			
2beta,3alpha,7alpha,12alpha-Tetrahydroxy-5beta-cholan-24-oic Acid	C24 H40 O6	424.279	10.963	-		-	-
Asn Phe Phe	C22 H26 N4 O5	426.189	6.655	-	-		
9-Hydroxyrisperidone	C23 H27 F N4 O3	426.207	7.016	-	-		
	C13 H8 N4 O9 S2	427.975	14.659	-		-	-
Pyridinoline	C18 H28 N4 O8	428.189	6.176	-			
Nitracridine	C22 H28 N4 O5	428.204	5.287	-	-		
(23S,25R)-25-hydroxyvitamin D3 26,23-lactone/(23S,25R)-25-hydroxycholecalciferol 26,23-lactone	C27 H40 O4	428.292	10.192	-		-	
Pro Trp Lys	C22 H31 N5 O4	429.237	6.398			+	+
Congocidin (Netropsin)	C18 H26 N10 O3	430.222	4.168		-		
Phe Glu His	C20 H25 N5 O6	431.18	6.308		+		
Arg Gln Glu	C16 H29 N7 O7	431.207	10.869	-			
Trp Asn Leu	C21 H29 N5 O5	431.218	6.107	-			
Hydroxysalmeterol	C25 H37 N O5	431.266	4.963		+		
vitexin	C21 H20 O10	432.103	6.064	-			
Asn Trp Asn	C19 H24 N6 O6	432.179	9.493	+			
Buclizine	C28 H33 Cl N2	432.236	6.782	+	+		
Trp Gln Thr	C20 H27 N5 O6	433.196	4.975		+		

	C22 H35 N5 O4	433.268	6.209	+	+		
2-Amino-3-hydroxy-5-nitrobenzophenone glucuronide	C19 H18 N2 O10	434.097	6.145	-			
SCANDENIN	C26 H26 O6	434.171	4.141	-	-		
MUNDULONE	C26 H26 O6	434.176	11.895	-			
Pro Arg Tyr	C20 H30 N6 O5	434.226	6.052	-			
nothofagin	C21 H24 O10	436.136	6.835	+	+	-	
Asp Phe Arg	C19 H28 N6 O6	436.207	7.059		+		
GPA(18:1(9Z)/0:0)	C21 H41 O7 P	436.259	10.207			-	
1-[[2-(2,3-dihydro-2-oxo-1H-indol-4-yl)ethyl]propylcarbamate] glucuronide	C20 H26 N2 O9	438.166	4.247	-			
Lys Glu Tyr	C20 H30 N4 O7	438.213	5.483	-			
Thr Tyr Arg	C19 H30 N6 O6	438.224	6.88			+	
GPA(18:0/0:0)	C21 H43 O7 P	438.274	10.548	-		-	-
Phe Phe Gln	C23 H28 N4 O5	440.205	6.654	-	-	-	
3beta-HYDROXYDEOXYDESACETOXY-7-OXOGEDUNIN	C26 H32 O6	440.224	6.064	-			
3"-HydroxyPravastatin	C23 H36 O8	440.24	6.438	+			
Hydroxydomperidone(M2a)	C22 H24 Cl N5 O3	441.155	3.966	-			
S-(4-Nitrobenzyl)glutathione	C17 H22 N4 O8 S	442.111	4.086	-			
Cortisol 21-sulfate	C21 H30 O8 S	442.167	1.266	-			
1alpha-hydroxy-18-(4-hydroxy-4-methyl-2-pentynyloxy)-23,24,25,26,27-pentanorvitamin D3/1alpha-hydr	C28 H42 O4	442.309	10.201	-		-	

Met Phe Phe	C23 H29 N3 O4 S	443.183	7.034	-		
Mitoxantrone	C22 H28 N4 O6	444.2	6.455	+	+	
Desmethylastemizole	C27 H29 F N4 O	444.234	6.156	-		
1(3)-glyceryl-6-keto-PGF1alpha	C23 H40 O8	444.274	9.991	-		
2-ETHOXYCARBONYL-5,7-DIHYDROXY-8,3',4',5'-TETRAMETHOXYISOFLAVONE	C22 H22 O10	446.13	4.352	-		
Trp Glu Leu	C22 H30 N4 O6	446.215	4.372	+/-		
Glu Trp Ile	C22 H30 N4 O6	446.215	5.448	-		-
Trp Glu Leu	C22 H30 N4 O6	446.216	4.379	-	+	
Fluocortin butyl	C26 H35 F O5	446.251	5.037	-	-	
(23S)-1alpha,23,25-trihydroxy-24-oxovitamin D3/(23S)-1alpha,23,25-trihydroxy-24-oxocholecalciferol	C27 H42 O5	446.303	10.521			-
Asp Trp Lys	C21 H29 N5 O6	447.211	4.372	+	+	
	C25 H12 N4 O5	448.08	7.4	-		
His His Arg	C18 H28 N10 O4	448.228	5.516	+	+	
luteolin	C21 H20 O11	448.101	6.649	-		
His His Arg	C18 H28 N10 O4	448.23	5.105	-		
Arg His His	C18 H28 N10 O4	448.23	5.533	-		
Algestone acetophenide	C29 H36 O4	448.265	5.179	-		
IRIGENIN, 7-BENZYL ETHER	C25 H22 O8	450.138	1.269	-		
Asp Met Trp	C20 H26 N4 O6 S	450.157	4.194	-		

aspalathin	C21 H24 O11	452.132	4.301	-		
ent-Copalyl diphosphate	C20 H36 O7 P2	450.188	4.881	+	+	
Fluocinolone acetonide	C24 H30 F2 O6	452.201	5.778	+	+	
2H-Indol-2-one, 4-[2-(dipropylamino)ethyl]-1,3-dihydro-7-hydroxy- glucuronide	C22 H32 N2 O8	452.216	6.37	-		
octadecanoic acid-1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl ester	C21 H35 F7 O2	452.254	10.963	-		-
GANGLEOIDIN ACETATE	C20 H16 Cl2 O8	454.02	4.539	-		
Carfecillin	C23 H22 N2 O6 S	454.111	2.539	-	-	
Bencianol	C28 H22 O6	454.138	9.609			-
Cys Trp Phe	C23 H26 N4 O4 S	454.169	1.258	-		
His Ile Trp	C23 H30 N6 O4	454.232	7.22	-		
Tyr Gln Phe	C23 H28 N4 O6	456.2	5.317	-		
Phe Tyr Lys	C24 H32 N4 O5	456.232	7.042	+	+	
Tyr Tyr Asn	C22 H26 N4 O7	458.178	6.418	-	+	
Arg Arg Gln	C17 H34 N10 O5	458.267	10.022	-		
Fluocortolone pivalate	C27 H37 F O5	460.266	7.465	-		
Hydrocortisone caproate	C27 H40 O6	460.282	9.816	-		
cefamandole nafate	C18 H18 N6 O5 S2	462.08	5.951	-		
3alpha,5beta-Tetrahydronorethindrone disulfate	C20 H30 O8 S2	462.138	4.026	-		
Trp Glu Glu	C21 H26 N4 O8	462.17	11.486	-		
Amcinatal	C26 H35 F O6	462.235	6.359	-		

Desoxycortone 21-(3-phenylpropionate)	C30 H38 O4	462.275	11.712	-		-	-
isoquercitrin	C21 H20 O12	464.097	5.27	+/-			
Loprazolam	C23 H21 Cl N6 O3	464.138	5.579	-			
Psychotrine	C28 H36 N2 O4	464.265	5.703		-		
Arg Gln Tyr	C20 H31 N7 O6	465.237	9.498		+		
BENFOTIAMINE	C19 H23 N4 O6 P S	466.111	5.262	-	-		
Staurosporine	C28 H26 N4 O3	466.201	11.876	-			
Glu Arg Tyr	C20 H30 N6 O7	466.219	6.141	-			
1,4-Benzodioxin, piperazine deriv	C23 H25 N5 O6	467.18	7.725				+
Trp Thr Tyr	C24 H28 N4 O6	468.196	6.109	-			
ANDIROBIN	C27 H32 O7	468.212	5.042	-			
(22E)-(25S)-26,26,26-trifluoro-1alpha,25-dihydroxy-22,23-didehydrovitamin D3/(22E)-(25S)-26,26,26-	C27 H39 F3 O3	468.284	10.524			-	
Cys Tyr Trp	C23 H26 N4 O5 S	470.164	1.264	-			
3beta-HYDROXYDEOXODIHYDRODEOXYGEDUNIN	C28 H38 O6	470.266	8.958	-		-	
(25R)-26,26,26-trifluoro-1alpha,25-dihydroxyvitamin D3/(25R)-26,26,26-trifluoro-1alpha,25-dihydrox	C27 H41 F3 O3	470.3	7.366			+	
5-Formiminotetrahydrofolic acid	C20 H24 N8 O6	472.181	1.146	-			
Lys Tyr Tyr	C24 H32 N4 O6	472.23	7.423	-		-	
	C22 H27 N11 O2	477.235	5.921	+			

	C17 H22 N10 O7	478.168	2.524	-			
Ipecac (Methylpsychotrine)	C29 H38 N2 O4	478.278	6.986	-			-
Trp Glu Phe	C25 H28 N4 O6	480.198	6.319		+		
Butoxylate	C32 H36 N2 O2	480.269	10.719	-		-	-
GPA(10:0/10:0)[U]	C23 H45 O8 P	480.285	10.534	-		-	-
Trp Glu Phe	C25 H28 N4 O6	480.199	9.137	+			
Acarbose (component 2)	C20 H34 O13	482.193	12.008	-			
Phe Met Trp	C25 H30 N4 O4 S	482.197	9.489	-			
Lithocholic acid taurine conjugate	C26 H45 N O5 S	483.303	10.972			-	
Glisindamide	C24 H28 N4 O5 S	484.172	6.32	+	+		
GPGro(16:0/0:0)[U]	C22 H45 O9 P	484.278	10.216	-		-	
(22S)-1alpha,25-dihydroxy-22-ethoxy-26,27-dimethyl-23,24-tetradehydro-20-epivitamin D3/(22S)-1alph	C31 H48 O4	484.353	3.61			+	
2-(8-[3]-ladderane-octanyl)-sn-glycero-3-phosphoethanolamine	C25 H46 N O6 P	487.302	9.813	-			
	C19 H36 O8 S3	488.157	3.746	-			
SERICETIN DIACETATE	C29 H28 O7	488.191	10.809	+/-	+		
Trp His Phe	C26 H28 N6 O4	488.226	5.782	-			
Prednicarbate	C27 H36 O8	488.234	5.15	-			
Salprotoside	C25 H30 O10	490.191	1.294	-			
Granisetron metabolite 4 glucuronide	C23 H30 N4 O8	490.207	5.529	-			
Bezitramide	C31 H32 N4 O2	492.256	6.208	-			

	C16 H32 N18 O	492.301	10.2		-	
Carindacillin	C26 H26 N2 O6 S	494.16	4.433	-		
harpagoside	C24 H30 O11	494.182	8.338	-		
diflorasone diacetate	C26 H32 F2 O7	494.215	5.014	-		
CYTOCHALASIN E	C28 H33 N O7	495.223	10.961	-	-	
Simetride	C28 H38 N2 O6	498.279	9.196	-	-	-
	C26 H15 N S5	500.982	11.696		-	
m-Hydroxycarvedilol sulfate	C24 H26 N2 O8 S	502.148	6.573	-		
Phenylbutazonetrimethoxy benzoate	C29 H30 N2 O6	502.205	6.385	-		
hypericin	C30 H16 O8	504.089	6.376	+		
Maltotriose	C18 H32 O16	504.169	1.188	-		
Granisetron metabolite 3 glucuronide	C24 H32 N4 O8	504.221	4.141	-		
betamethasone dipropionate	C28 H37 F O7	504.252	9.766	-	-	-
Digitoxigenin monodigitoxoside	C29 H44 O7	504.308	11.064	-	-	
Maltotriitol	C18 H34 O16	506.181	4.848	-		
Promethestrol dibenzoate	C34 H34 O4	506.249	6.083	-		
	C22 H36 O7 S3	508.162	6.021	-		
	C28 H44 O8	508.304	10.512		-	
Cefuroxime axetil	C20 H22 N4 O10 S	510.105	4.517	-		
GPGro(18:1(9Z)/0:0)	C24 H47 O9 P	510.295	11.12	-	-	-
3beta,7beta-DIACETOXYDEOXODEACETOXYDEOX	C30 H40 O7	512.276	11.047			-

YDIHYDROGEDUNIN							
	C22 H26 O14	514.132	4.062	-			
TELMISARTAN	C33 H30 N4 O2	514.24	5.137	+	+		
3beta-ACETOXYDEOXYANGOLENSIC ACID, METHYL ESTER	C29 H38 O8	514.257	11.881	-		-	
Cucurbitacin I	C30 H42 O7	514.293	10.737	-		-	-
	C22 H28 O14	516.148	3.492	-			
	C20 H16 N14 O4	516.148	4.156	-			
Cucurbitacin L	C30 H44 O7	516.307	9.405	-		-	
Gossypol	C30 H30 O8	518.189	0.437		-		
Cucurbitacin O	C30 H46 O7	518.325	8.519	-		-	
Medroxyprogesterone glucuronide	C28 H40 O9	520.271	4.978	-			
26,26,26,27,27,27-hexafluoro-25-hydroxyvitamin D2/26,26,26,27,27,27-hexafluoro-25-hydroxyergocalci	C28 H38 F6 O2	520.277	10.581				-
IRIDIN	C24 H26 O13	522.138	4.243	-			
	C29 H34 N10	522.297	11.12		+		
26,26,26,27,27,27-hexafluoro-1alpha,25-dihydroxyvitamin D3/26,26,26,27,27,27-hexafluoro-1alpha,25-	C27 H38 F6 O3	524.273	9.103			-	
26,26,26,27,27,27-hexafluoro-1alpha,24-dihydroxyvitamin D3/26,26,26,27,27,27-hexafluoro-1alpha,24-	C27 H38 F6 O3	524.276	10.943	-		-	-
KHAYASIN C	C30 H38 O8	526.254	10.846	-			
	C18 H20 N14 O6	528.169	1.29	-			

3beta-ACETOXYDEOXODIHYDROGEDUNIN	C30 H40 O8	528.273	9.728	-			+/-	
	C23 H30 O14	530.163	4.467	-				
	C35 H34 N2 O S	530.239	6.535	+	+		+	
Calotropin	C29 H40 O9	532.266	6.038	-				
Cucurbitacin J	C30 H44 O8	532.301	9.134	-			-	
	C21 H16 N10 O8	536.115	4.059		+			
Actodigin	C29 H44 O9	536.302	9.811	-				
12R-acetoxy-punaglandin 3	C27 H35 Cl O9	538.187	7.13	-				
Nafiverine	C34 H38 N2 O4	538.278	6.136	-				
Galbeta1-3GalNAcalpha-Thr	C21 H36 N2 O14	540.223	4.906	+/-	+			
3-DEACETYL KHIVORIN	C30 H40 O9	544.276	11.724	-			-	
Fetoxilate	C36 H36 N2 O3	544.278	4.912	+	+	+	+	+
	C18 H14 N2 O18	546.022	0.921				-	
Trp Trp Arg	C28 H34 N8 O4	546.266	6.065	-				
Sulindac sulfone glucuronide	C26 H25 F O10 S	548.117	5.661	-				
Murabutide	C23 H40 N4 O11	548.27	11.137	+				
CYMARIN	C30 H44 O9	548.297	8.827	-			-	
PERUVOSIDE	C30 H44 O9	548.298	7.214	-				
	C25 H26 N8 O5 S	550.175	1.155	-				
CONVALLATOXIN	C29 H42 O10	550.276	6.126	-				
	C18 H28 N6 O14	552.166	9.685	-				

	C18 H28 N6 O14	552.166	7.341	-				
	C17 H26 N6 O15	554.145	6.808	-				
	C19 H26 N10 O10	554.182	5.471	-				
	C21 H18 N18 O2	554.187	6.342	-				
KHAYASIN	C32 H42 O8	554.286	9.178	-	-		-	-
Betamethasone adamantoate	C33 H43 F O6	554.306	6.074	-				
	C22 H49 N7 O S4	555.288	10.535		+		+	
Niceritrol	C29 H24 N4 O8	556.159	4.735	-				
	C20 H31 N O11 S3	557.106	4.542	-				
	C24 H30 O15	558.158	4.497	-				
Cucurbitacin B	C32 H46 O8	558.318	10.011				+	
THEAFLAVIN	C29 H24 O12	564.13	6.342	-				
AKLAVINE	C30 H35 N O10	569.221	9.189				-	
2-O-p-Coumaroylaloesin	C29 H30 O12	570.177	7.233	-				
Diathymosulfone	C32 H34 N4 O4 S	570.232	10.715	+	+		+	
	C24 H32 N10 O7	572.246	9.177		+		+	+
	C17 H20 N8 O15	576.102	1.077			+		
Littoraloin	C28 H32 O13	576.186	7.532	-				
	C27 H28 N16	576.268	10.726		+			+
	C28 H24 N12 O3	576.209	5.403	-				
Trp Trp Trp	C33 H32 N6 O4	576.258	10.721	+/-			-	
	C18 H26 N8 O12 S	578.14	1.062			+		

