

AN INVESTIGATION INTO THE ANTIMICROBIAL AND
ANTICANCER ACTIVITIES OF *GERANIUM INCANUM*,
ARTEMISIA AFRA AND *ARTEMISIA ABSINTHIUM*

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this study is my own original work, and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

SUMMARY

It has been estimated that between 3000 and 4000 plant species are used for their medicinal properties throughout South Africa, with approximately 27 million South Africans making use of traditional medicines. Of this 27 million, 3 million South Africans rely on traditional medicine as their primary source of health care. Of the 250 000 to 500 000 known plant species, very few have been investigated for their pharmacological qualities, and compounds of significant medicinal value may still remain undiscovered in many plant species.

The aims of this study included investigating the antimicrobial properties of *Geranium incanum* and *Artemisia afra*, both plants traditionally used for their medicinal properties, and comparing the antimicrobial activity of the latter to that of *Artemisia absinthium*, as well as investigating the anticancer properties of *G. incanum* and *A. afra*, and comparing the anticancer activity of the latter to that of *A. absinthium*. Infusions, aqueous-, methanol- and acetone extracts of the three plants were prepared and used for anticancer and antimicrobial screening. Plant specimens used to prepare extracts for antimicrobial activity were collected and extracted over three seasons, while extracts used for anticancer screening were prepared from plants collected during the summer only. Considerable variation existed in the percentage crude extract yields obtained when different extractants were used, while the season in which the plants were harvested and extracted also appeared to play a significant role in the amount of extract obtained.

The plant extracts were screened for antimicrobial activity against various strains of *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Bacillus cereus*, using an agar dilution method. *G. incanum* and *A. afra* possessed activity for *C. albicans*, while all three plants showed activity for *S. aureus* and *B. cereus*. Activity was largely dependent on the extraction method used.

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used to screen for anticancer activity of the respective extracts, at varying concentrations, against MCF-7 (human breast adenocarcinoma) cells, HT-29 (human colonic adenocarcinoma) cells and HeLa (human cervical cancer) cells. All of the extracts showed cytotoxic activity in all three cell lines to varying extents, depending on the extract used and cell line screened. The acetone extract of *A. afra* proved to be the most effective inhibitor with the lowest IC₅₀ (2.65 ± 1.05 µg/ml) having been shown in MCF-7 cells. *A. afra* and *A. absinthium* showed similar inhibitory patterns, with the methanol- and acetone extracts having been the most potent inhibitors of each of the respective cell lines in general. Fluorescence microscopy employing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodide (PI) staining indicated that the acetone extract of *A. afra* induces apoptosis in MCF-7 cells as apposed to necrosis, and the results were comparable to those obtained for cells exposed to cisplatin.

Screening of the *A. afra* acetone extract for toxicity in normal human cells using the CellTiter-Blue® assay indicated the extract to be toxic to peripheral blood mononuclear cells (PBMC's) at concentrations comparable to that for MCF-7 cells, while cell cycle analysis of MCF-7 cells exposed to the *A. afra* acetone extract indicated the extract's ability to induce apoptosis comparable to that of cisplatin, with the extract exerting its activity at a point during or just prior to the S phase of the cell cycle.

Key words: anticancer, antimicrobial, apoptosis, *Artemisia absinthium*; *Artemisia afra*; *Bacillus cereus*; *Candida albicans*; cell cycle; DAPI; *Enterococcus faecalis*; *Escherichia coli*; *Geranium incanum*; HeLa; HT-29; IC₅₀; MCF-7; MTT; peripheral blood mononuclear cells; PI; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; traditional medicine

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celsius
µg	Microgram
µg/ml	Microgram per millilitre
<i>A. absinthium</i>	<i>Artemisia absinthium</i>
<i>A. afra</i>	<i>Artemisia afra</i>
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
APC	Anaphase promoting complex
ARV	Antiretroviral
ATCC	American type culture collection
AZT	Zidovudine
<i>B. cereus</i>	<i>Bacillus cereus</i>
<i>C. albicans</i>	<i>Candida albicans</i>
CDK	Cyclin dependent kinase
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DMAPP	Dimethylallyl pyrophosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diaminetetra-acetic acid
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
EGCG	Epigallocatechin gallate
FBS	Fetal bovine serum
FPP	Farnesyl pyrophosphate
g	Gram
GFPP	Geranylfarnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HeLa	Human cervical cancer cells
HHDP	Hexahydroxydiphenic acid
HIV	Human immunodeficiency virus

HMG-CoA	Hydroxymethylglutaryl-CoA
HPV	Human papilloma virus
HT-29	Human colonic adenocarcinoma cells
IC ₅₀	Substance concentration producing 50% inhibition
IPP	Isopentenyl pyrophosphate
MCF-7	Human breast adenocarcinoma cells
MDR-TB	Multi-drug resistant tuberculosis
mg	Milligram
mg/ml	Milligram per millilitre
ml	Millilitre
MH	Mueller Hinton
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NMMU	Nelson Mandela Metropolitan University
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBMC	Peripheral blood mononuclear cell
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PGG	Pentagalloyl glucose
PI	Propidium iodide
r ²	Coefficient of correlation
RT	Room temperature
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SD	Standard deviation
Spp	Species
TB	Tuberculosis
TEM-1	Temoneira
UV	Ultraviolet
VISA	Vancomycin intermediate <i>Staphylococcus aureus</i>
VRE	Vancomycin resistant <i>Enterococcus</i>
WHO	World Health Organisation
XP	Xeroderma pigmentosum

CHAPTER 1

INTRODUCTION

The past two decades have witnessed a renewed interest in research in the field of ethnopharmacology. The Western world acknowledges the use of traditional remedies by many cultures worldwide, and the need for new drug development has aimed the focus on an ethnobotanical approach to the discovery of new pharmacological agents (Light, Sparg, Stafford & Van Staden, 2005).

The history of traditional medicinal systems is as vast and varied as the cultures in which they have their roots, including those originating from Africa, North America, Central and South America, Australia, China, India, Arabia and Europe. The use of many early traditional medicines was influenced by irrational superstitions which, in some cases, continue today. The belief that the use of human body parts in conjunction with certain medicinal plants increases the potency of the medicinal qualities of these plants, has led to numerous ritualistic murders in South Africa, with children often falling victim to these “muthi” murders. However, not all traditional medicines were initially used based on superstitions. Modern scientific research in the fields of ethnobotany and ethnopharmacology has validated the traditional use of many plants for their medicinal properties by different cultures. Traditional African, Ayurvedic (Indian) and Chinese medicine systems are amongst the oldest known, and has undoubtedly influenced modern drug development and the isolation of novel compounds with therapeutic value (Gurib-Fakim, 2006).

Pollen and flower fragments of various medicinal plants discovered in the grave of a 60 000 year old Neanderthal man indicates that the human race has recognised and has been using plants for their medicinal properties for at least the last 60 millennia. Another example is the 5300 year old ice man discovered in the Italian Alps during 1991, whose possessions included, amongst other things, fragments of birch fungus, which was probably used as

a laxative or antimicrobial agent (Elgorashi, Taylor, Maes, Van Staden, De Kimpe & Verschaeve, 2003; Hart, 2005). A few plant species having been used by the Mesopotamian civilization for their healing properties some 4600 years ago are still being used today to treat a variety of conditions, ranging from colds to infection and inflammation. The recent development of the drug β -methoxypsoralen from *Ammi majus* (Bishop's weed), which is used to treat psoriasis and T-cell lymphoma, has validated the use of this plant by the Egyptian civilization for the treatment of vitiligo, a skin condition (Gurib-Fakim, 2006).

Knowledge of medicinal plants was passed on from one generation to the next and was eventually recorded in herbal journals (Balunas & Kinghorn, 2005). This knowledge is important and applicable to our times for various reasons. Firstly, it promotes the discovery of new alternatives to drugs currently being used. Secondly, it is important from a conservation point of view; if over exploitation of a medicinal plant species should occur, restrictive measures can and should be taken to ensure survival and sustainability of the specific species. From a cultural point of view, important knowledge regarding the traditional use of plants is lost as it is not being passed on from one generation to the next anymore. Thus it is important that this knowledge be documented to ensure that it is at the disposal of future generations who may benefit from it (Thring & Weitz, 2006).

It is estimated that 80% of the global population rely on plant derived medicines to address their health care needs (Gurib-Fakim, 2006). Consultation of traditional healers and the use of herbal medicine provided by them play an integral role in rural healthcare, with the majority of the South African population consulting traditional healers to address most, if not all, of their healthcare needs (Light *et al.*, 2005). This is mainly due to the high cost of Western medical care in developing countries such as South Africa, the inaccessibility of facilities rendering modern Western medical care to rural communities, unavailability of novel drugs and the cultural role that the use of traditional medicines play in these communities (Louw, Regnier & Korsten, 2002). Whereas modern Western medical treatment aims to treat the

immediate physiological processes and symptoms of a disease condition, traditional healers aim to restore balance and a general sense of well being of a patient on both a mental and physiological level. For this reason a combination of plants are usually prescribed to patients by traditional healers, who believe that the synergistic or additive effects of the correct combination of plants will be more successful in treating a patient than would be a single drug. The simultaneous use of traditional and modern Western medicines is common practice in most societies, with Western medicine being used to treat acute conditions, while traditional medicines are employed to provide relief from chronic disease conditions (Gurib-Fakim, 2006).

