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AN INVESTIGATION INTO THE ANTINEOPLASTIC POTENTIAL OF *BRIDELIA MICRANTHA* CONSTITUENTS

by

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MAGISTER SCIENTIAE (PHARMACOLOGY)

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DECLARATION BY CANDIDATE

I (D.J. Koot) declare that this dissertation (and the experimental work discussed within), which I hereby submit for the degree of MSc: Pharmacology, at the University of Pretoria, is my own work and has not previously been submitted for a degree at this or any other tertiary institution.

…… ……..

Dwayne Jonathan Koot Date

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SUMMARY

The fight against cancer is on going. This study provides a brief overview of the current status of cancer. Natural products as a source of clinically relevant, biologically active compounds, is the central theme of this study. Special reference has been made to antineoplastic (cancer-combating) compounds derived from botanicals.

Bridelia micrantha is a multipurpose tree used crudely by several African cultures for the treatment of a variety of ailments. Suggestive cytotoxic results attained from a preliminary toxicological screening along with the knowledge that the genus *Bridelia* has proven to be a source of novel cytotoxic aryltetralin lignan glycosides (structurally related to podophyllotoxin), served as motivation for undertaking this study.

The *in vitro* antineoplastic potential of several different solvent extracts of *B. micrantha* (bark) has been investigated. Five out of the six primary extracts displayed impressive cytotoxicity (IC_{50}<10µg/ml) towards three out of the four tested neoplastic cell lines. Indicative of good antineoplastic potential, these same extracts did not inhibit the growth of primary porcine hepatocytes and furthermore significantly (P<0.05) stimulated the proliferation of primary human lymphocytes at comparable, physiologically relevant concentrations. The presence of numerous structurally distinct cytotoxic compounds was shown by statistical comparisons (2way ANOVA and Bonferroni multiple comparison post-tests) of the IC_{50} values produced by each of the primary extracts on each of the tested neoplastic cell cultures.

The manner of cell death induction and points of cell cycle inhibition induced by the most promising of the primary extracts (H_20_{S1S} - lyophilised aqueous extract, soluble in EtOH) at a fixed concentration of 10µg/ml, was assessed flow cytometrically using COLO 320DM (human colorectal adenocarcinoma) cells. After 24hours incubation, extract H_20_{S1S} was shown to induce significant apoptosis (P<0.005) relative to appropriate controls. During that same period, the progression of the cell cycle was seemingly not hindered.

The active constituent/s (AC) within extract H_20_{S1S} were sought through bioassay-guided fractionation using the HeLa (human cervical epitheloid carcinoma) cell line as a monitor of bioactivity. Direct-infusion ESI-MS, HPLC/DAD/MS and TLC were used as monitors of fraction constituency. Fractions eluted from C18 and ion exchange sorbents were shown to possess decreased biological activity owing to incomplete distribution of the analytes and/or the separation of numerous compounds, acting

additively. Biological activity was enhanced through solvent-solvent partitioning between butanol (BuOH) and water (H_2O) - the BuOH fraction demonstrated a 2.5 fold increase in antiproliferative activity.

Attempts to further enrich the AC using normal phase preparative thin layer chromatography (pTLC) were met with a fair degree of separation. These fractions could unfortunately not be assayed due to appreciable solubilization of silica during analyte recovery and the resultant problems with accurate quantitation. Column chromatography employing silica as the stationary phase appears to be an attractive next fractionation step.

As yet the constituent/s responsible for the impressive antineoplastic activity have not been isolated nor structurally identified. Chemical (chromogenic) tests conducted on developed TLC plates do however suggest the possible presence of aryltetralin lignans. The results reported and discussed are of clinical relevance and certainly indicate that further investigation is required.

Key terms: Antineoplastic; bioassay-guided fractionation; botanicals; *Bridelia micrantha*; cancer; chemotaxonomy; chromatography; ethnobotanical; phytochemical; secondary plant metabolites

TABLE OF CONTENTS

GLOSSARY OF ABBREVIATIONS

AC	Active constituent/s
ATCC	American Tissue Culture Collection
Bp	Boiling point
$CHCl_3$	Chloroform
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
EMEM	Eagle's Minimal Essential Medium
EtOAc	Ethyl acetate
EtOH	Ethanol
ESI	Electrospray ionisation
HCl	Hydrochloric acid
HCO_2H	Formic acid
Hex	Hexane
HI FCS	Heat inactivated fetal calf serum
HNO_3	Nitric acid
HOAc	Acetic acid
HPLC	High pressure liquid chromatography
nm	Nanometer
MeCN	Acetonitrile
MRC	Medical Research Council
Mol. wt.	Molecular weight
MS	Mass spectrometry
MTT	(3-{4, 5-dimethylthiazol-2-yl}-2, 5-diphenyltetrazolium bromide)
PBS	Phosphate buffered saline
PDT	Podophyllotoxin
PHA	Phytohaemagglutinine
PI	Propidium Iodide
pTLC	Preparative thin layer chromatography
R*f*	Retardation factor (mobility relative to front)
RNA	Ribonucleic acid
SPE	Solid phase extraction
TDM	Total-dry mass
TIC	Total ion current
TLC	Thin layer chromatography
TWC	Total wavelength chromatogram
UV	Ultra violet

WHO	World Health Organization
XWC	Extracted wavelength chromatogram

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1. INTRODUCTION

1.1. The fight against cancer

The human race, endowed with the ability to consciously acknowledge that cancer kills and armed with scientific reason, is undoubtedly waging war. Ironically, it is a war that we wage against ourselves – the cellular part of ourselves that is out of control and has the means to grow and grow, regardless of boundaries and to a degree, free of inhibition.

Neoplastic growths can be either benign or malignant in nature (Pugh, 2000): Benign tumours are characterised by well-defined borders, a slow growth rate and the inability to spread. These attributes make benign tumours amenable to surgical procedures and in general, prognosis is good (although dependent upon the specific location). In contrast, malignant neoplasms are characterised by a fast growth rate, ill-defined borders, the ability to invade adjacent tissues and spread to other parts of the body where additional tumours may develop. As a result of this aggressive nature, malignant tumours are a major cause of mortality. Death ensues as result of vital organ or system obliteration and/or (owing to a progressive weakening state), as a consequence of opportunistic secondary infections (Stevens and Lowe, 2000).

Cancer is a general term frequently used to denote any type of malignant neoplasm of which there are more than 100 distinct types. Table 1.1 provides a basic classification of cancer according to the type of tissue from which it has originated (Alberts, 1993).

Cancer can be defined as uncontrolled and invasive growth of self-tissue, ultimately as result of diverse genetic mutations disrupting the control of normal cell proliferation. This is a micro-evolutionary, multi-step process. As this sequence of mutagenic events progresses (carcinogenesis), the cell evolves into a highly aggressive neoplastic cell possessing the following traits: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis (programmed self-death), limitless

potential to replicate, the ability to sustain angiogenesis (an adequate blood supply) as well as the ability to invade other tissue and metastasise to other areas of the body (Hanahan and Weinberg, 2000).

Table 1.1. Classification of cancer according to tissue of origin

Tissue of origin	**Name of malignant neoplasm (cancer)**
Epithelial tissue	Carcinoma
Connective and muscular tissue	Sarcoma
Nerve tissue	Glioma (brain) or Schwannoma (nerve)
Lymph node tissue	Lymphoma
Bone marrow and blood	Leukemia

Carcinogenesis occurs over time and involves the acquisition of continuous growth and/or anti-death stimuli through the inappropriate expression of certain mutant genes (oncogenes). Tumour suppressor genes normally function to inhibit cellular proliferation, repair damaged genes and initiate programmed cell death (apoptosis). The loss of tumour suppressor gene function, (through a mutation), thus circumvents an important anticancer defence mechanism. Each new mutation/step (progression) infers a proliferative advantage to a particular cell, culminating in its advantage over normal cells and consequent inappropriate growth (Lowe and Lin, 2000; Hanahan and Weinberg, 2000; Wyllie *et al.*, 1999).

Significant declines in mortality rates for cardiac, cerebrovascular and infectious disease has resulted in a higher human life expectancy (Varmus, 2006). Consequently, as cancer is a disease mainly of old age, the worldwide cancer mortality rate is expected to rise from the present estimate of 7.6 million cancer deaths per year to 9 million by the year 2015. Presently, cancer accounts for roughly 13% of all deaths (WHO, 2007)

In Southern Africa, the current cancer status is difficult to quantitate. The national cancer registry of South Africa is unreliable as result of large scale under reporting of incidence and furthermore is a number of years behind (Albrecht, 2006). The incidence rate, as reported by Mqoqi *et al.*, (2004), (of the National Cancer Registry) was 60 343

new cancer cases in 1999. The mortality rate, as reported by Bradshaw *et al*., (2000), (of the MRC Burden of Disease Unit) was 65 925 deaths in the year 2000. These two values (incidence and mortality rates) do not instil faith in the cancer surveillance infrastructure of South Africa, as it suggests that there are more people dying of cancer then there are people newly diagnosed with cancer. This is not a realistic representation of the situation. A concerted effort, extra funding and perhaps new legislation (on reporting of incidence) is required to help get a clear indication of the current status and where efforts need to be devoted (Albrecht, 2006).

In recent years the body of knowledge concerning cancer has grown tremendously. It is estimated that through several life style modifications over 40% of all cancers could be prevented. Preventative strategies include a healthy diet and avoidance of certain physical and environmental factors including cancer inducing substances (carcinogens), radiation and oncogenic viruses such a human papilloma and hepatitis B (WHO, 2007). Apart from preventative strategies, our arsenal of weaponry against cancer includes surgical intervention, radiation therapy and chemotherapeutic drugs. Through early detection and the use of such treatment modalities a further one-third of cancer deaths could be prevented (Albrecht, 2006).

The goal of modern day chemotherapeutic drug research is to discover or develop cytotoxic agents that possess greater (and if possible, exclusive) cytotoxic specificity for cancer cells relative to normal cells.

1.2. Natural products as a source of drugs

Throughout the history of drug therapy, natural products (produced by plants, micro-organisms, fungi and sessile animals) have played a fundamental role in that they are an autonomous source of diverse, evolutionary-shaped organic compounds that possess intrinsic biological activity (Houghton and Kaman, 1998). Living organisms may be thought of as biosynthetic laboratories for the manufacture of biologically relevant biochemicals (Nyiredy, 2004). Nature thus provides an enormous wealth of chemodiversity. Certain biochemicals, (which may be difficult and/or uneconomical to synthetically manufacture) may readily possess beneficial pharmacological activity

through specific, unspecific and/or semiochemical (combined) interactions with our own endogenous biochemical processes (Wink, 2003).

The potential therapeutic properties of plants and other organisms are predominantly attributed to secondary metabolites (Verpoorte, 1989). Unlike primary metabolites, secondary metabolites are not essential for the growth and reproduction of the organism but rather are responsible for interactions between the specific organism and it's surrounding ecosystem. They are produced to serve as effectors of ecological competition and symbiosis. Through this they form an integral part of a complex survival and propagation strategy (Williams and Maplestone, 1992).

Secondary metabolites, which are immensely diverse in both structure and function (Davies, 1992), are biosynthesised (Figure 1.1.) from primary metabolites (sugars, amino acids, lipids, nucleic acids) to take action within the same biochemical ecosystem in which we ourselves compete and interact chemically (Phillipson, 2001, Cordell, 2000). Through potent cytotoxic actions, many secondary metabolites (which are now of clinical relevance) for example, paclitaxel (Taxol) and podophyllotoxin (PDT) function as feeding deterrents (Oliva *et al*., 2001). Apart from defence against predators and pathogens, the function of secondary metabolites includes allelopathy (interspecies bio-messaging), nutrient recycling as well as attraction of pollinators and seed distributors.

Of the estimated quarter of a million higher plant species, only 10% have been investigated from a phytochemical or pharmacological point of view. These investigations have predominantly focussed only on the major constituents present (Harborne *et al*., 1999). Although in their infancy, phytopharmacological investigations have borne fruit in that an estimated 50 000 secondary plant metabolites have been characterized (Roessner and Scott, 1996) and that 119 of these are used globally as drugs (Farnsworth, 1984). In this regard, great urgency as well as multinational cooperation is required, as it is estimated that 60 000 higher plant species will be extinct by the year 2050 (Akerale *et al*., 1991).

More recently, Nyiredy, (2004) estimated that 35% of prescription drugs are of natural origin or are modelled after a phytochemical blue print and that 50% of over the counter drugs are of plant origin. If one extrapolates using the present trend, one can estimate the existence of half a million discreet secondary plant metabolites; a statement supported by Mendelson and Balick, (1995).

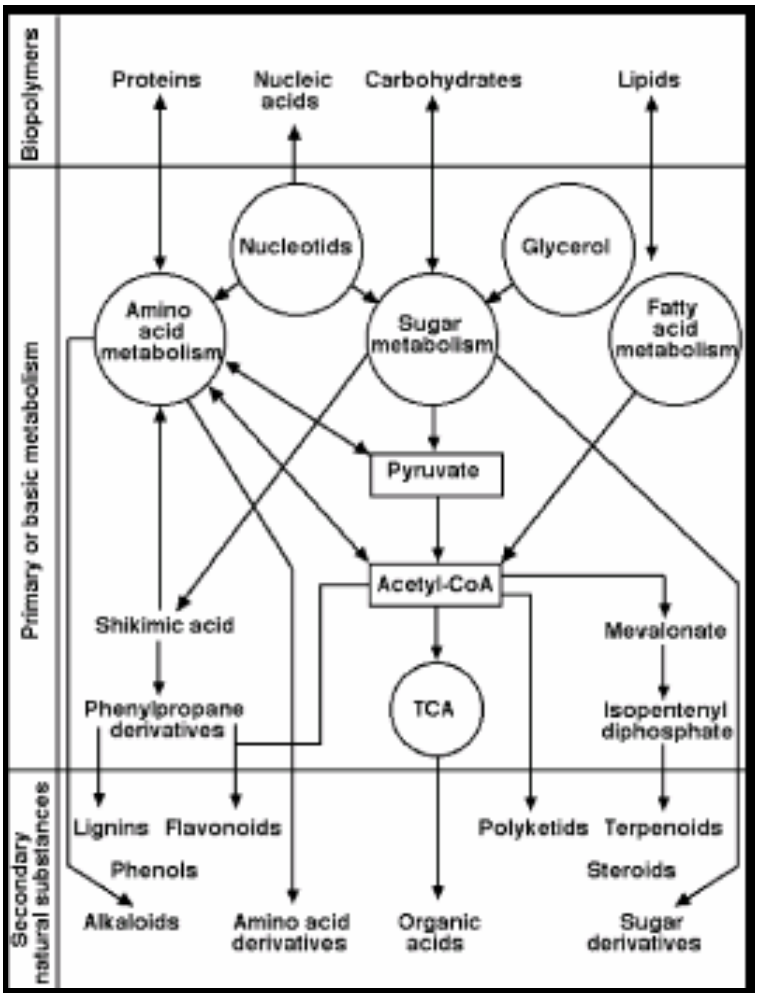


Figure 1.1. Biosynthetic pathways leading to the production of various secondary plant metabolites
(Nyiredy, S. 2004). Separation strategies of plant constituents - current status. Journal of Chromatography B 812 (1-2), pp.33-51)

To come to grips with such vast chemodiversity, not to mention the actual and potential biological mechanism of action of each one of these compounds, is a daunting task. As such, the discovery of pharmacologically active compounds (whether novel in structure or not) from flora and other natural sources is limited by both the sensitivity and specificity of a particular, normally narrow bioassay. To further confound the task,

additional constituents present within complex extracts may dull the potency or even antagonise the biological action of compounds worthy of recognition. Fortunately, tools such as ethnobotanical and chemotaxonomical data help direct where and what to look for.

Southern Africa contains more than 10% of the world's plant diversity (Van Wyk *et al*., 1997). Of this, 15% is reportedly used medicinally (Arnold *et al*., 2002). Considering this rich heritage of traditional medicine it seems likely that through utilising ethnobotanical data, novel compounds with clinically relevant biological activity will be discovered and perhaps synthetically improved upon for enhanced clinical efficacy and fewer unwanted side effects.

1.3. Botanicals as a source of antineoplastic compounds

Numerous antitumor compounds have been sourced from a variety of different plants and as such there is now no doubt that plants are a viable source of clinically relevant cancer combating compounds (Cragg *et al*., 1997; Mongelli *et al*., 2000). Several different structural classes of compounds including, but not exclusive to: terpenoids, steroids, lignans and alkaloids have been shown to have antineoplastic actions through diverse mechanisms (Cordell, 1978). The most successful antineoplastic compounds discovered and sourced from plants to date deserve a brief review:

Taxol (Figure 1.2.), is a water insoluble taxane (diterpene) sourced most abundantly, however not sustainably from the bark of the Western Pacific Yew, *Taxus Brevifolia*. Taxol and its semi-synthetic derivative Taxotere have been found to be effective in treating ovarian, breast and lung cancers. Taxol is an antimitotic agent that promotes the random assembly of polymerised tubulin into microtubules thus inhibiting the division of rapidly dividing mammalian cancer cells (Kingston, 1992; Phillipson, 2001). The molecular structure of Taxol is complex - it contains eleven chiral centres in four fused rings and an amino acid based side chain. Owing to this complexity, the likelihood of synthetically synthesising this molecule without a blue print is highly unlikely.

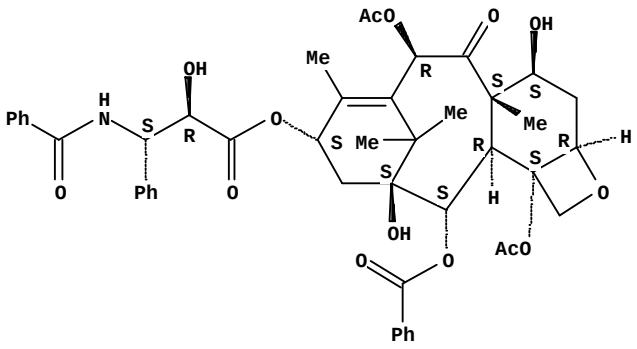


Figure 1.2. The structure of Taxol

Vincristine (Figure 1.3.) and Vinblastine (Figure 1.4.) are Vinca alkaloids sourced in small amounts from *Catharanthus roseus.* They are used extensively in combination with other drugs for the treatment of lymphomas, leukaemia, bladder cancer, testicular cancer and Hodgkin's disease. The antiproliferative mechanism includes inhibition of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein synthesis as well as the prevention of mitotic spindle formation through interactions with tubulin (Kingston, 1992).

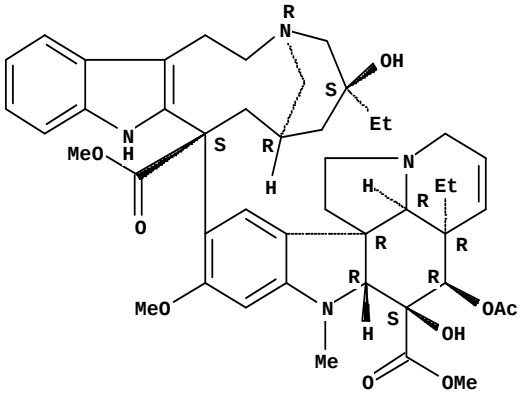


Figure 1.3. The structure of Vincristine

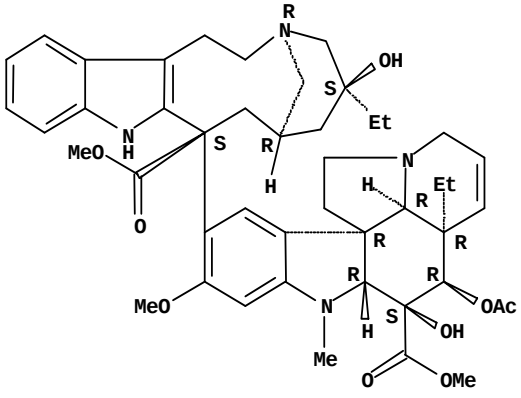


Figure 1.4. The structure of Vinblastine

Podophyllotoxin (PDT), (Figure 1.5.) is an aryltetralin lactone lignan derived through the phenylpropanoid pathway. PDT along with several precursors and derivatives thereof possess strong cytotoxic activity through inhibition of tubulin polymerisation (thus preventing microtubule assembly) and arresting the cell cycle at the beginning of metaphase. Due to unacceptable adverse side effect profiles, PDT is not used clinically as an anticancer drug (Srivastava *et al*., 2005).

However, extensive synthetic structure modifications of PDT resulted ultimately in etoposide (Figure 1.6.) and teniposide (Figure 1.7.). These two semi-synthetic compounds are currently used as front line chemotherapeutics against several cancers. Somewhat fortuitously, in contrast to PDT, these semi-synthetic derivatives are not active on the assembly of microtubules but rather exert their action on DNA synthesis via inhibition of topoisomerase II, thus inducing single or double strand breaks, halting the cell cycle at the end of S phase or at the beginning of the G_2 phase (Bruneton, 1995).

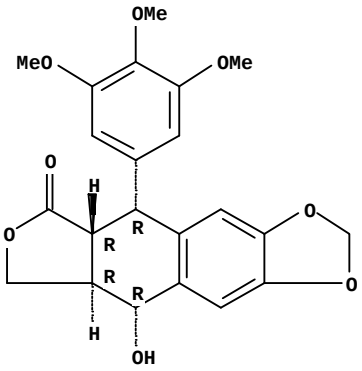


Figure 1.5. The structure of Podophyllotoxin

Both novel and new sources of known cytotoxic aryltetralin lignans are currently in high demand. PDT as the precursor for etoposide and teniposide is currently sourced from the rhizomes of the endangered Himalayan species *Podophyllum emodi* (Bedir *et al*., 2006). Both agricultural and biotechnological approaches have failed to produce a cost effective and renewable resource of aryltetralin lignans (Puri *et al*., 2006). As will be discussed later, compounds closely related to these structures have been found to be present in two species within the *Bridelia* genus.

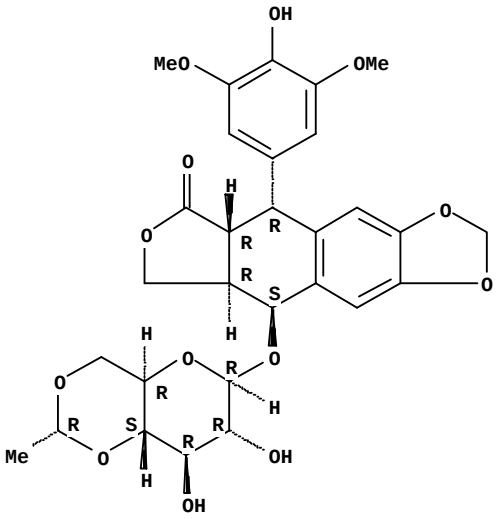


Figure 1.6. The structure of Etoposide

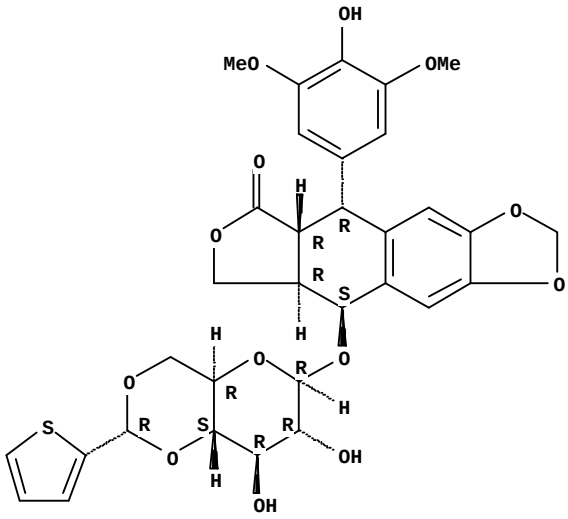


Figure 1.7. The structure of Teniposide

1.4. Botanicals as a source of primary medicine: global perspectives

The World Health Organisation claims that more than 3.3 billion people use plant material as their primary source of medicine (Farnsworth, 1994). Considering that 81% of the world's population has no access to synthetic drugs (Nyiredy, 2004), it goes to show that most of the developing world is reliant upon botanicals for pharmacological therapy of many ailments. Many cultures have for thousands of years collected ethnobotanical data and this can be considered as analogous with human bioassay data (Cox, 1994).

With the ever-growing demand for novel and affordable life saving drugs in mind, it is imperative that age-old herbal remedies of rapidly disappearing cultures be learnt, scientifically evaluated and perhaps adapted to suit modern medicines clinical requirements (Houghton and Kaman, 1998; Phillipson, 2001). Additionally, scientific evaluation of herbal remedies may serve to support or rebuke the traditional use thereof, thereby preventing potential long-term adverse side effects. Safety and efficacy are of paramount importance and should not be taken for granted.

1.5. Approaches to drug discovery

Out of necessity, early remedies to combat any number of ailments were undoubtedly derived from the natural environment to which man was exposed (Nyiredy, 2004). Through this need, influenced by cultural beliefs and limited by the availability of natural xenobiotics to particular geographical locations, ethnopharmacological practices were born and matured from generation to generation (Cox, 1994; Steenkamp, 2003).

As these folk remedies have proved anecdotally efficient and apparently non-toxic through generations of continued use, an investigation of the plant material commonly used by traditional healers is a justifiable and in fact, a sensible starting point when searching for novel biologically active compounds. The ethnobotanical/ethnomedical data approach to drug discovery has already yielded an estimated 122 drugs from 94 plant species (Fabricant and Farnsworth, 2001). Without doubt, the ethnobotanical data approach to drug discovery substantially increases the probability of discovering novel

biologically active compounds as appose to random botanical screening (Cordell, 1991).

Cancer, presenting with a complex set of signs and symptoms is not an easily diagnosed condition in traditional medicine (Mongelli *et al*., 2000). It is therefore common practise to investigate other ethnopharmacological uses that may combat a particular symptom of cancer. Indications that may be considered relevant include immune and skin disorders, inflammatory, parasitic and viral disease (Cordell *et al*., 1991; Popoca *et al*., 1998). In addition to ethnobotanical literature, anecdotal literature, field observations (whereby interactions between ecological inhabitants are observed) as well as case reports may all serve as indicators of a specific biological activity.

Further-more chemotaxonomical literature can motivate for the investigation of a particular plant species. For instance, when a particular type of phytochemical is sought, it is common practise to search within the same genus or botanical family from which similar compounds have been sourced (Hostettmann *et al*., 1995).

Advances in both knowledge and technology have afforded new routes of drug development. Advancements in the fields of biotechnology, synthetic chemistry and recombinant DNA technology has made it possible to systematically target specific receptors (Rang, 2006). Automated, high throughput screening is now common practise in industry when searching for a specific receptor agonists or antagonists. However, when assessing potential lead drugs/extracts through specific receptor-type assays, novel mechanisms (and thus the underlying systems) of action usually cannot be identified.

Semi-synthetic analogues and derivatives of known active natural products, is undoubtedly a viable source of new clinically relevant drugs with enhanced activity, better pharmacokinetic profiles and fewer undesirable side effects.

Motivation for undertaking of this study, the specific aim and major objectives thereof is discussed at the end of chapter 2.

CHAPTER 2

2. LITERATURE SURVEY

2.1. Taxonomy and chemotaxonomy of the *Bridelia* genus

The botanical family Euphorbiaceae (spurges), is known as a source of several structural classes of diterpenes possessing a host of different pharmacological properties including antitumour, cytotoxic, tumour-promoting, and anti-microbial activity (Grace *et al*., 2005). This family is known for its many toxic species (Cordell, 1978)

The *Bridelia* genus, of the Euphorbiaceae botanical family consists of roughly 60 species distributed through out Asia, Australia and Africa. Roughly one third of the *Bridelia* species can be found in Africa, of which four species in addition to *B. micrantha,* namely: *B. atroviridis, B. cathartica, B. mollis, B. tenuifolia* can be found within Southern Africa (Palgrave, 1977; Smith, 1987). Species within this genus are used extensively as medicinal plants.

Chemotaxonomy (Table 2.1.) is not widely reported although friedelane triterpenoids are thought to be common (Carpenter *et al*., 1980). Of particular interest and relevance to this study is that two African species namely *Bridelia ferruginea* and *Bridelia tulaneana* are known sources of cytotoxic aryltetralin lignans structurally related to PDT.

Table 2.1. Chemotaxonomical review of the *Bridelia* genus

Species	Part	Constituent	Reference
B. crenulata Roxb.	Bark	Luteoforol (3',4',4,5,7-pentahydroxyflavan)	Ramesh *et al*., 2001
B. ferruginea Benth.	Bark	3-O-methylquercetin; 3,7,3',4'-tetra-O-methylquercetin (rutisin); myricetin; 3',4',5'-tri-O-methylmyricetin (ferrugin); 3,3',4',5'-tetra-O-methylmyricetin, quercetin 3-O-glucoside and gallocatehin-[4'-O-7]-epigallocatechin.	Cimanga *et al*., 2001

	Root	5'-demethoxy-β-peltatin-5-O-β-D-glucopyranoside; β-peltatin; β-pelatin-5-O-β-D-glucopyranoside; deoxypodophyllotoxin; friedo-oleanan-3β-ol; friedo-oleanan-3-one; sitosterol; hexadecanoic acid; stigmasterol	Rashid *et al*., 2000; Addae-Mensah, 1985
	Leaves	Rutin; Quercetin 3-glycoside; quercetin; myricetin 3-rhamnoside; myricetin 3-glucoside; bridelilactone; bridelilactoside	Addae-Mensah, 1985; Iwu, 1993
B. moonii Thw.	Bark	Friedelan-3-one; friedelan-3β-ol; friedelan-3α-ol; sitosterol; glochidone	Carpenter *et al*., 1980
B. ovata Decne.	Braches	β-sitosteryl hexadecanoate; stigmateryl hexadecanoate; campesteryl hexadecanoate; friedelin; friedelan-3β-ol; trans-triacontyl 1,4 -hydroxy-3-methoxy cinnamate; β-sitosterol; stigmasterol; campesterol; B-sitosteryl 1-3-0-β-D-glucopyranoside; stigmastery 1-3-0-β-D-glucopyranoside; campestery 1-3-0-β-D-glucopyranoside; 24-methyllanosta-9-(11)-25-dien-3-one; 24,24-dimethyllanosta-9-(11)-25-dien-3-one; friedelin; friedelan-3β-ol; β-sitosterol; stigmasterol; campesterol; trans-triacontyl-4-hydroxy-3-methoxycinnamate	Kitchanachai, 1988; Boonyaratavej *et al*., 1992
B. retusa Spreng.	Bark	(*E*)-4-(1,5-dimethyl-3-oxo-1-hexenyl) benzoic acid; (*E*)-4-(1,5-dimethyl-3-oxo-1,4-hexadienyl) benzoic acid; (*R*)-4-(1,5-dimethyl-3-oxo-4-hexenyl) benzoic acid; (*R*)-4-(1,5-dimethyl-3-oxohexyl) benzoic acid (ar-todomatuic acid); (-)-isochaminic acid; 5-allyl-1,2,3-trimethoxybenzene (elemicin); (+)-sesamin; 4-isopropylbenzoic acid (cumic acid)	Jayasinghe *et al*., 2003
B. tomentosa Blume. (syn. *B. monoica* Merr.)	Root	24-methyllanosta-9(11),25-dien-3-one; 24, 24-dimethyllanosta-9 (11) , 25-dien-3-one; friedelan-3β-ol; β-sitosterol; stigmasterol; triacanthine; β-sitosteryl-3-O-β-D-glucopyranoside and stigmasteryl-3-O-β-D-glucopyranoside	Boonyaratavej and Petsom, 1991
	Leaves	Friedelan; friedelan-3β-ol; glutin-5-en-3β-ol; stigmasterol and β-sitosterol	Hui and Fung, 1968
B. tomentosa Blume.	Stems	Friedelan-3β-ol, stigmasterol	Hui and Fung, 1968
B. tulasneana	Twigs	Deoxypodophyllotoxin	Williams, 2002

2.2. Background information: *Bridelia micrantha*

Micrantha is a botanical epithet meaning "tiny flowered". Apart from the many vernacular names denoting *B. micrantha* there appears to be several scientific synonyms: *B. speciosa, B. stenocarpa, B. zanzibarensis, B. abyssinica, B. mildbraeddi,* as well as the basionym: *Candelabria micrantha* (Smith, 1987).