	C26 H28 O15	580.143	5.47	-				
	C26 H30 O15	582.159	4.23		+			
Carvedilol glucuronide	C30 H34 N2 O10	582.232	7.473		+			
	C23 H40 N2 O13 S	584.226	4.353	-				
	C31 H48 N6 O5	584.369	4.144				+	
8',10'-Dihydroxydihydroergotamine	C33 H39 N5 O5	585.303	5.794		+			
	C25 H26 N14 O4	586.225	10.715				+	
KHIVORIN	C32 H42 O10	586.275	8.824	-			-	
Etoposide	C29 H32 O13	588.184	4.503	-				
Rescimetol	C33 H38 N2 O8	590.257	5.796	-	-			
Dimethylprotoporphyrin IX dimethyl ester	C36 H38 N4 O4	590.293	5.044	-				-
D-Urobilinogen	C33 H42 N4 O6	590.307	7.666	-				
Ramipril glucuronide	C29 H40 N2 O11	592.256	10.717	+		+		
	C17 H30 N4 O15 S2	594.116	1.058			+		
	C24 H22 N10 O9	594.157	6.011	-				
BEBEERINE	C36 H38 N2 O6	594.281	9.159	+				
	C28 H20 N8 O8	596.141	3.63		-			
Metofenazate	C31 H36 Cl N3 O5 S	597.215	10.706				-	
	C28 H30 N12 O4	598.251	10.71		+		+	
	C26 H34 N10 O7	598.261	7.967	+	+	+		
	C33 H46 N10 O	598.386	5.243				+	

Nicofuranose	C30 H24 N4 O10	600.149	4.577	-			
	C30 H38 N10 O2 S	602.29	4.973	-			
	C27 H42 N10 O2 S2	602.293	6.653	-			
	C29 H30 N6 O9	606.208	6.614	+	+		
Harderoporphyrin	C35 H36 N4 O6	608.257	8.841				-
reserpine	C33 H40 N2 O9	608.283	4.973	-			
EPIGALLOCATECHIN 3,5-DIGALLATE	C29 H22 O15	610.096	5.886	-			
rutin	C27 H30 O16	610.153	5.658	+/-			
Rutoside	C27 H30 O16	610.157	4.358		-		
Manidipine	C35 H38 N4 O6	610.276	5.646	+			
Glutathione, oxidized	C20 H32 N6 O12 S2	612.147	5.871	-			
(3Z)-3-[(4-Nitrophenyl)hydrazono]-1-(2,3,4,6-tetra-O-acetylhexopyranosyl)-1,3-dihydro-2H-indol-2-one	C28 H28 N4 O12	612.168	5.235	+/-	-		
Novobiocin	C31 H36 N2 O11	612.241	5.794		+		
(3a,5b,7b,12a)-(1,3-dihydro-5-nitro-1,3-dioxo-2H-isoindol-2-yl)methyl ester-3,7,12-trihydroxy-Cholan	C33 H44 N2 O9	612.295	8.385		+	+	+
4-Oxomytiloxanthin	C40 H52 O5	612.387	8.818	-			-
PECTOLINARIN	C29 H34 O15	622.187	4.566	-			
	C32 H34 N10 O4	622.276	5.047	+			
Cefbuperazone	C22 H29 N9 O9 S2	627.143	5.654	-			
	C25 H20 N14 O7	628.164	5.106	-			

	C25 H20 N14 O7	628.165	3.181	-	
Diglucomethoxane	C15 H32 Hg O12 S	632.136	5.68	-	
	C22 H14 N6 O17	634.043	0.943		-
	C31 H18 N4 O12	638.091	5.886	-	
Nicomol	C34 H32 N4 O9	640.219	6.44	-	
	C31 H48 O10 S2	644.269	5.739	-	
11alpha-ACETOXYKHIVORIN	C34 H44 O12	644.284	7.909	+	
	C22 H26 N14 O10	646.196	1.517	-	
	C33 H45 N O12	647.293	6.102	-	
	C27 H18 N18 O4	658.176	4.525	-	
	C28 H18 N6 O14	662.089	5.873	+/-	
Syrosingopine	C35 H42 N2 O11	666.29	5.533	-	
Erythroxanthin sulfate	C40 H54 O7 S	678.352	7.051	-	
COSMOSIIN HEXAACETATE	C33 H32 O16	684.167	4.558	-	
	C30 H26 N14 O7	694.212	6.904	-	
SARMENTOSIDE B	C34 H48 O13	664.307	5.866	+	
	C34 H38 N10 O5	666.303	5.535	+	
	C34 H58 N6 O S4	694.355	7.008	-	
	C25 H42 N22 O3	698.382	6.979	-	
	C22 H46 N22 O5	698.401	5.937		+
Neu5Acalpha2-6Galbeta1-4Glcbeta-Sp	C25 H42 N4 O19	702.252	5.878	-	
	C30 H20 N14 O8	704.16	4.541	-	

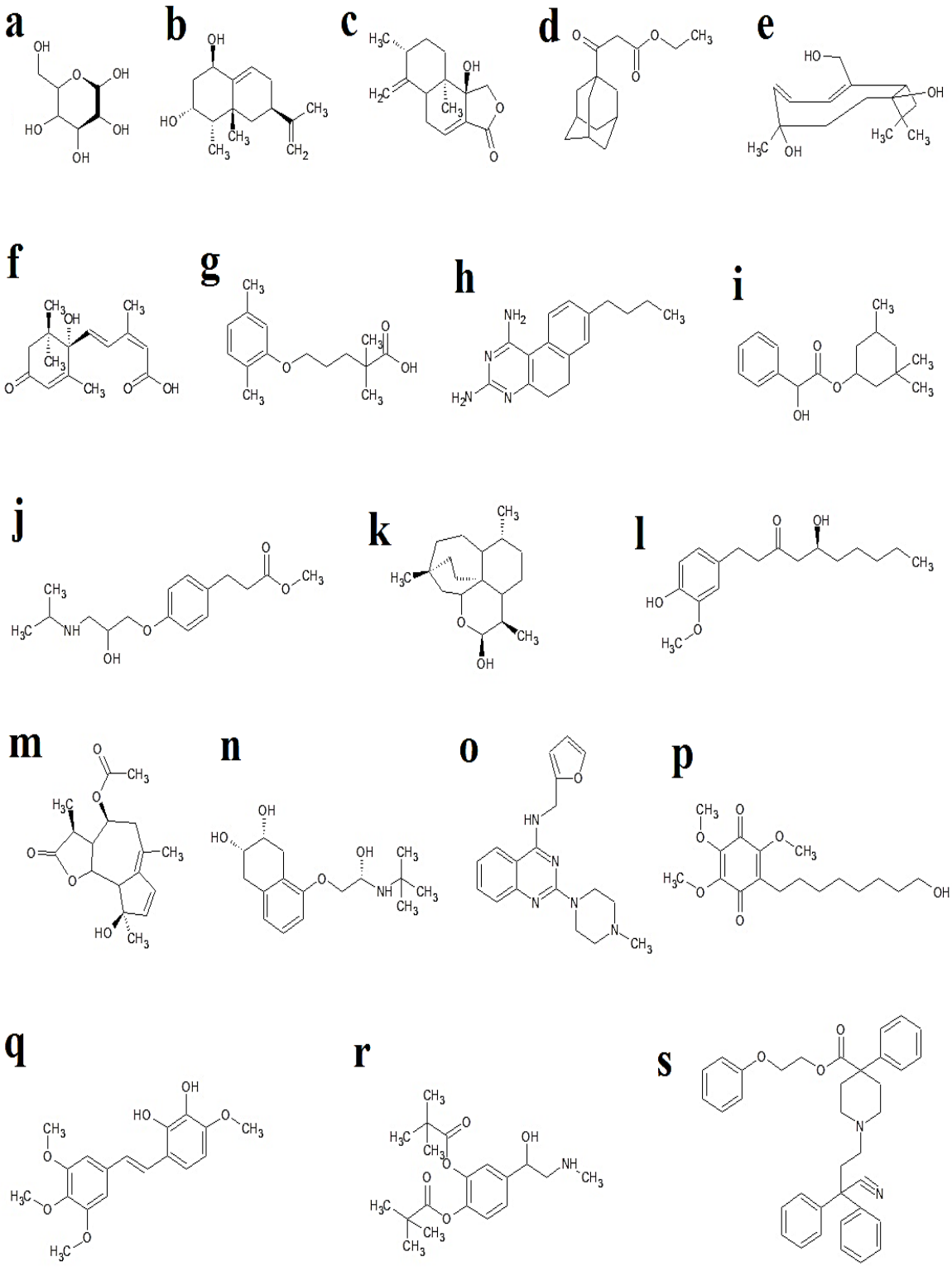
	C27 H28 N24 O	704.288	4.648	-			
	C30 H22 N14 O8	706.174	4.54	-	-		
	C32 H36 O18	708.19	4.532	-			
	C42 H87 N5 O3	709.682	4.525	-			
	C44 H95 N5 O	709.759	4.525	-			
	C29 H10 N8 O15	710.028	0.922			-	
	C28 H40 N8 O8 S3	712.213	4.493	-			
GPIns(12:0/13:0)	C34 H65 O13 P	712.429	10.549	-		-	-
	C23 H50 N14 O12	714.373	6.999	-			
	C25 H20 N2 O23	716.046	0.941		-	-	
Ritonavir	C37 H48 N6 O5 S2	720.3	7.03	-			
	C33 H40 O14 S2	724.186	4.537	-			
3-Formylrifampin	C38 H47 N O13	725.293	4.591	-			
	C26 H5 N3 O13 S5	726.84	6.507	-			
	C32 H20 N14 O8	728.159	4.57	-			
	C31 H26 N10 O12	730.173	8.345	+			
(Alginate)n	C24 H32 O26	736.127	5.93	-			
	C34 H28 N20 O2	748.27	4.725	-			
	C35 H44 N10 O9	748.329	7.045	-			
Benzquercine	C50 H40 O7	752.288	4.675	-			
Etoposide glucuronide	C35 H40 O19	764.222	1.325	-			
25-O-Desacetylrifampin	C41 H56 N4 O11	780.392	7.033	-			

Metildigoxin	C42 H66 O14	794.436	4.536	+	+		+	
Mocimycin	C43 H60 N2 O12	796.413	9.184	-			-	-
Acetyl-Digitoxin	C43 H66 O14	806.442	9.169	-			-	-
Uroporphyrin IV	C40 H38 N4 O16	830.237	2.744		-			
Rifapentine	C47 H64 N4 O12	876.437	11.697				-	
TG(22:0/22:0/22:0)	C69 H134 O6	1059.03	3.704	-				
beta-ESCIN	C55 H86 O24	1130.55	9.188	-			-	-

+ and - indicates the mass spectral mode in which the molecule is detected. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract.

As of the *T. lanceolata* berry extracts displayed anti-proliferative activity (Chapters 3 and 4), a comparison of the metabolomics profiles across these extracts was used to narrow the focus of phytochemicals which may contribute to this bioactivity. Of the 998 unique mass signals detected in the *T. lanceolata* berry extracts, only 19 were present in all of the anti-proliferative extracts. These are shown in order of increasing molecular mass in Figure 6.6. A noteworthy feature is the high proportion of sesquiterpenoids (capsidol, Figure 6.6 b; salutarisolide, Figure 6.6 c; punctaporin B, Figure 6.6 e; dihydroartmisinin, Figure 6.6 k; matricin, Figure 6.6 m) present across all extracts. A similarly high proportion of the compounds detected as being common between the *T. lanceolata* berry extracts were benzene and benzoic acid derivatives. These include 5-(4-hydroxy-2, 5-dimethylphenoxy)-2, 2-dimethyl-pentanoic acid (Figure 6.6 g), 3, 3, 5-trimethylcyclohexyl hydroxy(phenyl)acetate (cyclandelate) (Figure 6.6 i), 4-[2-hydroxy-3- [(1-methylethyl) amino] propoxy] -benzene propanoic acid (Figure 6.6 j), gingerol (Figure 6.6 l), [2-(2, 2-dimethylpropanoyloxy)-4- [1-hydroxy-2-(methylamino) ethyl] phenyl] 2, 2-dimethylpropanoate (Figure 6.6 r), 2-phenoxyethyl 1-(3-cyano-3, 3-diphenylpropyl)-4-phenylpiperidine-4-carboxylate (Figure 6.6 s). Whilst the stilbene combretastatin A1 (Figure 6.6 q) is also a benzene derivative, it warrants special mention as stilbenes have well established therapeutic bioactivities related inflammation and cancer. It is noteworthy that whilst combretastatin A1 was the only stilbene detected in all *T. lanceolata* berry extracts, other stilbenes (resveratrol, combretastatin, combretastatin A4) as well as the glycosylated stilbene piceid, were detected in one or more extracts, but not in all. The quinazoline alkaloids 8-butyl-5, 6-dihydrobenzo[f]quinazoline-1, 3-diamine (Figure 6.6 h), and N-(2-furylmethyl)-2-(4-methyl-1-piperazinyl)-4-quinazolinamine (Figure 6.6 o) were also putatively identified in all extracts. Also detected across all *T. lanceolata* berry extracts were glucose (Figure 6.12 a), 3-(1-adamantyl)-2-oxopropyl acetate (Figure 6.6 d), absinic acid (Figure 6.6 f), the naphthalene derivative 2, 3-

cis-1, 2, 3, 4-tetrahydro-5-[2-hydroxy-3-(tert-butylamino) propoxy]-2, 3-naphthalenediol (nadolol) (Figure 6.6 n), 2-(10-hydroxydecyl) -5, 6-dimethoxy-3-methyl-cyclohexa-2, 5-diene-1,4-dione (Figure 6.6 p).


a
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s

Figure 6.6: Chemical structures of *T. lanceolata* berry compounds detected in all solvent extractions: (a) glucose, (b) capsidiol, (c) salutarisolide, (d) 3-(1-adamantyl)-2-oxopropyl acetate, (e) punctaporin B; (f) absinic acid, (g) 5-(4-hydroxy-2, 5-dimethylphenoxy)-2, 2-dimethyl-pentanoic acid, (h) 8-butyl-5, 6-dihydrobenzo[f]quinazoline-1, 3-diamine, (i) 3, 3, 5-trimethylcyclohexyl hydroxy(phenyl)acetate (cyclandelate), (j) 4-[2-hydroxy-3- [(1-methylethyl) amino] propoxy] -benzene propanoic acid, (k) dihydroartmisinin, (l) gingerol, (m) matricin, (n) 2, 3-cis-1, 2, 3, 4-Tetrahydro-5-[2-hydroxy-3-(tert-butylamino)propoxy]-2,3-naphthalenediol (nadolol), (o) N-(2-furylmethyl)-2-(4-methyl-1-piperazinyl)-4-quinazolinamine, (p), 2-(10-hydroxydecyl) -5, 6-dimethoxy-3-methyl-cyclohexa-2, 5-diene-1, 4-dione (idebenone M8), (q) combretastatin A1, (r) [2-(2, 2-dimethylpropanoyloxy)-4- [1-hydroxy-2-(methylamino) ethyl] phenyl] 2, 2-dimethylpropanoate (dipivefrin), (s) 2-phenoxyethyl 1-(3-cyano-3, 3-diphenylpropyl)-4-phenylpiperidine-4-carboxylate (fetoxilate).

6.4.1.2 GC-MS head space analysis

The same optimised GC-MS parameters that were developed to examine the *T. ferdinandiana* fruit extract compound profiles were also used to examine the *T. lanceolata* berry extracts. The resultant gas chromatograms are presented in Figures 6.7-6.11. The methanolic extract GC-MS chromatogram (Figure 6.7) displayed numerous overlapping peaks throughout the middle phases of the chromatogram. A total of 202 peaks were detected in this chromatogram, with major peaks at approximately 14.1, 15.1, 16.4, 19.1, 21.0, 31.3, 31.8 and 35.0 min. Numerous overlapping peaks were also evident throughout the chromatogram, with a broad range of retention times between 10 - 40 min. The presence of peaks throughout the chromatogram attest to the wide range of compounds of widely varying polarity extracted with methanol.

Far fewer peaks were evident in the aqueous extract chromatogram (Figure 6.8). A total of 109 peaks were detected in the aqueous *T. lanceolata* berry extract, many at retention times corresponding to peaks in the methanolic extract. Of the major peaks, only the peaks at approximately 15.1 and 21.0 min corresponded with major peaks in the methanolic extract. A further major peak at 15.5 min in the aqueous extract corresponded with a minor peak in the methanolic extract. Many of the other major peaks that were present in the aqueous extract chromatogram were also present in the methanolic extract chromatogram, albeit with much reduced peak heights and areas. This indicates that methanol and water extracted many similar components, although many of the lower polarity compounds appear to be more effectively extracted into methanol than water.