South Africa has a remarkable botanical diversity with over 30 000 flowering plant species, which constitutes more or less one tenth of the global higher plant species. Of the 30 000 flowering plant species, ten families are endemic, with 80% of the species and 29% of the genera being endemic (Stafford, Jäger & Van Staden, 2005; Fennell, Light, Sparg, Stafford & Van Staden, 2004). It has been estimated that between 3000 and 4000 plant species are used for their medicinal properties throughout the country, with approximately 27 million South Africans making use of traditional medicines (Fennell *et al.*, 2004; Mulholland & Drewes, 2004). Of this 27 million, 3 million South Africans rely on traditional medicine as their primary source of health care (Louw *et al.*, 2002). Approximately 20 000 tons of plant material is sold on an annual basis on the South African market, be it on the street or by traditional healers, with 20 000 to 30 000 individuals deriving in income from the selling of medicinal plants in KwaZulu-Natal alone (Fennell *et al.*, 2004; Mulholland & Drewes, 2004). The number of traditional healers in the country is estimated at 200 000, with 60% of the population making use of their services (Light *et al.*, 2005; Thring & Weitz, 2006). Mulholland and Drewes (2004) estimated that the majority of South African households spend between 4% and 8% of their annual income on traditional medicinal plants and healers.

South Africa's increasing population has led to an increase in the demand and consumption of traditional botanical remedies during the latter part of the

twentieth century. Harvesting of medicinal plants often occurs under non-sustainable conditions, and it is estimated that as much as 85% of medicinal plants harvested are non-renewable sources such as bulbs, bark and rhizomes (Fennell *et al.*, 2004). The short term financial gains from the selling of these non-renewable plants and their parts appear to be more compelling to the harvester than the long term ecological impact and availability of scarce plants for use by future generations. This attitude has led to some plant species having become scarce and endangered. It would appear that the only remedy to this predicament is the small scale farming and production of medicinal plant species. However, medicinal plants cultivated and harvested artificially according to Western methods are often not acceptable to many conservative traditional healers, who claim that the plants are less effective and lose their potency if not grown and harvested naturally. A decrease in the availability of many plant species, along with an increase in the demand for and price of these plants has left the majority of traditional healers no alternative choice but to accept that agricultural cultivation is the only sustainable long term solution (Fennell *et al.*, 2004).

The use of plants for the treatment of various ailments has several advantages. Plant derived medicines are generally better tolerated by patients and have fewer side effects. If harvested correctly, medicinal plants are a sustainable natural source of medicine and the cultivation and processing of these plants do not lead to the inevitable pollution associated with the industrial processes used to produce many Western drugs. Cultivation and selling of medicinal plants could also provide needy families with a small income. The development of drug resistance is also less documented in plant derived medicines (Vermani & Garg, 2002).

Nature continues to play an integral role in the discovery and isolation of novel molecules with medicinal properties, with more than 50% of chemotherapeutic agents currently in use having been derived from natural products (Gurib-Fakim, 2006). Plants constitute about 25% of the total amount of natural agents which have yielded useful medicinal compounds (Gurib-Fakim, 2006; Rates, 2001). Eleven percent of the 252 drugs being considered by the World

Health Organisation as being basic and essential are of plant origin (Rates, 2001).

Detection, isolation and development of novel chemotherapeutic agents from plants is normally a time consuming, laborious and expensive task, with each new drug requiring US\$ 100-360 million in investment and at least 10 years of development and clinical trials. Furthermore, only 1 out of every 10 000 compounds tested will be considered for further development, and 1 out of every 4 of these promising compounds eventually reaches the status of a novel agent for medical use. However, of the 250 000 to 500 000 known plant species, very few have been investigated for their pharmacological qualities, with an estimated 5000 having been investigated for their medicinal properties (Rates, 2001). Thus it could be safe to assume that compounds of significant medicinal value still remain undiscovered in many plant species which have, to date, not been investigated. The rapid rate of deforestation of tropical areas in especially South America and Africa may lead to the extinction of many undiscovered plant species and the subsequent loss of compounds which may be the answer to some of mankind's most devastating disease conditions, such as HIV/AIDS, cancer, diabetes and malaria.

Examples of pharmacologically active compounds isolated from plants include morphine, cocaine, digitoxin, codeine, quinine, reserpine and pilocarpine. *Catharanthus roseus* (Rosy Periwinkle) has yielded two powerful anticancer agents, while three other major anticancer drugs were derived from plants traditionally used by North American Indians, including the Pawpaw (*Asimina* spp.), Mayapple (*Podophyllum peltatum*) and the Western Yew Tree (*Taxus brevifolia*) (Gurib-Fakim, 2006). In 2001 and 2002, a quarter of the best selling drugs globally were either natural products or derivatives thereof (Balunas & Kinghorn, 2005).

Though ethnopharmacology is progressing in South Africa, a lack of research that could be beneficial to traditional healers and their patients, as well as to Western medicine, still prevails. Scientific research done during the last century has proven the pharmacological activities of many plant species

traditionally used for their medicinal properties, thus validating the use of these plants (Louw *et al.*, 2002; Hart, 2005). The majority of current research is directed toward the discovery of commercially useful compounds from medicinal plants (Light *et al.*, 2005).

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

In the current chapter, main subgroups of phytochemicals as the biologically active compounds in medicinal plants are discussed. A brief overview of *Geranium incanum*, *Artemisia afra* and *Artemisia absinthium* is given, including botanical classifications and vernacular names, macroscopical morphology and geographical distributions. A brief summary of the known major phytochemical constituents of each of the respective plant species is also given, as well as the ethnopharmacological application of these plants by various cultures, and previous scientific research conducted on the efficacy of these plants in treating the conditions for which they are used.

The current chapter also features a brief discussion on cancer, its aetiology and pathogenesis on both macroscopical and molecular levels, as well as current plant derived anticancer agents being used in the clinical setting. Finally, the development of drug resistance amongst micro-organisms is briefly discussed, focussing on the mechanisms of the development of drug resistance and factors contributing to this.

2.2. Phytochemicals as the biologically active compounds in medicinal plants

In this section, an overview of some of the major phytochemical compounds found in plants, especially as relating to the three plants used in the current study, their synthesis and functions, and how they may be applied in industry and the clinical field, is given.

2.2.1. Flavonoids

Flavonoids refer to a group of phenolic compounds, the latter including a wide range of chemical constituents all having an aromatic ring which bears one or more common hydroxyl groups (Mukinda, 2005, p.27).

The basic nucleus of a flavonoid contains fifteen carbon atoms arranged in a C6-C3-C6 configuration, which forms two aromatic rings linked by a 3 carbon unit. This unit may or may not form a third ring structure. Flavonoids exist in either the free aglycone form, which is a flavonoid lacking a sugar moiety, or the conjugated glycoside form, consisting of a flavonoid aglycone conjugated to one or more sugar moieties. These glycosidic flavonoids may be one of two types, depending on the type of bond between the aglycone and sugar moiety:

- Flavonoid O-glycosides, in which case an acid labile hemiacetal bond connects one or more hydroxyl groups to a sugar moiety, and
- Flavonoid C-glycosides, in which case a carbon-carbon bond attaches a sugar moiety directly to the benzene nucleus.

(Mukinda, 2005, p.28)

Flavonoids are generally classified into three groups:

- Flavonoids, being derived from 2-phenyl-1,4-benzopyrone,
- Isoflavonoids, being derived from 3-phenyl-1,4-benzopyrone, and
- Neoflavonoids, being derived from 4-phenyl-1,2-benzopyrone.

Flavonoids are further subdivided into the following groups:

- Flavones, including the four subgroups flavones (e.g. luteolin), flavonols (e.g. quercetin), flavanones (e.g. hesperetin) and 3-hydroxyflavonones or 2,3-dihydroflavonols (e.g. dihydroquercetin),
- Isoflavones (e.g. genistein)
- Flavan-3-ols (e.g. catechins and epicatechins), and
- Anthocyanidins (e.g. cyanidin)

(Flavonoid, 2007)

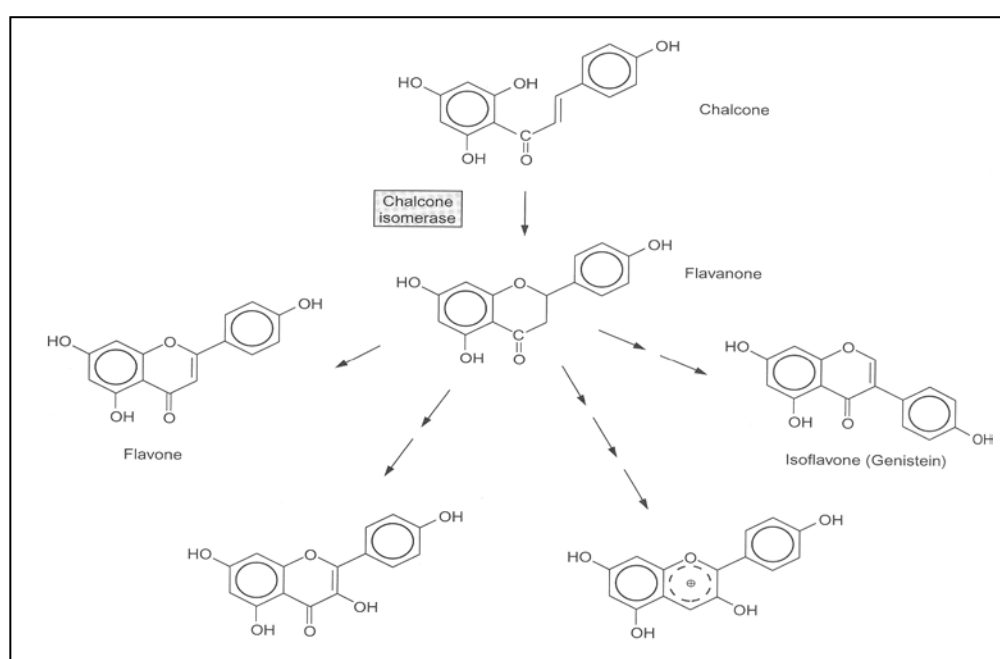


Figure 2.1: Various flavonoids, including flavanones, flavones, flavonols, anthocyanidins and isoflavones are derived from the precursor molecule chalcone (taken from Heldt, 2005, p. 448).