Bridelia micrantha (Hochst.) Baillon (Euphorbiaceae), known more commonly as Mitzeerie, Coastal golden-leaf, Bruinstinkhout or Wild coffee (Figure 2.1.) is becoming scarce due to over exploitation.

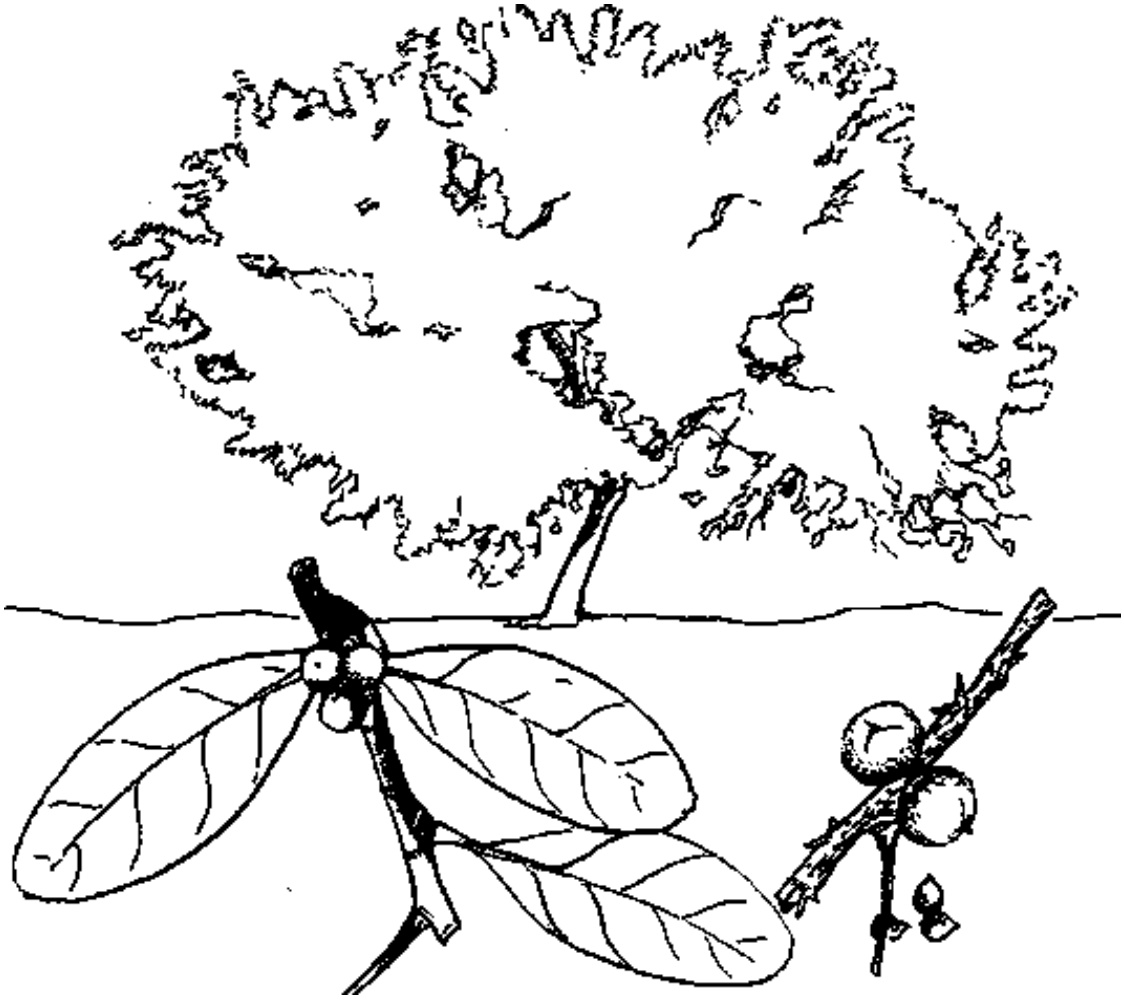


Figure 2.1. Sketch of *Bridelia micrantha* (Hochst.) Baillon

(Hines, D.A., Eckman, K., 1993. Indigenous multipurpose trees of Tanzania; uses and economic benefits for people)

This hardy, fast growing tree is found in a variety of water rich soils throughout South Eastern Africa. It is a small to medium sized deciduous tree, with varied sized leaves that are both simple and symmetrically arranged. The bark, which is brown to grey in colour, commonly cracks and peels off in small flat square flakes. The flowers occur in small groups in leaf axials. The ellipsoidal fruit, which is smooth-skinned and pitch-black when edible, ripens towards the end of the rainy season. This tree is used in the manufacturing of furniture, as a fuel wood and as a medicinal plant by African traditional healers (Hines and Eckman, 1993; Palgrave, 1977). Severe toxicity resulting in a patient death 4 hours after oral administration has been reported (Verdcourt and Trump, 1969).

2.3. Ethnobotanical data of *B. micrantha*

In order to facilitate and promote in depth scientific investigation, the traditional indications for the use of different morphological parts of *B. micrantha* warrant consideration and review (Table 2.2). Of particular interest is that the root material is indicated for the treatment of cancer and the leaf material is used as an abortifacient.

Table 2.2. Synopsis of the traditional indications for the use of *B. micrantha*

Part	Traditional indication	Reference
Bark	Colic	Noumi and Yomi, 2001
	Burn wounds, venereal disease, toothache, stomach pain and diarrhoea	Mabogo, 1990
	Typhoid fever, tuberculosis and dysentery	Abo and Ashidi, 1999
	HIV / AIDS	Bessong et al., 2004
Leaf	Painful eyes and conjunctivitis	Watt and Breyer-Brandwijk, 1962
	Fever and headache	Mabogo, 1990; Palgrave, 1977
	Used as an abortifacient	Van Wyk and Gericke, 2000
	Malaria	Ajaiyeoba et al., 2006
Root	Epigastric pain, tapeworm infection, used as a purgative	Hines and Eckman, 1993
	Paralysis and painful joints	Lin et al., 2002
	Cough, allergy, headache, gonorrhoea, prolapse of the rectum, diarrhoea, diabetes and cancer	Iwu, 1993; Kamuhabwa et al., 2000
	HIV / AIDS	Bessong et al., 2005

2.4. Phytochemical investigations of *B. micrantha*

A phytochemical screening (Abo and Ashidi, 1999) showed methanolic macerations of both the stem bark and leaf material to test positive for tannins and saponins, but negative for anthraquinones, cardenolides and alkaloids.

Pegel and Rogers, (1967) reported the following constituents to be present in the bark material (which was notably fresh):

- The triterpenoids: taraxerone, taraxerol, friedelin and epifriedelinol were identified from a hexane extract comprising 15 constituents as shown through thin layer chromatography (TLC).
- The tannins: gallic acid and ellagic acid were identified as the major constituents of both ether and acetone extracts.
- The anthocyanin, delphinidin, reportedly formed from leucodelphinidin or a related compound was identified as the major component of a hot hydrochloric acid (HCL) extract.

In addition to the above-mentioned phytochemicals, the presence of caffeic acid was indicated to be present in fresh leaf material. To date, no literature concerning phytochemical investigations conducted on the root material has been reported.

2.5. Investigations into the biological activity of *B. micrantha* extracts

Several of the most prominent traditional indications for the use of *B. micrantha* have been scientifically assessed. These investigations are briefly reviewed below:

2.5.1. Cytotoxicity screenings

Aqueous infusions prepared from dried bark material have been screened for cytotoxicity using the HeLa (human cervical) and MCF 12A (human mammary gland) neoplastic cell lines, as well as primary human lymphocytes and hepatocytes. EC_{50}'s (the specific concentration at which 50% of a particular effect is observed relative to untreated controls) of 9µg/ml and 35µg/ml were reported for the HeLa and MCF 12A

cell lines respectively. Only slight toxicity was observed for resting and phytohemagglutinin (PHA) stimulated lymphocytes >60µg/ml and insignificant toxicity was observed in the case of hepatocytes thus leading Mokoele *et al*., (2003) to conclude that aqueous infusions of *B. micrantha* (bark) are only antiproliferative to transformed, fast growing cell cultures. These results warranted further investigation of the bark material as a potential source of cancer combating compounds.

Additionally, Mokoele *et al*., (2003) carried out a vertebrate toxicity test in order to determine acute lethality. The test organisms, *Poecilia reticulata* (guppies) were placed in beakers with different concentrations ranging from 15µg/ml-1mg/ml of the aqueous infusion. One hundred percent mortality was recorded after 96 hours at all the tested concentrations.

The toxicity of *B. micrantha* (leaf) has been assessed using the Brine Shrimp Lethality (BSL) assay (Ajaiyeoba *et al*., 2006). The BSL assay is a simple, rapid and inexpensive bench-top method reportedly correlating well with antitumour activity (McLaughlin *et al*., 1998). At the highest tested concentration of 5mg/ml no LC_{50} value (the concentration at which 50% lethality is observed relative to untreated controls) could be attained for a methanolic extract. This lack in toxicity is further confirmed by Bessong *et al*., (2004), who reported no toxicity for either aqueous or methanolic extracts at a physiologically unattainable concentration of 600µg/ml using HeLa P4 cells (human epithelial carcinoma cell line, transfected with the CD4 transmembrane glycoprotein).

Methanolic macerations of the root material have been screened for cytotoxicity on three neoplastic cell lines: HeLa (human cervical carcinoma), HT29 (human colon carcinoma) and A431 (human skin carcinoma), (Kamuhabwa *et al*., 2000). The results of this investigation showed an inhibition in cell proliferation of between 0% and 25% relative to that of an untreated control for both the HeLa and HT29 cell lines and a inhibition of between 25% and 50% in the case of the A431 cell line at a concentration of 100µg/ml. This relative lack in cytotoxicity of the root material is further supported by Bessong *et al*., (2005), who reported no toxicity for either aqueous or methanolic extracts at concentrations of 400µg/ml using the HeLa P4 cell line.

These findings do not support the traditional use of *B. micrantha* (root) as an anticancer agent. It is however important to acknowledge that there are several variables (which are difficult to control for) that could influence the outcome of bioassays: the production of secondary metabolites is quantitatively determined by the particular plants stage of life (otogeny) and type (and degree) of environmental stresses (Nyiredy, 2004). Additionally, these limited *in vitro* (cells in culture) bioassays may not be an accurate representation of the action on all neoplastic cell types nor of the potentially complex *in vivo* (whole organism) situation. Therefore, although suggested, it cannot be concluded that the root material of *B. micrantha* has no antineoplastic action.

2.5.2. Antibacterial screenings

The presence of antimicrobial activity is thought to be indicative of far reaching bioactivity and thus should warrant broad-based pharmacological investigation (Demain, 1999).

Lin *et al*., (2002) using the disk diffusion method of Salie *et al*., (1996), (as part of an anti-diarrhoeal evaluation) assessed the antimicrobial activity of methanolic and aqueous extracts of *B. micrantha* (bark) against 17 isolates of *E. coli*, 13 isolates of *Salmonella* spp. and nine isolates of *Shigella* spp. The methanolic extract demonstrated weak inhibitory activities against *S. flexneri* and *S. plesiomona.*

Methanolic macerations of *B. micrantha* (both leaf and bark material) have been shown to weakly inhibit the growth of *Salmonella typhosa, Shigella dysenteriae* and *Escherichia coli* (Abo and Ashidi, 1999).

2.5.3. Antifungal screenings

Methanolic macerations of *B. micrantha* (both leaf and bark material) have been shown by Abo and Ashidi, (1999) to be practically inactive against the *Candida albicans, Aspergillus niger, Penicillium* spp. and *Microsporum* spp. at a concentration of 100mg/ml.

2.5.4. Antiviral screenings

Motivated by ethnobotanical data, Bessong *et al*., (2004) screened *B. micrantha* leaf material for inhibitory properties against HIV–1 reverse transcriptase (RT). Inhibition of the enzymes, RNA–dependent–DNA polymerase (RDDP) and ribonuclease H (RNase H) activities of HIV-1 RT were determined. An aqueous extract inhibited the activities of RDDP and RNase H with IC_{50}'s of 34.6µg/ml and 27.9µg/ml respectively. A methanolic extract inhibited the activity of RDDP and RNase H with IC_{50}'s of 23.5µg/ml and 18.9µg/ml respectively.

Similarly, Bessong *et al*., (2005) screened the root material for inhibitory properties against HIV–1 RT. An aqueous extract inhibited the activities of RDDP and RNase H with IC_{50}'s of 18.5µg/ml and 11µg/ml respectively. A methanolic extract showed relatively strong inhibitory properties against both RDDP and RNase H activities with IC_{50}'s of 10.5µg/ml and 10.3µg/ml respectively.

Of note is that the bark material, which is reported as being used for HIV/AIDS (Bessong et al., 2004), has not been scientifically evaluated for such activity.

2.5.5. Parasiticidal screenings

Clarkson *et al*., (2004) investigated the *in vitro* antiplasmodial activity of *B. micrantha* (twigs) against *Plasmodium falciparum* strain D10 using the parasite lactate dehydrogenase (pLDH) assay. A cold DCM (dichloromethane)/MeOH (methanol), (1:1) extract produced an IC_{50} of 59.3µg/ml. This was not considered promising but rather indicative of a non-specific general toxicity.

2.5.6. Anti-diarrhoeal properties

Both methanolic and aqueous extracts of *B. micrantha* bark have been shown to significantly reduce castor oil induced diarrhoea (by 60.97% and 63.75% respectively, $P<0.005$), to significantly inhibit PGE_2- induced increase in capillary permeability (by 58.49% and 54.71% respectively, $P<0.005$) and to significantly reduce gastro-intestinal

motility (by 57.31% and 25.56%, $P<0.05$) in the test animal (*Rattus norvegicus*) at a single dose of 400mg/kg. These results validate the traditional use of *B. micrantha* (bark) as an anti-diarrhoeal agent (Lin *et al*., 2002).

2.6. Motivation for study

The suggestive, neoplastic specific, cytotoxic literature pertaining to the bark of *B. micrantha* along with the knowledge that the *Bridelia* genus has in the past proven to be a source of novel aryltetralin lignans glycosides served as motivation to conduct this study.

2.7. Study aim

The primary aim of this study was to investigate the *in vitro* antineoplastic potential of *B. micrantha* (bark) constituents.

2.8. Major objectives

- Investigate the *in vitro* antineoplastic potential of all the primary extracts of *B. micrantha* (bark) through comparison of their: cytotoxic specificities for neoplastic cells versus that of normal primary cell cultures; cytotoxic selectivity's for different neoplastic cell cultures.

- Investigate the manner of cell death induction and points of cell cycle inhibition induced by the most promising of the primary extracts.

- Detect the presence of aryltetralin lignans (if any). Enrich / isolate the active constituent/s (AC) within the lead extract through bioassay-guided fractionation and attempt structural elucidation of the AC.

CHAPTER 3

3. EXTRACTION, EXTRACT PROCESSING AND PRELIMINARY PHYTOCHEMICAL INVESTIGATION

3.1. Introduction

Plants, like all living organisms are made up of a myriad of biochemicals that interplay in a controlled and ordered fashion to allow for life processes to occur. Plants contain several thousand secondary metabolites. The chemical structure of a particular biochemical determines it's reactive or interactive capability and thus it's particular biological function either within the organism (both intracellularly and extracellularly) or beyond, if exuded.

Compounds can be extracted into a suitable solvent based upon their respective solubility. The principle of "like dissolves like" applies - as such, a non-polar (lipophilic) solvent will solubilise non-polar compounds and polar (hydrophilic) solvents will solubilise polar compounds. Table 3.1 gives a generalised indication of the different types of phytochemicals that can be extracted with different solvents (Houghton and Kaman, 1998).

Table 3.1. Types of phytochemicals that can be extracted into different solvents

Polarity	Solvent	Chemical class extracted
Low polarity	Petroleum ethers, hexanes	Waxes, fats, fixed oils
↓	Toluene	Alkaloids, fats, fixed oils
	Chloroform, dichloromethane	Alkaloids, aglycones, volatile oils
	Ethyl acetate, acetone	Alkaloids, aglycones, glycosides
	Ethanol	Glycosides
	Methanol, water	Sugars, amino acids, glycosides
	Aqueous acid	Sugars, amino acids, bases
High polarity	Aqueous alkali	Sugars, amino acids, acids

There are numerous different extraction methods that may be implemented. Plant material (which may be fresh or dried) is typically immersed in a particular solvent with or without the addition of heat, pressure, mechanical maceration, sonication (acoustic vibrations) or microwaves for a period of time. The choice of extraction method is principally based upon availability of resources, cost of method, ease of use and what the aim of the extraction is.

Extraction using a Soxhlet apparatus has a distinct advantage over conventional macerations, decoctions and infusions in that fresh solvent is constantly recycled allowing for exhaustive extraction with the least amount of solvent. Sequential soxhlet extraction using several solvents of different polarity in order of increasing polarity is common practice should it be desired to extract a broad range of compounds (Stahl, 1969). Extraction proceeds in order of increasing polarity so that lipophilic coatings (which may impede extraction of polar constituents) may first be removed.

With any extraction, it is of paramount importance to consider the chemical nature of the desired compound as well as its potential susceptibility to decomposition under the standardised extraction and processing conditions. If the chemical nature and thus susceptibility of the active constituent/s is unknown then it is wise to implement the mildest possible conditions and to limit the variability of these conditions. This is done so that appropriate controls can be put in place in order to assess the preservation of biological activity under the set out, standardised conditions.

When screening natural products for biological activity it is wise to include several checkpoints within the study design in order to ensure that further investigation is indeed warranted. Time spent and costs incurred are of importance. As such, a small-scale pilot study was devised in order to assess the efficiency of the extraction and reconstitution methodology (discussed later) and importantly to confirm the presence of promising antineoplastic activity.

3.2. Materials

3.2.1. Procurement of plant material

For preliminary investigation, a small amount (roughly 20g) of the same *B. micrantha* material that had previously been investigated by Mokoele *et al.*, (2003) was made available. This material (both bark and leaf) had been collected during October of 2003 by a field collaborator of Dr. V. Steenkamp (Dept. of Pharmacology, University of Pretoria) from the village of Tshlamba (Venda). After authentication of the material by the South African National Botanical Institute (SANBI), voucher specimens were assigned specific identifier numbers (Bark - LT0022; Leaf - LT006) and were lodged at the Onderstepoort herbarium (Pretoria).

After confirmation of biological activity, a larger mass (roughly 1 kg) of bark material was collected (February, 2006) and authenticated by Dr. N. Hahn (Dept of Botany, University of Pretoria) from the Morningsun Farm, Soutpansberg, Limpopo Province, South Africa - identifier number 2229DD.

3.3. Methodology

3.3.1. Preparation of bark material

For preliminary (confirmatory) investigations, both the bark (LT0022) and leaf (LT006) material were received in a dried and finely ground state.

Newly sourced bark material (2229DD) was closely examined for insect damage, fungal infestation or any visible contaminant that may suggest possible deterioration. Any suspect material was removed. The material (Figure 3.1.) was air-dried at room temperature, in the dark for approximately one month, after which the material was sufficiently brittle to allow for grinding.

After visual inspection of the dried material two separate layers, an outer and an inner became apparent (Figure 3.2.). In order to ensure repeatability at a later stage by other

investigators the subjective nature of which portion of the bark material to use required standardisation and documentation. After consideration of what had apparently been done previously (Mokoele *et al*., 2003), only the inner layer was further investigated.

The inner layer was stripped from the outer layer and cut into small pieces using secateurs (Figure 3.3). Thereafter the material was finely ground using a Willey mill (Dept. of Plant Production and Soil Sciences, University of Pretoria). After grinding, the homogenised material was stored in a tightly sealed brown bottle at ambient room temperature.

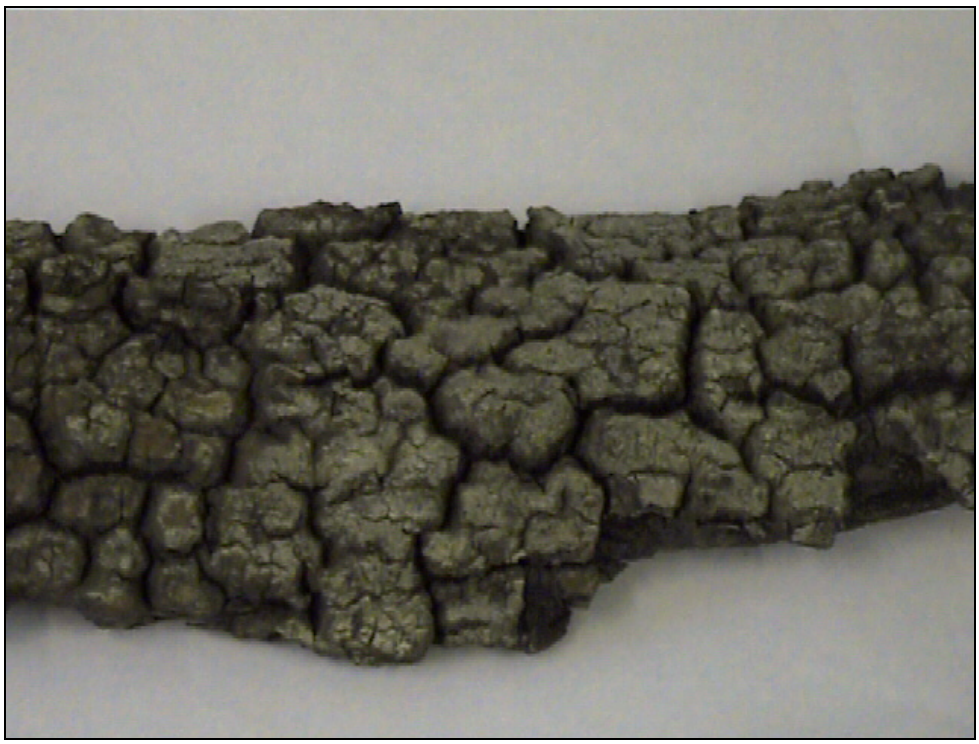


Figure 3.1. *B. micrantha* bark material (2229DD) as procured from Dr. N. Hahn

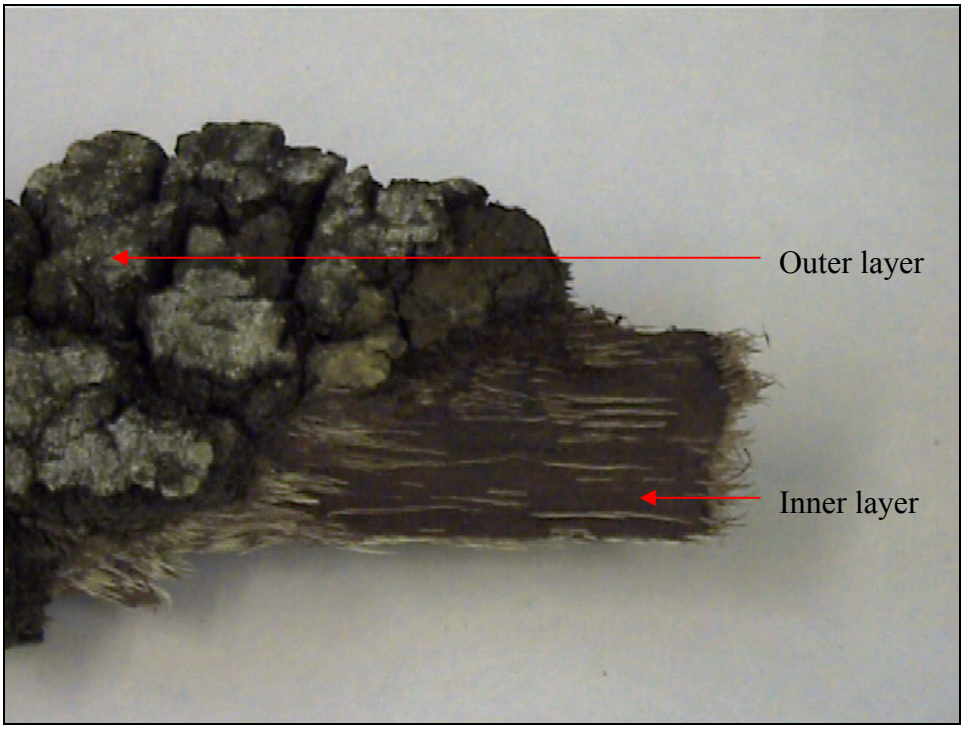


Figure 3.2. The apparent layers of *B. micrantha* bark material

Figure 3.3. Stripped and cut inner bark material

3.3.2. Extraction of plant material

All solvents were of analytical grade and were purchased from reputable suppliers. The sequential extraction methodology aimed to extract the broadest possible phytochemical range. Pertinently, the methodology incorporated methods used by San Feliciano *et al*., 1989, 1990 and Kim *et al*., 2002 to extract aryltetralin lignans.

Practical procedure: Soxhlet extraction

All glassware, before use was washed in an appropriate solvent to remove possible contaminates. Thereafter, where applicable the glassware was dried to constant weight and this weight recorded, thereby facilitating yield quantitation. Due to the risk of photodecomposition, where ever possible glassware was enclosed in foil.

- An appropriate mass of dried and finely ground plant material was weighed out into an extraction thimble (30x100mm, Macherey-Nagel).
- A Soxhlet apparatus (Technico) was employed to sequentially extract and separately collect hexane, ethyl acetate and aqueous extracts for a period of 4, 4 and 24 hours respectively.
- After each extraction and before the onset of the next extraction, the remaining plant material (the marc) was air-dried.
- After the three sequential extractions were complete, the remaining marc was air-dried and then stored in a sealed container in the dark at room temperature for the duration of the study.

3.3.3. Extract processing: Quantitation of extraction yields and reconstitution

Due to the different physicochemical properties of the different extractants used, processing of the two organic extracts (hexane and ethyl acetate) followed a different procedure to that of the aqueous extract. The practical aspects thereof are discussed accordingly.

Incorporation of the organic extracts into the polar environment of the *in vitro* bioassay necessitated the use of a water miscible and non-toxic (at a controlled concentration) carrier solvent. Owing to the presence of hydroxyl or glycosylated groups, lignans and their glycosides are expected to be appreciably soluble in alcohol (Wong *et al*., 2000). Correspondingly, ethanol (EtOH) was elected as the carrier solvent.

Owing to the lack in antiproliferative activity of an aqueous leaf extract on HeLa cell cultures, further investigation (i.e. EtOH reconstitution and *in vitro* bioassay) of the leaf organic extracts was not warranted and consequently abandoned.

Practical procedure: Organic extracts

- After Soxhlet extraction, the exact volume of the two respective organic extracts was noted using a pre-washed measuring cylinder.
- Three 1.00ml aliquots were removed and air-dried in pre-weighed petri dishes before being desiccated over night and finally weighed using an analytical balance (Precisa).
- The respective extraction yields were calculated (using the measured volume and the mean dry 1.00ml aliquot weight) and expressed as % total dry mass (TDM).
- The two extracts (denoted by the short-hand "Hex" and "EtOAc") were concentrated to approximately 30ml at temperatures < 40°C, under reduced pressure using a rotary evaporator.
- The extracts were transferred to pre-weighed centrifuge tubes and cooled overnight at roughly -10°C before being centrifuged (Sigma type 3K15) at 5°C for 30minutes at 2000g.
- The respective supernatants (denoted with the subscript "$_S$") were decanted into clean, pre-weighed centrifuge tubes and dried under nitrogen at < 40°C.
- The respective pellets (denoted with the subscript "$_P$") were dried under nitrogen at < 40°C.
- The weight of both the supernatants and pellets were documented and expressed as % (w/w) of TDM.

- Both the pellets and the supernatants of the two respective extracts were then extracted thrice with EtOH (10ml) with the aid of mild sonication within a water bath (185 watts at 42 KHz) for three successive 10minute periods. After centrifugation, the pooled supernatants were cooled overnight at roughly -10°C before again being centrifuged at 5°C for 30minutes at 2000g. The weight of the resultant supernatants (denoted with the subscript "$_{PS}$" or "$_{SS}$") and pellets (denoted with the subscript "$_{PP}$" or "$_{SP}$") was then determined and expressed as % (w/w) of the TDM.
- Three 1.00ml aliquots were taken of all the supernatants in order to determine their respective concentrations. Extracts were adjusted to a concentration of 10mg/ml. Several aliquots of these respective stock solutions were made and stored at -70°C.
- Non-soluble pellets were air-dried and their respective weights determined before being stored in the dark at ambient room temperature for the duration of the study.