Fewer peaks were evident in the ethyl acetate extract (Figure 6.9), chloroform extract (Figure 6.10) and hexane extract (Figure 6.11) chromatograms (80, 78 and 73 peaks respectively) than in the methanolic and aqueous extracts. Furthermore, many peaks in these extracts were at different retention times than seen for the more polar methanolic and aqueous extracts. The exception was the peak at 15.1 min, which corresponded to a peak in the methanolic extract chromatogram and the peak at 21.0 min. The ethyl acetate, chloroform and hexane chromatograms all had a major peak present at 19.7 min. In addition, the ethyl acetate and chloroform extracts had a further major peak present at approximately 15.6 min. This peak was lacking in the methanolic, aqueous and hexane extracts. A further peak was evident in the hexane extract at 28.9 min. This peak was present in all extracts, although it was only a major component in the hexane extract.

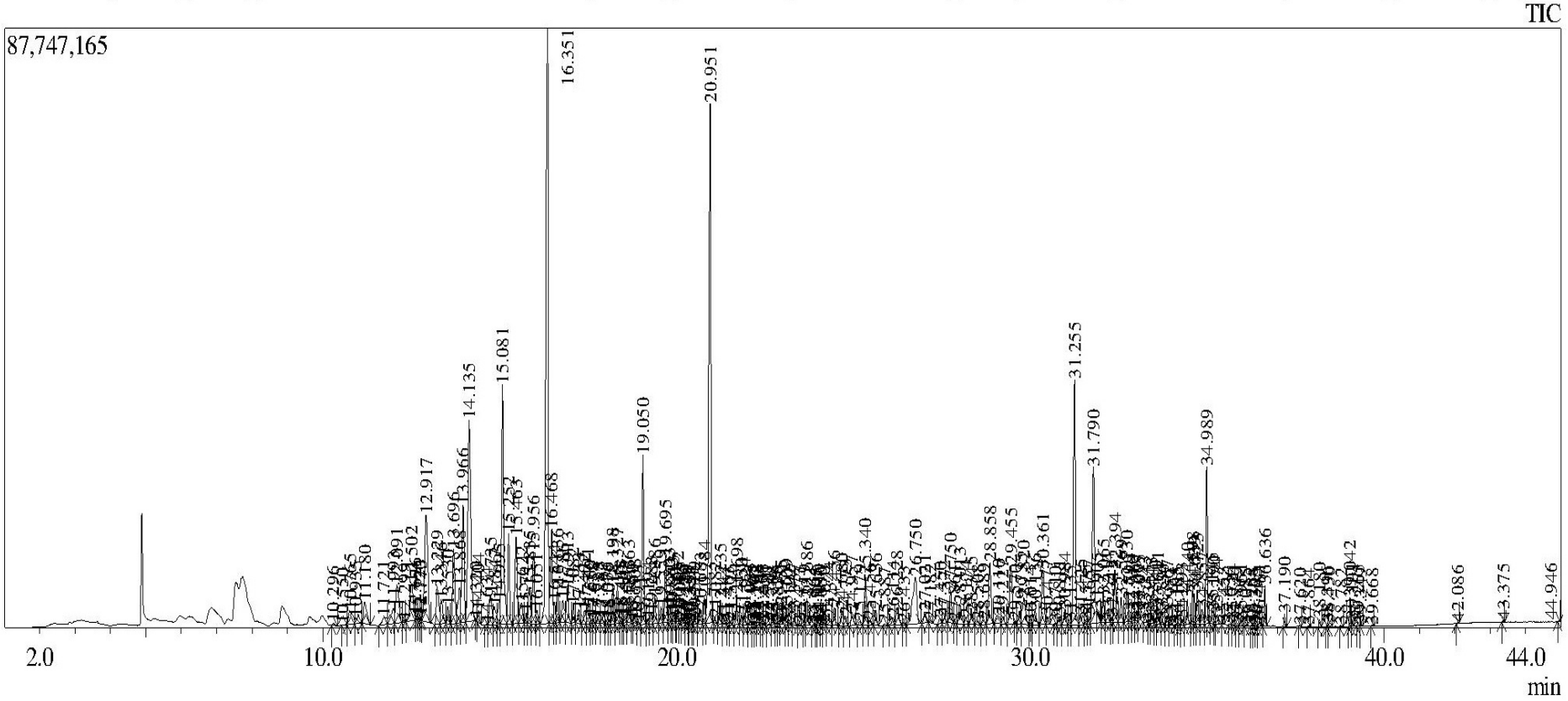

Figure 6.7: Head space gas chromatogram of 0.5 µL injections of *T. lanceolata* berry methanol extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

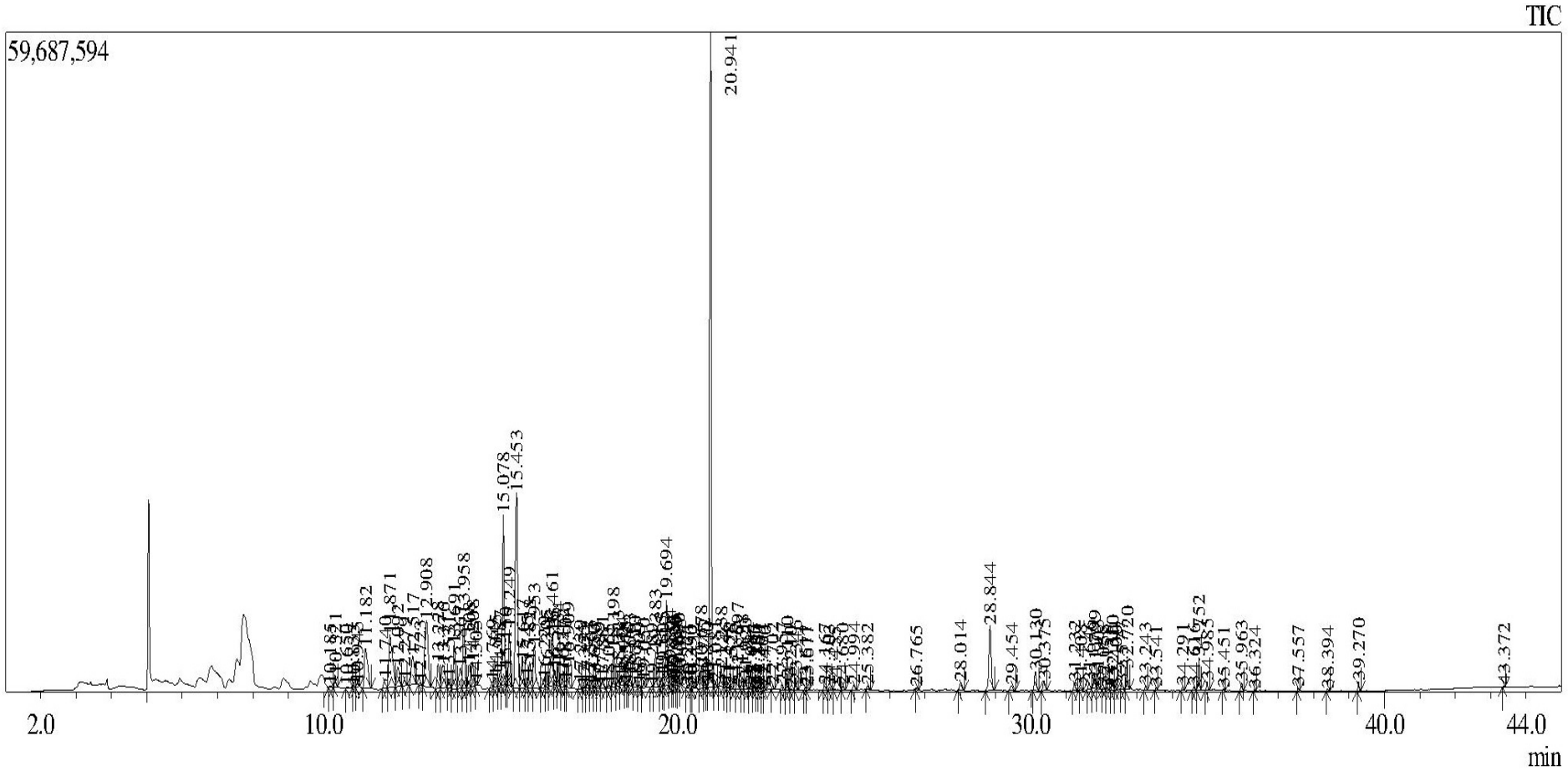

Figure 6.8: Head space gas chromatogram of 0.5 µL injections of aqueous *T. lanceolata* berry extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

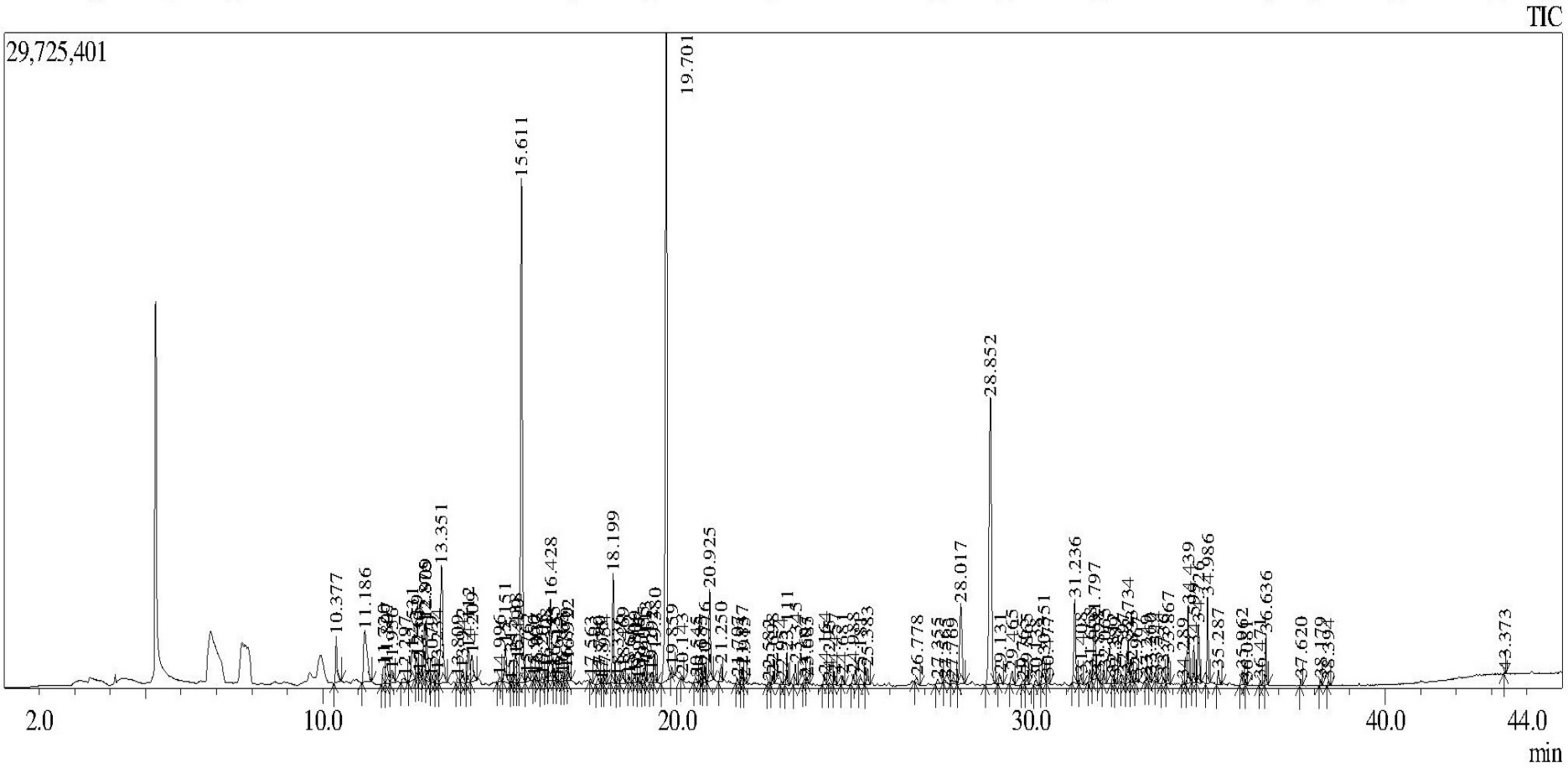

Figure 6.9: Head space gas chromatogram of 0.5 µL injections of *T. lanceolata* berry ethyl acetate extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

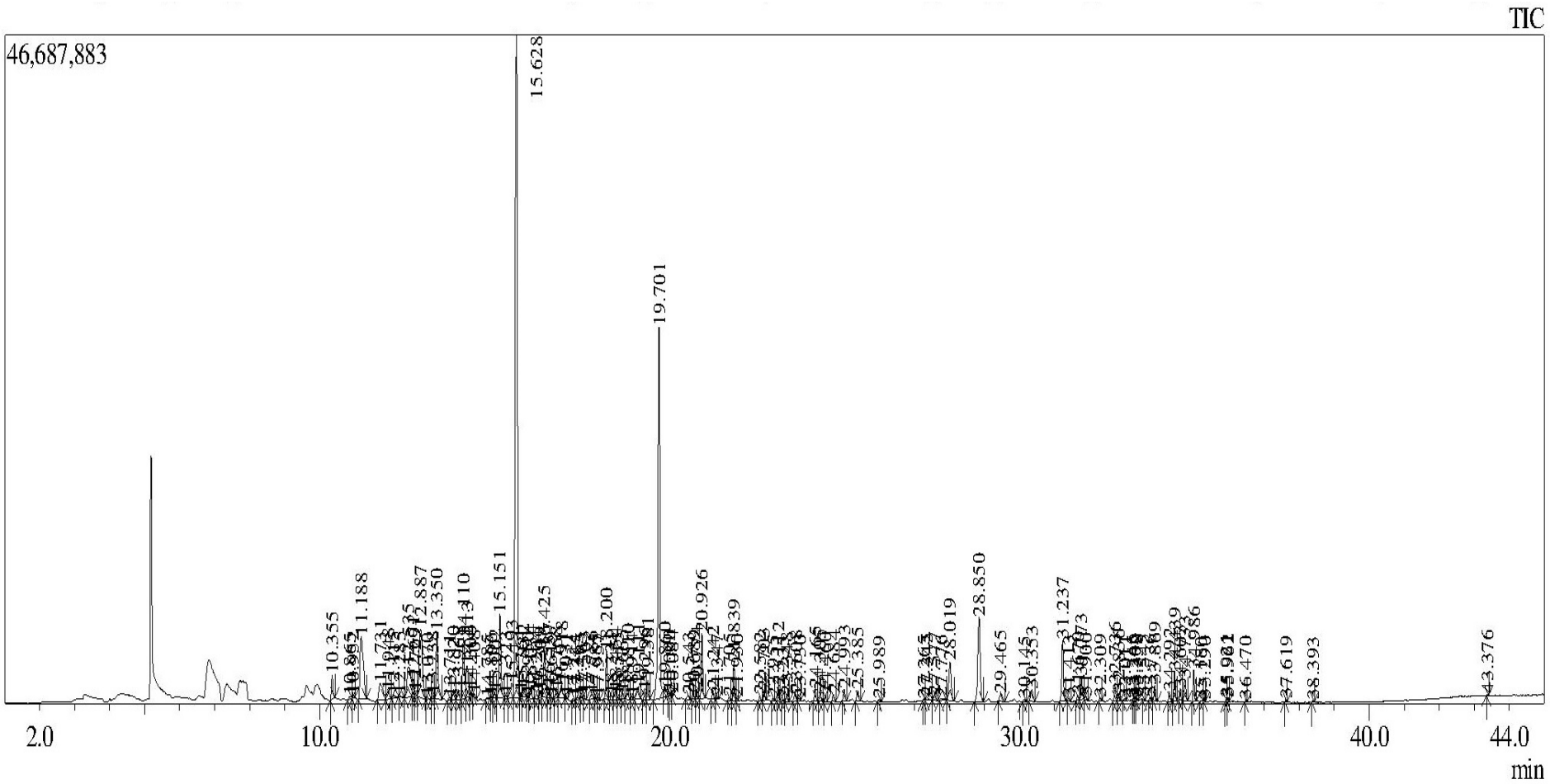

Figure 6.10: Head space gas chromatogram of 0.5 µL injections of *T. lanceolata* berry chloroform extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