Flavonoid synthesis occurs during the phenylpropanoid metabolic pathway. During this metabolic process, 4-coumaroyl-CoA is synthesised from phenylalanine. 4-Coumaroyl-CoA may then be combined with malonyl-CoA to form chalcones (Figure 2.1), which comprise the backbone of flavonoids.

Subsequent ring closure of the chalcones leads to the formation of a flavone. During the phenylpropanoid metabolic pathway, flavones are converted to flavonones, which are in turn converted to dihydroflavonols. Dihydroflavonols

are then converted to anthocyanins, which are the aglycones of anthocyanidins. Other compounds synthesised during the phenylpropanoid metabolic pathway include flavonols, flavan-3-ols and tannins, also known as proanthocyanidins (Flavonoid, 2007).

It would appear that the flavonoids possess a wide array of biological activity. Flavonoids are found in high concentrations in almost all green plants, and are responsible for the pigmentation of flowers and the protection of plants against microbes and insects. The anti-inflammatory, antimicrobial and anticancer properties of flavonoids have also been indicated (Flavonoid, 2007). Quercetin (Figure 2.2) is said to be the most active flavonoid and imparts many of the medicinal qualities to plants used for such purposes.

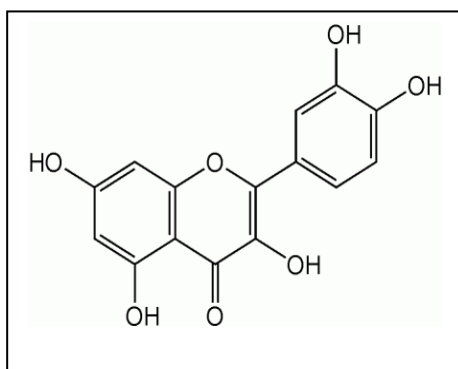


Figure 2.2: Structure of quercetin (taken from Quercetin, 2007).

It possesses anti-inflammatory qualities, for example, by inhibiting the release of histamine and other inflammatory mediators of allergic reactions, and is thought to have a vitamin C-sparing effect as well as anticancer properties. Proanthocyanidins are thought to increase intracellular vitamin C levels, eliminates oxidants and free radicals, prevents the destruction of collagen and decreases capillary fragility and permeability. Epicatechin (Figure 2.4) is said to improve blood flow and subsequently cardiovascular health. Cacao is especially rich in epicatechin, and cacao has been shown to possess antioxidant activity surpassing that of red wine or even green tea. However, a study conducted by the Linus Pauling Institute during 2007 (as cited in

Flavonoid, 2007) has indicated that a significant increase in the antioxidant capacity of blood after the ingestion of flavonoid-rich foods is not directly attributable to the flavonoids themselves, but rather to the increase in blood uric acid that occurs when these flavonoids are excreted. Thus flavonoids may indirectly function as inhibitors of cardiovascular disease and carcinogenesis by stimulating the mechanisms responsible for the inhibition of tumor invasion and killing off of cancerous cells (Flavonoid, 2007; Peterson, 1999, p.22).

Apart from red wine, cacao and green tea, other food sources rich in flavonoids include citrus fruits, onions, berries, legumes, parsley and dark chocolate with a cacao content of 70% or higher (Flavonoid, 2007).

2.2.2. Tannins

Plants are known to accumulate a wide variety of secondary metabolic compounds, including phenolics. The metabolism of phenolics is a complex process and yields compounds such as flower pigments and phenolics comprising plant cell walls. Tannins are a group of complex polyphenols which are distinguished from other plant phenolics based on their chemical reactivities and biological activities. They are normally divided into two groups: condensed tannins (proanthocyanidins) (Figure 2.5) and hydrolysable tannins (Figure 2.8). Geraniin ($C_{41}H_{28}O_{27} \cdot 7H_2O$) (Figure 2.11) is an example of a hydrolysable tannin (Hagerman, 2002). Red wine is said to be a good source of condensed tannins, which is found in grape skins and seeds, as well as the wooden casks in which wine is sometimes matured. Pomegranates are thought to be rich in hydrolysable tannins, which impart antioxidant qualities to pomegranate juice. Other food sources known to contain tannins include persimmons, cranberries, strawberries, blueberries and chocolate, due to its cocoa content (Tannin, 2007; Catechin, 2007).

2.2.2.1. Condensed tannins

Condensed tannins are polymers of flavonoids, which are based on a heterocyclic ring system which is derived from phenylalanine and polyketide synthesis. (+)-Catechin (Figure 2.3) and (-)-epicatechin (Figure 2.4), which are epimers, are flavan-3-ols which form the base for condensed tannins.

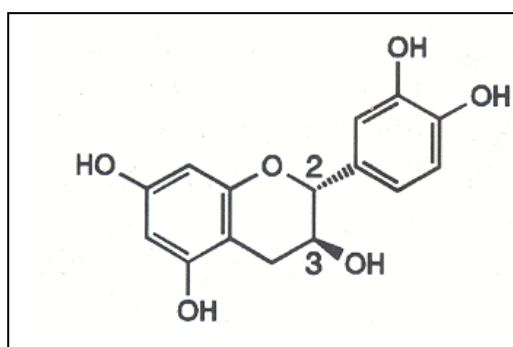


Figure 2.3: Structure of catechin (taken from Dey & Harborne, 1997, p.412).

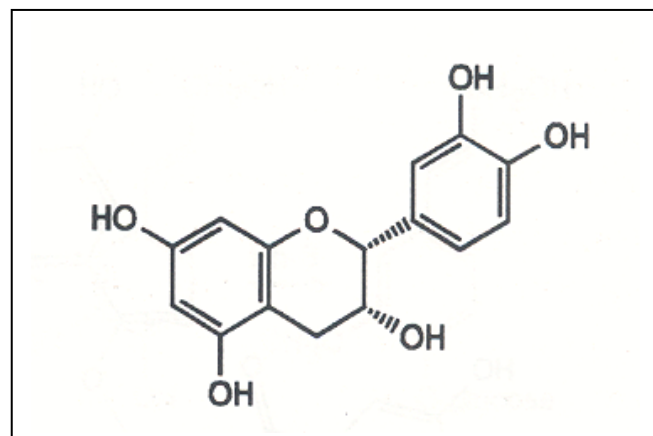


Figure 2.4: Structure of epicatechin (taken from Dey & Harborne, 1997, p. 412).

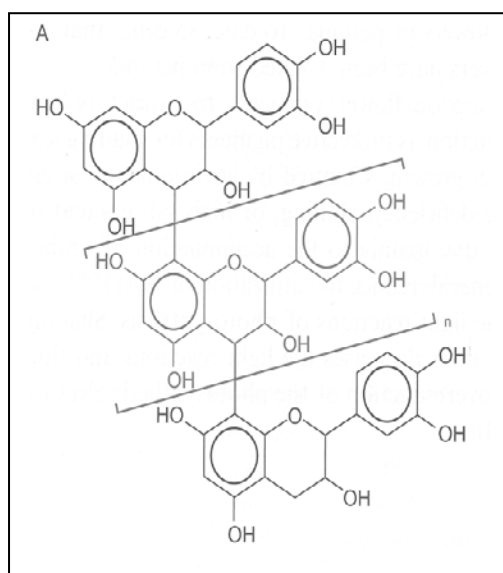


Figure 2.5: The general structure of a condensed tannin (taken from Heldt, 2005, p.452).

A wide variety of condensed tannins can be formed, depending on the carbon atoms involved in the carbon-carbon bonding. The addition of a third phenolic hydroxyl group to catechin and epicatechin yield the compounds gallocatechin and epigallocatechin, respectively. These catechin gallates are gallic acid esters of the catechins, such as epigallocatechin gallate (EGCG) (Figure 2.6), which is known to be the most abundant catechin in tea (*Camellia sinensis*).

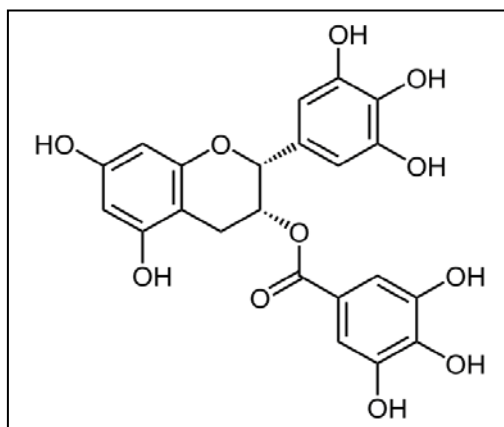


Figure 2.6: Structure of epigallocatechin gallate (taken from Epigallocatechin gallate, 2007).

Studies conducted on the possible health benefits of catechins and epicatechins have shown that catechins bring about a reduction in atherosclerosis and carcinogenesis, while the occurrence of stroke, heart failure, cancer and diabetes appear to decrease in individuals who ingest epicatechins on a regular basis (Catechin, 2007).