Practical procedure: Aqueous extract

- After Soxhlet extraction the exact volume of the aqueous extract was noted using a pre-washed measuring cylinder.
- Three 1.00ml aliquot was removed and air-dried in pre-weighed petri dishes before being desiccated over night and finally weighed using an analytical balance.
- The extraction yield was calculated (using the measured volume and the mean dry 1.00ml aliquot weight) and expressed as % (w/w) of TDM.
- The extract (denoted by the short-hand "H_20") was refrigerated overnight at 5-10°C.
- The extract was centrifuged at 5°C for 30minutes at 2000g.
- The supernatant (denoted with the subscript "$_S$") was decanted into a washed beaker of appropriate size and frozen at -70°C before being lyophilised using a Labconco (Freezone 6) freeze drying system.
- The pellet (denoted with the subscript "$_P$") was transferred to a pre-weighed centrifuge tube and dried under nitrogen at < 40°C before being desiccated overnight.

- With the aid of mild sonication within a water bath the pellet was extracted thrice with clean EtOH (10ml) for three successive 10minute periods. After centrifugation, the pooled supernatants were cooled overnight at 5-10°C before again being centrifuged at 5°C for 30minutes at 2000g. The yields of the resultant supernatant (denoted with the subscript "$_{PS}$") and pellet (denoted with the subscript "$_{PP}$") were then determined and expressed as % (w/w) of the TDM.
- The lyophilised supernatant (H_20_S) was divided in two and reconstituted in either EtOH (denoted by the subscript "$_{S1}$") or H_20 (denoted by the subscript "$_{S2}$") to a concentration of 15mg/ml.
- Both of the reconstituted supernatants were cooled overnight at 5-10°C before being centrifuged at 5°C for 30minutes at 2000g. Supernatants were then denoted by the subscript "$_{S1S}$" and "$_{S2S}$" respectively whilst the pellets were denoted by the subscript "$_{S1P}$" and "$_{S2P}$" respectively.
- Three 1.00ml aliquots were taken of all the supernatants in order to determine their respective concentrations. Extracts were adjusted to a concentration of 10mg/ml. Several aliquots of these respective stock solutions were made and stored at -70°C.
- Non-soluble pellets were dried, their respective weights determined before being stored in the dark at ambient room temperature for the duration of the study.

3.3.4. Analysis of extracts through thin layer chromatography (TLC)

Normal phase (Alugram® SIL G/UV$_{254,}$ Macherey-Nagel) TLC plates were used. Several different mobile phases (as discussed in section 3.4.3.) were used each with the intention of accomplishing a specific objective - TLC was performed in order to: assess the effectiveness of the EtOH reconstitution methodology; serve as a qualitative indicator of intra-species variance; detect the possible presence of aryltetralin lignans; assess the degree of complexity of the respective primary extracts; assess the possible coincidence of phytochemicals between extracts and give an indication of possible fractionation strategies.

Practical procedure: TLC

- The development chamber was saturated with mobile phase. Filter paper lining the back wall of the chamber was used to hasten the process.
- Typically 50-200µg (applied in 5µl increments of 10mg/ml stock solutions) of the extracts were spotted and allowed to air-dry 1cm from the bottom of the plate, 1cm apart and allowed to develop until approximately 1cm from the top.
- A pencil line was drawn to indicate the solvent front, thereby facilitating the calculation of respective retardation factors (R*f* values).
- After drying in a fume cabinet, separated components were visualized under visible and ultraviolet light (254nm and 360nm) using a Camag Universal UV lamp TL-600.
- Developed plates were stained with either iodine vapour (stains unsaturated and nitrogen containing compounds) or HOAc-conc HNO_3 (10:3) as suggested by Broomhead and Dewick, (1990) for the detection of podophyllotoxin derivatives.

3.3.5. Direct-infusion ESI-MS analysis of extracts (Dereplication)

An Applied Biosystems, MDS SCIEX 4000 Q-Trap, LC/MS/MS system was used to acquire mass fingerprint data of all the primary extracts. The 4000 Q-trap is a hybrid instrument affording the capabilities of both a triple quadrupole and a linear ion trap. Both positive and negative ion data was collected for all the primary extracts following infusion via a syringe pump directly into the electrospray ionisation (ESI) source. ESI is particular useful for the ionisation of thermally labile, polar molecules and offers an excellent mass range and sensitivity. Pertinently, ESI is a soft ionisation technique and is capable of producing unfragmented pseudo-molecular complexes.

Due to the low level of fragmentation, ESI is suitable to not only characterize single compounds but also complex mixtures. As such, a detailed fingerprint of plant extracts and other complex mixtures can be obtained through discernment (without chromatographic separation) of the compounds within the mixture based upon their respective mass to charge (m/z) ratios (Mauri and Pietta, 2000). This technique is fast and reliable (Araujo et al., 2005). When used in conjunction with post-analysis

chemometrical interpretation, this technique has the potential for semi-quantitation of specific plant metabolites (Favretto *et al*., 2001).

Direct-infusion ESI MS was used preliminarily as a method of dereplication - obtained pseudo-molecular weights were compared to a m/z database compiled of chemotaxonomical data of the genus *Bridelia* as well as common aryltetralin lignans (Appendix A). Further more, constituents present within different extracts could be qualitatively compared and potential decomposition monitored. In order to attain a global phytochemical profile of the respective extracts, mass spectra were acquired in both positive and negative ion mode. Standard conditions were used through out.

Practical procedure: Direct-infusion ESI-MS

- For positive ion mode analysis samples were adjusted to 0.2% formic acid; for negative ion mode analysis samples were adjusted to 0.2% ammonium hydroxide (Araujo *et al*., 2005).
- Before infusion the pH adjusted samples were centrifuged at 5°C for 10minutes at 14000g to remove any insoluble material.
- 1mg/ml samples (as suggested by Mauri and Pietta, 2000) were directly infused at a rate of 10µl/min for 1 minute, using a syringe pump (Harvard apparatus)
- The syringe and ion source were rinsed with EtOH before injection of the next sample.
- Mass spectra were acquired over a 100-1200 *m/z* range once a stable TIC (total ion current) had been attained.

3.4. Results and discussion

3.4.1. Extraction outcomes

Summarily, a known weight of bark material from two different trees was separately extracted using a Soxhlet apparatus. Leaf material from a third tree was extracted in the same way: Three extractants, namely: hexane (4 hours), ethyl acetate (4 hours) and water (24 hours) were employed sequentially to extract and separately collect non-

polar, moderately polar and polar compounds respectively. After drying aliquots of the extracts, the extraction yields were determined gravimetrically (Figure 3.4.).

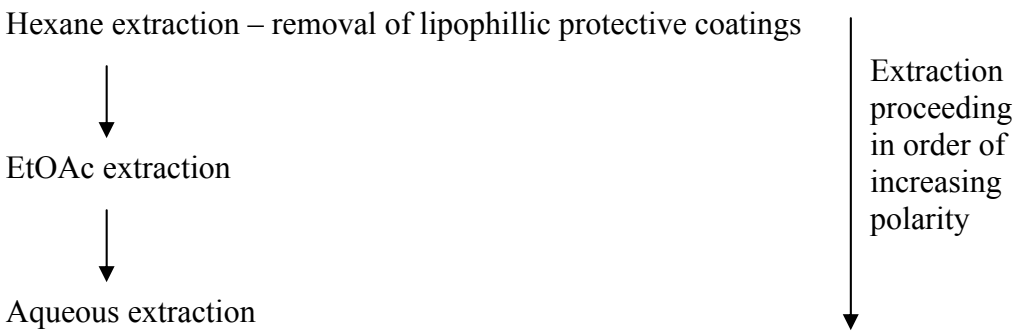


Figure 3.4. Scheme depicting the extraction process

Extraction yields as shown in Table 3.2 are expressed as the mean percentage weight (w/w) of the total-dry mass (TDM) ± SD (where repeats were performed). Extraction of the leaf material, (which considering previous reports, was not surprisingly found to be inactive), was only carried out once.

Table 3.2. Extraction yields in terms of percentage weight of the total dry mass (TDM). Sequential Soxhlet extraction was carried using hexane, ethyl acetate and water for periods of 4,4 and 24hours respectively.

Extract	Bark (LT0022)	Leaf (LT006)	Bark (2229DD)
Hexane	0.76 ± 0.05%, n=3	2.39%	0.48 ± 0.03%, n=2
Ethyl acetate	1.02 ± 0.17%, n=3	1.94%	0.61%
Aqueous	21.02 ± 2.61%, n=3	19.41%	15.23%

The yields attained for the aqueous extract of the bark (LT0022) far exceed that reported by others who used the same extraction instrumentation (Soxhlet apparatus) and duration (24 hours) - Lin *et al*., (2002) reported an aqueous extract yield of 14.1%. This difference could very well have been attributed to the sequential extraction methodology. However, the aqueous extract of bark (2229DD) more closely correlates with that reported by Lin *et al*., (2002) suggesting that the sequential extraction methodology had no influence on the extraction yields but rather that these outcomes are as a consequence of natural variance.

Considering that the same extraction methodology was used for the two bark specimens in this study, the intra-species variance could be assessed objectively through statistical comparison of the hexane extraction yields: there appears to be a significant difference between the hexane extracts taken from the two differently sourced bark materials (P 0.0062, $\alpha = 0.05$; two-tailed unpaired t test). This again demonstrates the natural variance of secondary metabolite expression. It is due to this variance that plant extracts need to be standardised in order to achieve a predictable and reproducible clinical outcome.

3.4.2. Reconstitution outcomes

As already mentioned, the *in vitro* assessment of the two organic extract's (hexane and ethyl acetate) cytotoxic capacities was dependent upon their incorporation into and thus miscibility with the polar environment of *in vitro* bioassays. As such, it was necessary to reconstitute these two extracts in a water miscible solvent that would not influence the bioassay at the specified, controlled for concentration. Ultimately and predictably however, this process of reconstitution might very well amount to sub-fractionation and the exclusion of certain classes of compounds as result of their inherit lack of solubility in the chosen carrier solvent. The use of liposomes or dimethyl sulphoxide (DMSO) to carry EtOH insoluble compounds into the cell culture medium was not motivated for, nor warranted.

The EtOH reconstitution yields for the organic extracts of bark (LT0022) were 0.51 ± 0.13% TDM (n=3) and 0.66 ± 0.08% TDM (n=3) for the hexane and ethyl acetate extracts respectively. For preliminary investigation using the LT0022 material, the insoluble precipitates formed upon cooling were not further processed and consequently their respective weight percentages were not determined. The EtOH reconstitution yields for the organic extracts of bark (2229DD) as well as a summary of the expanded methods employed are reported in Figure 3.5 and 3.6. The aqueous extract reconstitution yields along with a summary of the methods employed are reported in Figure 3.7.

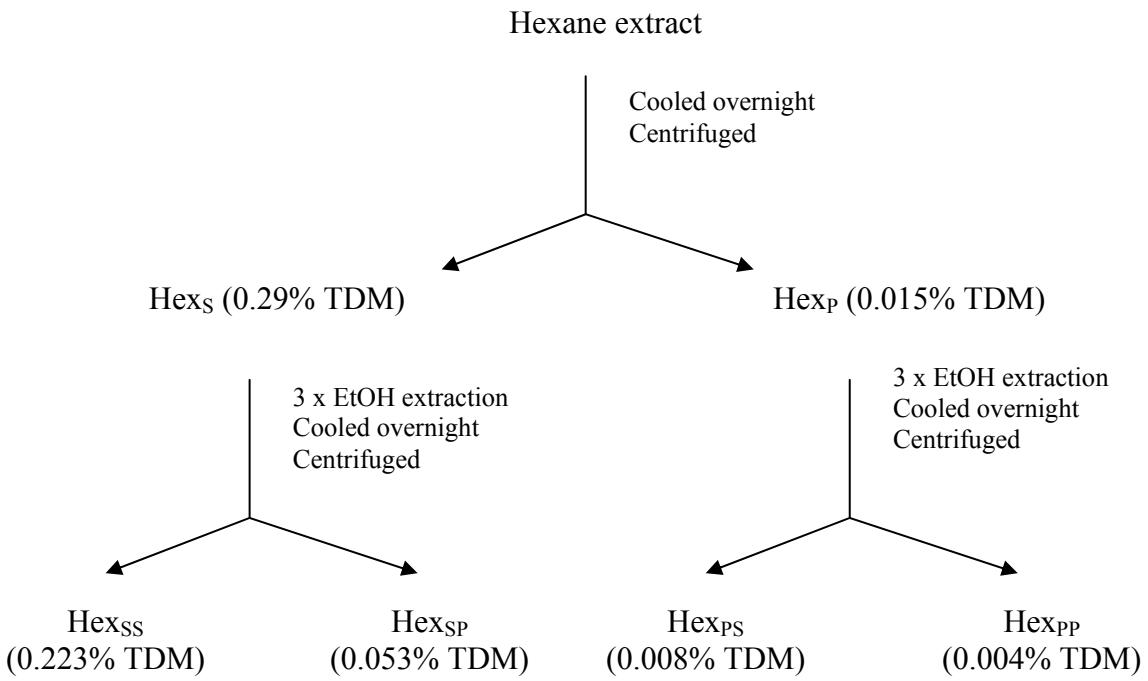


Figure 3.5. Scheme displaying hexane extraction and reconstitution outcomes. Supernatants are denoted by the subscript "S"; Pellets are denoted by the subscript "P"

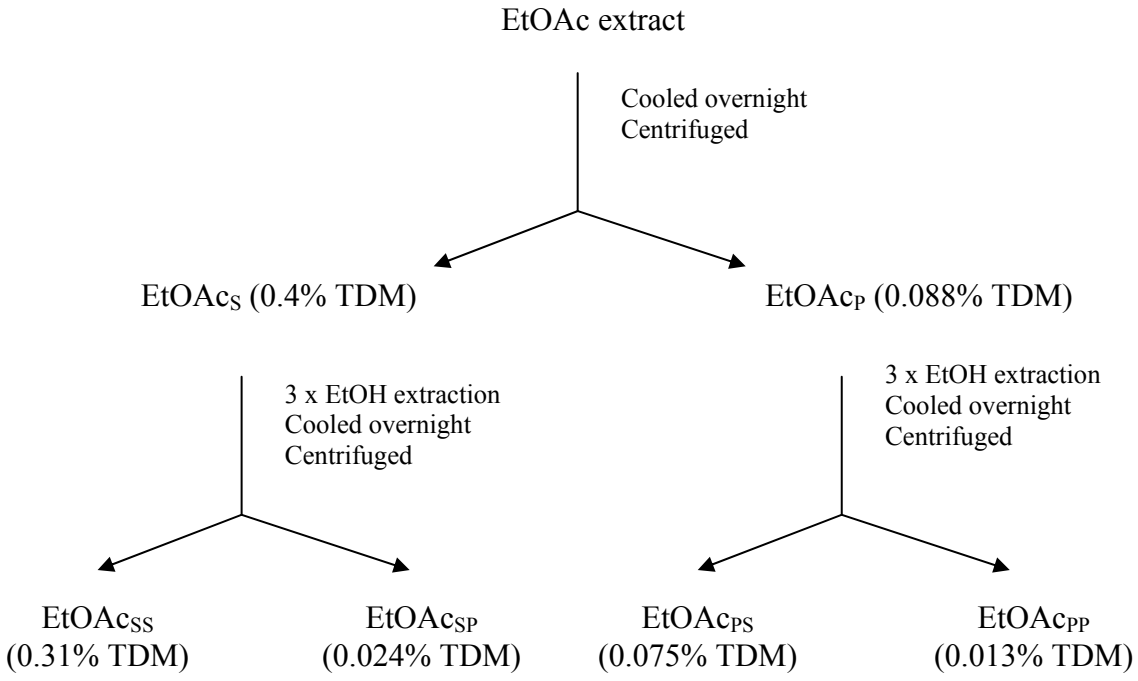


Figure 3.6. Scheme displaying ethyl acetate extraction and reconstitution outcomes. Supernatants are denoted by the subscript "S"; Pellets are denoted by the subscript "P"

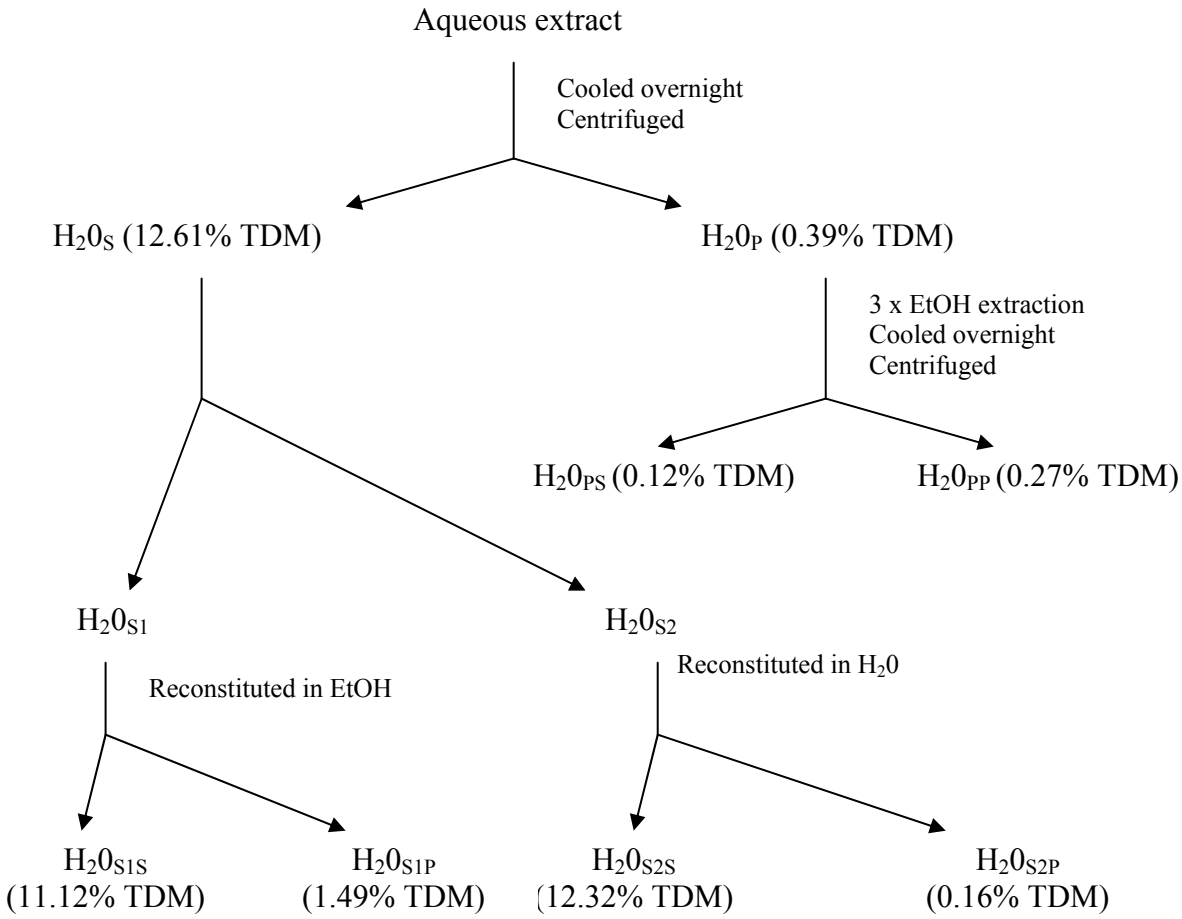


Figure 3.7. Scheme displaying aqueous extraction and reconstitution outcomes. Supernatants are denoted by the subscript "s"; pellets are denoted by the subscript "p"; Subscript "1" denotes EtOH reconstitution; Subscript "2" denotes H_20 reconstitution

3.4.3. TLC analysis

TLC plates were developed using a range of appropriate mobile phases to produce retainable qualitative fingerprints of the extracts. The extracts were all run using the same mobile and stationary phases thus allowing for common constituents within the different extracts to be identified.

Hexane extracts from the two different bark specimens were again used to assess intra-species variance, but this time qualitatively. Figure 3.8 displays similarities between the two hexane extracts as well as obvious differences, most notably the absence of

compounds from the 2229DD bark material corresponding to R*f* values of 0.14, 0.21 and 0.43 - 0.5.

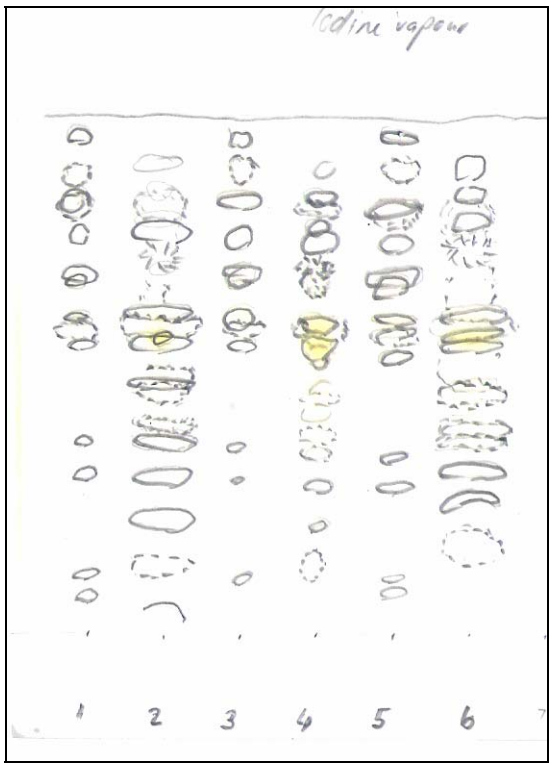


Figure 3.8. TLC comparison of hexane extracts taken from two different bark specimens of *B. micrantha*. Developed with Hexane: chloroform: acetic acid (5:4:1). Visualised at 254 and 360nm. Stained with iodine vapour. (Even numbered lanes: identifier number – LT0022; Odd numbered lanes: identifier number – 2229DD).

Due to a good degree of separation using only one mobile phase, in one dimension and the relative ease of visualization under UV light, this methodology is proposed as a tool to quickly and cost effectively, positively identify *B. micrantha* (bark) samples.

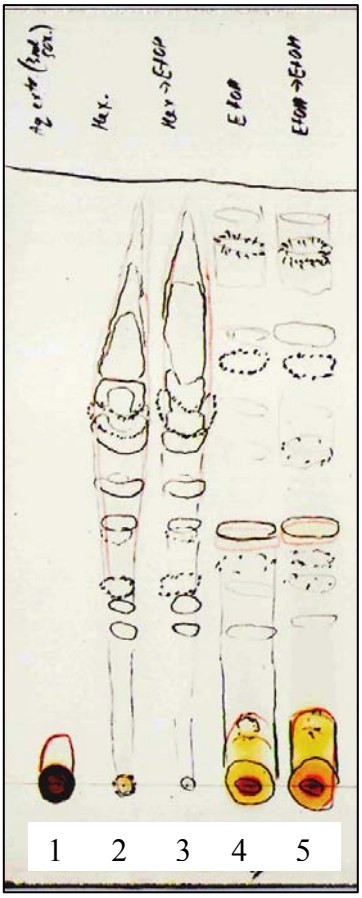
Extracts taken from bark material (LT0022) were used to demonstrate the efficiency of the EtOH reconstitution methodology. This was achieved by comparing the crude organic extracts with their reconstituted counterparts (Figure 3.9. & 3.10.). Observations of these two figures strongly suggest that the EtOH reconstituted extracts are good qualitative representatives of their original crude extracts. The use of the EtOH reconstitution methodology is thus validated. Additionally, it can be observed that a degree of overlapping of phytochemical components within the two organic extracts is probable, thus in all probability representing non-exhaustive extraction. Due

to the non-polar nature of the mobile phases used, the aqueous extract (which was not soluble in the mobile phase) did not migrate from the origin.

Mobile phases used by other researchers for the development of aryltetralin lignans were used. Acetic acid-nitric acid, (10:3) was used as a spray reagent to detect the possible presence of aryltetralin lignans (Broomhead and Dewick, 1990). It is important to acknowledge that there are no chromogenic reagents specific for lignans and that the general applicability of this reagent to detect aryltetralin lignans has not been fully evaluated. Following the methods of Broomhead *et al*., (1991) and Dewick and Jackson, (1981), plates (Alugram SIL G/ UV 254) were initially developed using $CHCl_3$-MeOH, (25:1) as shown in Figure 3.11. At a later stage $CHCl_3$-MeOH-H_20, (20:10:1) was used as a mobile phase in order to facilitate the migration of the more polar constituents (Figure 3.12.).

Developed plates were visualised under UV light before being sprayed with the $HOAc/HNO_3$ detection reagent. Plates were closely inspected for immediate reactions corresponding to the possible presence of 4'-demethyl aryltetralin lignan derivatives and then finally heated (100ºC, 5minutes) to detect the possible presence of trimethoxy derivatives (Jackson and Dewick, 1985).

Several brown colourations were observed (as indicated in the corresponding figures with arrows) after heating the sprayed plates. This brown colouration, as reported by Jackson and Dewick, (1985) is indicative of the possible presence of peltatins. Reactions at certain R*f* values were common among several of the extracts strongly suggesting the occurrence of the same compounds in these extracts.



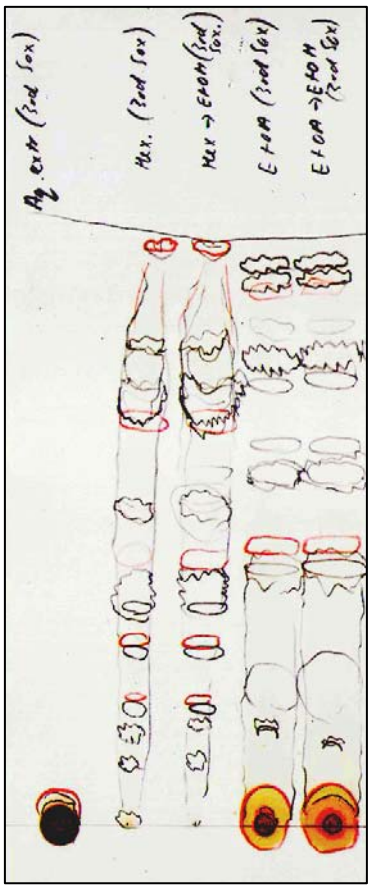


Figure 3.9.

Figure 3.10.

Figure 3.9. TLC (Alugram SIL G/ UV 254) chromatogram of bark (LT0022) extracts of *B. micrantha* developed with dichloromethane: chloroform: EtOAc (6:2:2) validating the EtOH reconstitution methodology. Visualised at 254nm and 360nm. Stained with iodine vapour. (From left to right, lane 1: Aqueous extract; lane 2: Crude hexane extract; lane 3: EtOH reconstituted hexane extract; lane 4: Crude EtOAc extract; lane 5: EtOH reconstituted EtOAc extract). Demonstrates that the EtOH reconstituted extracts are good qualitative representatives of the original crude extracts.

Figure 3.10. TLC (Alugram SIL G/ UV 254) chromatogram of bark (LT0022) extracts of *B. micrantha* developed with hexane: chloroform: acetic acid (5:4:1) validating the EtOH reconstitution methodology. Visualised at 254nm and 360nm. Stained with iodine vapour. (From left to right, lane 1: Aqueous extract; lane 2: Crude hexane extract; lane 3: EtOH reconstituted hexane extract; lane 4: Crude EtOAc extract; lane 5: EtOH reconstituted EtOAc extract).

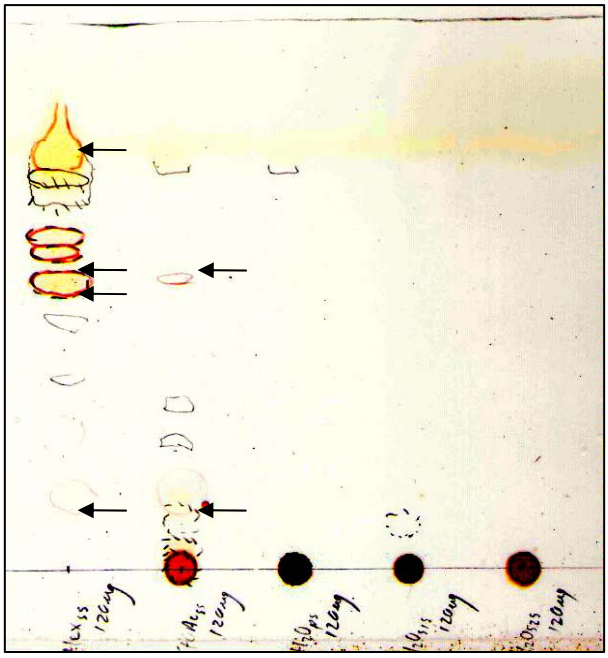


Figure 3.11. TLC chemical (chromogenic) test to detect the possible presence of aryltetralin lignans within the primary extracts of *B.micrantha* (bark). (From left to right, lane 1: Hex_{SS}; lane 2: $EtOAc_{SS}$; lane 2: H_20_{PS}; lane 4: H_20_{S1S}; lane 5:H_20_{S2S}. Plate (Alugram SIL G/ UV 254) developed in $CHCl_3$-MeOH, (25:1); Visualised at 254nm and 350nm. Plate sprayed with HOAc-conc HNO_3 (10:3) - arrows indicate the formation of a brown colour upon heating.