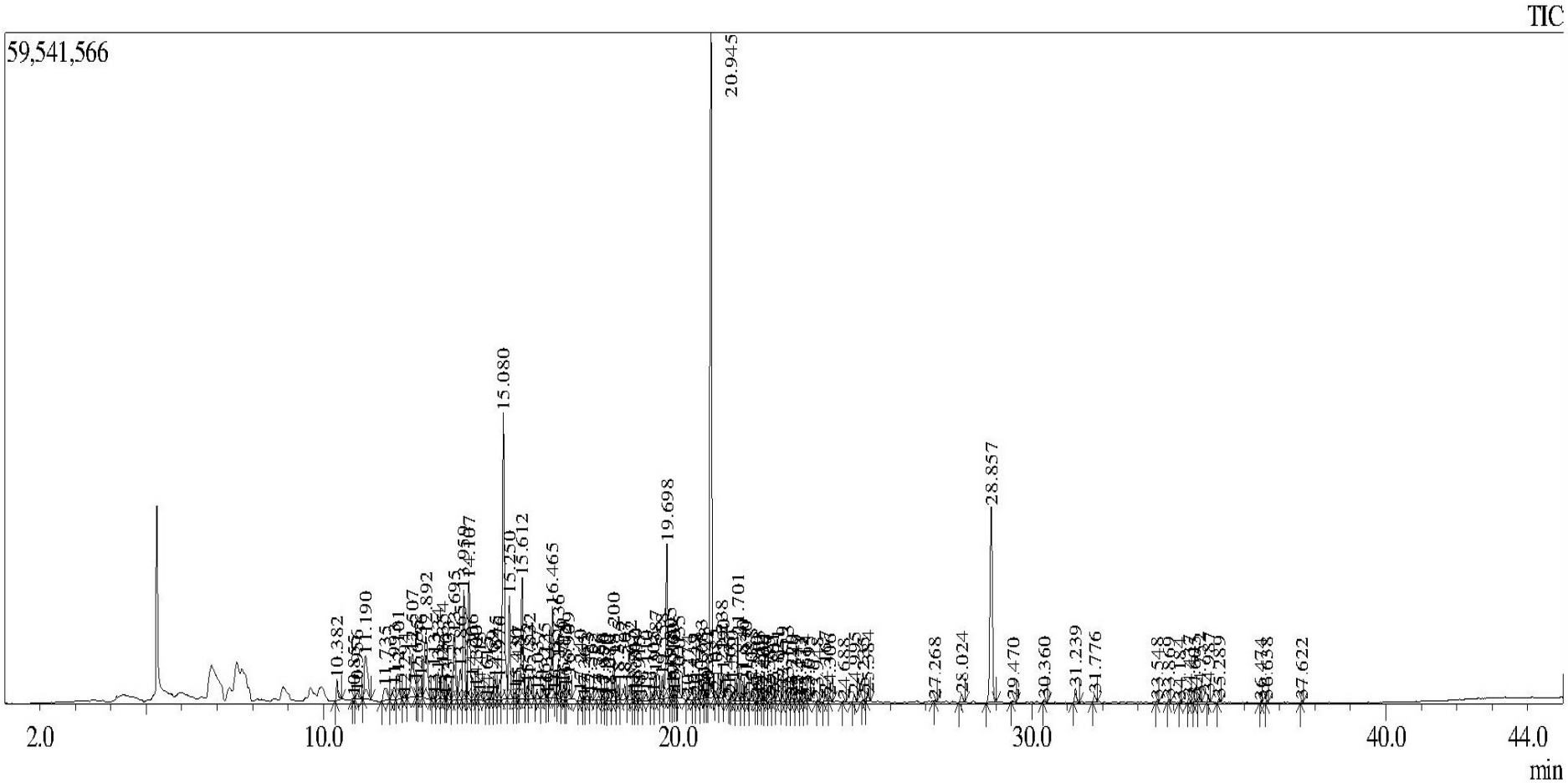

Figure 6.11: Head space gas chromatogram of 0.5 µL injections of *T. lanceolata* berry hexane extract. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

6.4.1.2.1. Qualitative GC-MS headspace analysis of the T. lanceolata berry extracts

In total, 239 unique mass signals were noted for the *T. lanceolata* berry extracts by GC-MS headspace analysis (Table 6.2). Putative empirical formulas were achieved for all of these compounds. Of these 239 mass signals, 169 compounds (70.1%) were putatively identified by comparison against the GC-MS mass and spectral database. A comparison of the metabolomics profiles across the GC-MS headspace chromatograms of these extracts was used to narrow the focus of phytochemicals which may contribute to this bioactivity. Of the 239 unique mass signals detected in the *T. lanceolate* berry extracts, only 20 were present in all of the anti-proliferative extracts. In total, 14 of these compounds were putatively identified. These compounds are shown in order of increasing retention time in Figure 6.12. Several terpenoids were detected in all *T. lanceolata* extracts. α-Terpineol (Figure 6.12 f) was the only monoterpenoid present in all extracts, although multiple other monoterpenoids were present in some but not all extracts. The sesquiterpenoid polygodial (Figure 6.12 h) was present in all extracts as either the major peak (at approximately 21 min), or one of the largest peaks. A further two sesquiterpenoids were also putatively identified as isomers of caryophyllene oxide. The GC-MS headspace analysis was unable to distinguish which of these isomers corresponded to each peak. For the purposes of this discussion, isomer 1 has been assumed to be caryophyllene oxide (Figures 6.12 m) and isomer 2 is assumed to be humulene (Figure 6.12 n). However, the order of these isomers may be reversed. A number of aliphatic keto- compounds were also detected. Of these, 4-methyl-2-heptanone (Figure 6.12 a), 2, 2, 6, 6-tetramethyl-3, 5-heptanedione (Figure 6.12 e) and butyl butoxyacetate (Figure 6.12 i) were present in all extracts. Similarly, the fatty alcohol (6Z)-3, 7-dimethyl-6, 11-dodecadien-1-ol, (Figure 6.12 k) and the aliphatic ester propanoic acid, 2-methyl-, 3-

hydroxy-2, 2, 4-trimethylpentyl ester (Figure 6.12 j) were also present in all anti-proliferative *T. ferdinandiana* extracts. All of the remaining compounds present in all extracts (cumene, Figure 6.12 b; 1, 2-dimethyl-4-ethylbenzene, Figure 6.12 c; p-cymene, Figure 6.12 d; 2-methyl-2-phenyl-oxirane, Figure 6.12 g; 3, 5-di-tert-butylphenol, Figure 5.12 l) were benzene derivatives.

Table 6.2: Qualitative GC-MS headspace analysis of the *T. lanceolata* extracts, elucidation of empirical formulas and putative identification (where possible) of each compound.

Molecular Mass	Molecular Formula	R.Time	Name	M	W	E	C	H
110	C6 H6 O2	10.19	Acetylfuran		Y			
151	C8 H9 NO2	10.375	Methyl N-hydroxybenzenecarboximidoate			Y	Y	Y
128	C8 H16 O	10.55	2,4,4-trimethyl-cyclopentanol	Y				
130	C7 H14 O2	10.765	Methyl caproate	Y				
130	C8 H18 O	10.892	4-Methyl-4-heptanol		Y			Y
170	C12 H26	10.935	3,8-Dimethyldecane	Y	Y		Y	Y
128	C8 H16 O	11.18	4-Methyl-2-heptanone	Y	Y	Y	Y	Y
126	C9 H18	11.721	1,1,3,4-Tetramethylcyclopentane	Y	Y	Y	Y	
156	C10 H20 O	11.805	2,2,5,5-Tetramethyl-3-hexanone			Y		
110	C6 H6 O2	11.87	2-Formyl-5-methylfuran		Y			
120	C9 H12	11.993	p-Ethylmethylbenzene	Y				Y
		12.091		Y	Y			
120	C9 H12	12.12	m-Ethyltoluene				Y	
156	C10 H20 O	12.299	Decanal	Y		Y	Y	Y
154	C10 H18 O	12.28	2,2,6-Trimethyl-6-vinyltetrahydropyran		Y			
114	C8 H18	12.502	2,4-Dimethylhexane	Y	Y			Y
186	C9 H14 O4	12.535	alpha.-D-Xylo-Hex-5-enofuranose, 5,6-dideoxy-1,2-O-(1-methylethylidene)-			Y	Y	
284	C16 H28 O4	12.626	Succinic acid, 2-methylpent-3-yl trans-he	Y		Y	Y	

126	C8 H14 O	12.72	6-Methyl-5-hepten-2-one	Y	Y	Y				
146	C7 H14 O3	12.773	Methyl 2-hydroxy-4-methylpentanoate	Y						
		12.917		Y	Y	Y				
170	C12 H26	13.229	Dodecane	Y	Y	Y				
128	C8 H16 O	13.235	Octanal				Y	Y		
		13.346		Y	Y					
142	C10 H22	13.53	3,3,5-Trimethylheptane	Y	Y			Y		
170	C12 H26	13.696	3,4,5,6-Tetramethyloctane	Y	Y			Y		
120	C9 H12	13.868	Cumene	Y	Y	Y	Y	Y		
140	C9 H16 O	13.94	Ethanone, 1-(2,2-dimethylcyclopentyl)-			Y				
142	C10 H22	13.966	3,3-Dimethyloctane	Y	Y		Y	Y		
154	C10 H18 O	14.135	Cineole	Y	Y		Y			
140	C9 H16 O	14.21	1,1,3-Trimethyl-2-cyclohexanone		Y	Y	Y			
		14.32		Y	Y		Y	Y		
136	C10 H16	14.404	alpha.-Pinene	Y			Y	Y		
126	C9 H18	14.625	n-Butylcyclopentane					Y		
		14.634		Y	Y					
136	C10 H16	14.735	.beta.-Ocimene	Y						
244	C13 H24 O4	14.847	Oxalic acid, isohexyl neopentyl ester	Y	Y		Y	Y		
278	C14 H30 O3 S	14.995	Sulfurous acid, nonyl pentyl ester	Y	Y					
114	C8 H18	15.081	3,3-Dimethylhexane	Y	Y			Y		

120	C8 H8 O	15.15	Bicyclo[4.2.0]octa-1,3,5-trien-7-ol			Y	Y		
128	C8 H16 O	15.325	trans-2-Octenol				Y		
242	C13 H22 O4	15.463	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	Y	Y				
162	C7 H14 O4	15.485	2,2-Dimethoxy-1-methylethyl acetate					Y	
120	C8 H8 O	15.61	Bicyclo[4.2.0]octa-1,3,5-trien-7-ol					Y	
212	C14 H12 O2	15.615	m-Toluic acid, phenyl ester		Y	Y			
134	C10 H14	15.622	m-Cymene	Y			Y		
200	C13 H28 O	15.744	11-Methyldodecanol	Y	Y			Y	
134	C10 H14	15.835	1,2-Dimethyl-4-ethylbenzene	Y	Y	Y	Y	Y	
170	C10 H18 O2	15.956	Linalool oxide	Y	Y	Y			
172	C 10 H20 O2	16.051	3-(Hydroxymethyl)-2-nonanone	Y					
228	C13 H24 O3	16.23	Carbonic acid, nonyl prop-1-en-2-yl ester		Y				
154	C10 H18 O	16.351	Linalool	Y	Y	Y			
156	C10 H20 O	16.425	dihydro-gamma.-Terpineol				Y		
170	C12 H26	16.468	4-Methylundecane	Y	Y	Y		Y	
140	C10 H20	16.544	1-Ethyl-1,4-dimethylcyclohexane	Y	Y				
156	C11 H24	16.636	6-Ethyl-2-methyloctane	Y				Y	
134	C10 H14	16.786	3,7,7-Trimethyl-1,3,5-cycloheptatriene	Y	Y				
134	C10 H14	16.913	p-Cymene	Y	Y	Y	Y	Y	
		17.082		Y			Y		
156	C10 H20 O	17.233	1-Methyl-4-(1-methylethyl)cyclohexanol	Y	Y		Y		

156	C10 H20 O	17.402	Neoisocarquejanol	Y					
152	C10 H16 O	17.491	4(10)-Thujen-3-ol	Y	Y		Y		
184	C11 H20 O2	17.56	2,2,6,6-Tetramethyl-3,5-heptanedione	Y	Y	Y	Y	Y	
		17.638		Y	Y				
		17.788		Y		Y	Y	Y	
		17.887		Y	Y	Y	Y	Y	
166	C12 H22	18.036	1-Hexyl-1-cyclohexene	Y					
		18.079		Y	Y			Y	
154	C10 H18 O	18.327	cis-2-Norbornanol	Y		Y		Y	
		18.418		Y	Y				
		18.465		Y	Y				
144	C8 H16 O2	18.47	Octanoic acid			Y	Y	Y	
170	C10 H10 O2	18.565	trans-Linalool 3,7-oxide	Y					
168	C12 H24	18.663	4,6,8-Trimethyl-1-nonene	Y	Y		Y		
		18.75		Y				Y	
		18.808		Y	Y	Y	Y	Y	
130	C8 H18 O	18.913	2,5-Dimethyl-2-hexanol	Y	Y		Y	Y	
154	C10 H18 O	19.05	.alpha.-Terpineol	Y	Y	Y	Y	Y	
		19.194		Y					
152	C10 H16 O	19.283	Myrtenol	Y	Y				
		19.386		Y	Y	Y	Y	Y	
156	C10 H20 O	19.559	5-Methyl-3-propyl-2-hexanone	Y	Y			Y	

134	C9 H10 O	19.695	2-methyl-2-phenyl-oxirane	Y	Y	Y	Y	Y
156	C11 H24	19.803	2,3,7-Trimethyloctane	Y	Y			Y
		19.863		Y	Y		Y	Y
		19.913		Y	Y			
184	C13 H28	20.032	4,8-dimethyl-undecane	Y	Y			Y
		20.085		Y	Y		Y	
196	C12 H20 O2	20.155	Nerol acetate	Y				
		20.27		Y	Y			Y
		20.335		Y				
		20.391		Y	Y			
184	C13 H28	20.445	3,3,5-trimethyl-decane	Y	Y			Y
		20.509		Y		Y		
252	C17 H32 O	20.675	13-Heptadecyn-1-ol		Y			
296	C20 H40 O	20.683	Phytol	Y				
232	C15 H20 O2	20.784	2,2,3,3-Tetramethylcyclopropanecarboxylic acid, 4-methylphenyl ester	Y	Y			
234	C15H22O2	20.951	polygodial	Y	Y	Y	Y	Y
184	C13 H28	21.082	4-methyl-dodecane	Y	Y			Y
158	C9 H18 O2	21.235	Nonanoic acid	Y	Y	Y	Y	
		21.344		Y	Y		Y	
		21.463		Y	Y			Y
114	C8 H18	21.698	3,3-Dimethylhexane	Y	Y			Y

188	C10 H20 O3	21.83	Butyl butoxyacetate	Y	Y	Y	Y	Y
		21.924		Y	Y	Y	Y	Y
198	C14 H30	22.083	4,6-Dimethyldodecane	Y	Y			
150	C10 H14 O	22.165	(2E,3Z)-2-Ethylidene-6-methyl-3,5-heptadienal	Y	Y			
		22.232		Y	Y			Y
222	C16 H30	22.408	(2-Cyclohexyl-1-methylpropyl)cyclohexane	Y	Y			
		22.457		Y	Y			
		22.59		Y		Y	Y	
140	C9 H16 O	22.708	2,3,4,5-Tetramethylcyclopent-2-en-1-ol	Y	Y	Y		Y
		22.825		Y				
194	C12 H18 O2	22.876	2-Pinen-10-ol, acetate	Y				
212	C15 H32	22.955	2,6,11-Trimethyldodecane	Y	Y	Y	Y	
158	C10 H22 O	23.215	2-Propyl-1-heptanol		Y			
		23.337		Y	Y	Y		
180	C13 H24	23.35	1,1,6,6-Tetramethylspiro[4.4]nonane				Y	Y
168	C12 H24	23.473	4-Methyl-1-undecene	Y				Y
286	C16 H30 O4	23.619	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate $$ Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(1-methylethyl)-1,3-propanediyl ester	Y	Y		Y	Y
164	C10 H12 O2	23.686	p-Eugenol	Y		Y		
		23.864		Y				
		23.945		Y				Y

180	C10 H12 O3	24.01	beta.-Phenyllactic acid methyl ester	Y				
		24.08		Y				
216	C12 H24 O3	24.17	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	Y	Y	Y	Y	Y
241	C13 H23 NO3	24.305	4-t-Butyl-2-(1-methyl-2-nitroethyl)cyclohexanone		Y	Y	Y	
204	C15 H24	24.344	.alpha.-Cubebene	Y				
180	C12 H20 O	24.456	4a,5-Dimethyloctahydro-2(1H)-naphthalenone	Y		Y	Y	
210	C14 H26 O	24.68	(6Z)-3,7-Dimethyl-6,11-dodecadien-1-ol	Y	Y	Y	Y	Y
204	C15 H24	24.773	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	Y				
		24.957		Y	Y	Y	Y	Y
		25.179		Y		Y		
204	C15 H24	25.34	(-)-Aristolene	Y				
226	C14 H26 O2	25.385	2,4,7,9-Tetramethyl-5-decyne-4,7-diol			Y	Y	Y
		25.456		Y				
204	C15 H24	25.636	Caryophyllene	Y				
202	C15 H22	25.887	(3R,4aS,8aS)-8a-Methyl-5-methylene-3-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene	Y				
204	C15 H24	26.114	Zingiberene	Y				
204	C15 H24	26.228	.alpha.-Guaiene	Y				
		26.433		Y				
220	C15 H24 O	26.75	(1R,2R,4S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decan-4-ol	Y				
166	C11 H18 O	26.78	Homomyrtenol			Y		