The best characterised condensed tannins are formed by bonding between C4 of the terminal flavonoid molecule, which may either be catechin or epicatechin, and C8 of the extender flavonoid molecule, which may also either be catechin or epicatechin. Further cross linking between carbon atoms is possible to yield linear 4,8 polymers, 4,6 polymers and branched polymers containing both 4,8 and 4,6 linkages. Oxidative coupling between the carbon and oxygen atoms of the flavonoid rings have also been described. It should be noted that unlike the case with hydrolysable tannins, carbon-carbon bonds

of condensed tannins are not susceptible to hydrolysis, but may be oxidatively cleaved (Hagerman, 2002).

2.2.2.2. Hydrolysable tannins

Hydrolysable tannins are derivatives of gallic acid (3, 4, 5-trihydroxyl benzoic acid) (Figure 2.7), and consist of gallic acid esterified to a core polyol. Further esterification or oxidative cross linking of the galloyl groups lead to more complex hydrolysable tannins.

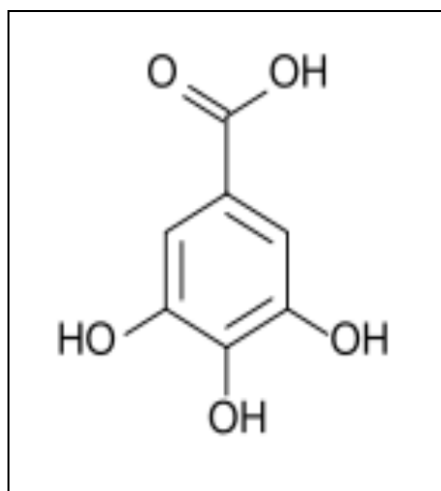


Figure 2.7: Structure of gallic acid (taken from Gallic acid, 2007).

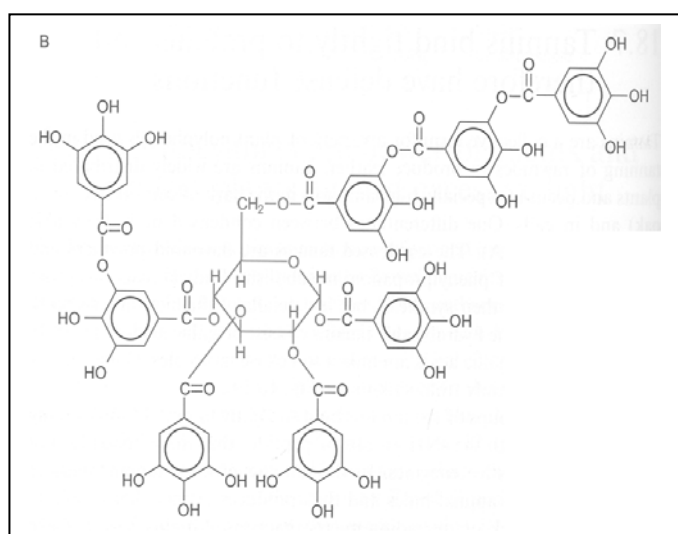


Figure 2.8: General structure of a hydrolysable tannin (taken from Heldt, 2005, p.452).

Gallotannins are the simplest form of hydrolysable tannins, and consist of phenolic gallic acid esterified to the hydroxyl groups of a carbohydrate, usually a glucose molecule, although it is known that in some cases the core glucose is substituted by molecules such as glucitol, hamamelose, shikimic acid, quinic acid and quercitol. Pentagalloyl glucose (PGG) (β -1, 2, 3, 4, 6-pentagalloyl-O-D-glucopyranose) would be a primary example of a gallotannin. More complex gallotannins may be formed by the addition of polygalloyl ester chains to PGG. This occurs through depside bonds involving the phenolic hydroxyl molecules of galloyl groups. These depside bonds are more readily hydrolysed than the aliphatic ester bonds between the core

Intramolecular carbon-carbon coupling between different carbon atoms of the galloyl groups yield a variety of different compounds, depending on the carbon groups involved and the stability of the conformation of the polygalloyl glucose molecule. Oxidative coupling of C2 with C4 and C3 with C6 will yield the ellagitannin geraniin. Ellagitannins may undergo further intermolecular oxidative coupling with other hydrolysable tannins to form dimers, as is the case with geraniin, which can undergo condensation with PGG to yield various euphrobins (Hagerman, 2002).

From a nutritional point of view, ingesting excessive amounts of tannin could be detrimental to the health of an individual, as tannins are known to be metal ion chelators. Chelated metal ions are not absorbed by the body, which could lead to mineral deficiency. Tannins are also used in a number of applications. The one characteristic that set tannins apart from other phenolic compounds, their ability to react with and precipitate proteins, is used in the tanning of hides to leather, with oak bark being the traditional source of tannin for this purpose, although industrial tannins are also employed. Other uses of tannin include its incorporation into medical anti-diarrheal, hemostatic, and anti-hemorrhoidal compounds, the reaction of iron (II)sulfate in the production of iron gall ink, and the use of tannin in industrial particleboard adhesive (Hagerman, 2002; Tannin, 2007).

2.2.3. Saponins

The term saponin is derived from the Latin word *sapo*, which means soap, and is a reflection of the tendency of aqueous solutions of saponins to froth. This is thought to be due to the amphiphilic nature of these molecules (Saponin, 2007). Ultimately, saponins are derivatives of terpenes or terpenoids, which is discussed in more detail in Section 2.2.4.

Terpenes are compounds consisting of variable amounts of five-carbon units, known as isoprene units (Figure 2.12), linked together. They are generally classified as hemiterpenes, monoterpenes, sesquiterpenes, diterpenes,

sesterterpenes, triterpenes, tetraterpenes and polyterpenes, depending on the amount of isoprene units present. Triterpenoids, which are formed from triterpenes through biological processes such as oxidation and other modifications, consist of six isoprenoid units. Squalene (Figure 2.13) is considered to be the parent isoprenoid of the triterpenes and triterpenoids. Modifications to squalene lead to the formation of different triterpenes and triterpenoids.

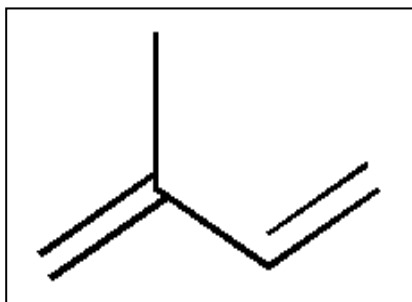


Figure 2.12: Structure of an isoprene unit (taken from Isoprene, 2007).

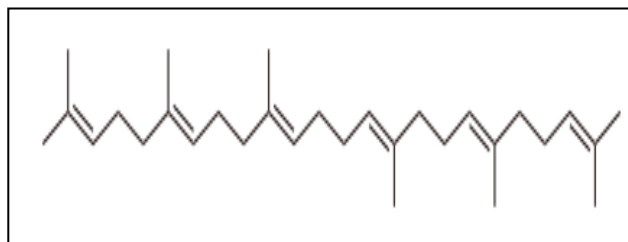


Figure 2.13: Structure of squalene (taken from Squalene, 2007).

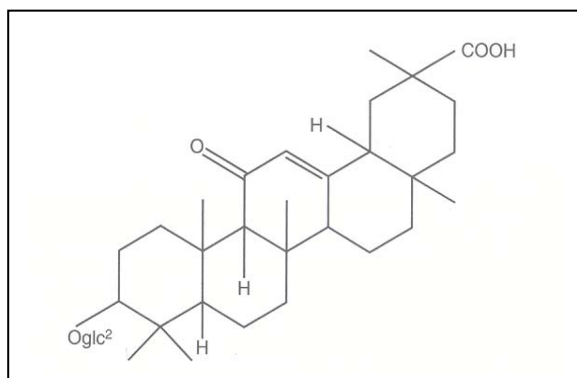


Figure 2.14: Glycyrrhizic acid, an example of a triterpenoid saponin (taken from Pengelly, 2004, p.75).

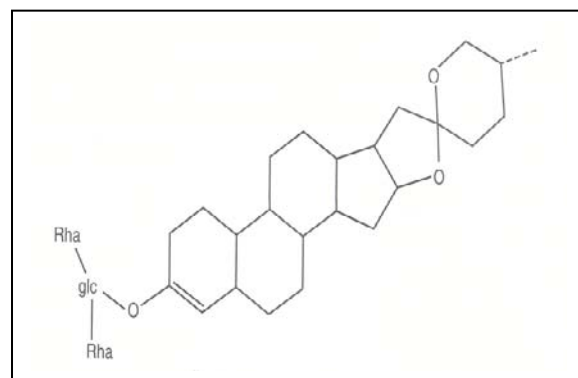


Figure 2.15: Dioscin, an example of a steroidal saponin (taken from Pengelly, 2004, p.75).

Saponins are glycosides of triterpenoids, meaning the presence of one or more sugar molecule. Secondary modifications to isoprene units of triterpenoids lead to the formation of different sterols and steroids, in which case the numbers of isoprene units are not so easily distinguishable. Saponins may also be glycosides of steroids and steroid alkaloids. Hydrolysis

of a saponin yields the triterpenoid or steroid as aglycone (Van Wyk *et al.*, 1997, p.22).