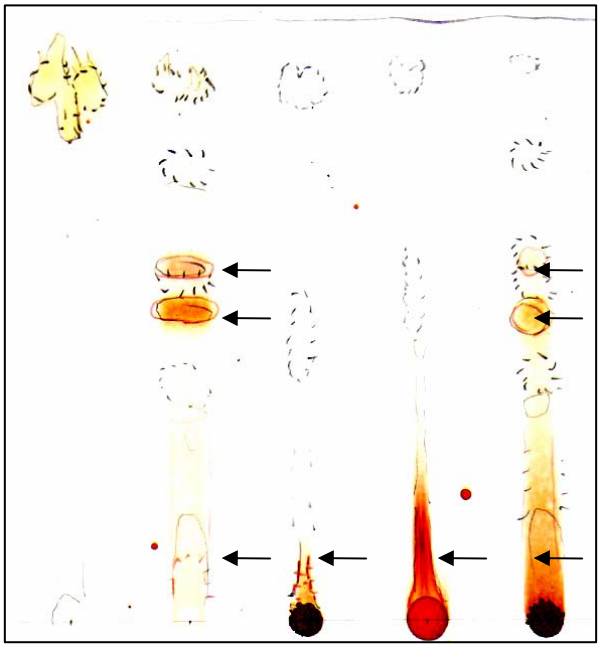


Figure 3.12. Detection of possible aryltetralin lignans. (From left to right, lane 1: Hex_{SS}; lane 2: $EtOAc_{SS}$; lane 3: H_20_{PS}; lane 4: H_20_{S2S}; lane 5:H_20_{S1S}. Plate (Alugram SIL G/ UV 254) developed in $CHCl_3$-MeOH-H_20, (25:10:1); Visualised at 254nm and 350nm. Plate sprayed with HOAc-conc HNO_3 (10:3) - arrows indicate the formation of a brown colour upon heating.

3.3.4. ESI-MS analysis

At this early stage of the study, this technique was used primarily as a method of dereplication. At a later stage this method was used to monitor fractionation outcomes and potential decomposition. Due to the chemical nature of the constituents not being known, wide ionisation parameters were opted for. Mass fingerprints of all the primary extracts were acquired in both positive and negative ion modes using continuous multichannel analysis.

None of the pseudo m/z data recorded (Figure 3.13-3.24.) corresponded to diagnostic ions of aryltetralin lignans within the compiled database (Appendix A). Assuming ionisation, one can only conclude that common aryltetralin lignans (i.e. those within the compiled database) are not present within this particular *B. micrantha* (bark) sample. Considering TLC observations, this does then also suggest the possible presence of uncommon and perhaps even novel aryltetralin lignans.

The presence of other compounds known within the genus is suggested (Appendix A), although not confirmed. A literature review did not reveal any cytotoxic activity associated with these compounds and as such tandem MS/MS experiments to confirm the presence of these compounds was not motivated for.

So as to allow for visual comparison of the various extracts, the phytochemical profile (both positive and negative ion mode), for each of the respective primary extracts is depicted in Table 3.3-3.4.. These qualitative profiles have taken into consideration only m/z intensity peaks > 20% of the maximum intensity (for each respective extract). It is important to acknowledge that these are representative qualitative profiles that typically depict only the major ionising compounds present and does not take into account the effect of ion suppression, particularly considering the amount of different compounds competing for a charge within the electrospray source.

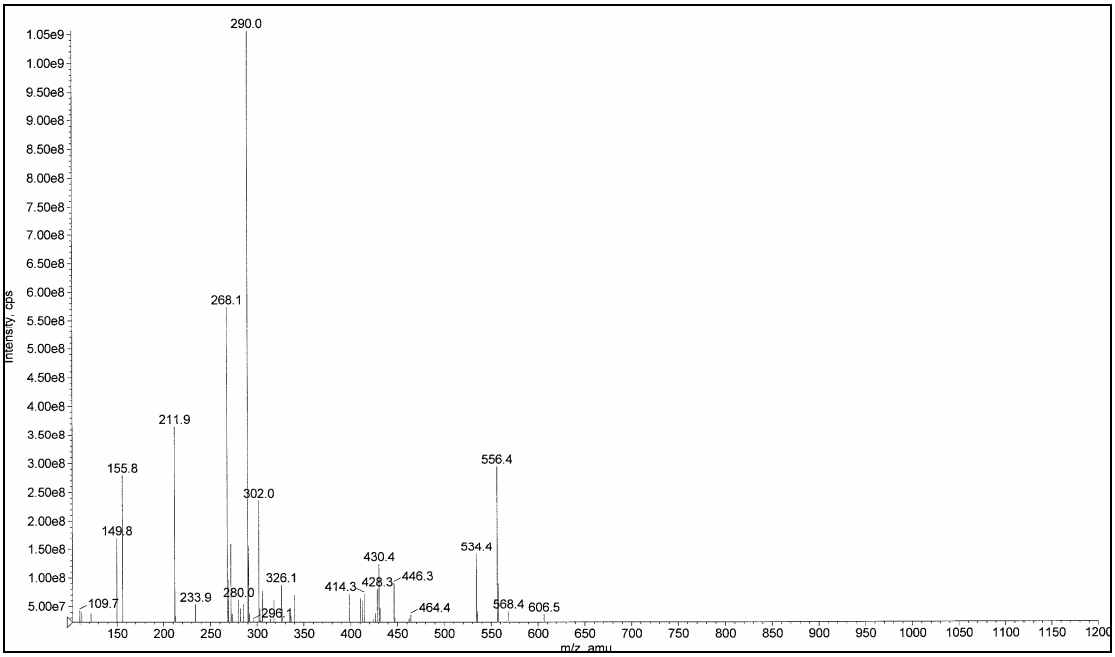


Figure 3.13. Positive ion mode ESI mass fingerprint of extract Hex_{ss}

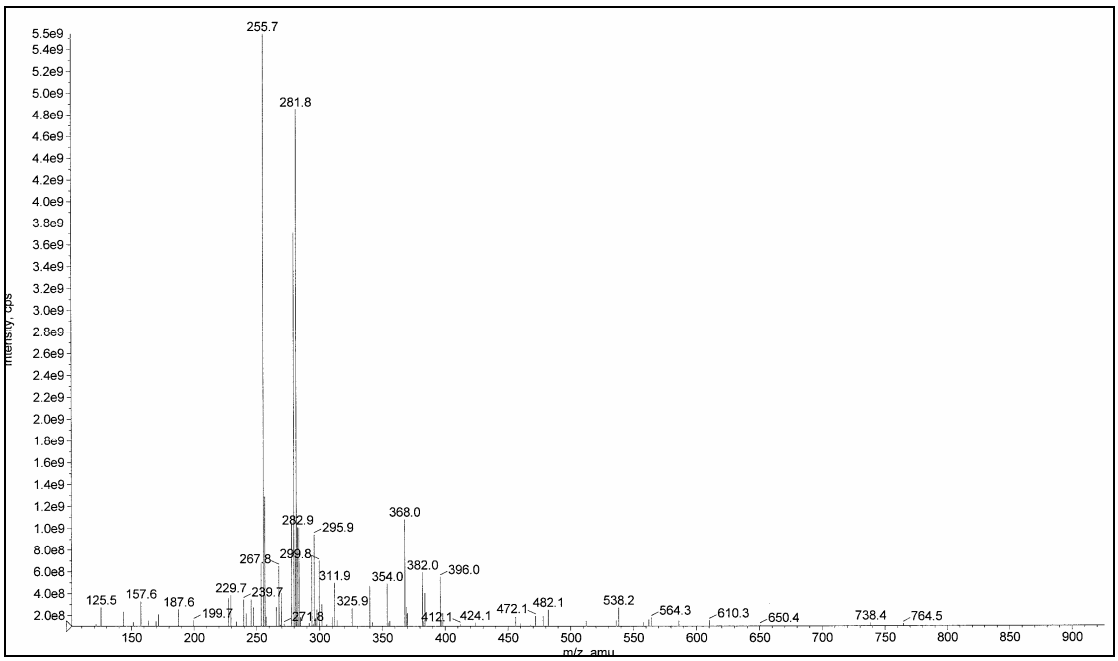


Figure 3.14. Negative ion mode ESI mass fingerprint of extract Hex_{ss}

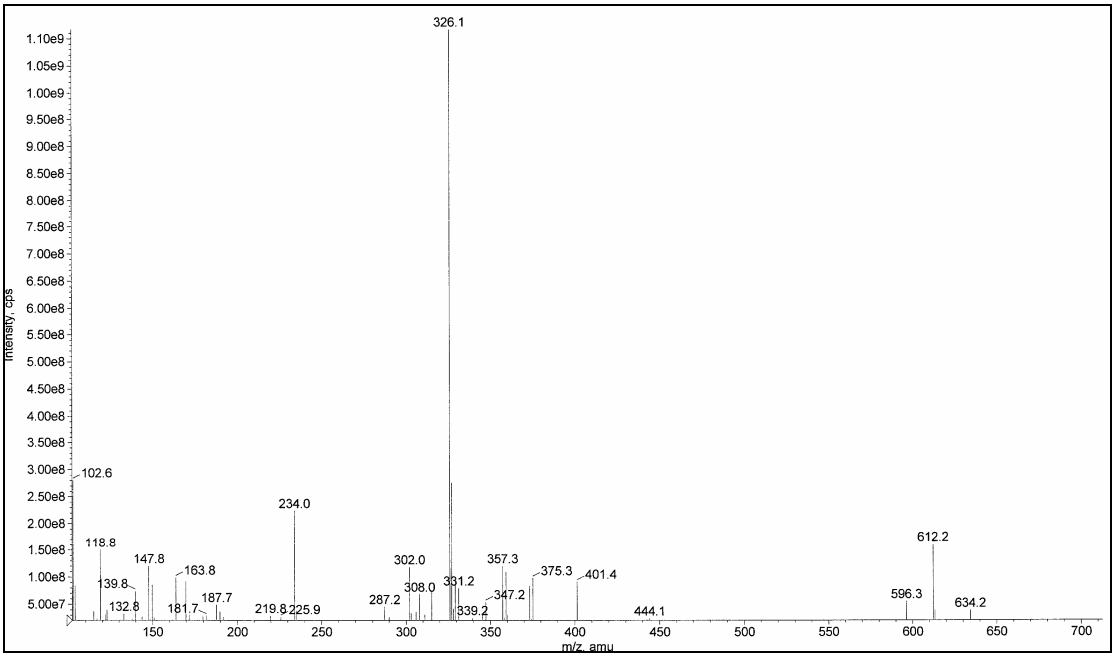


Figure 3.15. Positive ion mode ESI mass fingerprint of extract EtOAc$_{ps}$

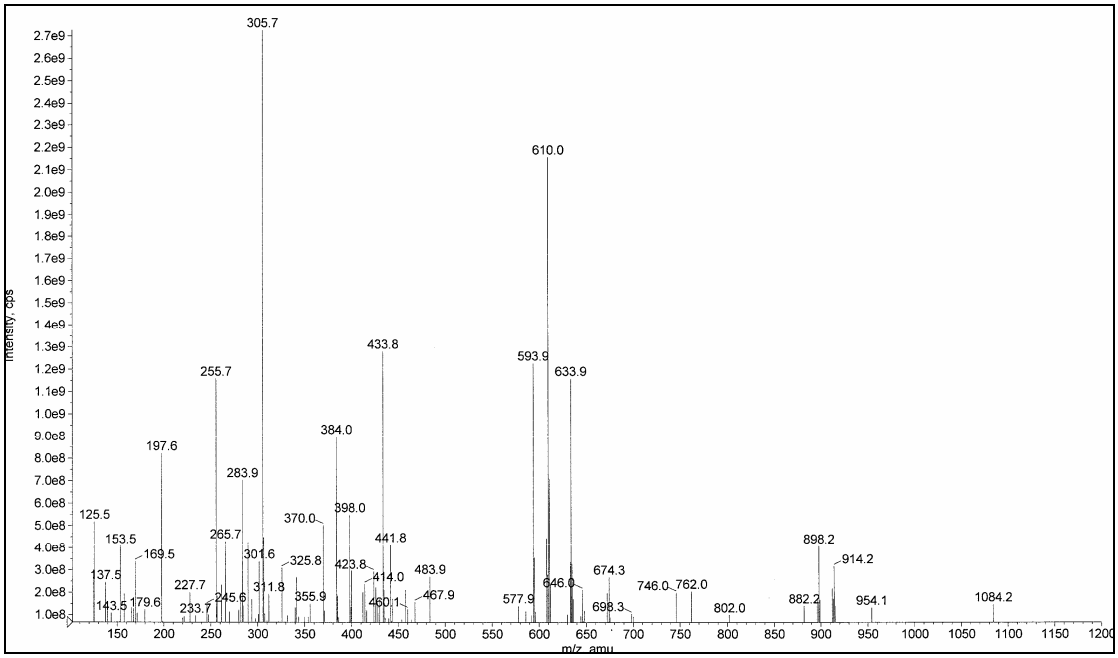


Figure 3.16. Negative ion mode ESI mass fingerprint of extract EtOAc$_{ps}$

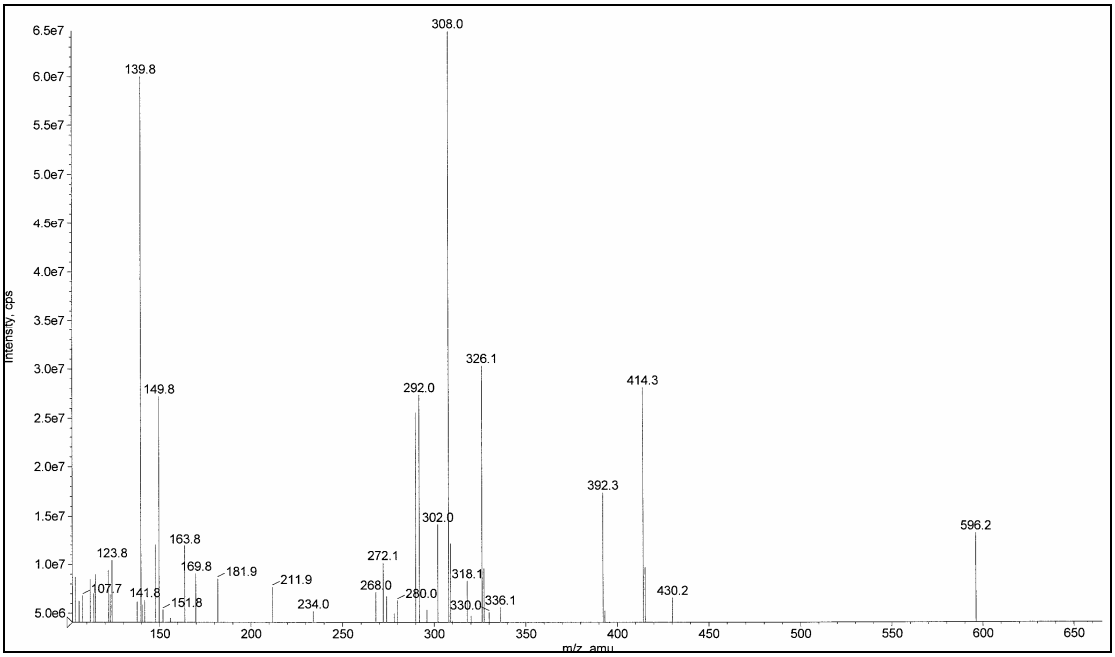


Figure 3.17. Positive ion mode ESI mass fingerprint of extract $EtOAc_{ss}$

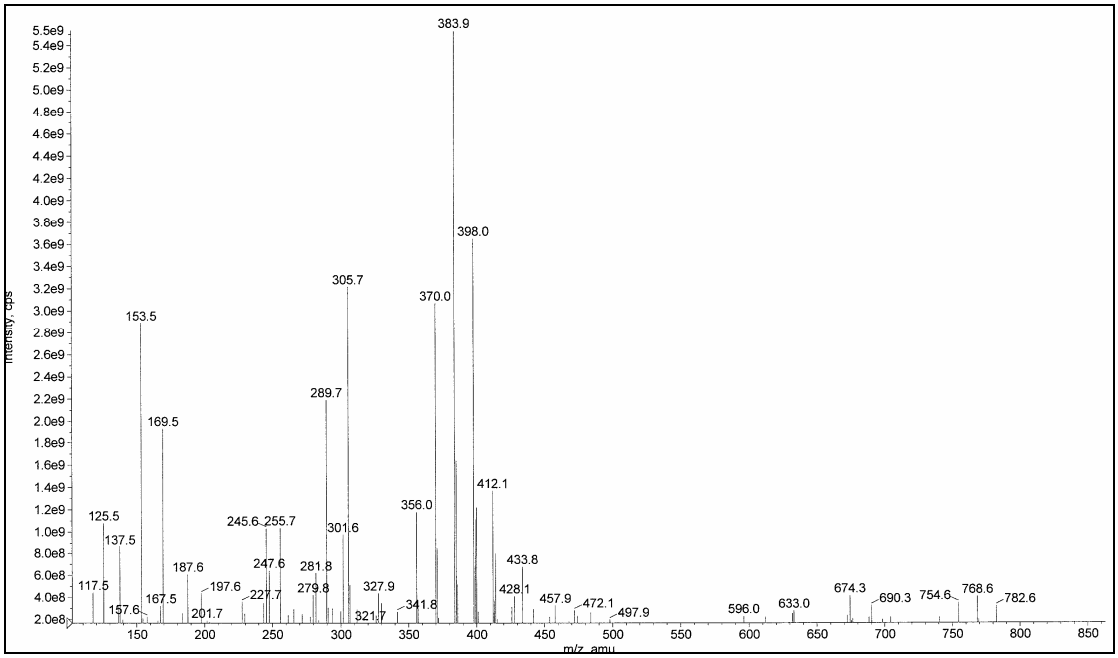


Figure 3.18. Negative ion mode ESI mass fingerprint of extract $EtOAc_{ss}$

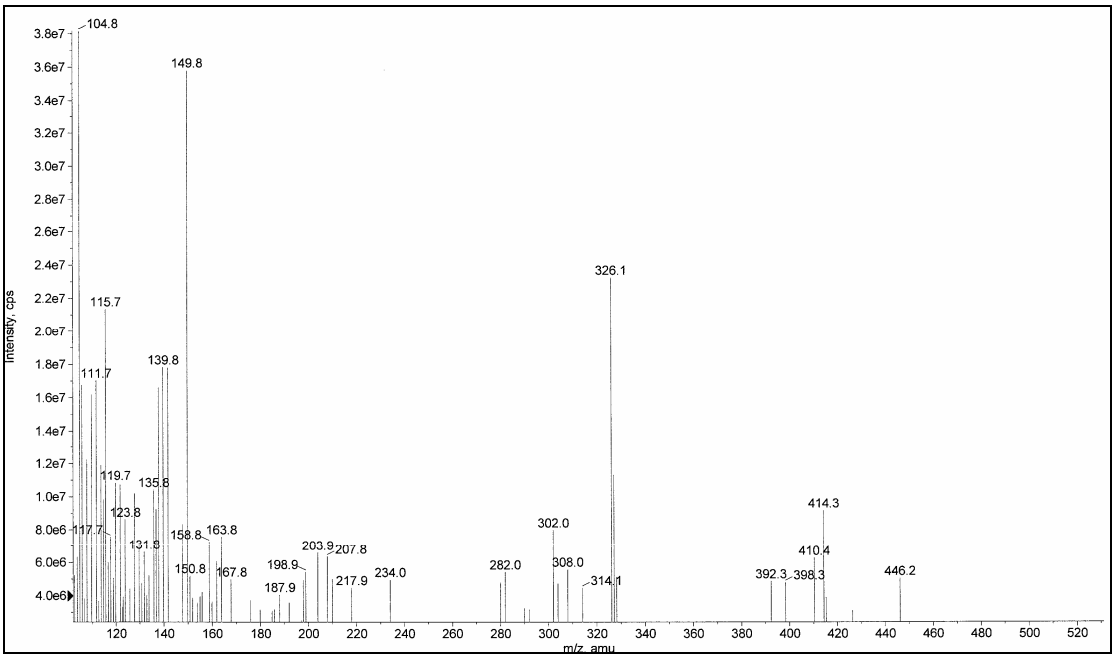


Figure 3.19. Positive ion mode ESI mass fingerprint of extract H_20_{ps}

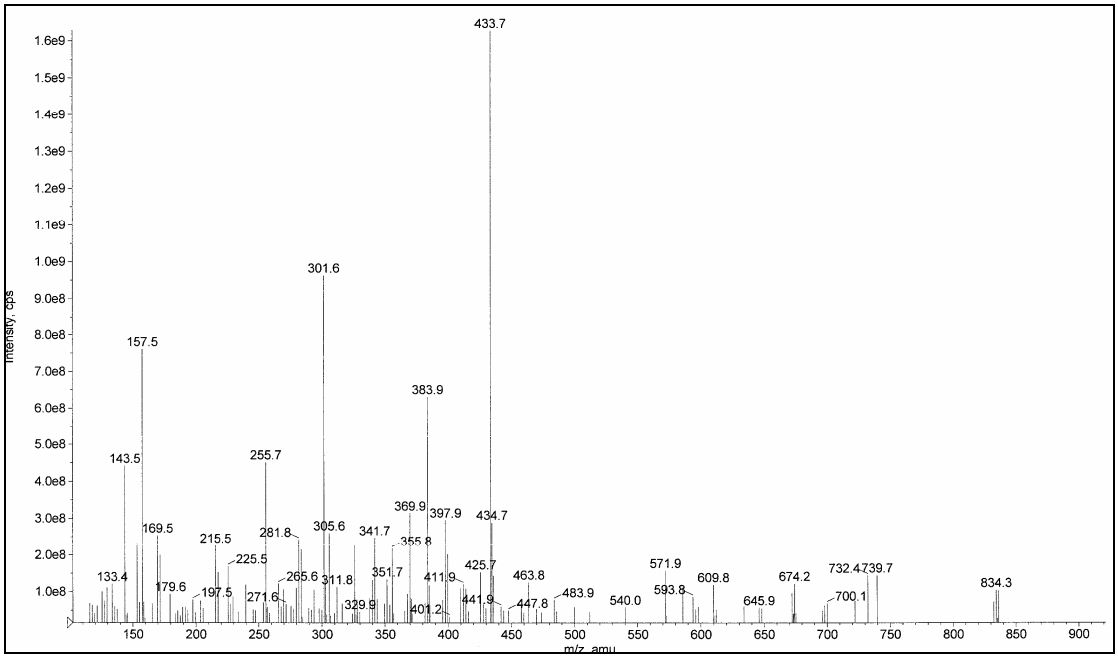


Figure 3.20. Negative ion mode ESI mass fingerprint of extract H_20_{ps}

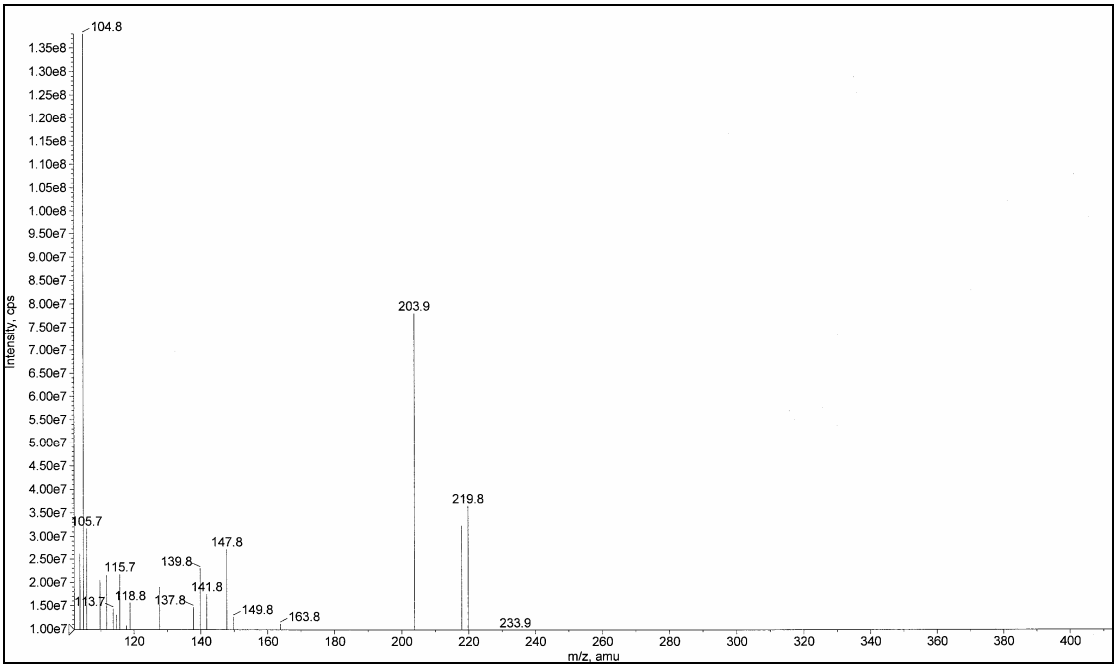


Figure 3.21. Positive ion mode ESI mass fingerprint of extract H_20_{s1s}

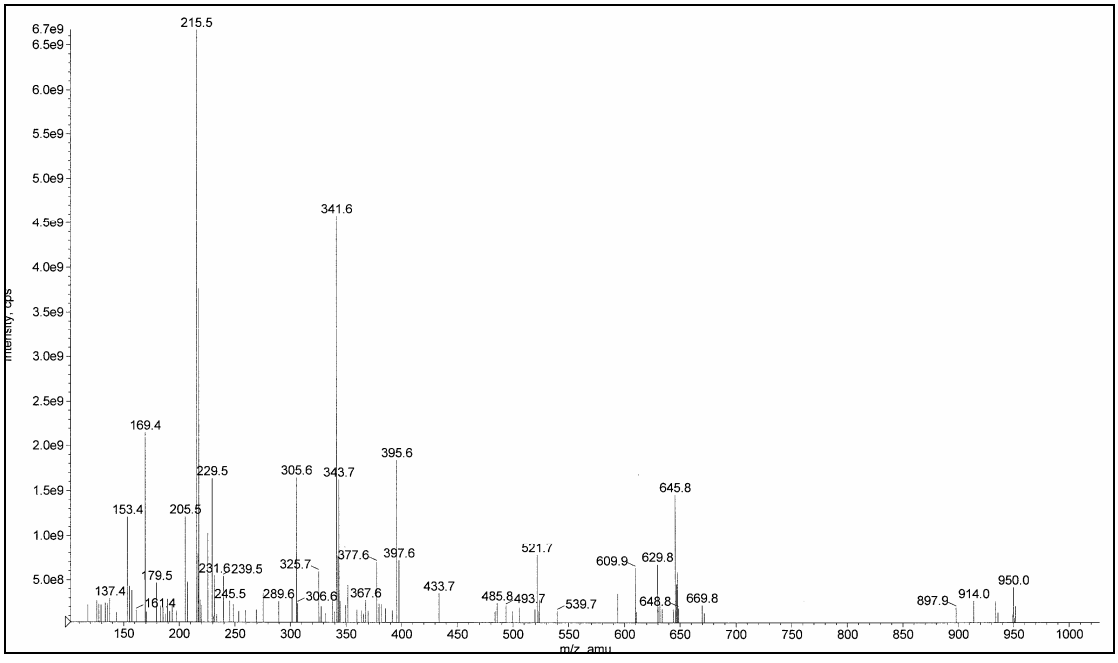


Figure 3.22. Negative ion mode ESI mass fingerprint of extract H_20_{s1s}

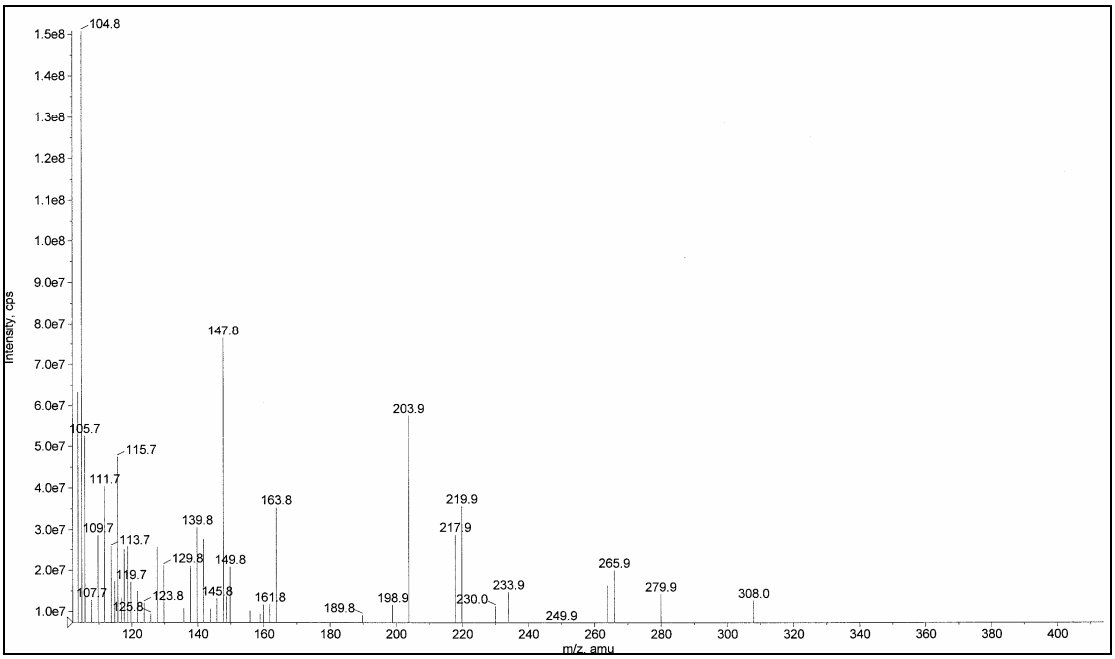


Figure 3.23. Positive ion mode ESI mass fingerprint of extract H_20_{s2s}

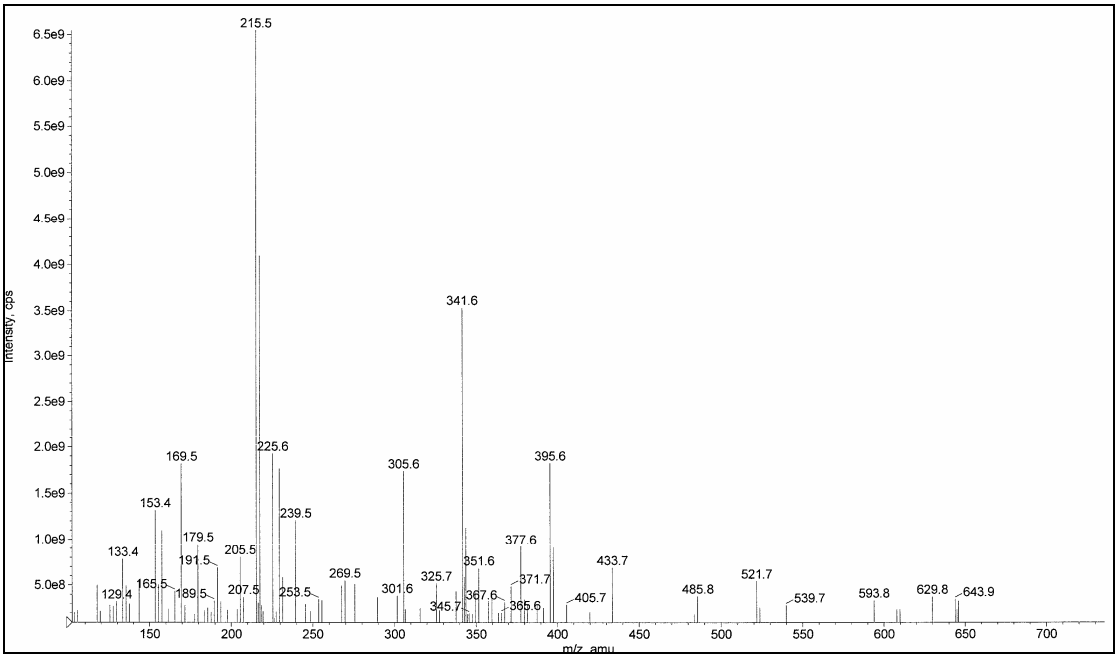


Figure 3.24. Positive ion mode ESI mass fingerprint of extract H_20_{s2s}

Table 3.3. Representative positive ion mode summary of the phytochemical profile for each of the primary extracts of *B. micrantha* (bark). Only M/z intensity peaks > 20% of the maximum intensity are displayed

+Q (Positive ion mode)					
H_2O_{S1S}	H_2O_{S2S}	H_2O_{PS}	$EtOAc_{PS}$	$EtOAc_{SS}$	Hex_{SS}
	103.7				
104.8	104.8*	104.7			
105.8*	105.8	105.8			
		107.8			
	109.7	109.7			
	111.7	111.7			
		113.7			
		114.7			
	115.7	115.7			
		119.7			
		127.7			
		135.9			
		136.7			
139.8	139.8	139.8		139.8	
		141.8			
147.8	147.8	147.8			
		149.8*		149.8	
					155.8
		158.8			
	163.8			163.8	
203.9	203.9				
					211.9
217.9	217.9				
219.9	219.9				
			234		
					268.1
				290	290*
				292	
					302
				308*	
				309	
		326.1	326.1*	326.1	
		327.1	327.1		
				392.3	
		414.3		414.3	
					556.4

*** Indicates M/z peak with maximum intensity**

Table 3.4. Representative negative ion mode summary of the phytochemical profile for each of the primary extracts of *B. micrantha* (bark). Only M/z intensity peaks > 20% of the maximum intensity are displayed

-Q (Negative ion mode)					
H_2O_{S1S}	H_2O_{S2S}	H_2O_{PS}	$EtOAc_{PS}$	$EtOAc_{SS}$	Hex_{SS}
			125.5	125.5	
		143.7			
153.4	153.4			153.4	
		157.6			
169.5	169.5			169.5	
			197.7		
205.6					
215.4*	215.4*				
217.7	217.7				
	225.7				
229.7	229.7				
	239.5				
				245.7	
		255.7	255.7	255.7	255.7*
					256.7
					277.9
					279.8
					281
					283
			284		284
				289.8	
					296
		301.8		301.8	
305.8	305.8		305.8*		
			306.8		
341.6	341.6				
343.8	343.8				
				355.9	
		370		370	370
				370.9	
		383.9	383.9	383.9*	
				385	
395.6	395.6				
				398	
				399	
				400	
				412.1	
		433.8*			
		434.8			
			594		
			610		
			611		
			633.9		
645.8					

*** Indicates M/z peak with maximum intensity**

3.5. Conclusions

The data available from this and other studies suggests that one can expect to attain a yield of between 14.1% and 23.6% (w/w) TDM for a 24hour aqueous Soxhlet extraction of *B. micrantha* (bark). This study demonstrates that extraction of *B. micrantha* (bark) with hexane and ethyl acetate yields <1% (w/w) TDM.