204	C15 H24	27.021	Aromandendrene	Y					
		27.182		Y					
210	C14 H26 O	27.36	(11E)-11,13-Tetradecadien-1-ol				Y		
204	C15 H24	27.45	Guaia-6,9-diene	Y					
166	C10 H14 O2	27.572	Cyclopentaneacetaldehyde, 2-formyl-3-methyl-.alpha.-methylene-	Y		Y	Y		
202	C15 H22	27.75	Curcumene	Y		Y	Y		
		27.861		Y					
154	C10 H18 O	28.013	Isogeraniol	Y	Y	Y			
220	C15 H24 O	28.174	(2S,3S,6S)-6-Isopropyl-3-methyl-2-(prop-1-en-2-yl)-3-vinylcyclohexanone	Y					
		28.345		Y					
204	C15 H24	28.508	4,5-di-epi-aristolochene	Y					
202	C15 H22	28.689	(3R,4aS,8aS)-8a-Methyl-5-methylene-3-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene	Y					
206	C14 H22 O	28.858	3,5-Di-tert-butylphenol	Y	Y	Y	Y	Y	
		29.11		Y		Y			
		29.227		Y					
166	C10 H14 O2	29.455	2-(1-Formylvinyl)-5-methylcyclopentanecarbaldehyde	Y	Y				
220	C15 H24 O	29.465	Caryophyllene oxide	Y	Y	Y	Y	Y	
		29.57		Y					
		29.735		Y					
		29.82		Y		Y			

		30.014		Y				
334	C22 H38 O2	30.136	Undec-10-ynoic acid, undec-2-en-1-yl ester	Y	Y	Y		
222	C15 H26 O	30.361	Cubenol	Y	Y			
		30.477		Y				
		30.71		Y				
264	C17 H28 O2	30.811	Nerolidyl acetate	Y				
222	C15 H26 O	30.984	Ledol	Y				
		31.135		Y				Y
302	C20 H30 O2	31.235	cis-5,8,11,14,17-Eicosapentaenoic acid		Y	Y		
220	C15 H24 O	31.255	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	Y			Y	
328	C22 H32 O2	31.405	cis-4,7,10,13,16,19-Docosahexanoic acid		Y	Y	Y	
220	C15 H24 O	31.425	2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	Y				
		31.575		Y				
208	C15 H28	31.657	Selinan	Y		Y	Y	
222	C15 H26 O	31.79	Guaiol	Y				
360	C24 H40 O2	31.905	Undec-10-ynoic acid, tridec-2-yn-1-yl ester	Y	Y		Y	
		32.065		Y	Y	Y		
		32.214		Y				
252	C17 H32 O	32.313	14-Methyl-8-hexadecyn-1-ol	Y	Y	Y	Y	
204	C15 H24	32.394	Guaia-6,9-diene	Y	Y	Y		

220	C15 H24 O	32.569	Dehydroxy-isocalamendiol	Y	Y				
222	C15 H26 O	32.595	Guai-1(10)-en-11-ol			Y			
220	C15 H24 O	32.73	(-)-Spathulenol	Y	Y	Y	Y		
		32.799		Y		Y			
224	C15 H28 O	32.835	2,10-Dodecadien-1-ol, 3,7,11-trimethyl-				Y		
222	C15 H26 O	32.91	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahy	Y					
222	C15 H26 O	33.007	2-Naphthalenemethanol, decahydro-.alpha.,.alpha.,4a-trimethyl-8-methylene-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	Y					
240	C15 H28 O2	33.075	Cryptomeridiol	Y					
152	C10 H16 O	33.243	2,4,6-Trimethyl-3-cyclohexene-1-carboxaldehyde	Y	Y		Y		
204	C15 H24	33.355	1H-3a,7-Methanoazulene, 2,3,6,7,8,8a-hexahydro-1,4,9,9-tetramethyl-, (1.alpha.,3a.alpha.,7.alpha.,8a.beta.)-	Y			Y		
		33.443		Y					
		33.54		Y	Y	Y	Y	Y	
		33.736		Y		Y	Y		
168	C11 H20 O	33.867	(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-methanol	Y		Y	Y	Y	
		33.957		Y					
		34.105		Y					
		34.185		Y				Y	
220	C15 H24 O	34.293	Caryophyllene oxide	Y	Y	Y	Y	Y	
204	C15 H24	34.44	Longicyclene	Y		Y	Y	Y	

222	C15 H26 O	34.598	1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1ar-(1a.alpha.,4.alpha.,4a.beta.,7.alpha.,7a.beta.,7b.alpha.)]-	Y		Y		
238	C15 H26 O2	34.75	Isocalamenediol		Y	Y		Y
		34.639		Y				
220	C15 H24 O	34.857	Longifolenealdehyde	Y				
218	C15 H22 O	34.925	,6,6-Trimethyl-2-(3-methylbuta-1,3-dienyl)-3-oxatricyclo[5.1.0.0(2,4)]octane			Y		
290	C20 H34 O	34.989	Copalol	Y	Y		Y	Y
222	C15 H26 O	35.145	Drim-7-en-11-ol	Y		Y		
		35.19		Y			Y	
124	C9 H16	35.274	(2E,4E)-6,6-Dimethyl-2,4-heptadiene	Y			Y	Y
		35.453		Y	Y			
		35.636		Y				
152	C10 H16 O	35.724	trans-Verbenol	Y				
222	C15 H26 O	35.849	Drimenol	Y		Y		Y
270	C17 H34 O2	35.92	Isopropyl myristate				Y	
180	C12 H20 O	35.961	2,2,6,8-Tetramethyl-7-oxatricyclo[6.1.0.0(1,6)]nonane	Y	Y			
		36.085		Y		Y		
206	C15 H26	36.268	Tricyclo[4.3.0.0(7,9)]nonane, 2,2,5,5,8,8-hexamethyl-, (1.alpha.,6.beta.,7.alpha.,9.alpha.)-	Y				
		36.323		Y	Y			
		36.365		Y				
278	C16 H22 O4	36.47	Diisobutyl phthalate			Y	Y	Y

		36.481		Y				
206	C14 H22 O	36.636	Norpatchoulenol	Y		Y		
270	C17 H34 O2	37.19	Methyl 14-methylpentadecanoate	Y				
334	C20 H30 O4	37.62	Butyl octyl phthalate	Y		Y	Y	Y
		37.864		Y				
152	C10 H16 O	38.18	cis-Chrysanthenol	Y		Y		
206	C15 H26	38.396	Patchoulane	Y	Y			Y
		38.43		Y				
		38.782		Y				
280	C18 H32 O2	39.042	cis,cis-Linoleic acid	Y				
296	C19 H36 O2	39.101	Methyl elaidate	Y				
234	C15 H22 O2	39.265	Drinenin	Y	Y			
284	C18 H36 O2	39.349	Methyl isoheptadecanoate	Y				
		39.668		Y				
		42.086		Y				
		43.375		Y	Y	Y	Y	
		44.946		Y				

Y indicates the presence of that the compound in the indicated extract.

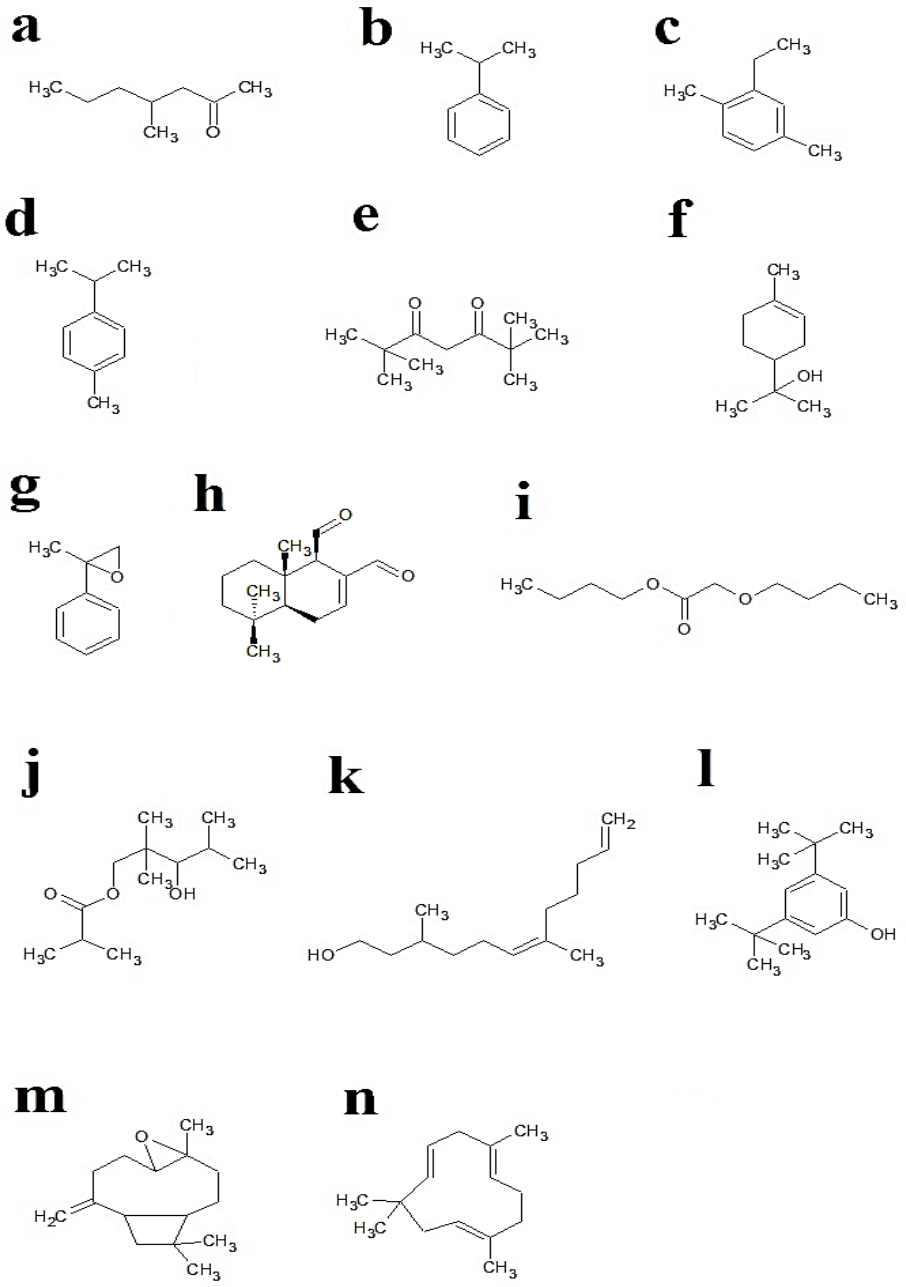

Figure 6.12: Chemical structures of *T. lanceolata* berry compounds detected in all solvent extractions using headspace GC-MS analysis: (a) 4-methyl-2-heptanone, (b) cumene, (c) 1, 2-dimethyl-4-ethylbenzene, (d) p-cymene, (e) 2, 2, 6, 6-tetramethyl-3, 5-heptanedione, (f) α-terpineol, (g) 2-methyl-2-phenyl-oxirane, (h) m-di-tert-butylbenzene, (i) butyl butoxyacetate, (j) propanoic acid, 2-methyl-, 3-hydroxy-2, 2, 4-trimethylpentyl ester, (k) (6Z)-3, 7-dimethyl-6, 11-dodecadien-1-ol, (l) 3, 5-di-tert-butylphenol, (m) caryophyllene oxide isomer 1, (n) caryophyllene oxide isomer 2 (humulene).

6.5 Discussion

A comparison between the metabolomics profiles of all *T. lanceolata* berry extracts displaying anti-proliferative activity highlighted a number of interesting compounds. An obvious feature was the number and diversity of terpenoids present in all extracts. From a comparison between the metabolomics profile comparisons obtained from LC-MS analysis, it was seen that the sesquiterpenoids (capsidol (capsaicin), Figure 6.6 b; salutarisolide, Figure 6.6 c; punctaporin B, Figure 6.6 e; dihydroartimisinin, Figure 6.6 k; matricin, Figure 6.6 m) were present in all extracts. It is likely that several of these contribute to the anti-proliferative activity against the carcinoma cell lines reported in Chapters 3 and 4. Capsidol has previously been reported to have potent anti-proliferative effects on prostate carcinoma cells, inducing apoptosis in both androgen receptor positive and negative cell lines [366]. Capsidol induced substantially increased levels of p53, p21 and Bax, whilst inhibiting NF-κB activation by preventing its nuclear migration and inhibited TNF-α stimulated degradation of IκBα in PC-3 cells, thus inhibiting proteasome activity. Furthermore, anti-proliferative activity was also demonstrated *in vivo* in a murine model in that study. Other studies have also demonstrated capsidol induced apoptosis in other cell lines including AsPC-1 and BxPC-3 pancreatic cancer [367] and HT-29 colon cancer cell lines [368]. Whilst reports of the other purified sesquiterpenoids putatively identified by LC-MS analysis in all of the *T. lanceolata* berry extracts are lacking, numerous studies have reported anti-proliferative activity for plant extract containing one or more of these sesquiterpenoid [369-371]. Furthermore, several sesquiterpenoids such as eupatoriopicrin [332, 333] and parthenolide [334] have also been reported to be potent anti-proliferative agents, inducing apoptosis via multiple mechanisms. The monoterpenoids piperitone (Figure 6.13 a) and gelsemiol (Figure 6.13 b), the

sesquiterpenoids and parthenolide (Figure 6.13 c), and the diterpenoid balletenol (Figure 6.13 d) were also putatively identified in one or more *T. lanceolata* berry extracts. However, none of these terpenoids were present across all of the extracts.

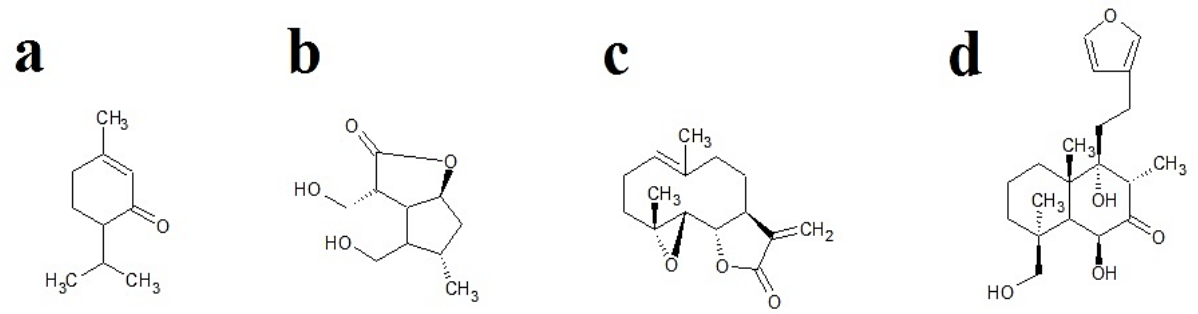

Figure 6.13: Terpinoid compounds detected in some, but not all the *T. lanceolata* berry extracts by LC-MS headspace analysis: (a) piperitone, (b) gelsemiol, (c) parthenolide, (d) ballotenol.

GC-MS headspace analysis also detected a diversity of terpenoids in the *T. lanceolata* berry extracts. The monoterpenoid α-terpineol (Figure 6.12 f), as well as the sesquiterpenoids polygodial (Figure 6.12 h), caryophyllene oxide (Figure 6.12 m) and humulene (Figure 6.12 n) and were detected in all *T. lanceolata* berry extracts. Polygodial was the major compound detected in the methanolic, aqueous and hexane extracts (based on peak area). This is in agreement with previous studies which frequently cite polygodial as a major component in *T. lanceolata* berries [372]. Indeed, some studies have reported that polygodial may account for nearly 40% of commercial *T. lanceolata* essential oil components [373]. Interestingly, the anti-proliferative activity of polygodial has been reported in several studies. A recent study reported that polygodial inhibited the proliferation of HT-29, MDA-MB231, DHF, MCF-7,

PC-3, DU-145 and CoN carcinoma cells, with IC_{50} values as low as 12.5 µM [374]. A further study reported polygodial to be a potent inhibitor of Ehrlich ascites tumour cells and mouse lymphocytic leukaemia-derived L1210 cells [375]. Polygodial and several other drimane sesquiterpenoids isolated from *Warburgia ugandensis* were reported to be potent inhibitors of KB cell proliferation (90% inhibition at 10 µg/mL, which equates to approximately 40 µM) [376]. This result is particularly significant as KB cells are a multidrug resistant cell line whose proliferation is not significantly inhibited by colchicine, adriamycin or vinblastine [377]. Unfortunately, whilst that study reported an IC_{90} value, it did not report an IC_{50} value, making it difficult to make comparisons with other anti-proliferative drugs. The anti-proliferative mechanism of polygodial has not yet been thoroughly studied. However, polygodial has been reported to inhibit the production of prostaglandin E2 [237], and is therefore likely to inhibit cyclooxygenase 2 (COX-2). As COX-2 synthesis is regulated via a TNF-α dependent mechanism [378], it is likely that an activation of TNF-α may be accompanied by a downregulation of NF-κB and subsequent induction of apoptosis. However, this is yet to be adequately tested. Furthermore, many plant extracts which contain polygodial have been reported to have anticancer properties [375, 376, 379, 380].