Saponins are said to be beneficial to human health as they may prevent or control hypercholesterolaemia and hyperglycaemia. They are also believed to act as antioxidants and blood cleansers, and to possess anticancer and anti-inflammatory properties due to their similarity in structure to steroids (Saponin, 2007). Inhibition of complement activity is said to be the anti-inflammatory mechanism of some saponins. Saikosaponins present in *Bupleurum falcatum*, as well as soyasaponins and kudzusaponins are said to have hepatoprotective qualities. Saponins may also be used as expectorants, laxatives and diuretics, and in the intestine they facilitate the absorption of some food and medicinal substances. Saponins themselves are generally poorly absorbed by the intestine, with an exception to those produced by the herbs paris and corn cockle, which may cause fatal haemolysis. Some saponins are sapotoxins, for example, those produced by the soapberry, and ingestion by humans may lead to reactions such as urticaria (Flück & Jaspersen-Schib, 1941/1976, p.10; Pengelly, 2004, pp.76-78; Peterson, 1999, p.22; Saponin, 2007).

Apart from their health benefits, saponins are also employed in other fields. They may be used as detergents, and are also employed in laboratory procedures to permeabilise the plasma membranes of cells and intracellular organelles in order to facilitate the uptake of antibodies and other peptides during histochemical staining (Saponin, 2007).

2.2.4. Terpenes

Terpenes are one of the most important groups of active compounds in plants, with over 20 000 structures known (Pengelly, 2004, p.59). Terpenes and terpenoids, which are also known as isoprenoids, consist of varying numbers of isoprene (2-methylbuta-1, 3-diene) units. An isoprene unit is a

five carbon structure (Figure 2.12), and is considered to be the building block of terpenes and terpenoids. During the formation of terpenes, isoprene units are linked to each other to form various isoprenoids, which are classified according to the number of isoprene units present:

- Hemiterpenes – These consist of a single isoprene unit, and have the molecular formula C_5H_8 . Isoprene itself is considered to be the only hemiterpene. Prenol and isovaleric acid, which are oxygen-containing derivatives, are called hemiterpenoids.
- Monoterpenes – Compounds in this group consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. Examples are geraniol, limonene, menthol and iridoids.
- Sesquiterpenes – These compounds consist of three isoprene units and have the molecular formula $C_{15}H_{24}$. The bitter principles, especially sesquiterpene lactones, are examples.
- Diterpenes – These compounds are composed of four isoprene units and have the molecular formula $C_{20}H_{32}$. Examples include cafestol, kahweol, cembrene and taxadiene (the precursor of taxol). Biologically active compounds such as retinol, retinal and phytol are linked to the diterpenes.
- Sesterterpenes – Having five isoprene units and a molecular formula of $C_{25}H_{40}$, these compounds are rare relative to the other classes.
- Triterpenes – These compounds consist of six isoprene units, with a molecular formula of $C_{30}H_{48}$. The reductive coupling of two farnesyl pyrophosphate molecules lead to the formation of squalene, a linear triterpene. Squalene is then processed to lanosterol, the structural precursor to all steroids. The addition of a sugar moiety to a triterpene or triterpenoid leads to the formation of a saponin, which has been discussed in Section 2.2.3.
- Tetraterpenes – Tetraterpenes consist of eight isoprene units and have the molecular formula $C_{35}H_{56}$. Lycopene, gamma-carotene and α - and β -carotene are biologically important tetraterpenes.

- Polyterpenes – These compounds have variable amounts of isoprene units linked together to form long chains. Rubber is an example of a polyterpene.

(Terpene, 2007)

In 1987 Wallach (as cited in Dey & Harborne, 1997, p.418) proposed the isoprene rule which was later formulated into the biogenetic isoprene rule proposed by Ruzicka. This rule stated that all isoprenoids are made from a biologically active isoprene. This biologically active isoprene turned out to be isopentenyl pyrophosphate (IPP), which is an intermediate in the hydroxymethylglutaryl-CoA (HMG-CoA) reductase metabolic pathway, as well as in the non-mevalonate pathway, where it is synthesised from (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) by the enzyme HMB-PP reductase. The latter pathway is mainly utilised by bacteria, malaria parasites and the plastids of higher plants (Isopentenyl pyrophosphate, 2007).

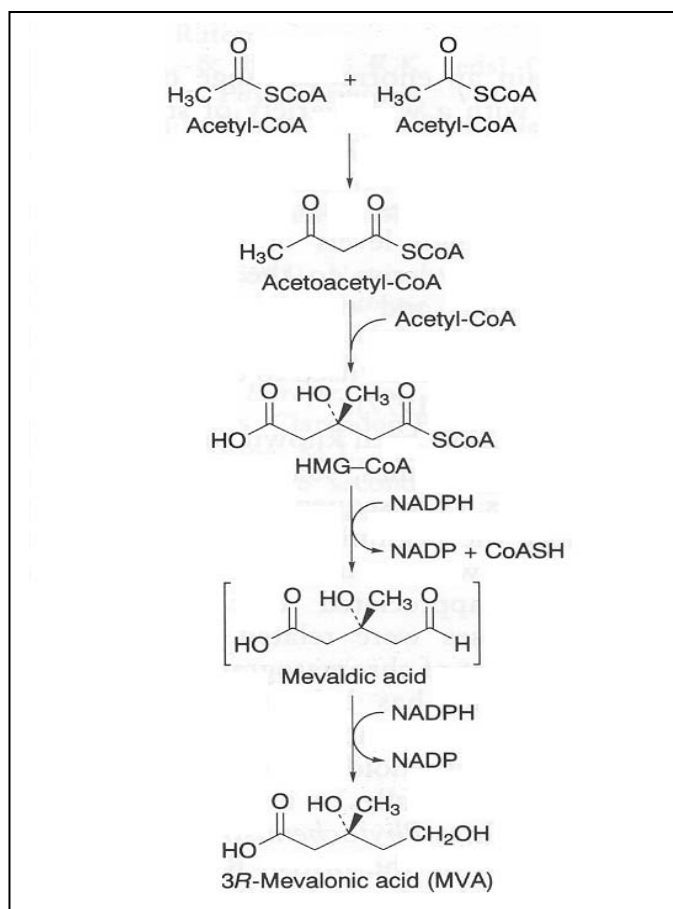


Figure 2.16: The conversion of acetoacetyl CoA to mevalonic acid (taken from Dey & Harborne, 1997, p.418).

During the HMG-CoA reductase pathway (Figure 2.16), two molecules of acetyl-CoA are combined to form acetoacetyl-CoA. Addition of a third acetyl-CoA leads to the formation of HMG-CoA. HMG-CoA is converted to mevalonic acid through the action of HMG-CoA reductase. During a three step process, mevalonic acid is converted to IPP which, through the working of IPP isomerase, is isomerised to dimethylallyl pyrophosphate (DMAPP). Either of IPP or DMAPP may be converted to a hemiterpene. Under the influence of prenyl transferase, DMAPP condenses with IPP to form geranyl pyrophosphate (GPP). GPP may be used for monoterpene synthesis or condensed with another IPP molecule to form farnesyl pyrophosphate (FPP). FPP, in turn, may be utilised in the synthesis of sesquiterpenes, may be extended in a chain fashion to form geranylgeranyl pyrophosphate (GGPP), or may undergo tail to tail dimerisation to form the triterpenes. GGPP may be utilised in the synthesis of diterpenes, undergo chain elongation to form geranylfarnesyl pyrophosphate (GFPP) or dimerise to form the tetraterpenes. GFPP may further undergo chain extension in the formation of polyprenyl pyrophosphates and polyterpenes, such as rubber (Figure 2.17) (Goodwin & Mercer, 1983, pp.423, 425).

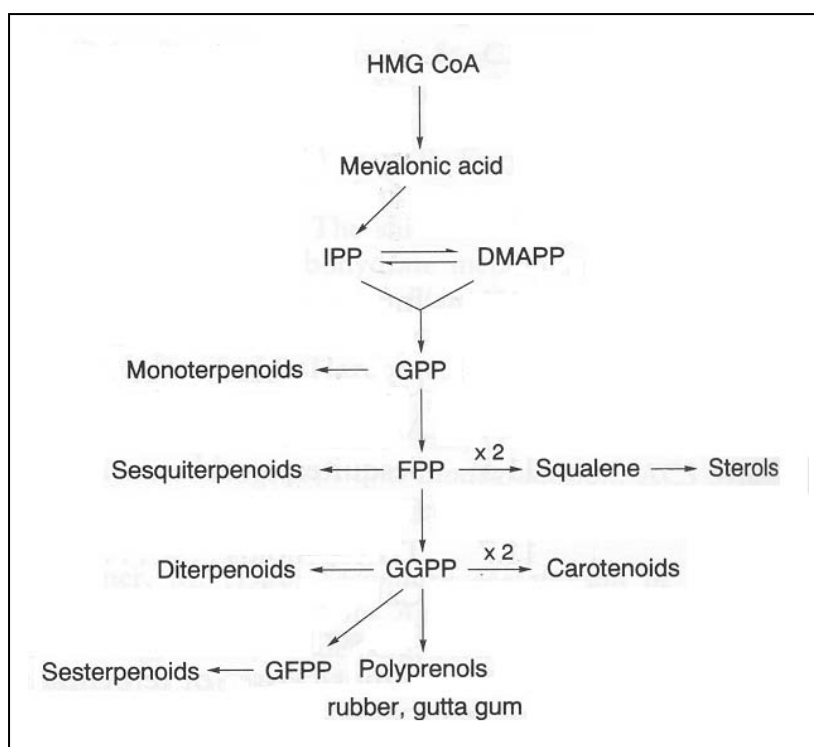


Figure 2.17: The general pathway of isoprenoid synthesis (taken from Dey & Harborne, 1997, p.418).