The EtOH reconstitution methodology was shown to be effective in that the phytochemical constituency/composition of the original crude organic extracts were qualitatively well represented by their EtOH reconstituted counterparts as shown through TLC.

Although a positive reaction (brown colouration) was observed to the HOAc/HNO_3 spray reagent suggesting the presence of peltatins, the specificity of this reagent to detect aryltetralin lignans exclusively is questionable, as many compounds are known to be susceptible to nitric acid oxidation. All of the primary extracts gave positive reactions to the spray reagent at various R*f* values. Several of these reactions were common among different extracts.

Direct-infusion ESI-MS was used to implement a dereplication strategy within the study design. Comparison of the mass spectra to a small mol. wt. database compiled from chemotaxonomical literature and including common aryltetralin lignans did not reveal the presence of known cytotoxic compounds.

CHAPTER 4

4. *IN VITRO* BIOASSAYS

4.1. Introduction

For ethical and financial reasons, *in vitro* bioassays (cells in culture) are commonly accepted as the first line of investigation as apposed to *in vivo* (animal) studies. However it is important to acknowledge that this is a reductionist point of view and may not necessarily accurately portray the response of a whole organism to the drug (plant extract) and furthermore the effect of the drug on a complex, multifaceted disease state (Houghton and Kaman, 1998).

Antiproliferative *in vitro* bioassays were performed in order to quantitate the effect of the primary extracts on the proliferation of both neoplastic and normal primary cultures. A standard number of a particular cell type was exposed to a concentration range of each extract and incubated for a standardised period of time. At the end of which, viable cells were discerned from dead cells through a suitable cell enumeration method. The antiproliferative effect of the different extracts was then quantified relative to untreated controls.

The *in vitro* antineoplastic potential of investigational compound/s (phytoconstituents) is commonly assessed through comparison of the *in vitro* antiproliferative effect on neoplastic cells with the antiproliferative effect on normal primary cell cultures (van Niekerk et al., 2001). This is done so that the degree of cytotoxic specificity (preference) for cancer cells can be assessed. Furthermore, as different types of cancer cells may possess differences in phenotypic expression (and thus response to cytotoxic agents), it is important to assess the degree of cytotoxic selectivity between neoplastic cells of different tissue origin. All the primary extracts were therefore screened against a panel of different neoplastic cells and primary cultures.

Additionally, flow cytometric bioassays were used to assess both the manner of cell death induction and to identify possible points of cell cycle blockage induced by the most promising of the primary extracts on a sensitive non-adherent neoplastic cell line.

A flow cytometer is a powerful instrument that illuminates cells (or other particles) as they progress in single file through a beam of focussed light. The emitted light, whether scattered or fluorescent, from each particle is detected, converted to a voltage and digitised allowing for interpretation. As such, statistical information can be obtained for large populations of cells in a short period of time (Hawley & Hawley, 2004). It is expected that an extract (complex mixture) displaying activity (thus no antagonism) would incur the same manner of biological action as a purified AC would, but just to a lesser degree owing to its dilution (w/w) within the mixture.

4.2. Materials

4.2.1. Preparation of reagents

Ammonium chloride solution

Prepared by dissolving 8,3g Ammonium chloride (NH_4Cl), 1g Sodium bicarbonate ($NaHCO_3$), (both purchased from Merck, JHB, SA) and 74mg EDTA (Sigma-Aldrich, JHB, SA) in 1000ml of distilled water. The solution was then filter sterilized using a 0.22 μm cameo filter and refrigerated until use.

Binding buffer

Prepared by dissolving 238mg HEPES, 876mg NaCl, 26.5mg $CaCl_2$, 37.3mg KCl and 9.5mg $MgCl_2$ in 100ml of deionised water. The pH was adjusted to 7.2 with NaOH.

Cell counting fluid

1ml of a 0.1% crystal violet solution and 2ml glacial acetic acid was dissolved in 97ml of distilled water. The solution was mixed well and refrigerated until use.

Essential Modified Eagle's Medium (EMEM)

EMEM powder (Sigma-Aldrich, JHB, SA) was dissolved in sterile water with the aid of a sterile stirrer. The pH of the solution was adjusted to 4.0 with 1N HCl to ensure complete solubilisation. Thereafter 2mg of $NaHCO_3$ was added to each litre of the medium. The pH was readjusted to 7.1 through the addition of either 1N HCl or 1N sodium hydroxide (NaOH). The medium was filter-sterilized through a 0.22µm filter and divided into 500ml aliquots. 55ml was removed from the 500ml aliquot and the remaining solution was supplemented with 5ml of a 1% penicillin/streptomycin solution (Adcock-Ingram, JHB, SA) and 50ml sterile heat inactivated fetal calf serum (HI FCS), (Adcock Ingram, JHB, SA). The medium was stored at 4ºC.

Dulbecco's Modified Eagle Medium (DMEM)

DMEM powder (Sigma-Aldrich, JHB, SA) was dissolved in sterile water with the aid of a sterile stirrer. The pH of the solution was adjusted to 4.0 with 1N HCl to ensure complete solubilisation. Thereafter 2mg of $NaHCO_3$ was added to each litre of the medium. The pH was readjusted to 7.1 through the addition of either 1N HCl or 1N sodium hydroxide (NaOH). The medium was filter-sterilized through a 0.22µm filter and divided into 500ml aliquots. 55ml was removed from the 500ml aliquot and the remaining solution was supplemented with 5ml of a 1% penicillin/streptomycin solution and 50ml sterile HI FCS. The medium was stored at 4ºC.

DMEM/Ham's F12 medium (1:1)

Supplemented with 20ng/ml epidermal growth factor, 100ng/ml cholera toxin, 0.01mg/ml insulin and 500ng/ml hydrocortisone.

Heparin

30g heparin (Sigma-Aldrich, JHB, SA) was dissolved into 90ml of distilled water. The solution was filter sterilized using a 0.22 µm cameo filter and refrigerated until use.

MTT solution

200mg MTT was dissolved in 40ml PBS solution. After solubilising the light sensitive solution was filter sterilized using a 0.22 µm cameo filter and stored (covered in tin foil) at 4ºC until use.

Phosphate buffered saline (PBS)

0.923g of FTA hemagglutinin buffer powder (The Scientific Group, JHB, SA) was dissolved in 100ml of distilled water. The pH was adjusted to 7.2.

Phytohemagglutinin (PHA) solution

5ml of distilled water was added to the freeze-dried PHA bottle content (Bioweb (PTY) Ltd., JHB, SA). 0.2ml aliquots were dispensed under sterile conditions and stored at -20°C.

Propidium Iodide

[50µg/ml] solution was made up in either PBS or sodium citrate.

Roswell Park Memorial Institute Medium (RPMI) 1640

RPMI 1640 powder (Sigma-Aldrich, JHB, SA) was dissolved in sterile water with the aid of a sterile stirrer. The pH of the solution was adjusted to 4 with 1N HCl to ensure complete solubilisation. Thereafter 2mg of $NaHCO_3$ was added to each litre of the medium. The pH was readjusted to 7.1 through the addition of either 1N HCl or 1N NaOH. The medium was filter-sterilized through a 0.22µm filter and divided into 500ml aliquots. 55ml was removed from the 500ml aliquot and the remaining solution was supplemented with 5ml of a 1% penicillin/streptomycin solution and 50ml sterile HI FCS. The medium was stored at 4ºC until use.

Sodium citrate

1.12% (w/v) solution was made up in distilled water.

4.2.2. Cell cultures

The cytotoxic selectivity and specificity of the different primary extracts was determined by comparing the cytotoxic effects exerted on a range of different neoplastic cells with a range of normal primary cultures. Normal primary cultures were included as predictors of potential toxicity (the therapeutic value of the AC) and used to demonstrate cytotoxic specificity (or lack thereof) for neoplastic cells.

Neoplastic cells lines

- HeLa (Human cervical carcinoma; ATCC number: CCL-2) is one of the most widely studied cell lines.
- MCF 7 (Human mammary carcinoma; ATCC number: HTB-22) is a hormone-dependent cell line.
- COLO 320DM (Human colorectal carcinoma; ATCC number: CCL-220) is a notoriously multi-drug resistant, non-adherent cell line.
- Jurkat (Human acute T cell leukaemia; ATCC number: TIB-152) is a non-adherent cell line and provides a mean of direct comparison with primary human lymphocytes.

Normal primary cultures

- Primary human lymphocytes (stimulated and resting) - procedures for isolation of lymphocytes are discussed appendix B.
- Primary chick fibroblasts - procedures for isolation of fibroblasts are discussed appendix B.
- Hepatocytes - provided by the Bio-liver Research Unit, Dept of Internal Medicine, University of Pretoria.

4.3. Methodology

4.3.1. Extract preparation

Prior to bioassay, aliquots [10mg/ml] of all the primary extracts were removed from storage (-70ºC) and allowed to thaw at ambient room temperature in the dark. All EtOH reconstituted extracts were diluted with the relevant medium for the specific cell line to make up appropriate volumes of the desired concentrations.

Water reconstituted extracts required filter-sterilization in order to prevent possible fungal contamination. As such, samples were diluted with distilled water to a concentration of 2mg/ml before being filter sterilized using a 0.22µm cameo filter

(0.25AS, AEC-Amersham P/L, JHB, SA). Aliquots were taken and dried in order to determine the exact concentration of the filtrate before adjusting the concentration to 1mg/ml. Several 1ml aliquots were dispersed into sterile eppendorfs before being stored at -70°C for later use. Aliquots were then further diluted with the appropriate medium for the specific cell line to achieve the required test concentrations.

4.3.2. *In vitro* antiproliferative bioassays

The following cells cultures were tested thrice in triplicate using sterile, round bottomed 96 well plates (AEC-Amersham P/L, JHB, SA): HeLa (cultured in EMEM), MCF 7 (cultured in DMEM) and COLO 320DM (cultured in RPMI). Stimulated and resting human lymphocytes as well as primary chick fibroblasts were cultured in RPMI. Primary porcine hepatocytes were cultured in DMEM/Ham's F12 medium.

Starting cell numbers that were inoculated, as determined through the use of a haemocytometer were standardised at 500 cells per well for each of the neoplastic cell lines; at 200 000 cells per well for the lymphocytes and at 20 000 cells per well for primary fibroblasts and hepatocytes.

All the primary extracts were tested at 4 different final concentrations (1, 10, 20 & 40µg/ml) in order to obtain a dose response effect. Concentrations higher than 40µg/ml are thought to be physiologically unattainable thus representing inadequate potency and were as such not used. Appropriate EtOH controls were assayed in parallel in order to compensate for any solvent cytotoxic effect. The highest final EtOH concentration in culture was 0.4% and this proved to have no influence on the proliferation of any of the cell cultures tested. In addition, Taxol was included as a positive control and screened in parallel in order to corroborate comparisons at a later stage. Data was analysed and statistically interpreted using GraphPad software (Prism, version 4).

Practical procedure: Sub-culturing and preparation of cell solutions

- Cells were typically grown in 75cm^2 (AEC-Amersham P/L, JHB, SA) culture flasks to achieve the required cell numbers for each assay.

- The medium was decanted before rinsing the flask with a small volume of trypsin/versene solution (Highveld biological, JHB, SA). Non-adherent cells i.e. COLO and Jurkat were simply decanted.
- Adherent cells were incubated with trypsin/versene until the cells detached from the flask (approximately 5-10 minutes, dependent on the specific culture).
- Fresh culture medium (5% FCS) was added and this solution transferred into 15ml tubes and spun down for 5minutes at 500g.
- The supernatant was removed and the cell pellet resuspended in 1ml of appropriate medium (10% FCS).
- 50µl of this cell suspension was added to 450µl counting fluid.
- A haemocytometer was used to count the cells.
- The remaining cell suspension was adjusted to a suitable volume to attain the desired cell concentration.

Bioassay procedure: Neoplastic cell lines, fibroblasts and hepatocytes

- 80µl of medium was added to all wells (experimental and control).
- 100µl of medium was added to the background wells.
- 100µl of the cell suspension (standardized number) were added to all wells except the background.
- Cultures were pre-incubated after inoculation for a period of one hour before the addition of the extracts.
- 20µl of each of the prepared, respective extract concentrations were added to each of the corresponding experimental wells.
- 20µl of medium was added to each aqueous extract control well.
- 20µl of 4% EtOH in the appropriate medium (highest concentration used) was added to each organic extract control well.
- Cultures were maintained in 5% CO_2 at 37ºC.
- After 7 days, MTT assays (as described below) were performed to order to quantitate the number of viable cells in each well.

Bioassay procedure: Resting and stimulated lymphocytes

- Lymphocytes were isolated as discussed in appendix B.
- 60µl of medium was added to all wells except the background wells.
- 100µl of medium was added to the background wells.
- 100µl of the cell solutions (standardized concentration) were added to all wells except that of the background.
- Cultures were pre-incubation for 1hour.
- 20µl of each prepared extract concentration was added to each experimental well.
- 20µl of medium was added to each aqueous extract control well.
- 20µl of 4% EtOH in medium was added to each organic extract control well.
- 20µl of PHA [25µg/ml] was added to all stimulated wells.
- 20µl of medium was added to all resting wells.
- Cultures were incubated in 5% CO_2 at 37ºC
- After 3 days, MTT assays (as described below) were performed to order to quantitate the number of viable cells in each well.

Practical procedure: MTT assay (Methods of Mossmann, 1983 with modifications)

- 20µl of 5mg/ml, MTT (Sigma-Aldrich, JHB, SA) in PBS was added to each well.
- Plates were incubated for 3-4 hours in 5% CO_2 at 37ºC. Fibroblasts required incubation for 24hours.
- Plates were centrifuged at 500g for 10minutes.
- Supernatant were carefully removed without disturbing the pellet.
- Pellets were washed with 150µl PBS.
- Plates were again centrifuged at 500g for 10minutes.
- The supernatant was again carefully removed before drying the pellet.
- 100µl of DMSO was added to each well.
- Plates were gently shaken on a model VRN-200 shaker for at least an hour.
- Plates were read spectrophotometrically using a microplate reader (ELx 800 UV) at 570nm, using 630nm as a reference to determine the relative number of viable cells.

4.3.3. Flow cytometric bioassays

As it was first necessary to determine which of the cell lines were sensitive to which of the primary extracts, flow cytometric bioassays were conducted secondary to the *in vitro* antiproliferative bioassays. The purpose of such investigations was to serve as an initial indicator of the underlying molecular mechanism of action. Only extract H_20_{S1S}, which possessed the most antineoplastic potential was investigated. After obtaining poor reproducibility using the HeLa cell line (possible as result of the scrapping technique employed to detach the cells from their growth substrate, which as reported by Hawley and Hawley, (2004) may in itself induce apoptosis), the COLO 320DM cell line (which showed comparable sensitivity, as reported later) was used for all subsequent flow cytometric bioassays. Cell death induction and cell cycle perturbations induced by extract H_20_{S1S} at a fixed concentration of 10µg/ml was assessed in triplicate after 24 and 48hour incubation times.

The two main regulators of cell population size are mitosis (cellular replication) and apoptosis (programmed cell death). Cancerous cells have acquired genetic traits that typically promote mitosis and evade apoptosis. The balance between cell death and cell replication determines the rate at which a tumour will grow (Stevens and Lowe, 2000). Most anticancer drugs exhibit their cytotoxic action through the induction of apoptotic pathways (Arya *et al*., 2000).

Apoptosis is programmed cell death. It is an active process (requires energy) that is under genetic control. Apoptosis is characterised by membrane blebbing, cellular shrinkage, condensation of cytoplasm and chromatin as well as fragmentation of DNA, all whilst maintaining intracellular order and cohesion allowing for the formation of apoptotic bodies. Apoptosis, unlike necrosis (which is a passive death process, as result of injury or damage to many contiguous cells), does not result in inflammation (Kuan and Passaro, 1998).

Annexin V-FITC (Fluorescein isothiocynate conjugated Annexin V - fluoresces in the green region of the spectrum) selectively binds to the externalised phospholipid, phosphatidylserine. Annexin V (BD Bioscience, JHB, SA) was used to quantitate the

percentage of cells undergoing early apoptosis, as the translocation of PS from the inner to the outer layer of the plasma membrane is indicative of such a process. Propidium iodide (PI), (BD Bioscience, JHB, SA), a membrane impermeable nuclear stain (intercalates in the double helix), which excites at 488nm and emits in the 570-630nm range (fluoresces in the orange/red region) of the spectrum was used to quantitate cells in which the plasma membrane had been compromised through either apoptotic or necrotic pathways. Cells staining positive for Annexin V are undergoing early apoptosis. Cells staining positive for PI only, are undergoing necrosis. Cells staining positive for both probes are said to be undergoing late stage apoptosis, undergoing necrosis or already dead. Cells staining negative for both probes are live viable cells.

The cell cycle is the ordered (and regulated) sequence of events that culminates in cellular division. The eukaryotic cell cycle is divided into four distinct phases. During S phase DNA replication occurs allowing for the creation of two identical daughter cells through mitosis occurring during M phase. These two phases are separated by gap phases, G_1 before S phase and G_2 before M phase where the cell prepares for the forthcoming processes. There are multiple checkpoint mechanisms that ensure the correct and unadulterated progression from one phase to the next. Mutation of these checkpoint effectors is often oncogenic (Bartek *et al*., 1999).

The cell cycle profile (DNA histogram) of COLO 320DM cell cultures treated with the most promising of the primary extracts was investigated and compared to the profile of untreated cells: After permeabilisation of the cell membranes, a RNase (Ribonuclease-A) solution was used to prevent staining of double stranded RNA. PI was added and the cell suspension analysed by flow cytometry to quantitate the amount of DNA present in each cell and thus determine the % of cells within each phase of the cell cycle. Data was analysed using a data analysis program (Multicycle AV software) to give the mean percentage of cells within each stage of the cell cycle.

Practical procedure: Cell death pathway induction

- For each of the two respective time periods, COLO 320DM cells (100 000) were seeded into three 25cm^2 culture flasks.
- Flasks were incubated for an hour at 37°C in 5% CO_2.
- Extract H_2O_{S1S} was added to make up a final concentration of 10µg/ml.
- The respective flasks were incubated for 24 and 48 hours at 37°C in 5% CO_2.
- Cells suspensions were decanted from the flasks and washed twice with PBS.
- Cells were resuspended in 1ml binding buffer.
- 100µl of each cell suspension was transferred into two flow cytometry tubes.
- 5µl of Annexin V and 10µl of PI was added to each of the stained tubes.
- Cell suspension were mixed gently and kept at 20-25ºC for 15minutes in the dark.
- 400µl of binding buffer was added to each tube and this solution analysed using a Beckman Coulter, Cytomics FC 500 flow cytometer within 1 hour.
- Each analysis was based on at least 10 000 events.

Practical procedure: Cell cycle analysis

- For each of the respective time periods, COLO 320DM cells (100 000) were seeded into three 25cm^2 culture flasks.
- Flasks were incubated for an hour at 37°C in 5% CO_2.
- Extract H_2O_{S1S} was added to make up a final concentration of 10µg/ml.
- The respective flasks were incubated for 24 and 48hours at 37°C in 5% CO_2.
- Cells were decanted from the flasks and centrifuged at 200g for 5minutes.
- Cell were resuspended in 500µl of PBS and chilled well on ice.
- Flow cytometry tubes containing 500µl of ice-cold EtOH were prepared.
- The cell suspension were rapidly pipetted into the ice-cold EtOH and aspirated before being kept on ice for 15minutes.
- The cell suspension was then centrifuged for 3minutes at 300g before carefully removing the supernatant.

- A volume of 125µl of RNase [2mg/ml], (BD Bioscience, JHB, SA) made up in a sodium citrate solution [1.12% w/v] was added to each tube followed by incubation in a water bath at 37°C for 15minutes.
- 125µl of PI [50µg/ml] made up in a sodium citrate solution [1.12% w/v] was added to each tube and mixed well.
- Samples were allowed to stain at room temperature in the dark for at least 30 minutes before being analysed on the flow cytometer
- Each analysis was based on at least 10 000 events.

4.3.4. Ethical considerations

The animal use and care committee (AUCC) approved the use of chicken embryo fibroblasts for cytotoxicity assays (Protocol H22/06, June 2006). Ethics committee approval for the use of human lymphocytes was obtained in June 2002 (Project number 164 of the Ethics committee of the University of Pretoria).

4.4. Results and discussion

4.4.1. *In vitro* antiproliferative bioassays

For preliminary (confirmatory) investigation, an aqueous extract of the bark material (LT0022) was shown to have an impressive IC_{50} of 6.44µg/ml against the HeLa cell line. Both resting and PHA stimulated primary human lymphocyte cultures were shown to be stimulated at concentrations up to 100µg/ml. This selective cytotoxic activity was superior to that reported by Mokoele *et al*., (2003) and undoubtedly warranted further investigation. Additionally (considering that the same sample material was used, as had been used by Mokoele *et al*., (2003) - all be it approximately 2 years older) this result attests to the long-term storage stability of the un-extracted dry plant material. Further more, the AC has seemingly been enriched. This enrichment may represent degradation of inert constituents or be as a consequence of the sequential extraction methodology.

An aqueous extract of the leaf material was shown to have an insignificant effect on the proliferation of HeLa cells at concentrations up to 100µg/ml (Figure 4.1.). This is in

agreement with other reports and serves as a negative control. Consequently, further investigation of the leaf material was consequently abandoned.

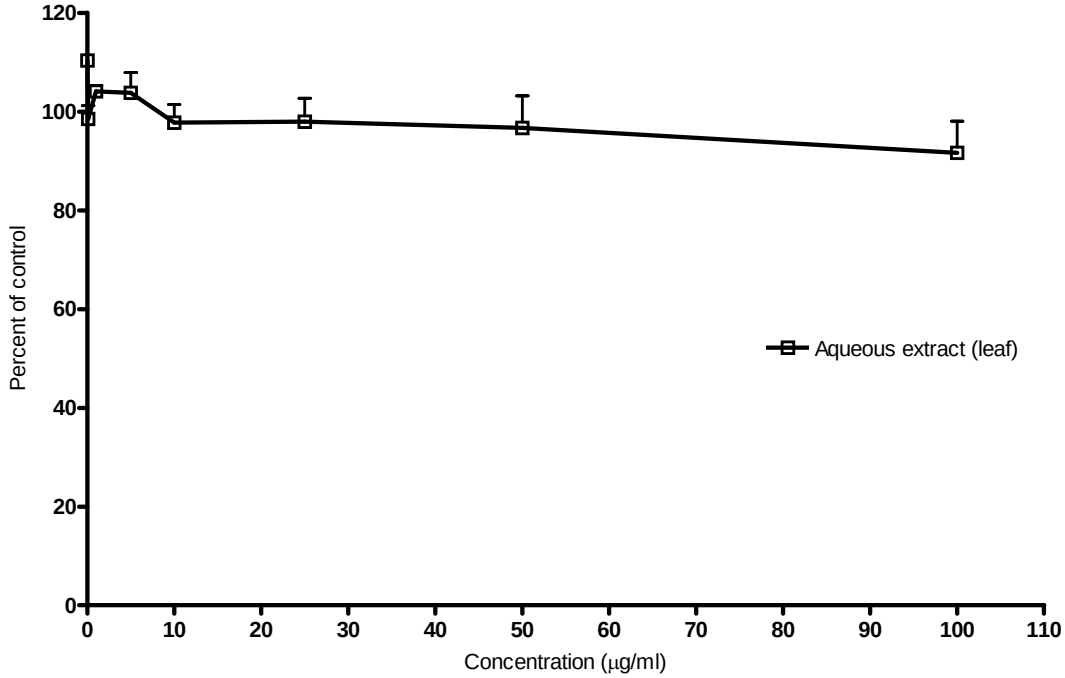


Figure 4.1. The effect of an aqueous extract of *B. micrantha* (leaf) on HeLa cell culture growth.

Using the validated extraction and processing procedures, five out of the six primary extracts of the bark material (2229DD) namely: H_20_{S1S}, H_20_{S2S}, H_20_{PS}, $EtOAc_{SS}$ and $EtOAC_{PS}$ showed exceptional antineoplastic potential in that they demonstrated pronounced *in vitro* cytotoxicity to three out of the four tested neoplastic cell lines namely: HeLa, COLO 320DM and Jurkat at concentrations below 10µg/ml (Figure 4.2 - 4.5). The cytotoxic effects produced by the different primary extracts demonstrated different selectivity's to different neoplastic cell lines suggesting the presence of more than one (different) active constituents (Appendix C).

What was particularly impressive is that in stark contrast these same five extracts did not inhibit the proliferation of hepatocytes (Figure 4.8) and had a significant stimulatory effect on resting and PHA stimulated human lymphocytes cultures (Figure 4.6 & 4.7, respectively). The proliferation of primary chick fibroblasts (Figure 4.9) was inhibited to a similar degree to that of the neoplastic cells.

IC_{50} values were determined for each of the primary extracts on each cell culture (Table 4.1.). The American National Cancer Institute recommends that an IC_{50} value of below 30µg/ml warrants bioassay-guided fractionation in order to identify the AC (Suffness and Pezzuto, 1990). As such, extract H_20_{S1S}, H_20_{S2S}, H_20_{PS}, $EtOAc_{SS}$ and $EtOAc_{PS}$ all deserved further investigation.

In order to assess variance in biological activity of the different primary extracts or to look at it from a different point of view, commonality - these values were statistically assessed through the use of a 2way ANOVA and Bonferroni multiple comparison post-tests (α =0.05), (Appendix C). Statistical interpretation of the different cytotoxic specificities exerted by different primary extracts strongly suggests the presence of numerous (functionally distinct) biologically active compounds.

Considering the much larger extraction yield of the aqueous extract compared to the organic extracts, the equally active extract H_20_{S1S} and H_20_{S2S} (no significant difference for any of the tested neoplastic cell cultures, appendix C) were deemed to possess the most promise.