Caryophyllene oxide has been reported to inhibit cell growth and induce apoptosis in human PC-3 prostate and MCF-7 breast cancer cell lines [381]. The anticancer effects are mediated by 2 mechanisms: an inhibition of PI3K/AKT/mTOR/S6K1 signalling cascade, as well as by activating cellular ERK, JNK and p38 MAPK. Caryophyllene oxide exposure induces increased levels of mitochondrial ROS, loss of mitochondrial membrane potential, release of cytochrome c, activation of caspase-3 and cleavage of PARP. Furthermore, caryophyllene oxide down regulated the expression of cyclin D1, bcl-2, bcl-xL, survivin, IAP-1 and IAP-2,

whilst stimulating the expression of p53 and p21. Also noteworthy, caryophyllene oxide can potentiate the apoptotic effects of other sesquiterpenoids, including humulene and isocaryophyllene [382]. The anti-proliferative activity of α-terpineol via a downregulation of NF-κB has already been discussed in Chapter 5 so will not be further discussed here.

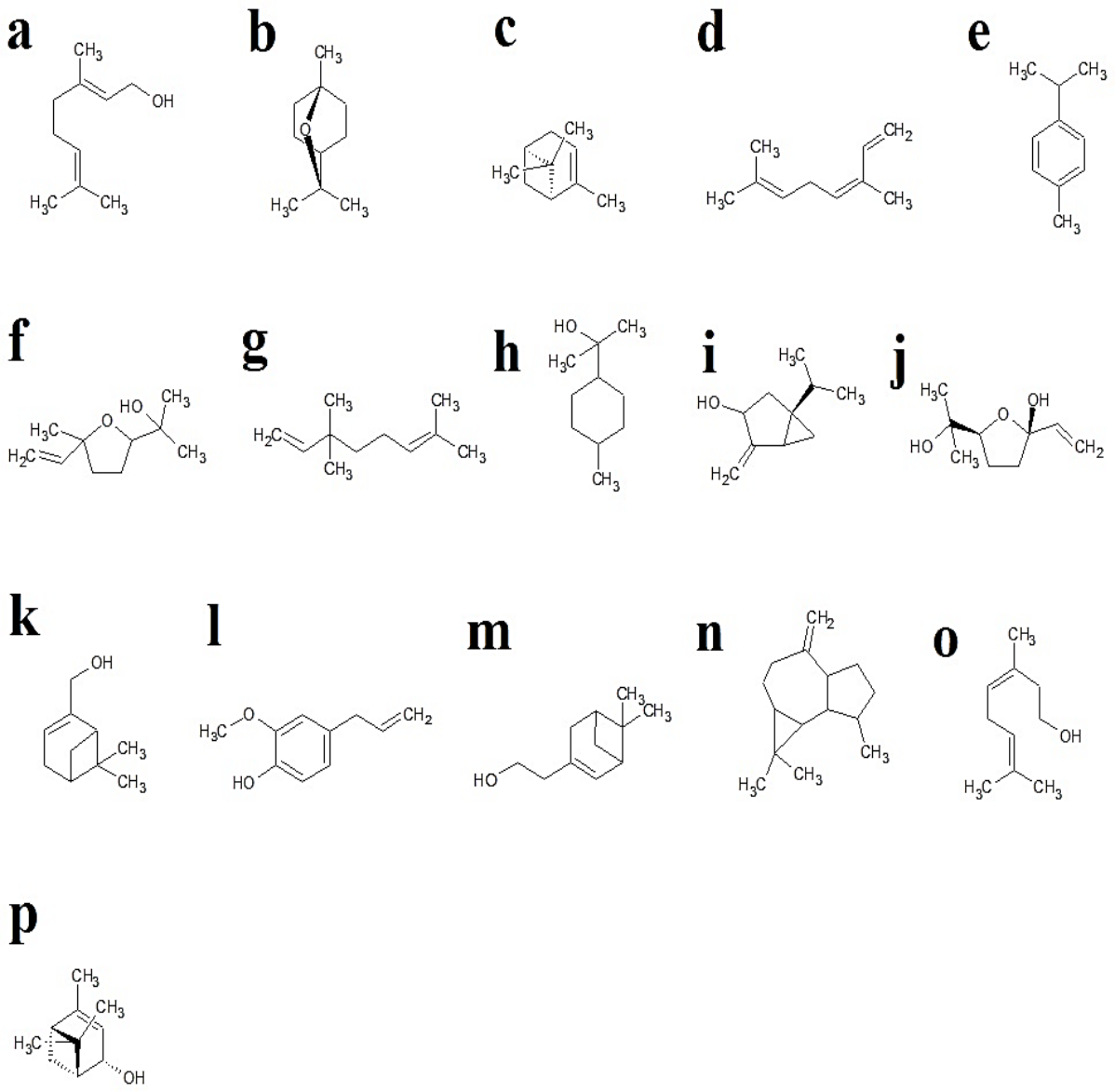

Figure 6.14: Monoterpenoids components which were detected in some, but not all the *T. lanceolata* berry extracts by GC-MS headspace analysis: (a) geraniol, (b), cineole (c), α-pinene, (d) β-ocimene, (e) p-cymene, (f) linalool oxide, (g) linalool, (h) dihydro-γ-terpineol, (i) sabinol, (j) trans-linalool-3,7-oxide, (k) myrtenol, (l) p-eugenol, (m) homomyrtenol, (n) aromandendrene, (o) isogeraniol, (p) verbenole.

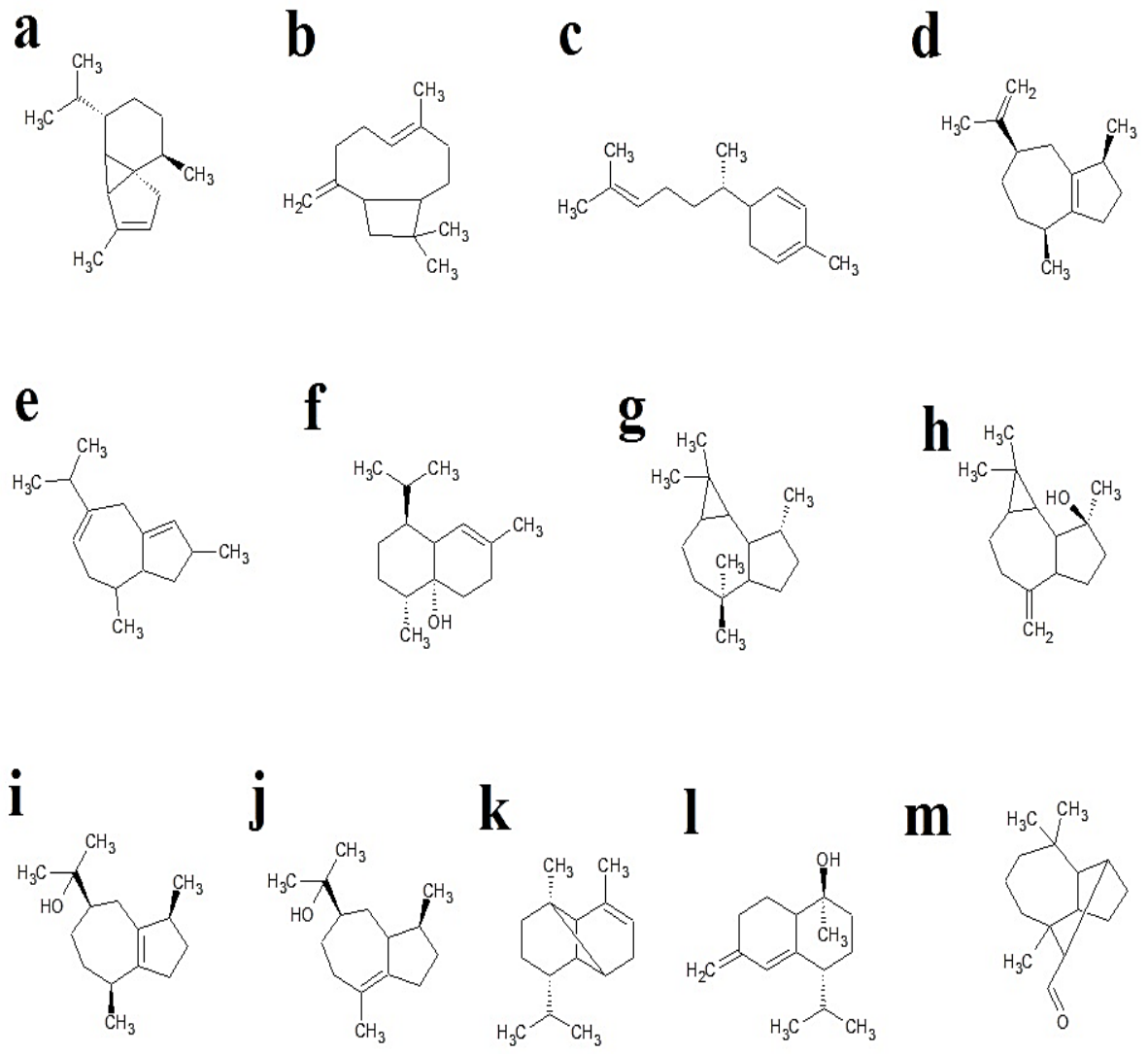

Figure 6.15: Sesquiterpenoid components detected in some, but not all the *T. lanceolata* berry extracts by GC-MS headspace analysis: (a) α-cubenene, (b) caryophyllene, (c) zingiberene, (d) α-guaiene, (e) guaia-6,9-diene, (f) cubenol, (g) ledol, (h) spathulenol, (i) guaiol, (j) guai-1(10)-en-11-ol, (k) longicyclene, (l) isocalamenediol, (m) longifolenealdehyde.

A number of other terpenoids were detected in some, but not all the *T. lanceolata* berry extracts by GC-MS headspace analysis. Monoterpenoids were particularly prevalent, with 2,2,6-trimethyl-6-vinyltetrahydropyran (geraniol, Figure 6.14 a), cineole (Figure 6.14 b), α-pinene (Figure 6.14 c), β-ocimene (Figure 6.14 d), p-cymene (Figure 6.14 e), linalool oxide (Figure 6.14 f), linalool (Figure 6.14 g), dihydro-γ-terpineol (Figure 6.14 h), 4(10)-thujen-3-ol (sabinol) (Figure 6.14 i), trans-linalool-3,7-oxide (Figure 6.14 j), myrtenol (Figure 6.14 k), p-eugenol (Figure 6.14 l), homomyrtenol (Figure 6.14 m), aromandendrene (Figure 6.14 n), isogeraniol (Figure 6.14 o) and verbenole (Figure 6.14 p) detected in all extracts. The anticancer properties of many of these monoterpenoids have been extensively documented. Geraniol and iosgeraniol inhibit the proliferation of murine leukaemia, hepatoma and melanomas *in vivo* [383, 384]. Cineole induces apoptosis in HCT116 and RKO human colon carcinoma cells via an inactivation of Akt and survivin, and an activation of p38 and of caspase-3 [385]. Treatment of metastatic melanoma cells with α-pinene treatment disrupts mitochondrial potential, increases production of ROS, increases caspase-3 activity and DNA fragmentation, resulting in the induction of apoptosis [386]. Linalool and several linalool derivatives have both cytostatic and cytotoxic effects and have been reported to activate p53 and cyclin dependent kinase inhibitors, suggesting that they induce apoptosis as well as arresting the cell cycle in a panel of leukaemia cell lines [387]. Furthermore, linalool exposure reverses doxorubicin resistance in MCF7 AdrR multidrug resistant human breast adenocarcinoma cells, thereby improving the therapeutic index of doxorubicin [388]. Many of these monoterpenoids have also been identified in plant extracts with anticancer activities [389-393].

Several sesquiterpenoids including α-cubenene (Figure 6.15 a), caryophyllene (Figure 6.15 b), zingiberene (Figure 6.15 c), α-guaiene (Figure 6.15 d), guaia-6, 9-diene (Figure 6.15 e), cubenol (Figure 6.15 f), ledol (Figure 6.15 g), spathulenol (Figure 6.15 h), guaiol (Figure 6.15 i), guai-1(10)-en-11-ol (Figure 6.15 j), longicyclene (Figure 6.15 k), isocalamenediol (Figure 6.15 l), longifolenealdehyde (Figure 6.15 m) were also present in some but not all *T. lanceolata* berry extracts. Caryophyllene has been reported to inhibit cell growth and induce apoptosis in human PC-3 prostate and MCF-7 breast cancer cell lines with similar efficacy to caryophyllene oxide [381]. Zingiberene supresses the proliferation of rat N2a-NB neuroblastoma cells [394]. The authors of that study examined markers of oxidative stress. They reported that zingiberene treatment induced oxidative stress and postulated that this results in an induction of apoptosis. Whilst reports of anticancer activity are lacking for the other sesquiterpenoids, many have been identified in anti-proliferative plant extracts [395-403]. Parthenin (Figure 6.16 a), which was detected only in the methanolic extract, has been reported to inhibit NF-κB production [404] and induce apoptosis in acute myelogenous leukaemia [405]. Furthermore, a single diterpenoid (phytol, Figure 6.16 b) was putatively identified only in the methanolic extract by GC-MS headspace analysis. Exposure of Molt 4B human leukaemia cells to phytol inhibits cellular proliferation and induces apoptosis [406]. Furthermore, phytol has been identified in several anti-proliferative plant extracts [407].

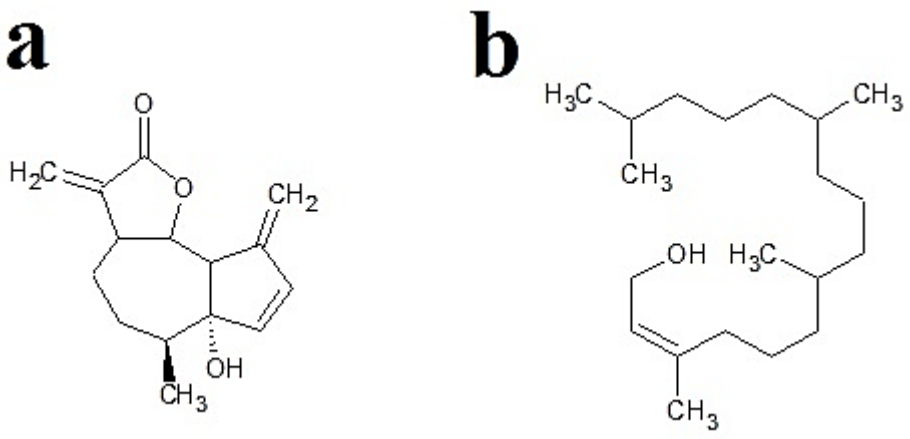

Figure 6.16: Structures of terpenoid components detected only in the *T. lanceolata* berry methanolic extract by GC-MS: (a) parthenin and (b) phytol.

Several other interesting compounds were also putatively identified in the *T. lanceolata* berry extracts. Of particular note, was the presence of multiple stilbenes. Combretastatin A1 was detected in all extracts by LC-MS analysis. Other stilbenes (resveratrol, pentahydroxy stilbene, combretastatin, combretastatin A4) as well as the glycosylated stilbene piceid, were detected in one or more extracts, but were not present in all. Stilbenes have well documented anti-proliferative properties and have mechanisms that confer both cytostatic and cytotoxic properties towards cancer cells. The anticancer properties and mechanisms of resveratrol and piceid are particularly well studied and have been thoroughly discussed already in Chapter 5 and will not be further discussed here. Pentahydroxy stilbene is a resveratrol derivative and has similar activity to resveratrol. A recent study reported that pentahydroxy stilbene has potent cytotoxicity towards HT-29 colorectal carcinoma cells [408]. The authors of that study reported that pentahydroxy stilbene induces apoptosis via a caspase dependent mechanism and regulates the oxidative effects via an up regulation of ROS generation and a depletion of intracellular glutathione. A different study examined the signal pathways involved in

pentahydroxy stilbene induced apoptosis and reported MEK/ERK signalling to be the direct molecular target [409].