α -And β -thujone (Figure 2.20 and Figure 2.21), 1,8-cineole (Figure 2.22), camphor (Figure 2.24), α -pinene (Figure 2.25) and borneol (Figure 2.23), the major chemical constituents of the volatile oil of *A. afra* (Scott, Springfield & Coldrey, 2004, p.209; Van Wyk *et al.*, 1997, p.44), are monoterpene derivatives, as are most constituents of volatile plant oils. Thus, for the scope of this study, only the monoterpenes will be briefly discussed next.

2.2.4.1. Monoterpenes and their derivatives

Monoterpenes are components of essential oils, and are probably ubiquitous in higher plants. They are especially valued in the perfume industry and are also used as food flavourants (Dey & Harborne, 1997, p.419).

As previously stated, GPP is known to be the precursor molecule of monoterpene synthesis. Monoterpene cyclases (synthases), of which more than twenty have been identified, are the enzymes responsible for the conversion of GPP to cyclic, oxygenated monoterpenes during oxidation and reduction reactions (Dey & Harborne, 1997, p.419).

Monoterpenes are classified according to 38 types, and are divided into four broad structural categories:

- Acyclic
- Cyclopentanoid,
- Cyclohexanoid, which may either be mono-, bi- or tricyclic, and
- Irregular monoterpenes

(Dey & Harborne, 1997, p.419; Goodwin & Mercer, 1983, p.401)

An example of a monocyclic, cyclohexanoid monoterpene would be limonene, which is formed during the cyclisation of GPP (Figure 2.18) (Monoterpene, 2007)..

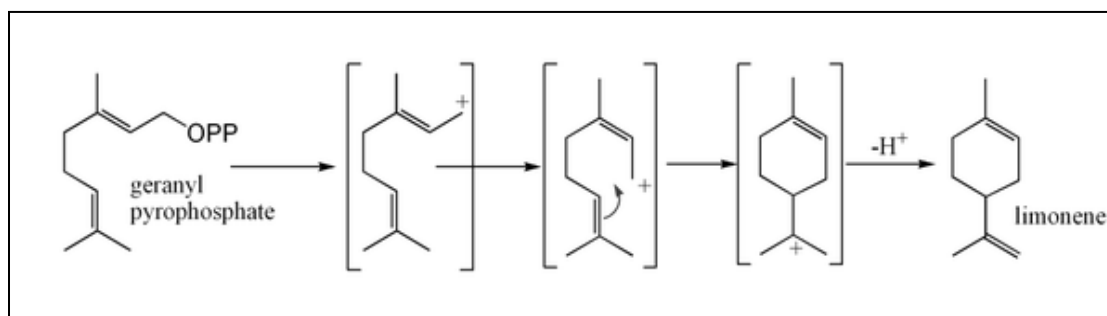


Figure 2.18: The conversion of GPP to limonene (taken from Monoterpene, 2007).

Pinene is an example of a bicyclic, cyclohexanoid monoterpene, and is formed during two sequential cyclisation reactions of GPP (Figure 2.19) (Monoterpene, 2007)

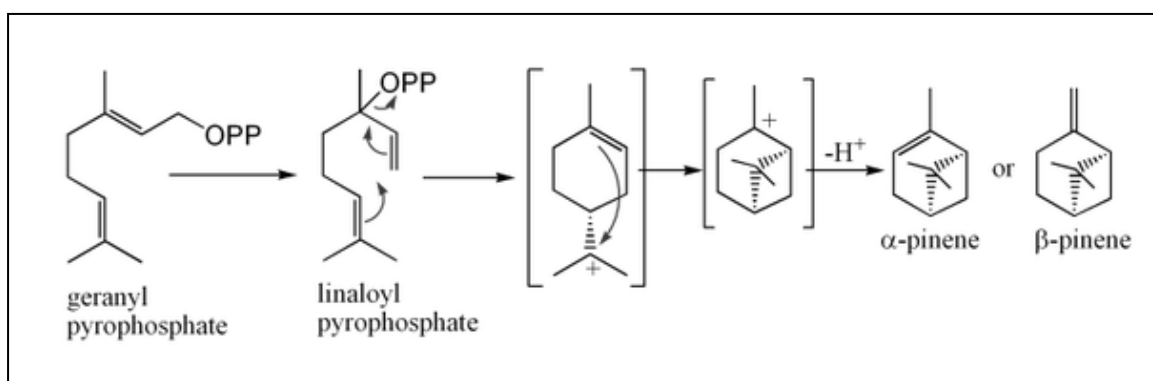


Figure 2.19: The conversion of GPP to pinene (taken from Monoterpene, 2007).

The properties of monoterpenes are dependent on the oxygen containing radicals bound to the carbon skeleton. Monoterpenes may be simple hydrocarbons, or they may occur as oxygenated derivatives, such as ketones, aldehydes, alcohols, oxides or phenols (Pengelly, 2004, p.87). Examples of these include:

- Ketones – Thujone and camphor
- Aldehydes – Geranial
- Alcohol – Borneol
- Oxide – Cineole
- Phenol – Thymol

- Hydrocarbon - α - and β -Pinene

2.2.4.1.1. Thujone

Thujone is classified as a bicyclic, monoterpenoid ketone, i.e. it is a monoterpene with a carbonyl group (C=O) connected to two carbon atoms. Two stereoisomeric forms are known to exist; (+)-3-thujone or α -thujone (Figure 2.20), and (-)-3-thujone or β -thujone (Figure 2.21) (Thujone, 2007).

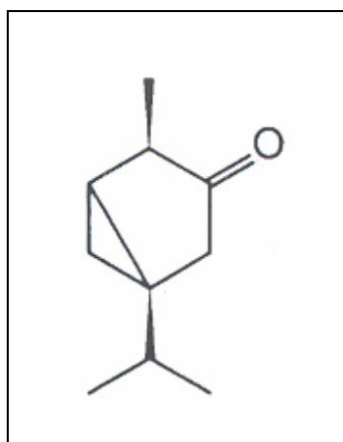


Figure 2.20: Structure of α -thujone (taken from Van Wyk *et al.*, 1997, p.44).

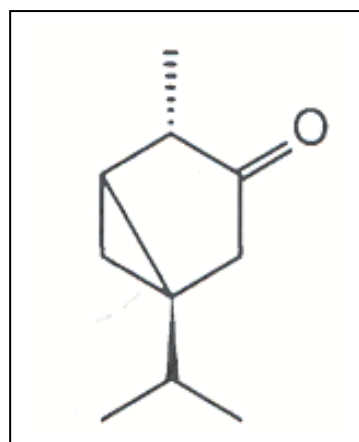


Figure 2.21: Structure of β -thujone (taken from Van Wyk *et al.*, 1997, p.44).

Thujone is probably best known for being a chemical constituent of the popular alcoholic drink absinth, of which *A. absinthium*, also known as European wormwood or Grand wormwood, is a main ingredient. The volatile oil of *A. absinthium* has been shown to contain both α - and β -thujone in varying ratios amongst different populations (Gambelunghe & Melai, 2002; Padosch, Lachenmeier & Kröner, 2006; Thujone, 2007).

Up until recently, it was believed that, due to their structural similarities, thujone and tetrahydrocannabinol (THC), the chemical responsible for the euphoric sensations brought on by cannabis usage, shared the same mechanism of action in the brain. Today this is known to be false. Thujone blocks the γ -aminobutyric acid type A (GABA_A) receptor chloride channel.

Inhibition of GABA receptor activation leads to neurons firing more rapidly and consequential muscle spasms (Thujone, 2007). This could possibly explain the convulsions which have been seen in individuals suffering from absinthism, a neurological syndrome characterised by hallucinations, epilepsy, convulsions and psychiatric illness, amongst others. Absinth consumption and absinthism is discussed in Section 2.5.1.

2.2.4.1.2. 1, 8-Cineole

1,8-Cineole (Figure 2.22), also known as cineole, cineol, limonene oxide, cajeputol, 1,8-epoxy-p-menthane, 1,8-oxido-p-menthane, eucalyptol, eucalyptole or 1,3,3-trimethyl-2-oxabicyclo(2,2,2)octane, is classified as a cyclic, monoterpenoid oxide or monoterpene ether. A secondary ring containing an oxygen atom is attached to the primary ring structure at positions 1 and 8.

Cineole is a major constituent in eucalyptus oil, and has a spicy, pleasant aroma. This has made it popular in flavourings, fragrances and cosmetics. It also has mucolytic properties and is used as an expectorant. It is almost universal in mouth washes and cough lozenges (Eucalyptol, 2007; Pengelly, 2004, p.98).

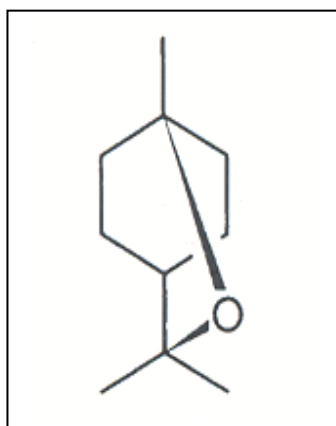


Figure 2.22: Structure of 1,8-cineole (taken from Van Wyk *et al.*, 1997, p. 44).

Furthermore, cineole has been shown to possess anti-inflammatory properties and to relieve pain. It has also been shown to have anticancer properties (Eucalyptol, 2007). Eucalyptus oil in general is known for its antimicrobial properties, while some are said to possess antiviral qualities as well. Oil rich in cineole and terpene hydrocarbons are said to be the most effective against influenza, for example (Pengelly, 2004, p.99).