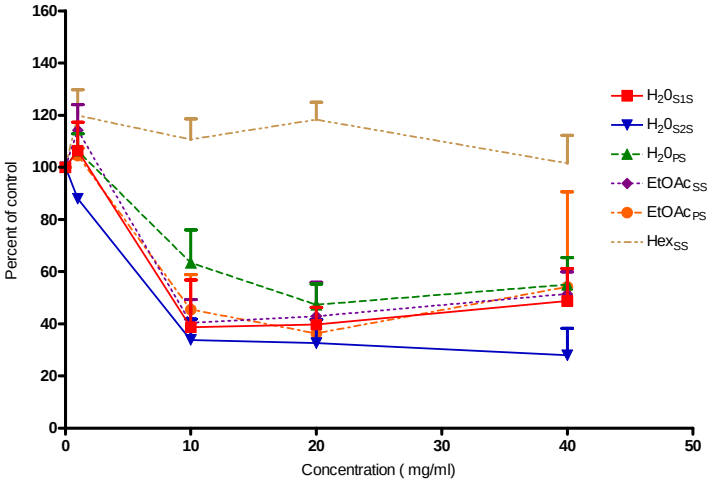


Figure 4.2. The antiproliferative effect of various primary extracts of *B. micrantha* (bark) on Jurkat cell cultures

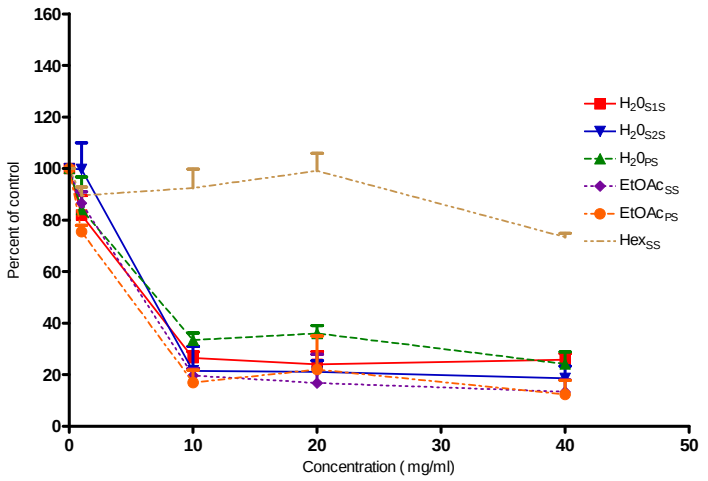


Figure 4.3. The antiproliferative effect of various primary extracts of *B. micrantha* (bark) on COLO320DM cell cultures

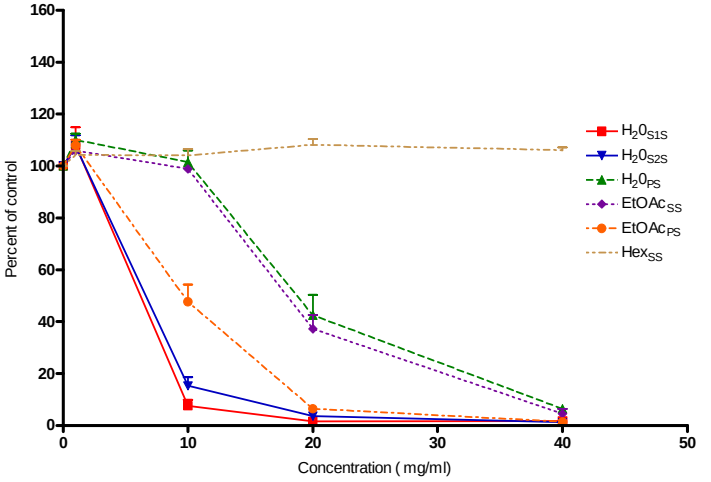


Figure 4.4. The antiproliferative effect of various primary extracts of *B. micrantha* (bark) on HeLa cell cultures

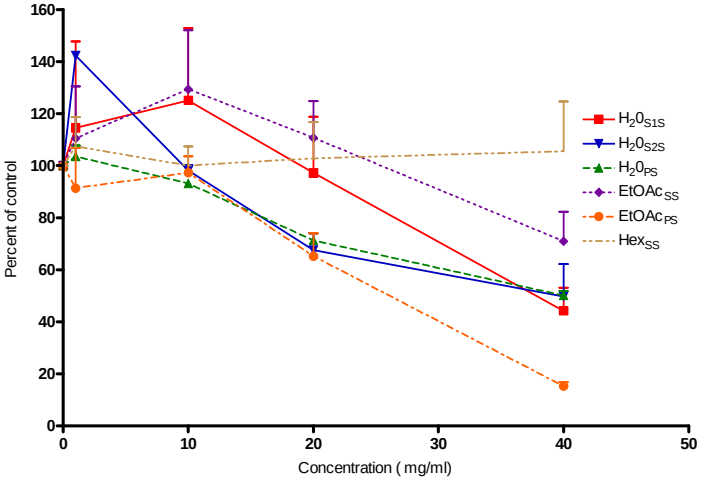


Figure 4.5. The antiproliferative effect of various primary extracts of *B.micrantha* (bark) on MCF 7 cell cultures

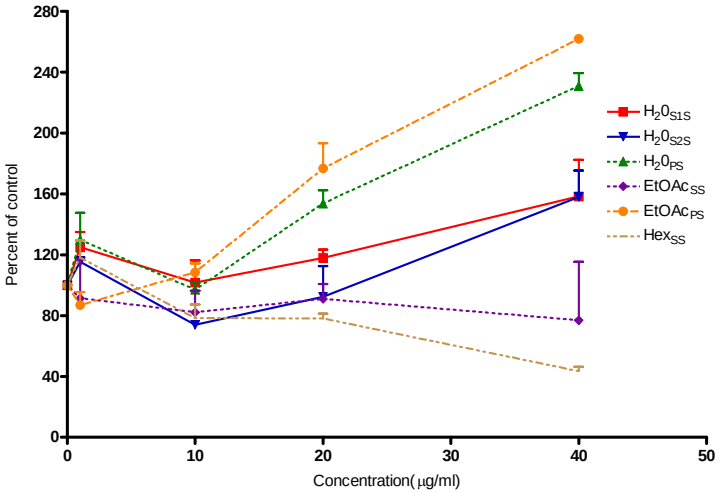


Figure 4.6. The effect of various primary extracts of *B. micrantha* (bark) on resting primary human lymphocyte cultures

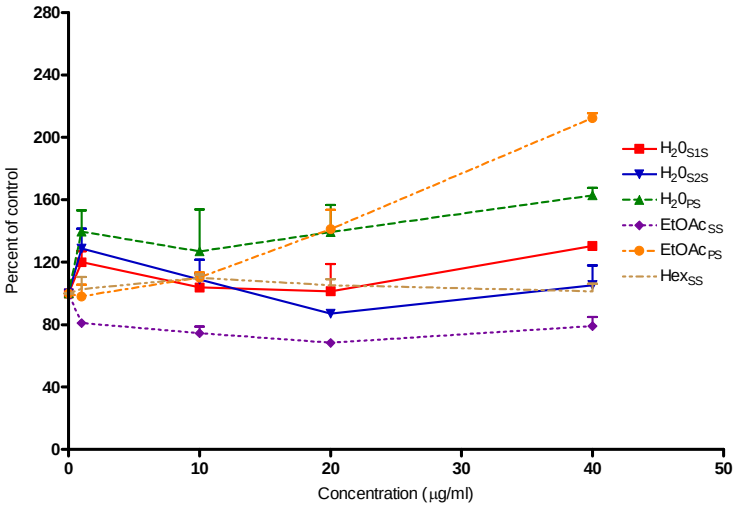


Figure 4.7. The effect of various primary extracts of *B. micrantha* (bark) on PHA stimulated primary human lymphocyte cultures

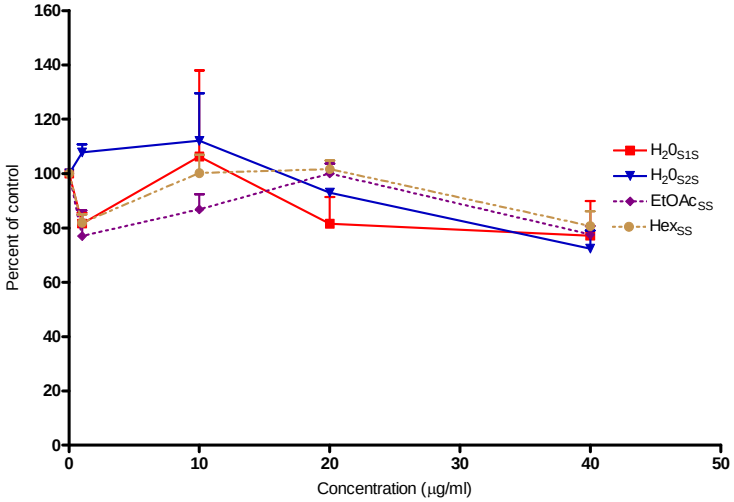


Figure 4.8. The effect of various primary extracts of *B. micrantha* (bark) on primary hepatocyte cultures

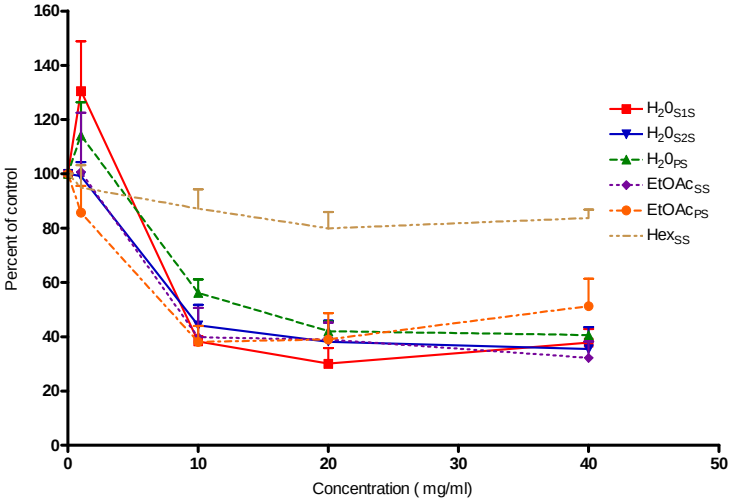


Figure 4.9. The antiproliferative effect of various primary extracts of *B.micrantha* (bark) on primary fibroblast cultures

Table 4.1. Cytotoxicity data (IC_{50} in µg/ml) produced by various primary extracts of *B. micrantha* (bark) on various cell cultures

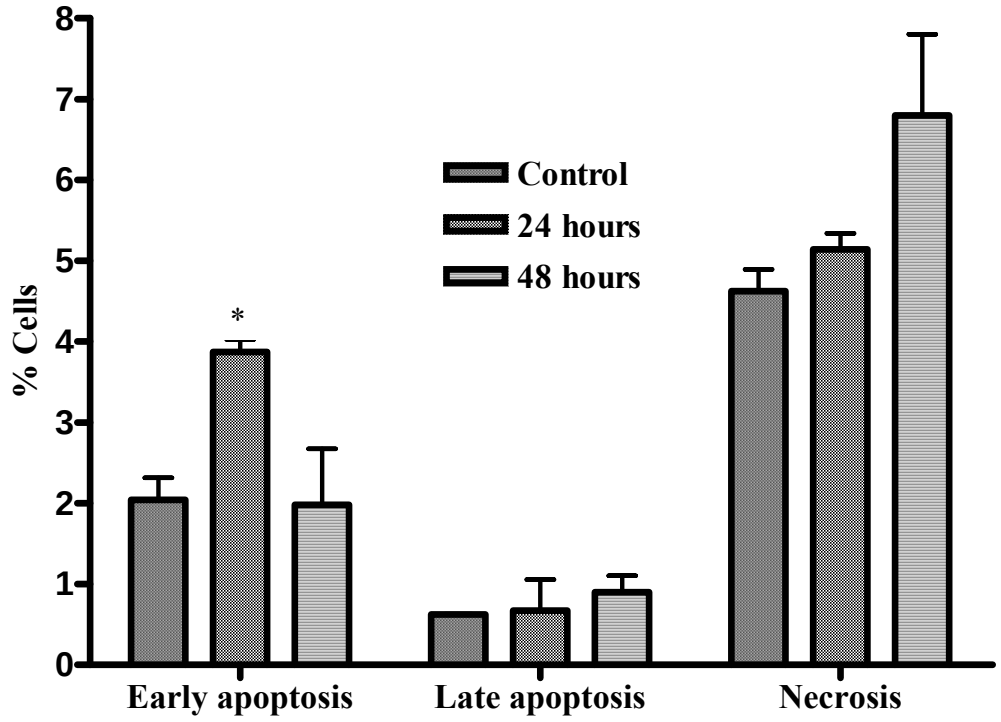
Extract	Neoplastic cells				Normal cells			
	HeLa	COLO	MCF 7	Jurkat	Rest. Lymph	Stim. Lympho	Hepato.	Fibro.
H_20_{S1S}	6.1[a] ± 0.6	6.1[a] ± 0.8	37.8[a] ± 4.7	8.5[a] ± 3.5	>40[a]	>40[a]	>40[a]	8.7[a] ± 0.6
H_20_{S2S}	6.6[a] ± 0.4	8.1[a] ± 1.7	38.3[a] ± 2	7.6[a] ± 1.5	>40[a]	>40[a]	>40[a]	11[a] ± 4.2
H_20_{PS}	19.1[a] ± 2.3	6.7[a] ± 1.7	39.8[a] ± 2.7	17.2[a] ± 1.5	>40[a]	>40[a]	>40[a]	13.5[a] ± 3.8
$EtOAc_{SS}$	18.1[a] ± 1.3	5.9[a] ± 0.7	>40[a]	8.8[a] ± 1.8	>40[a]	>40[a]	>40[a]	7.2[a] ± 1.9
$EtOAc_{PS}$	10[a] ± 1.6	5[a] ± 1	25.3[a] ± 4.4	8.1[a] ± 1.1	>40[a]	>40[a]	>40[a]	7.5[a] ± 2.5
Hex_{SS}	>40[a]	>40[a]	>40[a]	>40[a]	36.5[a] ± 1.4	>40[a]	>40[a]	>40[a]

[a] IC_{50} ± SD - Specific concentration causing 50% inhibition of cell proliferation relative to appropriate controls

4.4.2. Assessment of cell death induction

After 24hours incubation, extract H_20_{S1S} at a fixed concentration of 10µg/ml was shown in triplicate to significantly (*P*-value = 0.023, α = 0.05, one-tailed unpaired *t* test) increase the amount of Annexin V staining to COLO 320DM cells (relative to appropriate EtOH treated controls). This is suggestive of early apoptosis induction as the flip of PS to the extracellular environment where Annexin V can bind to it, is indicative of such a process. This effect is not seen after 48hours incubation.

Although not significant (*P*-value = 0.053, α = 0.05, one-tailed unpaired *t* test), after 48hours a larger percentage of cells (relative to appropriate EtOH treated controls) stained positive for PI. This trend towards necrosis suggests that a larger percentage of cells had progressed to a point where their cell membranes had been compromised allowing PI access to DNA fragments (Figure 4.10).



***P* 0.0023, one-tailed unpaired *t* test compared to appropriate controls**

Figure 4.10. Cell death route induced by extract H_20_{S1S} at a fixed concentration of 10µg/ml on the COLO 320DM cell line over different exposure times.

4.4.3. Cell cycle analysis

COLO 320DM cells were incubated in the presence of extract H_2O_{S1S} at a fixed concentration of 10µg/ml for a period of 24 and 48hours. DNA concentration profiles were produced through PI staining of membrane permeabilised cells. The profile (DNA histogram) of treated cells was compared to the profile of appropriate EtOH treated control cells. No significant differences were evident between treated and untreated cells for any of the cell cycle stages at either time period suggesting that progression of the cell cycle had not be hindered (Figure 4.11).

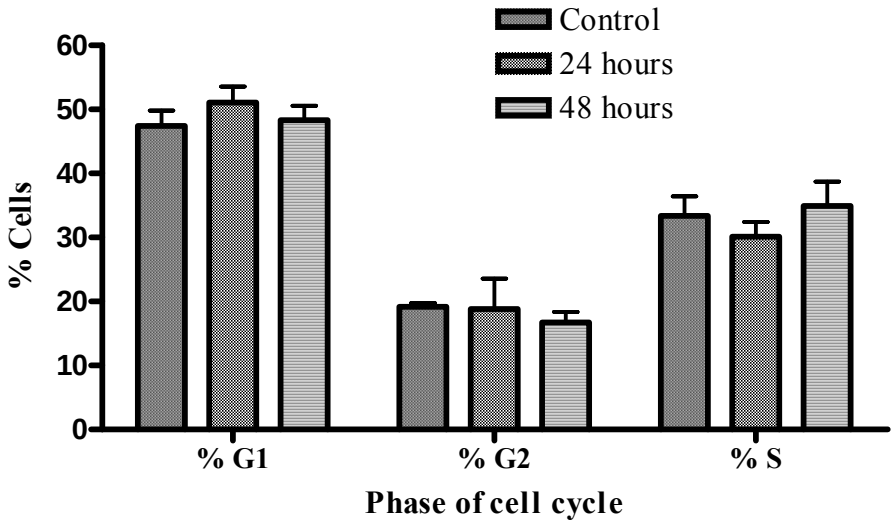


Figure 4.11. Cell cycle analysis of COLO 320DM cell cultures after exposure to extract H_20_{S1S} at a fixed concentration of 10µg/ml over different exposure times

4.5. Conclusions

Five out of the six primary extracts of *B. micrantha* (bark) possess impressive *in vitro* antineoplastic activity. Considering the different extractants that had been used to attain the respective primary extracts and the different selective biological activities that were demonstrated in different cell cultures, it is apparent that there are numerous different constituents within *B. micrantha* (bark) that exhibit antineoplastic potential.

On comparison of the antiproliferative effects of the aqueous extracts from the two different bark samples, it could be seen that they produced much the same IC_{50} values for the HeLa cell line despite their different extraction yields.

Based largely on extraction yields and expected distribution behaviour, extract H_20_{S1S} was deemed to be the most promising and as per protocol was therefore assessed though flow cytometric bioassays and later subjected to bioassay-guided fractionation (as discussed in chapter 5).

Multi-parametric assessment of cell death induction revealed that extract H_20_{S1S} exerted its cytotoxic effect through an apoptotic pathway. Further work is required to elucidate the underlying molecular biology leading to this death induction.

CHAPTER 5

5. BIOASSAY-GUIDED FRACTIONATION

5.1. Introduction

As *in vitro* bioassays are a quick and efficient means of determining biological activity, they can be employed to direct fractionation of an active extract. The aim of bioassay-guided fractionation is to isolate (or allow for structural elucidation of) the AC as quickly and as cost effectively as possible. The advantage of employing bioassay-guided fractionation is that it avoids having to isolate and screen each individual compound present within a complex mixture.

There are numerous different fractionation techniques employing numerous different chemical and chromatographic principles that afford separation of compounds based on their respective physiochemical characteristics. Differences in distribution coefficients between solvents or chromatographic phases were exploited. Typically, several selective differences need to be exploited in sequence, in order to isolate a single compound (Verpoorte, 1989).

Chromatography is a separating technique that relies on the differences in partitioning behaviour of the respective constituents within a mixture. It is the most widely used method of separating and purifying compounds from a complex heterogenous mixture. Generally speaking there are two forms of chromatography, either column or planar, both of which rely on the distribution of the analytes between a flowing mobile phase and a stationary phase to separate the components of a mixture based on one or other physicochemical selectivity.

Adsorption chromatography utilizes a liquid or gaseous mobile phase to carry analytes/solutes, which adsorb onto the surface of a solid stationary phase. Equilibration of the analytes between the mobile and stationary phase accounts for the separation of the analytes. Examples of adsorbents (have the property of holding molecules on its surface) include silicic acid and aluminium oxide. Charcoal is an

example of an adsorbent that can be used in reverse phase mode as apposed to those mentioned earlier, which are typically used in normal phase mode. Normal phase mode refers to the mobile phase being less polar than the stationary phase and consequently the more lipophillic analytes elute earlier. The opposite applies for reverse phase mode where the more polar constituents elute earlier.

Partitioning chromatography relies on the analytes equilibrating between a mobile phase and a thin film of liquid on the surface of a solid support commonly being cellulose or silicic acid.

Ion exchange is a type of chromatography that employs ionic charged resins as the stationary solid support. Analyte ions within the mobile phase with the opposite charge to the resin are attracted through electrostatic forces.

A successful fractionation procedure would translate to selective and complete accumulation of specific constituents into a single fraction. It is therefore expected that an increase in biological activity (proportionate to the enrichment of the AC within that fraction) would be observed after each successful fractionation step.

It is important to acknowledge that fractionation often results in a reduction of biological activity due to decomposition of the AC, separation of a synergistic combination or incomplete separation (Houghton *et al*., 2005). Where activity was lost or reduced, all the resulting sub-fractions were typically screened in unison in an attempt to distinguish between the break up of active compounds or ineffiecient separation. Potential decomposition was controlled for through out, by subjecting an aliquot of the full (un-fractionated) extract in parallel, to the same conditions as those for the produced sub-fractions.

5.2. Methodology

5.2.1. Fractionation methods

The choice of fractionation methods was determined by the availability of equipment and by the suspected chemical nature of the AC considering what extraction solvent had been used. As little was known about the chemical nature of the AC within the most promising extract, a number of small-scale (pilot) experiments were devised in order to determine which techniques work best. Accordingly, as method development, fractionation was firstly carried out on a small scale (in order to motivate for preparative scale fractionation at a later stage) using solid phase extraction (SPE).

SPE is a technique used typically to clean up and concentrate analytes from various mixtures. Numerous types of sorbents (stationary phases) are available that offer diverse mechanisms of retention and thus selectivity. Considering the polar nature of the sample matrix, extract H_20_{S1S} was subjected to SPE in reverse phase mode using octadecylsilane (ODS, C18). Thereafter, extract H_20_{S2S} was subjected to SPE using a multimode (C18 combined with strong anion and cation exchange resins) cartridge.

After collection, drying and quantitation of all the respective fractions (as discussed in the relevant practical procedures), they were reconstituted in EtOH. To aid solubilization, fractions were subjected firstly to vortex mixing, followed by sonication and gentle heating (<40ºC) if necessary. After centrifugation, insoluble portions (if any) were collected as pellets and considered as separate sub-fractions and were stored at -70ºC for later use (should bioactivity have been completely lost from the remaining supernatants). The concentration of all the EtOH reconstituted fractions was adjusted to 10mg/ml.

To ensure that these procedures had not in some way altered the biological potency of the AC, “experimental conditions controls” (aliquots of the un-fractionated extract) were subjected in parallel to the same drying and reconstitution conditions.

Practical procedure: C18 SPE

- The vacuum manifold and all associated equipment was washed with 70% EtOH to prevent possible contamination of the samples.
- Cartridges (Alltech EV, 500mg, C18) were solvated with 6ml of MeOH before being equilibrated with 6ml of H_2O.
- After concentration of the extract, 20mg (i.e. <5% of the sorbent weight) of the sample (in a volume less than 1ml) was loaded onto each cartridge bed and allowed to flow via gravity.
- The cartridges were washed with 6ml of 2% MeCN (0.1% HCO_2H) and this fraction collected.
- The cartridge was dried under full vacuum for 2 minutes.
- The cartridge was then sequentially eluted with 3ml of 25% MeCN (0.1% HCO_2H); 50% MeCN (0.1% HCO_2H) and 100% MeCN (0.1% HCO_2H). Applied as 3 x 1ml aliquots with the first aliquot allowed too soak through the bed for approximately 2minutes.
- All the eluted fractions were separately collected in pre-weighed test.
- Final drying of all the fractions was carried out under nitrogen at <40ºC.
- Fraction weights were recorded and expressed as percentage (w/w) of the full amount of extract loaded on the cartridge.
- An aliquot of the full, un-fractionated extract was dried and reconstituted under the same conditions, thus serving as an experimental conditions control.

Practical procedure: Multimode SPE

- The vacuum manifold and all associated equipment was washed with 70% EtOH to prevent possible contamination of the samples.
- Cartridges (Isolute, 500mg, multimode) were solvated with 6ml of MeOH before being equilibrated with 6ml of H_2O.
- After concentration of the extract, 20mg (i.e. <5% of the sorbent weight) of the sample (in a volume less than 1ml) was loaded onto each cartridge bed and allowed to flow via gravity.

- The cartridge was sequentially eluted with 3ml of H_20; 50% MeCN; 100% MeCN; 50% MeOH (0.1% $HC0_2H$); 50% MeOH and 50% MeOH (1% NH_4OH). Applied as 3 x 1ml aliquots with the first aliquot allowed too soak through the bed for approximately 2 minutes.
- All the eluted fractions were separately collected in pre-weighed test tubes.
- Final drying of all the fractions was carried out under nitrogen at <40ºC.
- Fractions weights were recorded and expressed as percentage (w/w) of the full amount of extract loaded on the cartridge.
- An aliquot of the full, un-fractionated extract was dried and reconstituted under the same conditions, thus serving as an experimental conditions control.

Solvent-solvent partitioning (between BuOH and H_2O) was also attempted as it was reasoned that large amounts of inert compounds could be removed easily and quickly through this simple procedure. Immiscible liquid phases can be used to separate solubilized analytes according to their respective partition coefficients between the different phases. By increasing the surface interaction of the two phases (i.e. shaking the flask), the analytes will reach equilibrium fairly quickly. Through repeating the extraction procedure several times with fresh solvent, near complete separation can be achieved.

Practical procedure: Solvent-solvent partitioning (H_2O- BuOH)

- Extract H_20_{S1S} was dried too a slurry consistency before being taken up in 10ml of H_20.
- This solution was extracted in a separating flask (with the aid of shaking for 10 minutes) with an equal volume of butanol (BuOH).
- The solution was transferred to a clean measuring cylinder and the two phases allowed to separate.
- The two-phase mixture was placed in -70ºC conditions until the H_20 phase had frozen.
- The upper liquid BuOH phase was decanted into a clean beaker.

- The remaining H_20 phase was twice more subjected to this extraction procedure with clean BuOH.
- The pooled BuOH fractions were washed twice with clean H_20.
- Fractions were allowed to concentrate in suitable-sized beakers in a fume cupboard before final drying in pre-weighed test tubes under nitrogen at <40ºC.
- An aliquot of the full un-fractionated extract was dried under the same conditions, thus serving as a conditions control.

An analytical normal phase TLC mobile phase was optimised to give the best separation of compounds within the BuOH sub-fraction of extract H_20_{S1S}. This optimised mobile phase was then used with preparative TLC plates in an attempt to further enrich the AC.

Practical procedure: Preparative thin layer chromatography (pTLC)

- The BuOH fraction (of extract H_20_{S1S}) was dried to a minimum volume under vacuum using a rotary evaporator at <40ºC.
- The optimised mobile phase: $CHCl_3$, MeOH, BuOH, H_20 (10:10:10:1) was allowed to saturate the developing chamber.
- Using the edge of a clean sheet of glass, roughly 40mg of the BuOH sub-fraction (in solution) was loaded, in a straight line across the width of the preparative plate, approximately 1cm from the base of the plate.
- The plates were developed to a suitable height before being air-dried and visualised under UV light.
- Migration zones were marked and respective R*f* values determined.
- Silica representing the different zones was meticulously scrapped off the glass plate and each zone separately collected in 50ml centrifuge tubes.
- The collected silica fractions were separately extracted thrice with a suitable volume of EtOH each time centrifuging for 30minutes at 5ºC at 2000g and decanting the supernatant.

- Pooled supernatants were dried to a small volume under vacuum at <40ºC using a rotary evaporator. Final drying of all the fractions was carried out under nitrogen at <40ºC.
- Fractions were desiccated overnight before their respective weights were determined.

5.2.2. Bioassay methods

Each sub-fraction of lead extract produced through the methods discussed above was screened using the already described *in vitro* bioassay methods on the HeLa cell line. Taxol was used as a positive control through out in order to provide uniformity, quickly distinguish between erroneous bioassay outcomes and the loss of biological activity. In addition, in all cases, experimental condition controls were screened along-side the respective sub-fractions in order to ensure that the employed experimental procedures did not have a deteriorative effect on the constituent/s responsible for the observed biological activity. The bioactivity of each sub-fraction could thus be quantitatively compared to both the appropriate experimental conditions control and earlier bioassay results.

5.2.3. Monitoring methods

Apart from monitoring the success of a particular fractionation procedure through bioassays, it is important to monitor the phytochemical profile (constituency) of the produced fractions. This gives an indication of the successes and limitations of a particular fractionation procedure. Qualitatively and in certain instances semi-quantitatively, the separation of constituents present within a mixture can be monitored through techniques such as TLC, HPLC (with hyphenated techniques, i.e. LC/MS and LC/UV) and direct infusion ESI-MS.

For the purpose of monitoring fractionation outcomes and potential decomposition, direct infusion ESI-MS was thought to be simple, reproducible, cost and time efficient. Positive and negative ion mode mass fingerprints of all fractions were obtained using the same methods as discussed previously. As such, global

phytochemical profiles of each fraction (depicting constituents present or not present) could be visually compared. For practical comparative purposes, only m/z peak intensities >10% of the maximum intensity (for each respective fraction) were considered.

HPLC coupled to diode array detection (DAD) and MS was used to demonstrate the complexity of certain fractions and to collect large amounts of spectroscopic and spectrometric data. HPLC analysis was performed on an Agilent 1100 series system controlled through Analyst (Applied Biosystems-Sciex) software that integrated the different modules of the LC/UV/MS system. All mobile phases were vacuum filtered (0.45µm) under suction to remove trapped gases. DAD data was typically collected between 205nm and 600nm and the first quadrupole mass spectra were acquired over a 100-1200 *m/z* range.

5.3. Results and discussion

5.3.1. Solid phase extraction (SPE)

Reverse phase SPE using a C18 cartridge was the first fractionation method employed. Apart from attempting to enrich and confine the AC to a narrow polarity range, this method was used to clean the extracts prior to HPLC using a C18 column.

The fractionation outcomes are shown in Figure 5.1. A loss of 14.4% reveals that an appreciable amount of the extract could not be eluted from the C18 stationary phase using the described eluent mixtures. It was therefore important to demonstrate that the AC had not irreversible bound to the stationary phase as this would strongly abrogate the use of a C18 column for HPLC analysis.

Even after pooling of the replicate fractions eluted from 5 cartridges the yields attained for the 50% MeCN (0.1% HCO_2H) and 100% MeCN (0.1% HCO_2H) fractions were too low to allow for reconstitution at an adequate concentration required for bioassays. The antiproliferative activity of these two fractions could therefore not be determined.