Perhaps the most promising stilbenes for the treatment of cancer are the combretastatins. Combretastatins are well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin, thereby disrupting microtubule formation [246]. Of particular interest, combretastatin A4 (Figure 6.17 a) has been reported to inhibit tumour growth in a murine bladder model [410]. The same study reported that the combretastatin A4 anti-proliferative mechanism was due to an inhibition of microtubule polymerisation. This results in IC_{50} cytotoxicity values in human bladder cancer cells of less than 4 nM. It is believed that combretastatin A4 acts by a similar mechanism to that of colchicine (N-[(7S)-1, 2, 3, 10-tetramethoxy-9-oxo-5, 6, 7, 9-tetrahydrobenzo[a]heptlen-7-yl] acetamide), by binding the colchicine binding site on the tubulin peptide and inhibiting polymerisation [247]. The anticancer properties of combretastatin A4 are believed to be of such interest that the drug is currently in phase 1 trials for the treatment of some cancers [411] and phase 2 trials for the treatment of others [412].

Also noteworthy in *T. lanceolata* berry extracts were the diversity of tannins present. Whilst no tannin was detected in all of the extracts, 7-deshydroxypyrogallin 4-carboxylic acid (Figure 6.17 b), gallic acid (Figure 6.17 c), gallocatechin (Figure 6.17 d), exifone (Figure 6.17 e), ellagic acid (Figure 6.1 f), trimethyl ellagic acid (Figure 6.17 g) and corilagen (Figure 6.17 h) were detected only in the more polar extracts. The pseudotannin ferulic acid (Figure 6.17 i) was also detected in the methanolic extract, as well the less polar ethyl acetate and chloroform extracts. The potential of tannins in cancer therapy has already been described

earlier in Chapter 5. It is likely that these tannins may have also contributed to the anticancer activities of the extracts in which they are present.

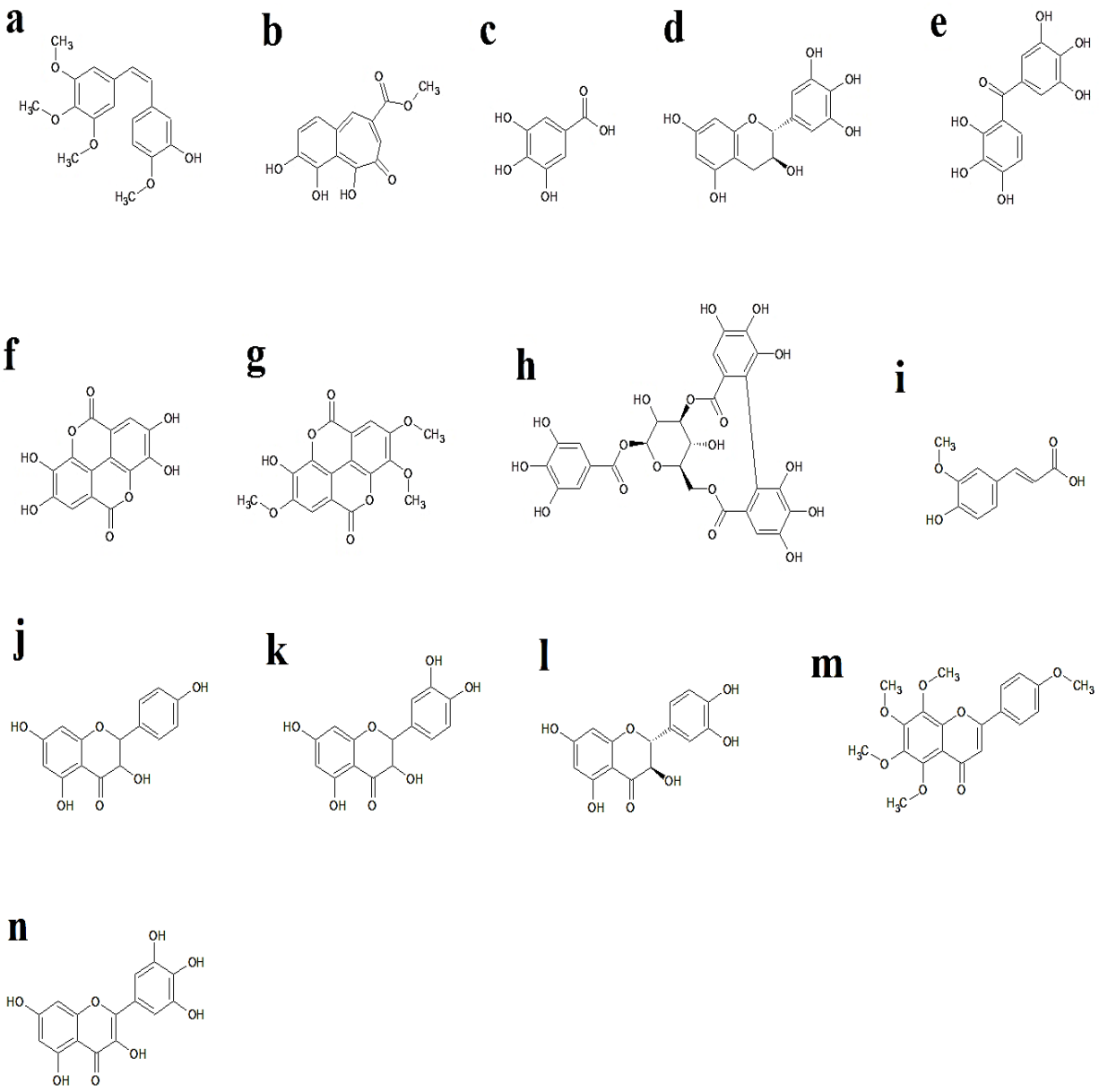

Figure 6.17: Non-terpenoid components of the *T. lanceolata* berry extracts: (a) combretastatin A4, (b) 7-deshydroxypyrogallin 4-carboxylic acid, (c) gallic acid, (d) gallocatechin, (e) exifone, (f) ellagic acid, (g) trimethyl ellagic acid, (h) corilagen, (i) ferulic acid, (j) dihydrokaempferol, (k) quercetin, (l) taxifolin, (m) tangeritin, (n) myricetin.

A number of flavonoids were also detected by LC-MS analysis in some but not all of the *T. lanceolata* berry extracts. In particular, dihydrokaempferol (Figure 6.17 j), quercetin (Figure 6.17 k), taxifolin (Figure 6.17 l), tangeritin (Figure 6.17 m) and myricetin (Figure 6.17 n) were putatively identified in the polar extractions. A recent study reported good anti-proliferative properties for isolated dihydrokaempferol and quercetin [413]. When tested at a concentration of 100 µg/mL against human CAL-27 and KB oral carcinoma cell lines, HT29 and HCT-116 colorectal cancer cells, and LNCaP and DU145 prostate cancer cells, these flavonoids inhibited cell proliferation by as much as 80% of the untreated cell proliferation rate. Unfortunately, this study did not report IC_{50} values, making it difficult to compare the efficacy with other compounds/other studies. A different study reported that kaempferol, taxifolin and myricetin induce apoptosis in human LNCaP prostate cancer and MDA-MB-231 breast cancer cells [414]. This study reported a strong relationship between the induction of apoptosis and an inhibition in fatty acid synthesis. When exogenous palmitate was added to the treated cells, apoptosis was supressed, indicating that the mechanism by which flavonoids induce apoptosis is associated with their inhibition of fatty acid synthesis. Quercetin, kaempferol and taxifolin have also been reported to inhibit glutathione S-transferase P1-1 and GS-X pump activity in MCF7 cells, with IC_{50} values between 0.8 and 8 µM, indicating that these flavonoids may be useful for potentiating apoptosis in multidrug resistant cells [415]. Furthermore, these flavonoids have been identified in many plant extracts with anti-proliferative activity [416-419].

The studies presented in this chapter indicate that the *T. lanceolata* berry extracts contain a variety of different compounds with anti-proliferative activity. Furthermore, a comparison between the metabolomics profiles of the extracts has highlighted several compounds of

particular interest. Despite this, it is unlikely that any single molecule is responsible for the *T. lanceolata* berry extracts anti-proliferative activity reported in Chapters 3 and 4. Instead, it is more likely that a number of compounds contribute to this activity. Furthermore, it is possible that synergistic interactions between the various bioactive components may be potentiating the anti-proliferativity of the individual components, increasing their efficacy. At the very least, the presence of numerous molecules with a wide variety of anticancer mechanisms indicates that these extracts are likely to function by pluripotent pathways.

CHAPTER 7: DISCUSSION

The intake of plant based food or extracts has the potential to protect against and decrease the incidence of some cancers [156]. A recent epidemiological study which examined over 200 individual cases reported that the intake of fruit and vegetables delivered significant protection against the incidence of lung, colon, breast, cervix, oesophagus, oral cavity, stomach, bladder, pancreas and ovary cancers [123, 420-423]. The phytochemical content of high antioxidant plants confers cellular protection against oxidative stress. Plant phenolic compounds exhibit antioxidant potential and thus possess redox capability to inhibit mutagenesis and carcinogenesis [168]. Furthermore, antioxidant plant compounds may selectively affect the redox state of a carcinoma cell, providing further protection against cancer [424-427].

Plant-derived compounds already account for a major portion of the current chemotherapeutic treatments for cancer. Past studies have revealed that the plant's secondary metabolites have great anticancer potential. Indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last 50 years have been directed towards combating cancer [428-430]. There are 4 main classes of plant-derived anti-cancer drugs consisting of vinca alkaloids, epipodophylltoxins, taxanes and campothecins [289, 431-435] that are currently being used as a front line drugs in cancer treatment and management. These metabolites are toxic and part of the host plant's defence as evidenced by alkaloids present as essential oils in the Genus *Acronychia* that have been known to function as DNA

intercalating agents [289]. Structural elucidation of these alkaloids indicates that they are polyphenolic compounds with redox potential.

Taxus brevifolia (taxol) and *Catharanthus roseus* (vincristine and vinblastine) are perhaps the most commonly used anticancer drugs. These compounds have been used continuously over the last 5 decades for cancer chemotherapy due to lack of other effective options. Taxol is a semi-synthetic product which is manufactured from the bark of the of the *Taxus brevifolia* (Pacific yew). Vincristine and vinblastine are alkaloids from the leaves of *Catharanthus roseus* (Madagascar periwinkle). All of these compounds are in short supply due to lack of raw materials. Although biotechnology has alleviated much of the need through tissue culture, semi synthesis and total synthesis, demand is still on the rise and medical science is struggling to supply adequate quantities of these drugs [436]. Unfortunately, the harvesting of bark from yew trees to meet medicinal demand has resulted in deforestation, further exacerbating the problem. Additionally, many of the current retinue of cancer chemotherapeutics are inadequate to the treatment of the myriad of aggressive cancers. For example, pancreatic cancer often requires a concurrent high dosage of chemotherapeutics and radiotherapy to arrest angiogenesis and metastasis. This is ultimately detrimental to the patient as these treatments are highly toxic and patients may suffer from acute chemical poisoning from the treatment.

Hence there is a critical need to develop novel cancer drugs that are highly potent but relatively nontoxic. As high antioxidant activity has been linked with anticancer properties, a major focus of the present study was to identify high antioxidant Australian native plants and

to screen them for anti-proliferative activity. The results of this studies demonstrated a correlation between high antiproliferative potential against the carcinoma cell lines and high antioxidant content present in the plant extracts. The study further aimed to narrow the focus to identify species with the most promising potential for further development as cancer chemotherapies.

The initial selection of Australian plants of high antioxidant capacity, were based on an extensive literature search. Following selection, the high antioxidant content of these plants was verified by using a DPPH reduction assay before trialling the extracts for their antiproliferative activities. Interestingly, all of the high antioxidant Australian plants displayed strong anti-proliferative activities against HeLa and Caco-2 carcinoma cell lines. Indeed, some of the extracts were particularly potent anti-proliferative drugs. In our initial screening studies, the *Syzygium* spp. were classed as potent antiproliferative agents against HeLa and Caco-2 cells due to their low IC_{50} values. Notably *T. ferdinandiana* recorded negative proliferation, whilst *T. lanceolata* extracts were able to inhibit > 80% cellular proliferation of HeLa and Caco-2. All these 3 plants had high antioxidant capacity and were good inhibitors of HeLa and Caco-2 proliferation, supporting the hypothesis that a positive correlation exists between potent antiproliferative activity and high antioxidant capacity.

Interestingly, several extracts (particularly those prepared from *T. ferdinandiana* and *T. lanceolata*) not only completely blocked cellular proliferation, but resulted in cell metabolic rates lower than those at the commencement of the experiment (as determined by an MTS colorimetric assay). This indicates that not only had the cell number not increased over the time-course of the experiment, but the metabolic rate (which is indicative of cellular activity and thus cell number) had decreased. This is a noteworthy result as such decreases in metabolic activity are indicative of cell death. Thus, it is likely that the anti-proliferative mechanism of these extracts may involve the induction of apoptosis. Indeed, some of the extracts were particularly potent anti-proliferative drugs. Several other plant species were also good anti-proliferative agents. In particular, the *Syzygium* spp. and *D. pruriens* produced very low IC_{50} values of (43 µg/mL, 169 µg/mL), which is indicative of potent anti-proliferative activity.

A further interesting trend was noted. Whilst all of the plant species screened inhibited HeLa and Caco-2 proliferation, there were quite dramatic differences between the different solvent extractions within a plant species (or plant part). Generally, the anti-proliferative activity was greatest in the extracts with the highest measured antioxidant capacity (the aqueous and methanolic extracts). Furthermore, the extracts of some species with low antioxidant capacity and reducing power (the ethyl acetate and hexane extracts), not only failed to inhibit cellular proliferation, but instead stimulated cell growth. This is evidenced with the *D. pruriens* ethyl acetate leaf extracts > 75% cellular proliferation was observed for both HeLa and Caco-2.

Similar trends have also been reported in previous studies examining the effects of other plant extracts on cell lines. A recent study reported that apples with skin possessed a far superior radical scavenging activity compared to apples without skin. The greater phenolic content present in the skin as (quercetin glycosides) could have been responsible for this increased activity against radicals. The same study also trialled apples with and without skin against Caco-2 (human colorectal carcinoma) and HepG2 (human liver tumour) cells. Interestingly, both apple extracts induced cell proliferation at low concentrations, but significantly reduced tumour cell proliferation at higher concentrations. The authors postulated that these effects may be due to the presence of both phenolic acids and flavonoids in the flesh and skin of the apple. Thus, it is likely that polyphenolic compounds can induce proliferation of carcinomas at lower concentrations, but inhibit at their growth at higher concentrations as a result of their redox potential [437]. Thus, the first objective of the project was achieved: high antiproliferative activity correlated with high antioxidant content in the extracts, whilst proliferation was induced in in extracts with lower antioxidant content.

A further aim of my project was to identify the most promising plant species through anti-proliferative screening against HeLa and Caco-2 to narrow the focus for further studies. As all plant species tested displayed anti-proliferative activity, criteria were developed to select the best candidate species for further evaluations. The selection criterion required the extracts to have high antioxidant content (> 100 mg AA equivalents per g extracted plant material), good antiproliferative potential with IC_{50} (< 1000 µg/mL) and low toxicity (< 1000 µg/mL). Utilising these criteria, *T. ferdinandiana* and *T. lanceolata* were chosen for further investigations as they both were potent anti-proliferation, both had high antioxidant capacities and both were non-toxic. Whilst all other species fulfilled one or more of these criteria, only

these two species fulfilled all of the selection criteria. Much of the high antioxidant capacity of *T. ferdinandiana* has previously been attributed to its extremely high ascorbic acid content [179]. In contract, the same study reported that *T. lanceolata* (which also had a high antioxidant capacity) had only low levels of ascorbate, below the detection threshold. This difference between the 2 species provides an interesting comparison between the anti-proliferative activities of these species. However, the potent antiproliferative activity in several other plant species attests to their potential testing. The *Syzygium* spp. displayed potent antiproliferative activity against HeLa and Caco-2, and were only excluded from further testing due to their toxicity. *D. pruriens* and *A. acidula* were also not selected despite their good antiproliferative activity and low toxicity, as they had relatively low antioxidant content in comparison to *T. ferdinandiana* and *T. lanceolata*. Although the *Syzygium* spp., *D. pruriens* and *A. acidula* were not selected for the course of this study due to the selection criteria, these plants warrant further investigation and studies are already planned within our group. Thus, this fulfilled the aim of narrowing the field of study to two plants namely *T. ferdinandiana* and *T. lanceolata* for testing against an extended panel of cell lines to determine antiproliferative mechanistic detail.