2.2.4.1.3. Borneol and camphor

Borneol (Figure 2.23) is classified as a bicyclic, monoterpenoid alcohol, i.e. it has a hydroxyl group attached to the terpene skeleton. Alcohols rank amongst the most potent antimicrobial components of essential oils. Terpene alcohols are generally highly valued for their fragrance, healing properties and gentle reactions on skin and membranes. A well known example of a monoterpenoid alcohol is menthol, which is found in peppermint oil, and which is used in the pharmaceutical industry as well as in the food industry. Borneol is easily oxidised to the monoterpenoid ketone, camphor (Borneol, 2007, Pengelly, 2004, pp.90-92).

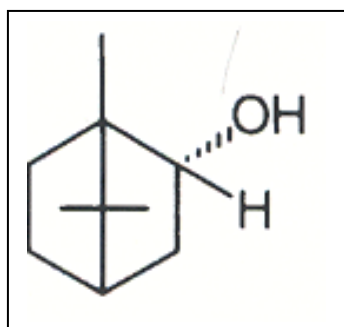


Figure 2.23: Structure of borneol (taken from Van Wyk *et al.*, 1997, p.44).

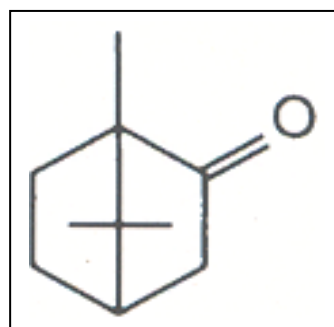


Figure 2.24: Structure of camphor (taken from Van Wyk *et al.*, 1997, p.44).

Camphor (Figure 2.24) generally occurs as a waxy, white to transparent solid with a strong aromatic odour. It occurs mainly in the wood of *Cinnamomum*

camphora, and is known to be a central nervous system stimulant. It is used in the medical industry for its mucolytic, antipruritic and rubifacient properties, and is an active ingredient in Vicks VapoRub®. Camphor is known to be toxic when ingested in large amounts, and may lead to seizures, confusion, irritability and neuromuscular hyperactivity. Non-medicinal uses of camphor include embalming, the manufacturing of fireworks, as a plasticizer for cellulose nitrite and as an insect repellent. In the Orient it is used in the flavoring of confectionary and culinary dishes (Camphor, 2007; Pengelly, 2004, p.97).

Camphor may be biosynthetically produced through the cyclisation of linaloyl pyrophosphate to bornyl pyrophosphate. This is then followed by hydrolysis to borneol, and oxidation to camphor (Camphor, 2007).

2.2.4.1.4. α -Pinene

α -Pinene (Figure 2.25) is one of two isomers of pinene, the other being β -pinene. It is classified as a bicyclic, monoterpene hydrocarbon, and lacks oxygen-containing functional groups. Pinenes are produced from geranyl pyrophosphate via cyclisation of linaloyl pyrophosphate. Pinenes are widely distributed in plants, and occur in high concentrations in oil of turpentine, which is derived from different species of *Pinus*. Medicinally, oil of turpentine is used as a rubifacient and linament in rheumatic disease. Pinene is also an important constituent of many culinary spices, including black pepper (Pengelly, 2004, p.90; Pinene, 2007).

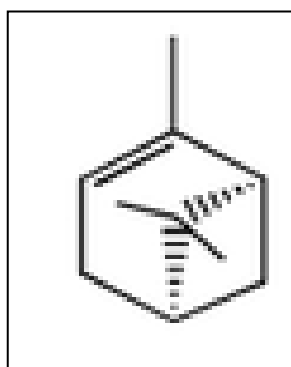


Figure 2.25: Structure of α -pinene (taken from Pinene, 2007).

Other terpenes which are important to mention relating to the scope of this study with regards to being chemical constituents of *A. absinthium*, include cadinine, camphene, myrcene, phelandrene and sabinene. These are briefly discussed next.

2.2.4.1.5. Cadinene

Cadinenes (Figure 2.26) are classified as bicyclic sesquiterpenes named after the Cade Juniper, the compound being first isolated from the oil of the wood (Cadinene, 2008). The yellow or light green oily liquid is used as a flavouring agent in baked goods, candy and chewing gum, as well as providing fragrance to cosmetics and detergents (Safety data for cadinene, 2005). The name cadinene has been used to describe any sesquiterpene with the cadalane carbon structure. However, a large number of double bond and stereochemical isomers exist, and the group of compounds have subsequently been divided into four subclasses according to stereochemistry of the isopropyl group, as well as the two bridgehead carbon atoms. These subclasses include cadinene, muurolene, amorphene and bulgarene (Cadinene, 2008).

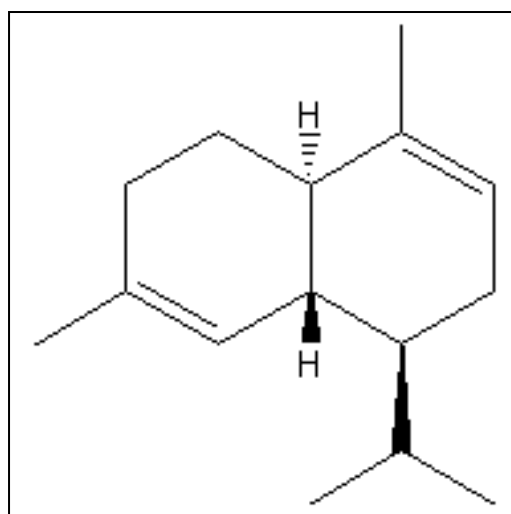


Figure 2.26: Structure of cadinene (taken from Cadinene, 2008).

2.2.4.1.6. Camphene

Camphene (Figure 2.27) is classified as a bicyclic monoterpene, and is produced in industry by the catalytic isomerisation of α -pinene. Camphene can be found in a variety of essential oils such as camphor oil, cypress oil, turpentine, citronella oil and ginger oil, and is used in fragrance preparation and as a flavouring agent in food (Camphene, 2008).

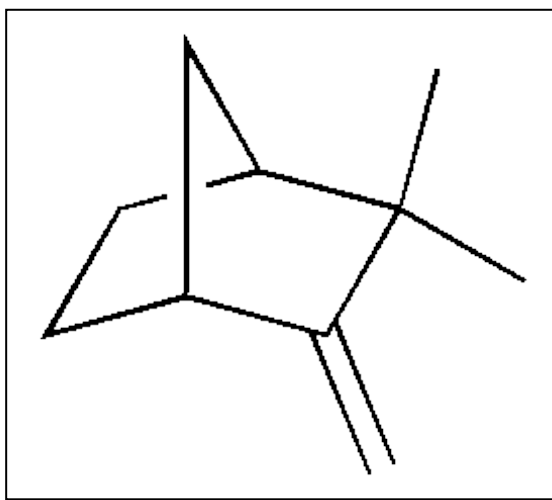


Figure 2.27: Structure of camphene (taken from Camphene, 2008).

2.2.4.1.7. Myrcene

Myrcene (Figure 2.28) is classified as a monoterpene, and is a constituent of the essential oils of bay, verbena and myrcia. The pyrolysis of pinene may also be used to synthesise this compound. Two isomers exist, namely α -myrcene and β -myrcene, only the latter being found in nature. Myrcene possesses a very pleasant odour, and is an extremely important compound used in the perfume industry, as well as in the synthetic preparation of menthol, citral, citronellol, geraniol, nerol and linalool.

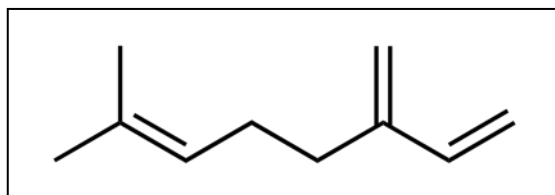


Figure 2.28: Structure of myrcene (taken from Myrcene, 2008).

2.2.4.1.8. Phellandrene

Phellandrene refers to two compounds, α -phellandrene (Figure 2.29) and β -phellandrene (Figure 2.30), which are cyclic monoterpenes and double bond isomers. α -Phellandrene possesses two endocyclic double bonds, while β -phellandrene has one endocyclic- and one exocyclic double bond. α -Phellandrene is found in the essential oils of *Eucalyptus radiata* and *Eucalyptus dives*, while β -phellandrene is a constituent of the essential oil of water fennel and Canada balsam oil. Phellandrenes are said to possess a pleasant aroma and are subsequently used in the fragrance industry (Phellandrene, 2008).

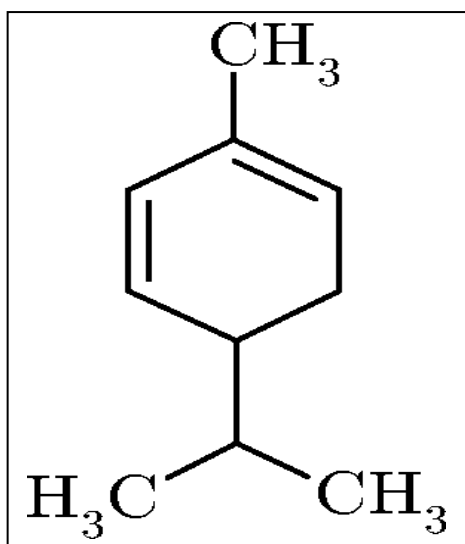


Figure 2.29: Structure of α -phellandrene (taken from Phellandrene, 2008).