The antiproliferative activity of fraction 2% MeCN (0.1% HCO_2H) and fraction 25% MeCN (0.1% HCO_2H) versus that of the experimental conditions control is shown in Figure 5.2. The experimental conditions control had an IC_{50} value of 7.3µg/ml whilst the two fractions had IC_{50} values of 16.6µg/ml and 9.1µg/ml respectively thus demonstrating a decrease in biological activity. This was thought to be indicative of incomplete distribution of the analytes owing to poor separation efficiency or alternately the break-up of dualistically or synergistically acting compounds. No significant difference in antiproliferative activity was evident between the experimental conditions control and extract H_20_{S1S} (as assayed previously). This indicates that the conditions under which drying and reconstitution were conducted had no deteriorative effect on the biological activity of the AC.

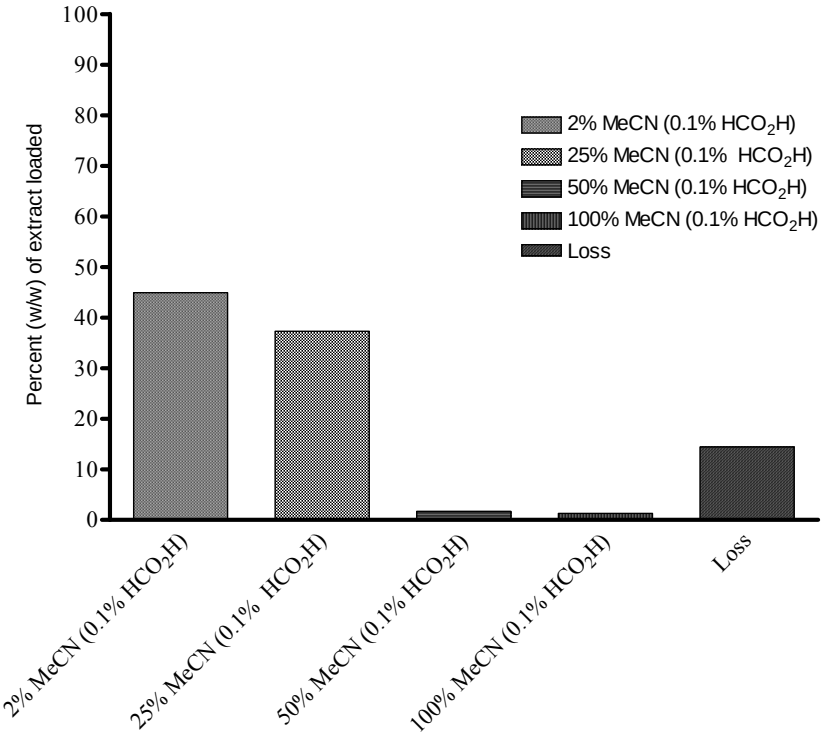


Figure 5.1. C18 SPE fractionation yield outcomes expressed as percentage (w/w) of the original extract loaded onto the cartridge. 5 replicate cartridges; pooled before individual weight determination.

To test whether this decrease in bioactivity was attributable to the break-up of a synergistic combination, the 2% MeCN (0.1% HCO_2H) fraction was combined in a 50:50 ratio with the 25% MeCN (0.1% HCO_2H) fraction and again screened for antiproliferative activity using HeLa cultures. The combination of the two fractions

was shown to produce an IC_{50} value of 8.9µg/ml. This result, although difficult to interpret does suggest the presence of more than one active constituent.

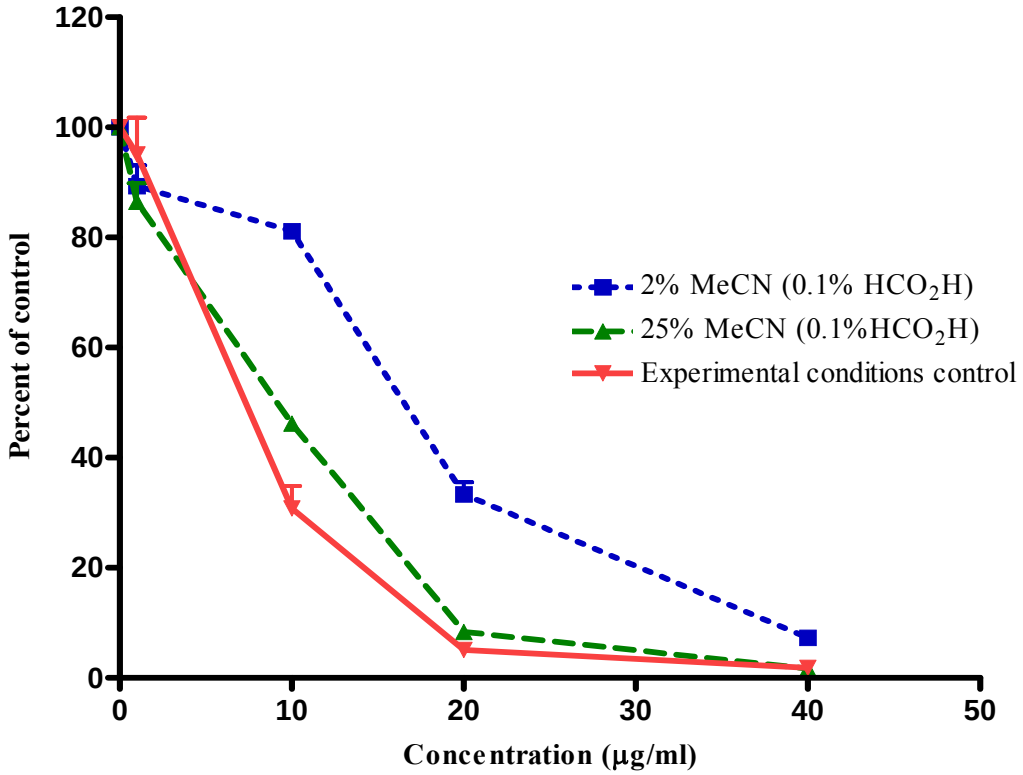


Figure 5.2. The antiproliferative effect of various sub-fractions of extract H_2O_{S1S} produced through successive elution from a C18 SPE cartridge and tested on HeLa cell line cultures

Fractionation was monitored firstly through using reverse phase (Alugram RP-18W/UV_{254}) TLC plates (data not shown) but met with little success owing to a streaking effect that could not be rectified with modifications to either the mobile phase (organic strength or pH) or the amount of sample spotted on the plate.

An optimised gradient elution HPLC method using a C18 column (Altech Apollo, 5µm, 15mm x 4.6mm) was developed considering the physicochemical characteristics of potential eluents and attempting to mimic the SPE elution conditions. Using an autosampler, 10µl of 1mg/ml solutions were injected. The flow rate was set at 1ml/min with the column's temperature at 40°C.

Extracted wavelength chromatograms (XWC) at 260nm and 280nm allowed for visual comparison of the two fractions as shown in Figures 5.3-5.6. Apart from

notable differences: the absence of early eluting compounds (with retention times of between 1 and 6 minutes) from the 25% MeCN (0.1% HCO_2H) fraction; the absence of compounds eluting with a retention time of 15.52 and 17.71 minutes from the 2% MeCN (0.1% HCO_2H) fraction, there are also notable similarities at retention times of 11.73, 20.28, 20.97, 21.52, 22.35 and 24.97 minutes demonstrating that the fractionation procedure was not as selective as had been hoped. Visual interpretation of these complex chromatograms was unable to provide a clear explanation for the bioassay outcomes, although incomplete distribution of the analytes is clear.

Direct-infusion ESI-MS data was further used to evaluate the success of the C18 SPE fractionation procedure. Table 5.1 compares the representative qualitative phytochemical profiles of extract H_2O_{S1S} to that of the two fractions assayed and the experimental conditions control. In support of the HPLC visual observations, a degree of incomplete distribution of the analytes is evident in both ion polarity modes. In positive ion mode the pseudo-molecular complexes of 105,1; 115,7; 139.8 and 149.8 m/z were common among all the mixtures. The same can be said for the pseudo-molecular complexes of 169.5; 217.7; 225,7; 229.7 and 341.7 ionising in negative ion mode. The presence of certain complexes existing in the experimental conditions control but not in extract H_2O_{S1S} is suggestive of possible decomposition.

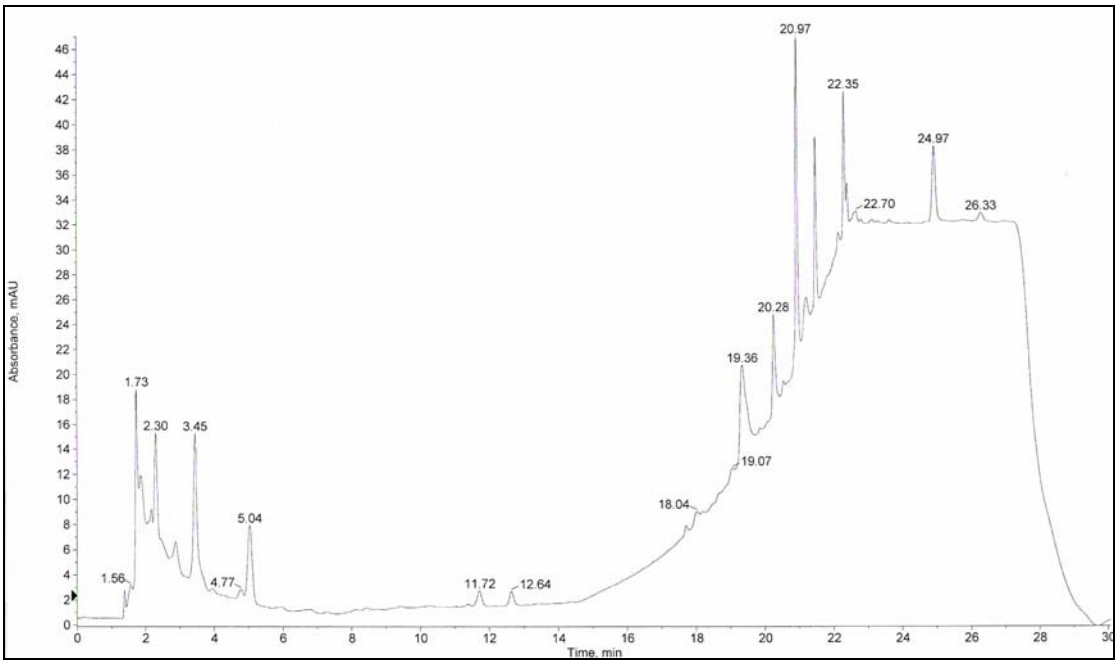


Figure 5.3. Extracted wavelength chromatogram (XWC) of DAD spectral data at 260nm of the 2%MeCN (0.1% HCO_2H), (SPE, C18) sub-fraction of extract H_20_{S1S}. Gradient mixture: A, H_20 (0.1%HCO_2H); B, MeCN (0.1%HCO_2H). Elution: time 0min, 10% B; time 5min, 10% B; time 12min, 25% B; time 20min, 98% B; time 25min, 98% B; time 27min, 10% B.

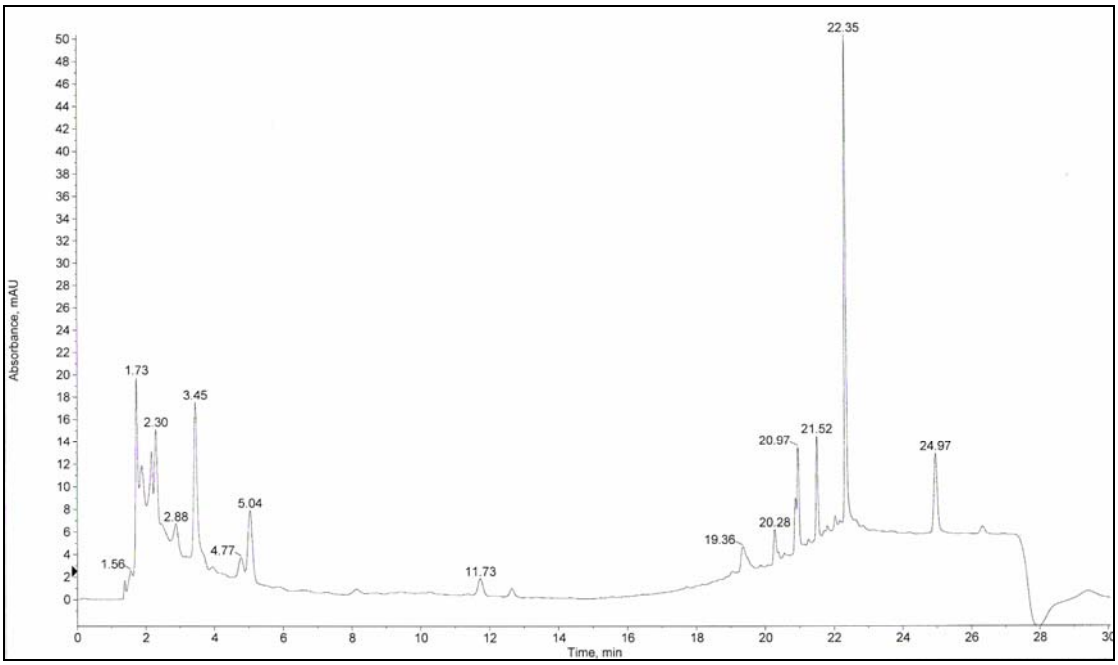


Figure 5.4. XWC of DAD spectral data at 280nm of the 2%MeCN (0.1% HCO_2H), (SPE, C18) sub-fraction of extract H_20_{S1S}. Gradient mixture: A, H_20 (0.1%HCO_2H); B, MeCN (0.1%HCO_2H). Elution: time 0min, 10% B; time 5min, 10% B; time 12min, 25% B; time 20min, 98% B; time 25min, 98% B; time 27min, 10% B.

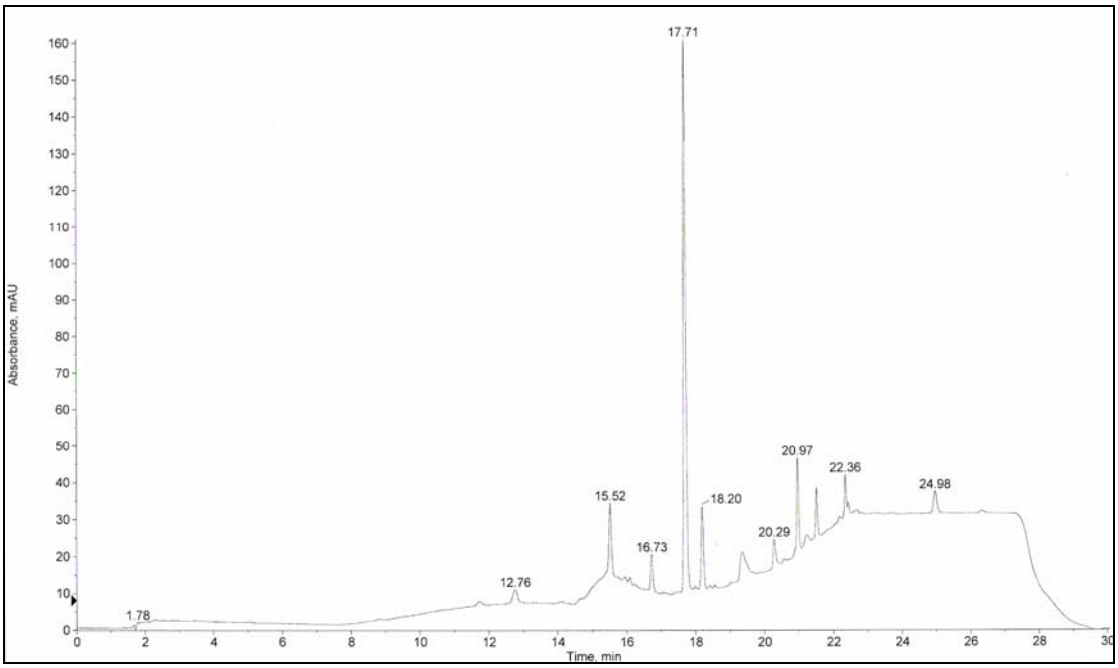


Figure 5.5. XWC of DAD spectral data at 260nm of the 25%MeCN (0.1% HCO_2H), (SPE, C18) sub-fraction of extract $H_20_{S1S.}$ Gradient mixture: A, H_20 (0.1%HCO_2H); B, MeCN (0.1%HCO_2H). Elution: time 0min, 10% B; time 5min, 10% B; time 12min, 25% B; time 20min, 98% B; time 25min, 98% B; time 27min, 10% B.

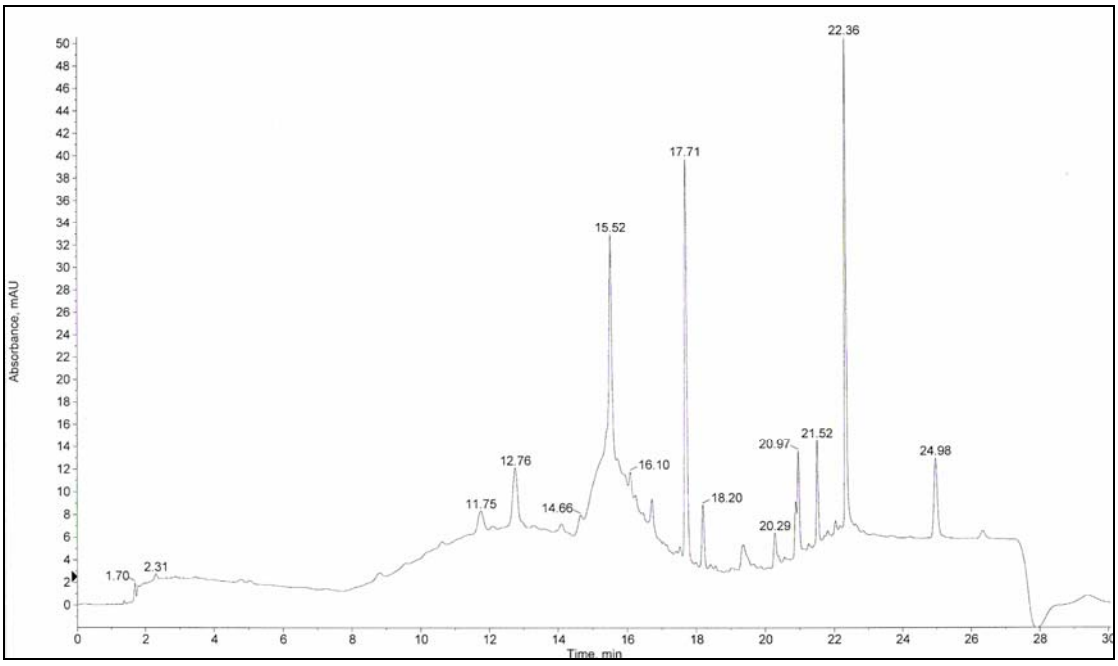


Figure 5.6. XWC of DAD spectral data at 280nm of the 25%MeCN (0.1% HCO_2H), (SPE, C18) fraction of extract $H_20_{S1S.}$ Gradient mixture: A, H_20 (0.1%HCO_2H); B, MeCN (0.1%HCO_2H). Elution: time 0min, 10% B; time 5min, 10% B; time 12min, 25% B; time 0 min, 98% B; time 25min, 98% B; time 27min, 10% B.

Table 5.1. Representative qualitative (global) phytochemical profiles of extract H_2O_{S1S}, C18 SPE eluted sub-fractions thereof and the appropriate experimental conditions control. Comparison of the M/z ions found by direct infusion into an ESI-MS system. Only M/z intensity peaks > 10% of the maximum intensity are displayed

+Q1 (Positive ion mode) ESI				-Q1 (Negative ion mode) ESI			
H_2O_{S1S}	2%MeCN eluted fraction	25%MeCN eluted fraction	Experimental conditions control	H_2O_{S1S}	2%MeCN eluted fraction	25%MeCN eluted fraction	Experimental conditions control
103.7	103.7		103.7		105.4	105.4	
104.8*	104.7		104.7		113.4	113.4	
105.7	105.7	105.7	105.7				117.5
		107.8			129.3	129.3	
109.8		109.8	109.8				133.4
111.7		111.7					135.4
		114.8					143.6
115.7	115.7	115.7	115.7	153.3		153.3	153.3
	117.7		117.7				157.5
118.8							165.5
		121.8		169.5	169.5	169.5	169.5
		123.8			179.7		179.7
127.8		127.8	127.8				191.7
		135.8				203.7	
137.8		137.8	137.8	205.6			
139.8	139.8	139.8	139.8			212.7	
141.8		141.8	141.8	215.5*	215.5		215.5
147.8	147.8*		147.8	216.7			
	148.8			217.7	217.7	217.7	217.7
149.8	149.8	149.8	149.8	225.7	225.7*	225.7	225.7
		158.8			226.7		
163.8	163.8		163.8	229.7	229.7	229.7	229.7
203.9	203.9		203.9	231.7			
217.9	217.9		217.9		239.6		239.6
219.9	219.9		219.9			258.5	
	225.9					289.6	
		230			293.8		
	233.9				305.8	305.8*	305.8
	263.9					335.7	
	265.9			341.7	341.7	341.7	341.7
	289.2	289.2*	289.2	342.8			
		290.2	290.2	343.8			343.8
		308.0			347.7		
			317.2		350.7		
		612.1			351.7	351.7	351.7
					377.7		
					387.7		
				395.6	395.7		
				397.6			
						433.8	433.8
				521.7			
						593.9	
						610.0	
						634.0	
				645.8			

*** Indicates M/z peak with maximum intensity**

Considering that water had been used as the extractant and possible presence of ions, a multimode SPE cartridge was used in an attempt to enrich the AC within extract H_2O_{S2S}. The mean fraction yields (± SD) of three repeats are shown in Figure 5.7.

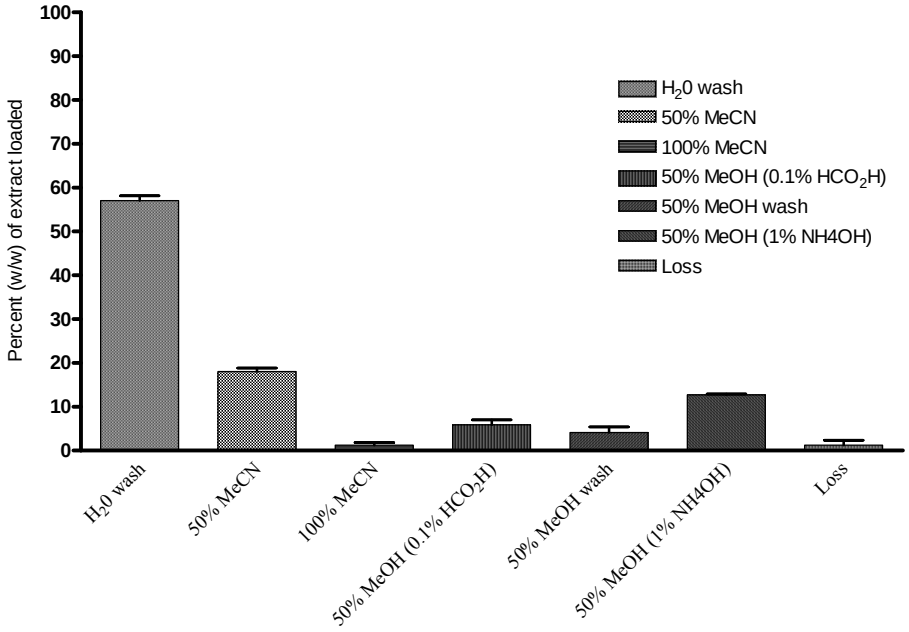


Figure 5.7. SPE (multimode) fractionation yields of extract H_2O_{S2S}

As was with the C18 fractionation, the AC was not enriched in any of the eluted fractions (Figure 5.8). IC_{50} values for each of the respective sub-fractions against HeLa cell cultures are shown in Table 5.2. These results again attest to the possible separation of active compounds or incomplete distribution of the analytes. As enrichment of the AC had not been achieved a change in fractionation strategy was opted for.

Table 5.2. IC_{50} values of various SPE (multimode) eluted fractions of extract H_2O_{S2S} on the HeLa cell line

Extract	IC_{50} value (µg/ml)
Experimental conditions control	6.6
H_2O wash	11.4
50% MeCN	7.8
50% MeOH (0.1% HCO_2H)	10.7
50% MeOH wash	>40
50% MeOH (1% NH_4OH)	>40

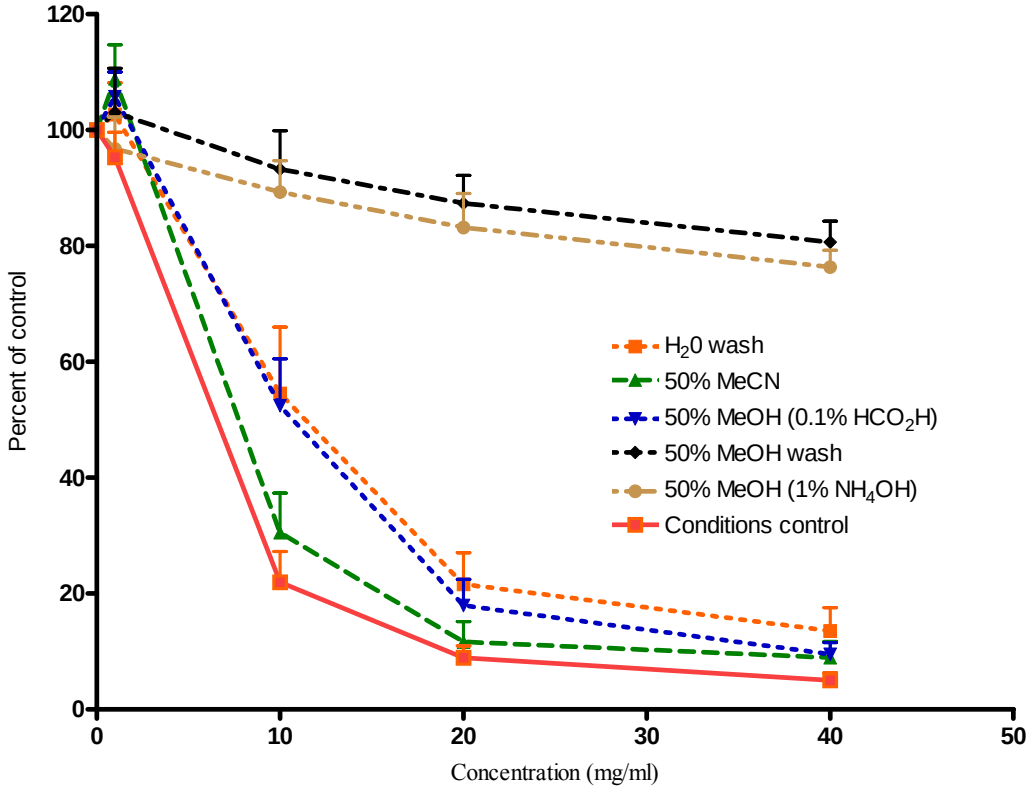


Figure 5.8. The antiproliferative effect of various sub-fractions of extract H_2O_{S2S} produced through successive elution from a multimode SPE cartridge and tested on HeLa cell line cultures

5.3.2. Solvent-solvent partitioning (BuOH-$H_2$0 partitioning)

Successive solvent-solvent partitioning of extract H_2O_{S1S} between equal volumes of BuOH and $H_2$0 was attempted in the hope of removing large amounts of inert constituents. Fractionation yields expressed as percentage (w/w) of the original extract are shown in Figure 5.9. After drying, the BuOH fraction was shown to readily solubilise in absolute EtOH, whilst 7.8% of the aqueous fraction was EtOH insoluble and was consequently considered as a unique sub-fraction. This sub-fraction was successfully reconstituted in H_2O.

The antiproliferative activity of the BuOH fraction (Figure 5.10) was shown to be superior to that of the experimental conditions control - an impressive IC_{50} value of 2.7µg/ml was produced, representing an increase in bioactivity by a factor of roughly 2.5. The EtOH soluble portion of the dried aqueous partition (IC_{50} value of 6.9µg/ml) was shown to possess no statistically significant differences in biological activity at

any of the tested concentrations to that of the experimental conditions control (IC_{50} value of 6.7µg/ml), strongly suggesting a similar percentage (w/w) of the AC. The bioactivity of the EtOH insoluble portion (of the aqueous partition) was shown to be reduced compared to that of the experimental conditions control, but still active with an IC_{50} value of 8.5µg/ml. This again strengthens the hypothesis of numerous active compounds being present within the primary extract.

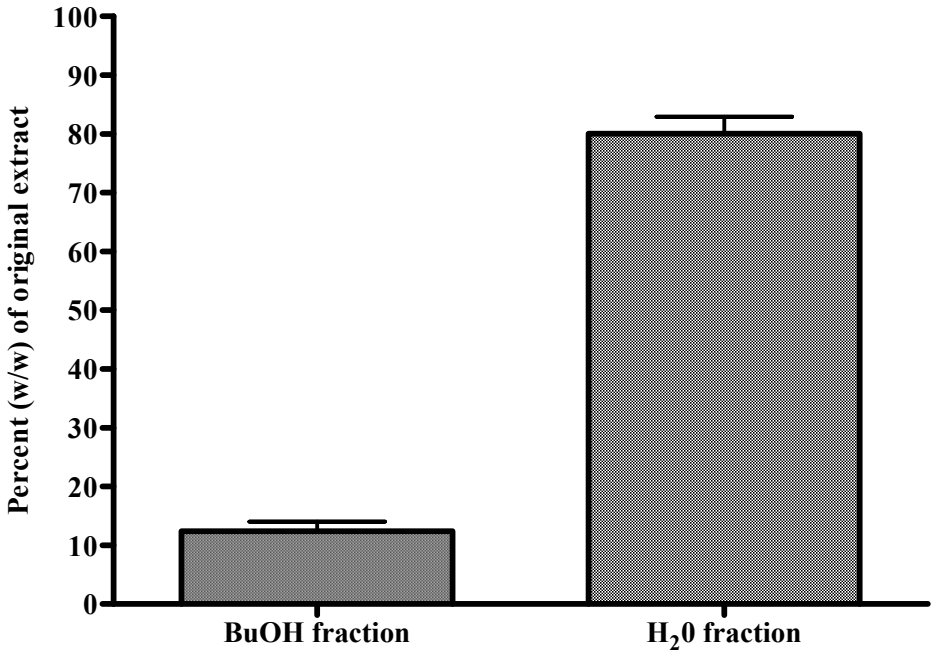


Figure 5.9. Fractionation yield outcomes: solvent-solvent (BuOH-H_20) partitioning of extract H_2O_{S1S}

TLC using a normal phase (Alugram® SIL G/UV_{254}) plate was used to demonstrate what had transpired through solvent-solvent partitioning. The BuOH and H_2O (EtOH soluble) fractions were compared to extract H_2O_{S1S}, Figure 5.11 shows that the fractionation procedure successfully distributed the more lipophilic compounds (those that had migrated further) into the BuOH phase. The more polar compounds (those not migrating far from the baseline) appear to have remained in the H_2O phase.