The *T. ferdinandiana* and *T. lanceolata* extracts were further examined using an expanded cell panel. The cell lines used in this study were selected, not only to expand the number of carcinoma lines, but also to gain detail into the antiproliferative mechanism of these extracts. The well-characterised diagnostic cell lines (JEG-3/JAR, MC3T3-E1/MG-63) were chosen due to their differential production/secretion of proteins associated with cellular redox state and apoptotic mechanisms were used. The JEG-3 and JAR cell pair varies in the relative levels of expressed thioredoxin and TNF. Notably, JEG-3 cells are known for their

production of higher relative levels of thioredoxin JAR cells [304]. Conversely, JAR cells produce substantially higher levels of TNF than JEG-3 cells do. It can be postulated that actively proliferating JAR cells produced TNF at levels that would be subsequently higher compared to JEG-3 that has relatively constant levels of TNF. The MC3T3-E1 and MG-63 cell pair varies in their susceptibility to TNF, with the MC3T-E1 being the more substantially susceptible of the two.

Surprisingly, *T. ferdinandiana* extracts were significantly more potent against JEG-3 cells than against JAR cells. Indeed, exposure to *T. ferdinandiana* extracts resulted in negative proliferation (i.e. a metabolic rate below that at the start of the experiment), suggesting cytotoxicity. It was postulated that an extrinsic pathway may not have been involved in apoptosis due to the involvement of TNF as a promoter of cellular proliferation in JAR cells. In contrast, the *T. lanceolata* fruit and leaf extracts displayed more potent inhibition of JAR cell proliferation than JEG-3 cells. Indeed, with a decrease in proliferation to below the starting point (i.e. negative proliferation). Such a dramatic decrease in proliferation is indicative of cytotoxic mechanisms in the JAR cells. As JAR cells express relative high levels of TNF, this may function as a promoter of apoptosis in the JAR cells. Conversely, thioredoxin may have had a protective role in JEG-3 cells, and as such would possess a greater role with the *T. lanceolata* extracts (with ascorbic acid below the level of detection) compared to the *T. ferdinandiana* extracts which have very high ascorbic acid levels [179]. Thus, the results suggest that an extrinsic apoptotic pathway may be involved.

T. ferdinandiana extracts also inhibited MC3T3-E1 cellular proliferation to a greater degree compared to MG-63 cells, as evidenced by negative proliferation indicative of cytotoxicity. It was postulated that a TNF induced extrinsic apoptotic pathway might have been involved. Similarly, *T.lanceolata* fruit and leaf extracts were also noted to induce negative proliferation in MC3T3-E1 cells possibly implicating TNF as a promoter of apoptosis through an extrinsic pathway.

The induction of negative proliferation following exposure of the JEG-3, JAR, MC3T3-E1, MG-63, HeLa and Caco-2 carcinoma cells to the *T. ferdinandiana* and *T. lanceolata* extracts (despite their lack of toxicity in the toxicity screening assays) suggests that these extracts act via apoptotic mechanisms. This hypothesis is supported by the cell imaging studies presented in chapter 4. Apoptogenic morphology indicating cytotoxicity was clearly observed in Caco-2 cells on exposure to the plants extracts. The findings reported here examined the morphological changes of key organelles in Caco-2 cells on exposure to the extracts at sub lethal concentrations. Characteristics consistent with apoptosis such as an intact membrane, marginalisation of cytoplasm, extensive 'blebbing', and presence of apoptotic bodies were observed. Similarly, morphological changes consistent with necrosis which included the loss of membrane integrity, extensive chromatin condensation, presence of numerous vacuoles and appearance of 'ghosts' (cells without cellular contents) were also observed. Hence, both phenomena caused by the *T. ferdinandiana* and *T. lanceolata* extracts to varying degrees are indicative of the potency of the extracts and further confirms all results obtained through the MTS cell proliferation assays.

Phytochemical evaluations of *T. ferdinandiana* and *T. lanceolata* extracts revealed a diversity of compounds which have potential as cytotoxic agents. Indeed, a number of phytochemical classes in *T. ferdinandiana* extracts were putatively identified were inclusive of tannins, pseudo-tannins, stilbenes, sphingolipids, imidazole-alkaloids, lactones, aliphatic acids, and monoterpenoids whilst others may truly be novel compounds. Alternatively, *T. lanceolata* extracts, had a comparatively different profile which included classes such as sesquiterpenoid polygodials, benzene/benzoic acid derivatives, stilbenes, quinazoline alkaloids and monoterpenoids. This study will attempt to report a few of the interesting compounds that were putatively identified, since most compounds did report some manner of antiproliferative activity in the literature search. Hence, the results of the metabolomic analysis and subsequent phytochemical profiling are consistent with cytotoxic activity observed.

QTOF HPLC-MS identified the presence of C17 sphingosine in the active *T. ferdinandiana* extracts known to be a potent inhibitor of sphingosine-1- phosphate (S1P) [329], thereby suppressing carcinoma proliferation, angiogenesis and inducing apoptosis [438, 439]. Some carcinoma cells inclusive of Caco-2 been known to possess elevated levels of S1P [440, 441] which has been implicated in TNF induction [325-328] resulting in NF-κB activation and suppression of apoptosis [327]. Thus, C17 sphingosine may have in part been responsible for negative proliferation observed in Caco-2 cells. In addition, several monoterpenoids (linalool oxide, terpineol, camphor) were detected in the extracts of which linalool and α-terpineol deserves special mention. Some studies have reported linalool's cytotoxic potential via TFN-α production [359], likewise α-terpineol's antiproliferative ability via NF-κB inhibition [360].

Similarly, many other phytochemical components in the *T. lanceolata* extracts are consistent with cytotoxic mechanisms. Polygodials form a major component of phytochemical profile of the *T. lanceolata* extracts, and many studies have reported much antiproliferative activity in cancer lines even against multi drug resistant lines [376, 377]. This might be possibly through the activation of TNF-α [378] followed by a downregulation of NF-κB and subsequent induction of apoptosis. Another cytotoxic agent was the sesquiterpenoid caryophyllene oxide which was detected via GC-MS headspace. It has the potential to induce apoptosis in cancer cell lines [381] via the induction of increased mitochondrial ROS, leading to the activation of caspase 3 and ultimately apoptosis. Also detected, was the penta-hydroxy stilbene which is a resveratrol derivative in the *T. lanceolata* extracts. It has similar activity to resveratrol. It was reported that penta-hydroxy stilbene acts through a cytotoxic mechanism [408] via an upregulation of ROS generation and a depletion of intracellular glutathione leading to the induction of apoptosis via a caspase dependent mechanism.

Whilst apoptotic pathways were indicated by the antiproliferative screening and cell imaging studies, other antiproliferative pathways may also be involved. The *T. ferdinandiana* and *T. lanceolata* extracts are complex mixtures containing a large variety of phyto-compounds. It is possible that some of these may have cytostatic effects, thereby providing pluripotent anticancer mechanisms. Indeed, the *T. ferdinandiana* and *T. lanceolata* extract were also found to contain a number of compounds with cytostatic capacity, which can arrest the cell cycle, subsequently arresting carcinoma growth. A number of examples are presented here to illustrate the different mechanisms by which cell cycle arrest could be achieved by compounds present in the extracts.

Our initial phytochemical studies indicated the presence of moderate levels of tannins and later our metabolomic studies also confirmed the presence of gallic and chebulic acid (tannins) in *T. ferdinandiana* extracts. Both gallotannins and ellagitannins have been known to inhibit proliferation through various mechanisms possibly via a cytostatic capacity [318-321]. The metabolomic study also identified an imidazo-alkaloid compound, 1-(3H-imidazol-4-yl)-ethanone possessing an imidazole moiety. Since, being structural isomers of naturally occurring nucleotides, the imidazole alkaloid compound is in a unique position to disrupt DNA and RNA synthesis [349] thereby inhibiting carcinoma proliferation via a cytostatic capacity.

Another interesting compound, resveratrol (phytoalexin) which is well-known for its redox potential in preventing carcinogenesis, its ability to induce cell cycle arrest and promote apoptosis was detected in our studies [442]. Apparently, resveratrol potentially blocks NF-κB activation via its induction by TFN-α and IL-1β subsequently inducing apoptosis [244]. Likewise piceid (glycosylated stilbene) of resveratrol was also detected in *T. ferdinandiana* extracts, which also functions in a similar manner [427]. Another class of compounds with similar cytostatic potential were the stilbenes (combretastatin, combretastatin A-1), which were detected in the *T. ferdinandiana* extracts and well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tubulin via a cytostatic mechanism [246]. Similarly combretastatin A1, combretastatin A4 also detected in T. lanceolata extracts have the potential to act in a similar manner. Whilst this class of compounds, the pseudo-tannins (chlorogenic acid, caffeic acid, ferulic acid and ferulic acid dihydro dimer) were detected in some, but not in all extracts, these may not act directly as

such but potentiate the activity of other compounds and therefore have the potential to act as cytostatic agents.

Although the metabolomics profiling in chapter 6 and 7 were able to effectively narrow the focus of the compounds of interest within the extracts, further investigation is required to elucidate the mechanisms and pathways by which these compounds act. Notably, these extracts tested against the selected cell lines are essentially crude mixtures, very complex and may illicit multiple mechanisms that may be occurring simultaneously within cells. Further studies utilising a bioactivity driven separation may be required in isolating the compounds and their associated mechanisms independently. Unfortunately on isolation, these compounds could suffer a loss of activity and their inhibitory potential could be undermined. Indeed, further studies would require combinatorial/synergistic approach to appreciate the interactions between these compounds and their combined potency to induce negative proliferation in cancer cells.

Synergistic and combinatorial studies may actually be the new trend in phytotherapeutic research given that novel compounds are difficult to isolate. Current medical treatment for cancer has begun to shift towards a combinatorial approach involving chemotherapy where two or more dugs are co-delivered, hormonal therapy, immunotherapy and radiotherapy for better outcomes. Past strategies that have relied heavily on the use of single drugs agents that have failed considerably with poor outcomes for the patient. These anticancer drugs over time have lost their potency due to poor bioavailability at tumour site, non-specific targeting of cancer cells, cumulative toxicity, and multidrug resistance (MDR) [443-447].

Although efforts have been focussed on the development of new novel drugs, either from synthetic approaches or from foraging for natural novel chemo-types the success of this approach may be limited. Combinational approaches towards chemotherapy through the use of chemosensitizers, adjuvants and excipients may prove to be a more effective treatment strategy. Thus, plant extracts may be ideally suited for this purpose. Plants are a mixture of compounds that may be used as combinational therapies against cancer. They are a rich source of phytochemicals, and those plants that have a higher antioxidant content have a greater potential as therapeutic agents. The antiproliferative activity witnessed in studies in this lab is a testament of this. Phenolic compounds, ascorbic acid and vitamin E and their analogues contribute to the overall antioxidant potential of the extracts. Notably this antioxidant potential can contribute towards altering the redox state of the cellular environment in both normal and cancer cell with specificity, where normal cells may experience proliferation whilst the reverse may apply to cancer cells. For example, the ascorbic acid present in fruit extracts has the ability to function as an electron donor by restoring the reduced state of an active phytochemical compound, potentiating it, thereby allowing it to continue its antiproliferative activity (Figure 7.1).

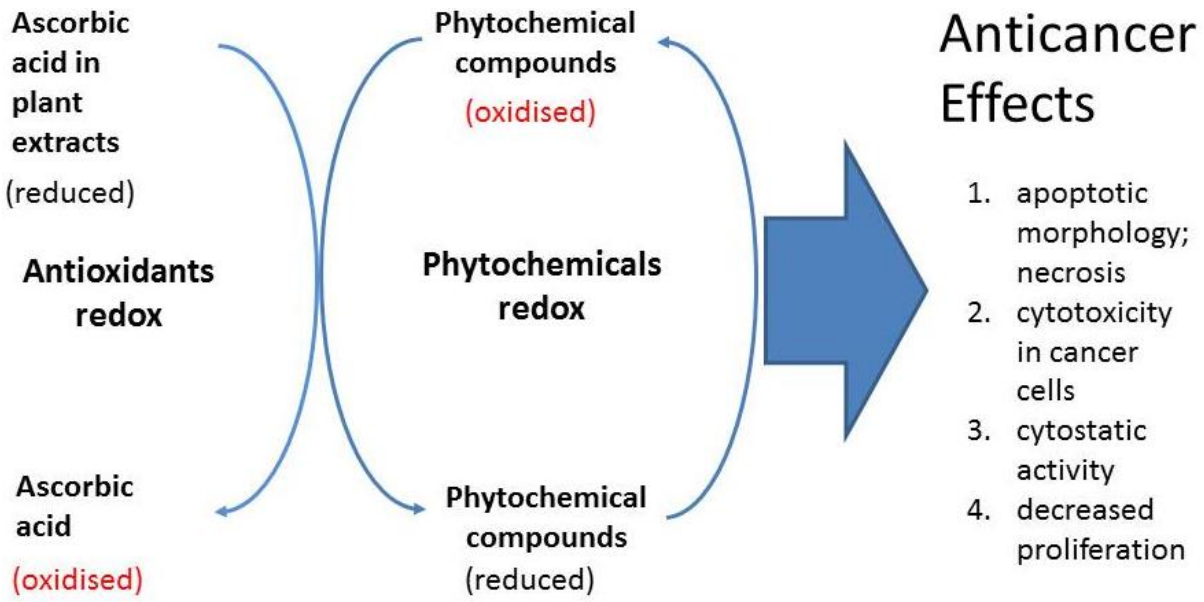

Figure 7.1 Ascorbic acid and phytochemical redox cycle that promotes anticancer activity.

Caffeic acid has been known to act as a chemosensitizer that potentiates the therapeutic property of chemotherapeutic agents by up to 13-fold [448]. Likewise, a recent study also reported that linalool (terpene alcohol) has the potential to act as an adjuvant in reversing doxorubicin (cancer drug) resistance in MCF-7 breast cancer cell line AdrR multidrug resistant human breast adenocarcinoma cells, thereby improving the therapeutic index of doxorubicin [388]. Another study reported that curcumin facilitated retention of doxorubicin in nuclei of K562 cells, reversing the drug resistance of doxorubicin and enhanced its antiproliferative activity [449]. Ginsenosides (saponins) have been reported in a patent to not only be an anticancer agent but a chemosensitizer as well. Indeed, ginsenosides (protopanaxadiol and protopanaxatriol) have been effective in sensitising multi-drug resistant (MDR) cancer cells to paclitaxel, mitoxantrone or cisplatin *in vitro* [450, 451]. Plants are already well placed as natural combinatorial therapies because they are complex mixtures which

work in concert to provide enhanced effects far above what pure compounds may allow. Plant extracts are able to function concurrently via several different mechanisms and target multiple active sites whilst current therapeutics are restricted to a single active site.

A total of 1243 compounds were identified in *T. ferdinandiana* fruit extracts through QTOF LC-MS and GC-MS with putative identification. Likewise, a total of 1237 compounds were also identified in *T. lanceolata* fruit extracts. The metabolomic studies for QTOF LC-MS and GC-MS were invaluable in serving to narrow the focus of phytochemicals responsible for antiproliferative activity. Although this provided much needed understanding of the compounds that may be responsible for the activity, it is not by any means a confirmed identity of the compounds. Hence identification of the compounds would require more investigations inclusive of NMR studies to elucidate these compounds with absolute certainty. Furthermore, in addition to all compounds that have been putatively identified via the metabolomics studies, there are many other compounds that remain to be elucidated and their activities subsequently studied. Much of the analysis that remains to be done is beyond the scope of this project. The studies undertaken hence far have been inclusive of initial phytochemical screening, DPPH profiling, antiproliferative studies (MTS), toxicity determination, cell imaging and metabolomic profiling. In addition, more investigations using normal cell lines against the extracts *in vitro* may have to be undertaken to determine their relevant toxicities to the carcinoma lines.

Also, further studies utilising a bioactivity driven separation may be required in isolating the compounds and their associated mechanisms independently. Although these compounds

could subsequently suffer a loss of activity and inhibitory potential, synergistic studies would be the next step in understanding the interactions that exist between the compounds in the raw extract. In addition, further investigations would be required into the pathways and mechanisms of the extracts of the *T. ferdinandiana* and *T. lanceolata* fruit extracts. Caspase and apoptotic protein determination assays would essentially provide information into the identity of pathways and mechanisms involved apoptotically. Furthermore, more information via cytostatic studies is needed to isolate the critical stages at which cell cycle arrest occurs by cell imaging and differential staining. Other plants of interest were identified during the course of studies inclusive of *A. acidula, D. pruriens* and *Syzygium* spp, and studies have been planned for future investigations.

The results from the present study require ongoing investigations into the understanding of the action of the compounds, their interactions involved and the pluripotent mechanisms involved. The extracts are highly complex due to the number of compounds present and possibly the ratios in which they may be present in. Synergistic studies may also help to establish the quantification of these compounds once positive identification of their identities are confirmed. Studies to date have been restricted to *in vitro* investigations and may have to be extended to include *in vivo* with mice. The antiproliferative effect of the extracts, and subsequently the isolated compound(s) may have to be trialled on mice that have had carcinogenesis induced. The weight of tumours induced and their regression will require correlation to their efficacy. Investigations into the pharmacokinetics and pharmacodynamics of the active compounds isolated in the present study require future investigation to advance these studies further.

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