Direct-infusion ESI-MS data was further used to evaluate the success of the solvent-solvent fractionation procedure. Table 5.3 compares the representative qualitative phytochemical profiles of the respective fractions.

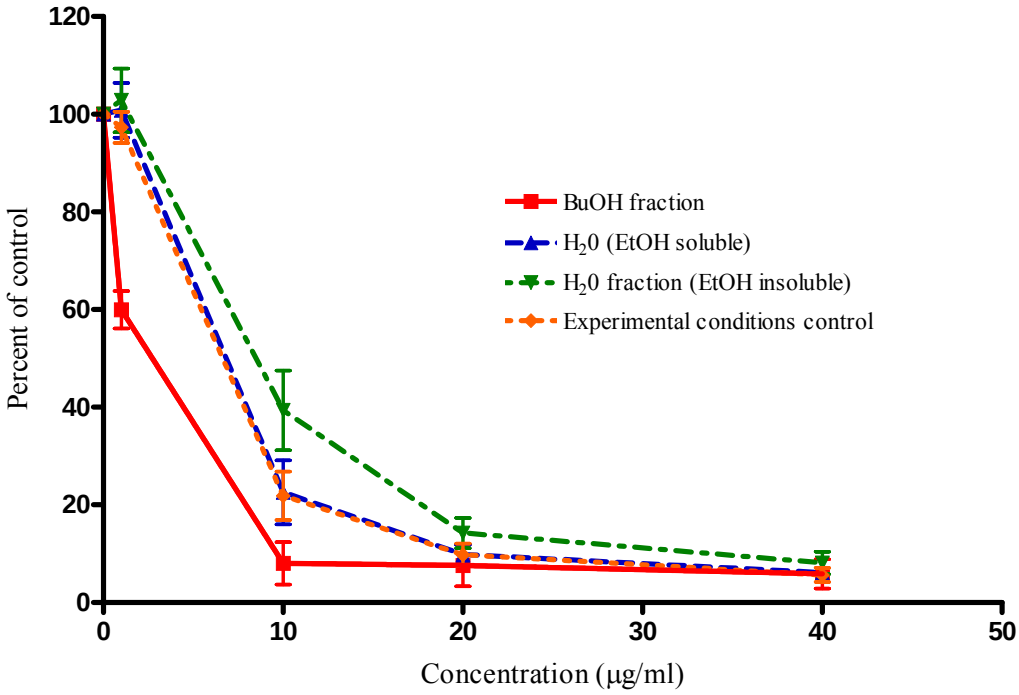


Figure 5.10. The antiproliferative effect of various solvent-solvent (BuOH-H_20) partitioning sub-fractions of extract H_2O_{S1S} on HeLa cell line cultures

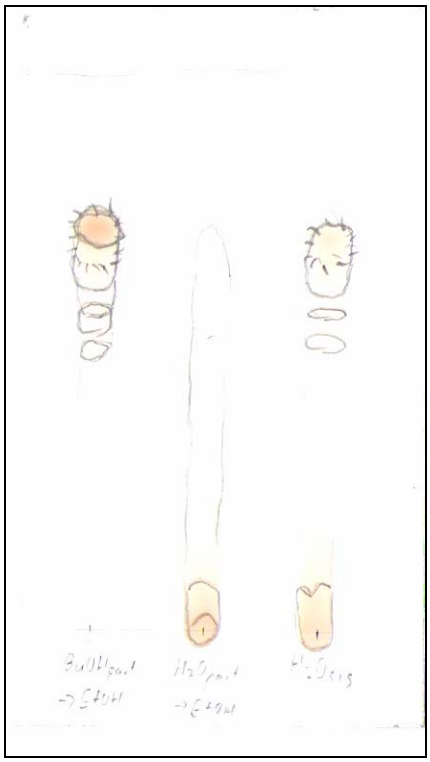


Figure 5.11. TLC chromatogram demonstrating the effect of solvent-solvent (BuOH-H_20) partitioning of extract H_2O_{S1S}. The BuOH and H_2O (EtOH soluble) sub-fractions were compared to the original un-fractionated extract H_2O_{S1S}. Developed with EtOH: BuOH: H_2O (10:5:5). Visualised at 254 and 360nm. (From left to right, lane 1: BuOH fraction; lane 2: H_2O (EtOH soluble) fraction; lane 3: Un-fractionated extract H_2O_{S1S}

Table 5.3. Representative qualitative (global) phytochemical profiles of sub-fractions produced through solvent-solvent partitioning (H_2O-BuOH) of extract H_2O_{S1S}. Comparisons of the M/z ions found by direct infusion into an ESI-MS system. +Q1 are the ions fond in positive on mode whilst –Q1 are those found in negative ion mode. Only M/z intensity peaks > 10% of the maximum intensity are displayed.

+Q1 (Positive ion mode) ESI			-Q1 (Negative ion mode) ESI		
BuOH	**H_2O (EtOH soluble)**	**H_2O (EtOH insoluble)**	**BuOH**	**H_2O (EtOH soluble)**	**H_2O (EtOH insoluble)**
	104.7	104.7	125.5	125.5	
105.7		105.7			141.5
		109.7	153.4		
		111.7	169.5		
		114.8			212.8*
121.8				215.7*	
122.8				217.7	
123.8			289.8		
		127.7	301.7		
		135.7	305.7*	305.7	
136.8			306.8		
137.8			341.6	341.6	
139.8*				377.6	
141.8		141.8	433.7		
		149.8	610	610	
		158.8		611	
		198.9		898.1	
203.9	203.9*	203.9*		914.1	
	217.9	217.9			
	219.8	219.8			
242					
275.1					
292					
302					
307.9					
326.1					

*** Indicates M/z peak with maximum intensity**

5.3.3. Preparative thin layer chromatography (pTLC)

An optimised analytical scale, normal phase TLC mobile phase was developed for the separation of the BuOH fraction (of extract H_2O_{S1S}). Visual inspection under UV light revealed a good separation using $CHCl_3$: MeOH: BuOH: H_2O (10:10:10:1) as a mobile phase (Figure 5.12.) R*f* values of 0.04; 0.27; 0.6; 0.66; 0.74; 0.79 and 0.9 were recorded for the respective zones. This mobile phase was then used with preparative TLC plates.

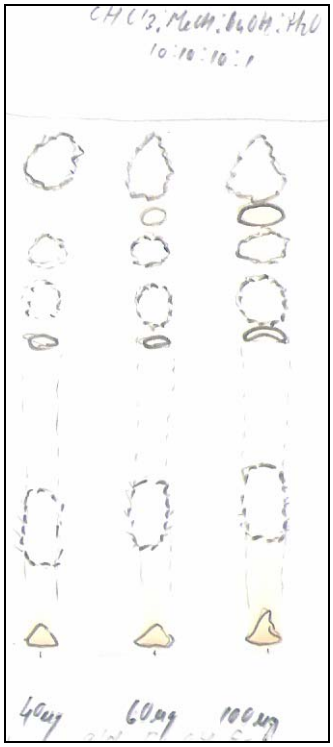


Figure 5.12. Analytical silica (normal phase mode) TLC plate showing the migration of different constituents within the BuOH sub-fraction of extract H_2O_{S1S}. Developed with $CHCl_3$-MeOH-BuOH-H20, (10:10:10:1). Visualised at 254 and 360nm. (From left to right: Amounts spotted - 40, 60, 100µg)

Figure 5.13 shows a photograph taken of a developed pTLC plate. Development to 15cm took approximately 6 hours. Eight zones corresponding to R*f* values of 0; 0.1; 0.15; 0.44; 0.5; 0.62; 0.73 and 100 were visualised under UV light as encircled in the accompanying figure. A streaking effect was evident through out the entire length of the run. The demarcated zones were individually scrapped, separately collected and extracted with EtOH. After gravimetrically determining the fraction yields it became apparent that a substantial amount of silica had been solubilized – a 280% increase in dry mass was recorded. This made accurate quantitation of the respective fractions impossible with out a suitable clean up method. As result thereof, the antiproliferative activity of the various pTLC fractions could not be determined. This however did not prevent further chromatographic, spectroscopic and spectrometric analysis of the various pTLC fractions.

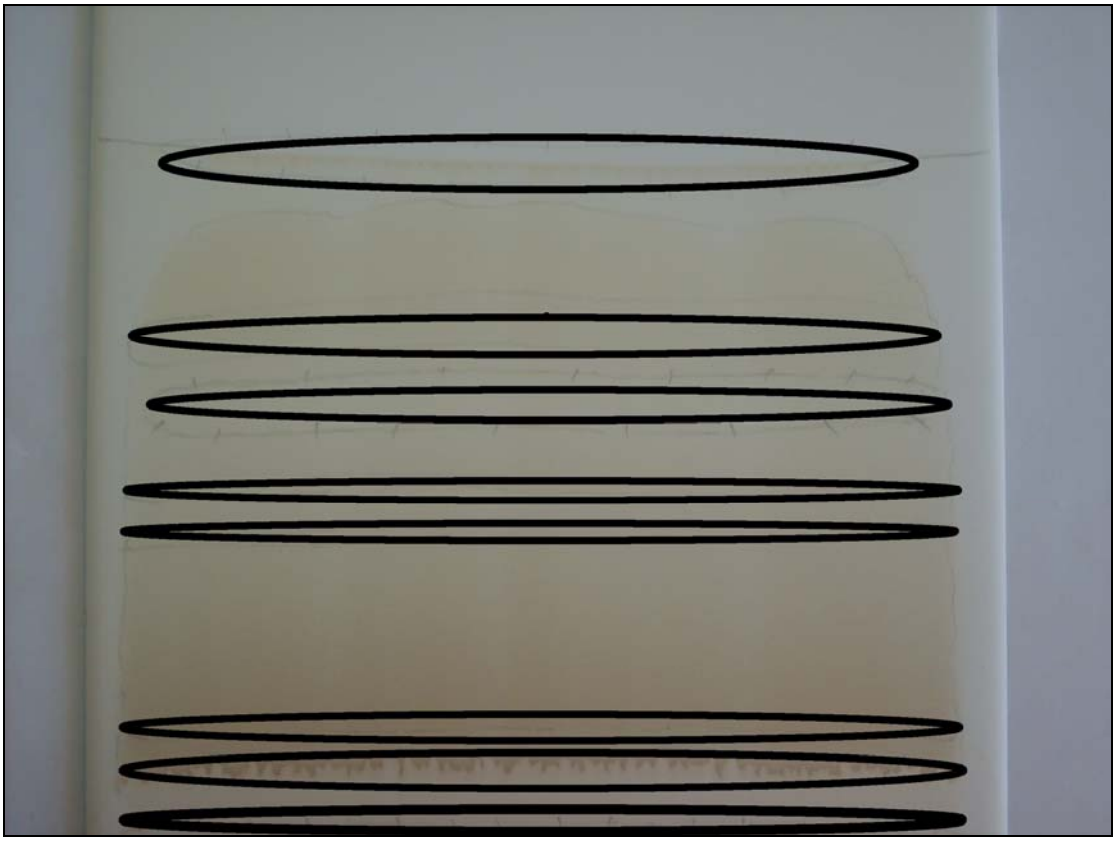


Figure 5.13. Photograph of pTLC plate highlighting UV visualised zones. Developed with $CHCl_3$:MeOH:BuOH:H20, (10:10:10:1)

TLC was used to qualitatively compare the constituents within each of the pTLC fractions. The same mobile phase used to produce the fractions was again used with analytical TLC. On visual inspection of the chromatogram (Figure 5.14.), common constituents can be found within certain of the fractions suggesting that the fractionation procedure was less efficient than had been hoped. The developed and UV visualised plate was stained with iodine vapour - the zones correlating to R*f* values of roughly 0.77-0.83 found in fraction 2 and 3 were marked before allowing the iodine to evaporate. These spots are clearly discrete from those in fraction 6 and 7 (with similar R*f* values), which fluoresce purple under UV light.

The plate was then sprayed with a 0.1N solution of silver nitrate as suggested by Stahl, 1969 for the visualisation of peltatins. β-peltatins reportedly yield jet-black spots whilst α-peltatins yield brown-black spots. Subjectively, a blackish colour change was observed (particularly prominent in fraction 2 at a R*f* value of 0.73). This observation further strengthens the hypothesis of the presence of such compounds.

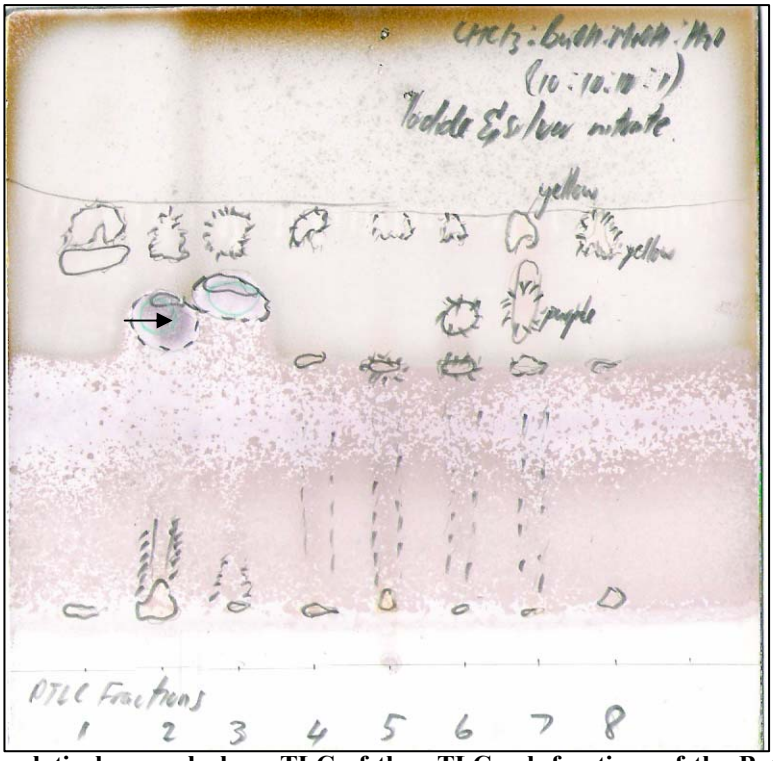


Figure 5.14. Analytical normal phase TLC of the pTLC sub-fractions of the BuOH fraction of extract H_2O_{S1S}. Developed with $CHCl_3$:MeOH:BuOH:H20, (10:10:10:1). Sprayed with a 0.1N silver nitrate solution - black arrow indicates a prominent blackish colour appearance (From left to right, pTLC fractions 1 through 8).

5.4. Conclusions

SPE was selected as an easy and cost effective means of evaluating different mechanisms of retentions (selectivity). SPE (without the benefit of the knowing the physicochemical nature of the desired compound/s) proved to have poor resolving power. Disappointingly, the AC was not enriched through either C18 or multimode SPE cartridge elution. Bioassay results supported through on-line monitoring of the respective fractions, suggests the possibility of there being more than one active compound present. Incomplete distribution of analytes was clearly evident as shown through comparison of HPLC retention times and common M/z ions found through direct-infusion ESI-MS.

Ideally, a fractionation strategy should take into consideration the amount of material required in order to allow for sufficient product to progress further with fractionation and carry out the required bioassays at each step. Accordingly the first fractionation procedure should provide a means of removing large amounts of biologically inert

compounds and be amendable to large-scale operation. Solvent-solvent partitioning between BuOH and water meet these criteria and successfully enriched the antiproliferative activity. The antiproliferative activity of the BuOH fraction of extract H_2O_{S1S} was shown to produce an impressive IC_{50} of 2.7µg/ml against HeLa cell line cultures. The potency of the AC present within this fraction is exemplified by considering the prevailing complexity of this fraction as demonstrated by an HPLC total wavelength chromatogram (TWC) / full spectrum chromatogram, (Figure5.15.).

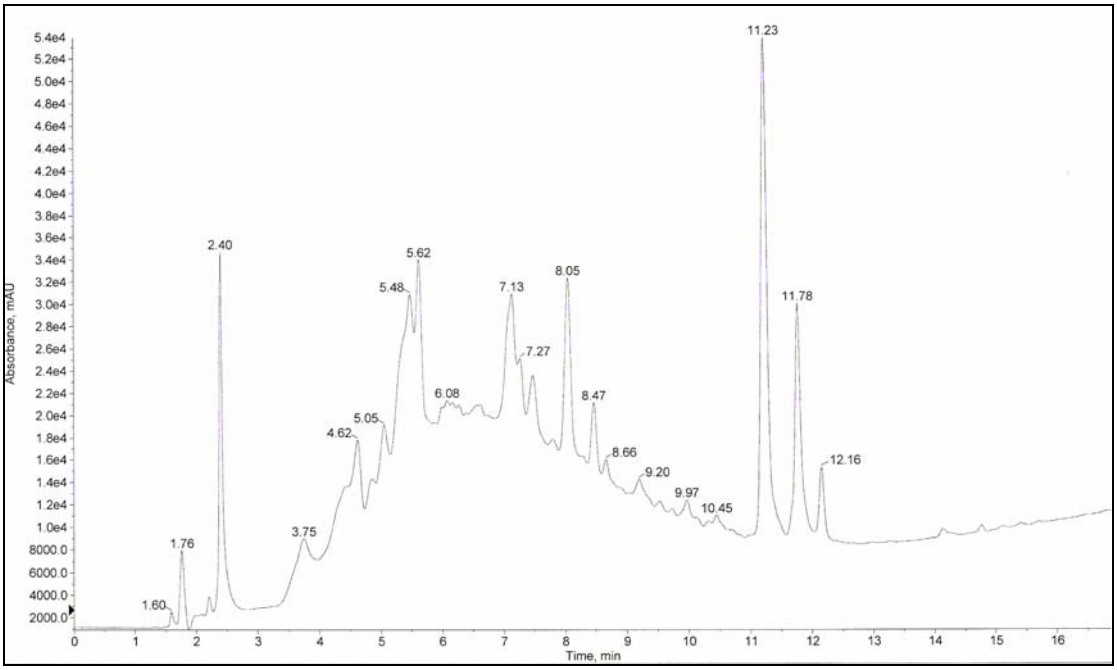


Figure 5.15. Total wavelength chromatogram (TWC) of the BuOH fraction of extract H_2O_{S1S}. Mobile phases: A, H_20 (0.1% HCO_2H); B, MeOH (0.1% HCO_2H). Elution: time 0-0.5min, 10% B; 14.5min linear gradient to 95% B; held for 1min before returning to starting conditions at 19min. Column re-equilibrated for 5min at 10% B. DAD (205-600nm) and MS data collected for 17min.

Normal phase pTLC proved to be a partially efficient next fractionation step. Further optimisation of the mobile phase is required to avoid a streaking effect witnessed when large quantities of material are loaded onto the plate. Moreover, this normal phase method could easily be adapted for preparative column chromatography using silica as a sorbent.

CHAPTER 6

6. FINAL CONCLUSIONS

The genus *Bridelia* is used extensively in the traditional medicine of many indigenous African and Asian cultures. Numerous biological activities are associated with constituents present within this genus. This study, brought about through suggestive results attained in a preliminary toxicological screening, focussed on the antineoplastic potential of extracts taken from the bark of *Bridelia micrantha*. As foundation, ethnobotanical and chemotaxonomical literature were reviewed.

Considering chemotaxonomical data pertaining to the presence of cytotoxic aryltetralin lignans, this study was designed in a way so as to avoid replication (i.e. identification of already known compounds) and the costs associated therewith. Apart from extracting a broad phytochemical range, the sequential extraction methodology aimed to extract aryltetralin lignans. Preliminary phytochemical investigations, in the form of chemical (chromogenic) tests performed on developed TLC plates revealed the possible presence of several aryltetralin lignans within various primary extracts. The presence of such compounds could not be confirmed through comparison of mass spectra attained through direct-infusion ESI-MS with a small mol. wt. database compiled from chemotaxonomical literature and including common aryltetralin lignans.

The intra-species variance of two bark samples of *B. micrantha*, sourced from different geographical locations at different times of year was assessed through comparison of their phytochemical compositions (both quantitative and qualitative) and respective biological activities. The time of year and the location from which samples are sourced does not appear to have an effect on the biological activity of aqueous extracts of *B. micrantha* (bark).

The *in vitro* antineoplastic activity of six primary extracts was evaluated through comparison of their antiproliferative activity for neoplastic cell lines versus that for normal primary cultures. Five out of the six primary extracts displayed impressive *in*

vitro antineoplastic activity in that they showed impressive cytotoxic specificity for neoplastic cell cultures as compared to primary human lymphocytes and porcine hepatocytes. The presence of numerous (functionally and therefore structurally distinct) cytotoxic constituents within the bark material of *B. micrantha* is strongly suggested by the differing patterns of cytotoxic selectivity displayed by various primary extracts.

The most promising of the primary extracts was deemed to be H_2O_{S1S}. Multi-parametric flow cytometric analysis revealed that death induced by this extract followed an apoptotic route, whilst not altering cell cycle progression. However, the mechanism leading to induction of apoptosis was not elucidated and further investigation is required in this regard.

Bioassay-guided fractionation of extract H_2O_{S1S} using SPE (C18 and multimode) cartridges did not result in any of the eluted sub-fractions possessing superior cytotoxicity against HeLa cell cultures as compared to the un-fractionated extract and the experimental conditions control. The cytotoxicity data, in conjunction with the employed monitoring methods suggests the possible break-up of numerous active compounds and/or incomplete distribution of the analytes. In this regard, the use of an additional neoplastic cell line to guide fractionation is proposed for further investigation as differential cytotoxic selectivity may be demonstrated, thus allowing for different active compounds to be separately targeted for elucidating.

The cytotoxic activity against HeLa cell cultures was ultimately improved by a factor of 2.5 in a BuOH sub-fraction of extract H_2O_{S1S} produced through solvent-solvent partitioning between H_2O and BuOH. Further attempts to enrich the active constituent/s using normal phase pTLC was met with marginal success in that a fair degree of separation was evident but that the sub-fractions were severely contaminated with solubilised silica from the pTLC plate. As such, the antiproliferative activity of the produced sub-fractions could unfortunately not be determined. Normal phase column chromatography appears to be an attractive next fractionation step.

Direct-infusion ESI-MS mass spectra were acquired throughout the study as a monitor of the global (both positive and negative ion mode) qualitative phytochemical profiles of the different extracts and fractions thereof. As complex samples were analysed without chromatographic separation, the potential for ion-suppression and the generation of inaccurate data had to be borne in mind. It is therefore important to acknowledge that these qualitative profiles are only representative. Mass spectra attained through direct-infusion proved to be quick, cost effective and reproducible. The actual value of this technique as a monitor of fractionation can only be assessed retrospectively once the active constituent/s have been identified.

LC hyphenated techniques (DAD and MS) were used to acquire large amounts of spectroscopic and spectrometric data of the pTLC sub-fractions. Without suitable libraries and not knowing which particular compound (peak) is responsible for the biological activity, this data is of little value. It is hoped that future investigations correlating semi-quantitative on-line data (through chemometrical discrimination using pattern recognition software) with differential bioassay outcomes will serve as a predictor capable of assigning biological activity to particular constituents.

As bioassay-guided fractionation was conducted over the course of time it provided a convenient means of assessing the stability of extract H_2O_{S1S} under the standardised storage conditions. Up to a period of 6 months, no significant loss of activity has been observed for 10mg/ml samples of extract H_2O_{S1S} stored at -70ºC.

As to whether aryltetralin lignans are responsible for the observed cytotoxic activity is not yet clear. TLC chromogenic observations suggest that peltatins are present, however selective ion-monitoring did not reveal the presence of any common pseudo-ionic forms of peltatins. The possibility of novelty is thus suggested.

Undoubtedly, the antineoplastic activity reported here within has potential clinical relevance and should be further explored to elucidate the biologically active constituent/s present.

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Appendix A

Molecular weight database

M/z	Compound and Empirical formula
152	Methyl salicylate ($C_8H_8O_3$)
164	Cumic acid ($C_{10}H_{12}O_2$)
166	Isochaminic acid ($C_{10}H_{14}O_2$)
170	Gallic acid ($C_7H_6O_5$)
208	Elemicin ($C_{12}H_{16}O_3$)
290	Luteoferol; Catechin ($C_{15}H_{14}O_6$)
302	Ellagic acid ($C_{14}H_6O_8$)
302	Quercetin ($C_{15}H_{10}O_7$)
306	Gallocatechin ($C_{15}H_{14}O_7$)
316	3-O-methylquercetin ($C_{16}H_{12}O_7$)
318	Myricetin ($C_{15}H_{10}O_8$)
322	Leucodelphinidin ($C_{15}H_{14}O_8$)
338	Delphinidin ($C_{15}H_{11}O_7$)
354	Sesamin ($C_{20}H_{18}O_6$)
398	Deoxypodophylltoxin ($C_{22}H_{22}O_7$)
400	Demethylpodophyllotoxin ($C_{21}H_{23}O_{23}$)
413	Stigmasterol ($C_{29}H_{48}O$)
414	Podophyllotoxin; Peltatins ($C_{22}H_{22}O_8$)
425	Taraxerone ($C_{30}H_{48}O$)
427	Taraxerol; Friedelin ($C_{30}H_{50}O$)
429	Epifriedelinol ($C_{30}H_{52}O$)
434	Ferrugin ($C_{26}H_{26}O_6$)
464	Myricetin 3-rhamnoside; Quercetin 3-glucoside ($C_{21}H_{20}O_{12}$)
480	Myricetin 3-glucoside ($C_{21}H_{20}O_{13}$)
547	5-demethoxy-β-peltatin 5-O-β-D glucopyranoside ($C_{27}H_{31}O_{12}$)
562	4’ Demethylpodophyllotoxin glucoside ($C_{27}H_{31}O_{13}$)
576	Podophyllotoxin glycoside ($C_{28}H_{32}O_{13}$)
610	Rutin ($C_{27}H_{30}O_{16}$)

Appendix B

Isolation of primary human lymphocytes

Methods:

- Carefully load 30ml heparinized (0.1ml heparin / 1ml blood) blood onto 15ml Histopaque.
- Centrifuge for 25minutes, 500g at ambient room temperature.
- Remove top plasma layer.
- Transfer the lymphocyte/ monocyte layer to sterile 50ml tubes.
- Fill tubes with RPMI 1640 medium.
- Centrifuge for 15minutes at 500g.
- Discard supernatant; fill up with sterile RPMI 1640 and centrifuge for 10minutes at 500g.
- Discard supernatant and fill up with sterile, cold ammonium chloride.
- Place on ice for 10minutes.
- Centrifuge for 10minutes at 500g.
- Discard supernatant; fill up with sterile RPMI 1640.
- Centrifuge for 10minutes at 500g, discard supernatant.
- Resuspend cells in 1ml RPMI supplemented with 10%FCS.
- Dilute cells in RPMI (10%FCS) for experiment.

Isolation of primary chick fibroblasts

Methods:

- Remove 7day embryo from egg.
- Transfer embryos to sterile petri dish.
- Remove fat and necrotic material from embryo/s
- Transfer embryos to new sterile petri dish.
- Chop embryos finely with crossed scalpels into small pieces.

- Transfer tissue to sterile 50ml centrifuge tube.
- Wash tissue with RPMI; Allow pieces to settle and remove supernatant. Repeat twice.
- Transfer tissue to sterile flask; Remove most of the residual fluid.
- Add the following: 45ml PBS (filter sterilized) + 2.5% trypsin. Add magnetic stirrer.
- Stir at 100rpm for 30minutes at 37°C.
- After 30minutes allow pieces to settle; Pour supernatant (with disaggregated cells in suspension) into sterile 50ml centrifuge tubes and place on ice. Repeat until disaggregation is complete (3-4 hours).
- Centrifuge the supernatant at 500g for 5 minutes.
- Discard supernatant and resuspend cell pellet in RPMI (10%FCS) medium.
- Seed cells in culture flask.
- Change medium / passage regularly as dictated by growth or depression of pH.

Appendix C

Bonferroni multiple comparison post-tests

IC_{50} values for all the neoplastic cell cultures and primary fibroblasts produced by the all the primary extracts (except Hex_{SS}) were statistically assessed through the use of a 2-way ANOVA and Bonferroni multiply comparison post-tests (α=0.05). Such statistical comparisons allow the biological activity exerted by each primary extract on each cell culture to be compared with each other. Through this, selective biological actions exerted by the respective extracts can be discriminated. Significant differences seen between various extracts for a particular cell culture contrasted by non-significant differences between the same extracts for a different cell culture indicate the presence of functionally distinctive (different) active compounds.

Culture	**H_2O_{S1S} vs. H_2O_{S2S}**	**H_2O_{S1S} vs. H_2O_{PS}**	**H_2O_{S1S} vs. $EtOAc_{SS}$**	**H_2O_{S1S} vs. $EtOAc_{PS}$**	**H_2O_{S2S} vs. H_2O_{PS}**	**H_2O_{S2S} vs. $EtOAc_{SS}$**	**H_2O_{S2S} vs. $EtOAc_{PS}$**	**H_2O_{PS} vs. $EtOAc_{SS}$**	**H_2O_{PS} vs. $EtOAc_{PS}$**	**$EtOAc_{SS}$ vs. $EtOAc_{PS}$**
HeLa	*P*>0.05	*P*<0.001	*P*<0.001	*P*>0.05	*P*<0.001	*P*<0.001	*P*>0.05	*P*>0.05	*P*<0.001	*P*<0.001
COLO	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05
MCF 7	*P*>0.05	*P*>0.05	*P*<0.001	*P*<0.001	*P*>0.05	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001
Jurkat	*P*>0.05	*P*<0.001	*P*>0.05	*P*>0.05	*P*<0.001	*P*>0.05	*P*>0.05	*P*<0.001	*P*<0.001	*P*>0.05
Fibro.	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*>0.05	*P*<0.01	*P*<0.05	*P*>0.05