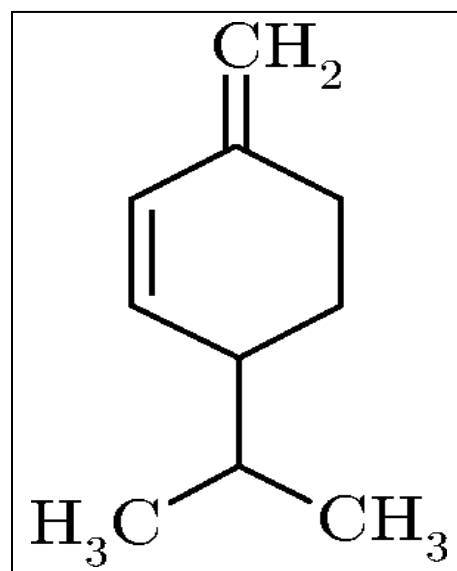


Figure 2.30: Structure of β -phellandrene (taken from Phellandrene, 2008).

2.2.4.1.9. Sabinene

Sabinene (Figure 2.31) is classified as a bicyclic monoterpene and has been isolated from a variety of plants. Its chemical structure is characterised by the fusion of a cyclopentane ring to a cyclopropane ring. Sabinene is a constituent of carrot seed oil and tea tree oil, and contributes to the spicy taste of black pepper (Sabinene, 2008).

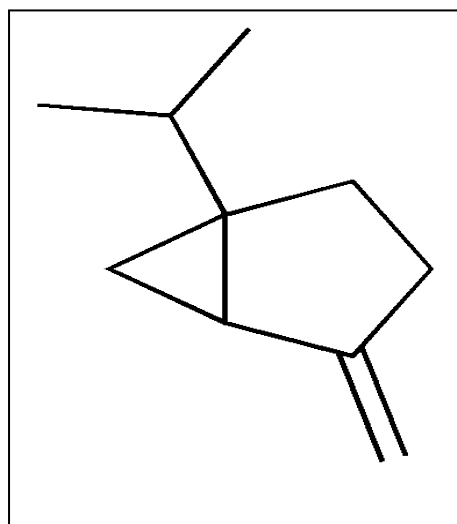


Figure 2.31: Structure of Sabinene
(taken from Sabinene, 2008).

2.3. *Geranium incanum* (Burm. f.)

2.3.1. The medicinal qualities and health supporting properties of *G. incanum* and its use as a traditional medicinal plant

Although information regarding the use of *G. incanum* as a medicinal plant and its active chemical compounds is limited in the literature, it is known that the plant, as well as other *Geranium* species, have been used by individuals to address a wide array of ailments.

G. incanum's vernacular Afrikaans name of *vrouebossie* (*vroue* = women; *bossie* = small bush or shrublet) is most probably derived from its use by the early Dutch Cape settlers to treat ailments of the genitourinary tract, particularly in females. These include bladder infections and cystitis, venereal diseases and menstrual related disorders. The plant may also be useful in aiding in the expulsion of afterbirth and the stimulation of lactation. *G. incanum* is also used in the treatment of various other disorders, including diarrhoea, colic, fever and bronchitis. A lotion may be prepared which can be used to treat dry, itchy skin, while individuals whom are prone to oily hair may benefit from rinsing the hair with an extract of the plant. Furthermore, the plant may also be effective in the treatment of dandruff and psoriasis of the scalp. Its properties as an anthelmintic have also been described (Mountain Herb Estate, 2006a; Schwegler, 2007; Scott & Springfield, 2004a, p.3; Scott *et al.*, 2004, p.192; Van Wyk *et al.*, 1997, p.134; Van Wyk & Gericke, 2000, p. 186; Watt & Breyer- Brandwijk, 1962, p.451)

Traditionally, an infusion of *G. incanum* leaves is made by adding a quarter cup fresh leaves to 180 ml boiling water. This is then left for ten minutes, strained and drunk while warm. For the treatment of intestinal parasites, a teacupful is taken once daily on an empty stomach for ten days. One teacupful is taken three times daily for three days to relief colic, bladder infections and diarrhoea, while for the treatment of dysmenorrhoea, one teacupful is taken three times per day for two to three days (Schwegler, 2007; Scott & Springfield, 2004a, p.4; Van Wyk *et al.*, 1997, p.134).

During a study conducted by Scott *et al.* (2004, p.198) on the pharmacognostical properties of several plants used as traditional medicines in South Africa, it was found that the aqueous extract of *G. incanum* expressed inhibition to the growth of *Staphylococcus aureus*, but no significant results were obtained regarding the inhibition of *Pseudomonas aeruginosa*, *Candida albicans* or *Mycobacterium smegmatis*. A separate study conducted by Treurnicht (as cited in Scott *et al.*, 2004, p.208), showed cytotoxicity at all concentrations of aqueous extracts of *G. incanum* to HeLa (human cervical cancer) cells, Vero (African Green monkey kidney epithelial)

cells, Jurkat E6.1 (human T-cell lymphoblast) cells, AA-2 (human CD4⁺ lymphocyte) cells and CEM-SS (human T-lymphoblast) cells. This may have been due to the presence of tannins. Similar extracts also exhibited a reduction in the infectivity of both Coxsackie B2 virus and Herpes Simplex Virus 1 during an antiviral cell culture assay.

This, in conjunction with the historical use of the plant by the early Dutch Cape settlers as well as the use of the plant by indigenous South African peoples such as the Southern Sotho, warrants further investigation into the medicinal properties of *G. incanum* (Mountain Herb Estate, 2006a; Schwegler, 2007; Watt & Breyer-Brandwijk, 1962, p.450).

2.3.2. Botanical classification and vernacular names

The botanical classification of *G. incanum* is as follows:

Kingdom	: Plantae
Phylum	: Magnoliophyta
Class	: Magnoliopsida
Order	: Geraniales
Family	: Geraniaceae
Genus	: <i>Geranium</i>
Species	: <i>G. incanum</i>

Vernacular names: Carpet Geranium [English]; Vrouebossie, Amarabossie, Bergtee, Horlosies [Afrikaans]; Ngope-sethsoha, Tlhako [Sotho]; Tlako [Xhosa]; Mlako [Tswana].

(Scott & Springfield, 2004a, p.1; Van Wyk *et al.*, 1997, p.134)

It should be noted that many individuals often refer to different species of the genus *Geranium* as *Pelargoniums* and vice versa. When botanical collectors from the United Kingdom collected different species of the genus *Pelargonium*, which are indigenous to South Africa, a few centuries ago, it was noted that the South African *Pelargoniums* were in many aspects similar to the different species of *Geranium* being cultivated in Europe at the time. This led to the South African plants being erroneously referred to as *Geraniums*. This miss-nomenclature seems to have endured, and today one may walk into a nursery enquiring about *Geraniums*, only to be shown a South African *Pelargonium*. Thus, in the South African context, *Geraniums* are almost always in truth *Pelargoniums*. However, *G. incanum*, which is indigenous to South Africa, is classified as a true *Geranium*, and should not be confused with the genus *Pelargonium* (*Geranium* or *Pelargonium*, n.d.).

2.3.3. Macroscopical morphology

G. incanum is a low spreading, multiple branching, perennial shrublet, roughly 10-25 cm in height. The plant possesses a long, thickened tap root, while stems are slender. The leaves are borne on slender petioles, each up to 5 cm wide and up to 7 cm long. Leaves have 3 to 7 lobes, each lobe being pinnatisect and covered in fine silver hair which lies flat



Figure 2.32: *G. incanum*
(taken from Scott & Springfield, 2004a, p.1).

against the leaf surface, unlike other *Geranium* species in which case the hair is spreading. The plant normally flowers between September and November, with flowers being white to pale pink with dark veins or light violet to magenta in colour, and up to 4 cm in diameter. Flowers are borne on slender pedicels which are also covered in fine silver hair. The characteristic fruit is an elongated capsule which resembles the bill of a stork. Due to the similarities between different species of *Geranium*, confusion has arisen in the past as to the medicinal records of the different species. Subsequently, *G. incanum* has been divided into two varieties; var. *incanum*, which possesses the white to pale pink flowers, and var. *multifidum*, which bears the violet to magenta coloured flowers (Scott & Springfield, 2004a, p.1; Van Wyk *et al.*, 1997, p. 134).

2.3.4. Geographical distribution

G. incanum commonly occurs on flats, hill slopes and dunes along the Southern coasts of the Western and Eastern Cape provinces of South Africa. Although both var. *incanum* and var. *multifidum* have been recorded from the Cape peninsula eastwards towards Knysna, only the latter occurs in the Eastern Cape as far as Port Alfred (Scott & Springfield, 2004a, p.2).

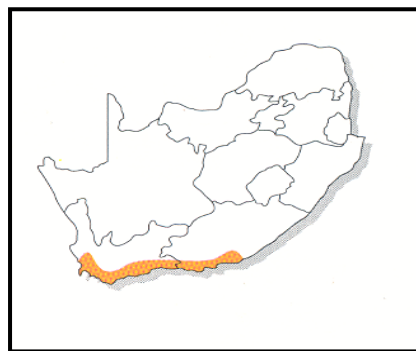


Figure 2.33: South African geographical distribution of *G. incanum*, the orange area indicating the regional occurrence (taken from Van Wyk *et al.*, 1997, p.134).

2.3.5. Major chemical constituents

According to Van Wyk *et al.* (1997, p.137) as well as Watt and Breyer-Brandwijk (1962, p.451), the leaves of *G. incanum* are said to contain tannins, of which geraniin (Figure 2.11) is the best known example, while Scott *et al.* (2004) detected the presence of saponins. Different flavonoids have also been detected in *Geranium* species, but other than this, very little or no published information on the phytochemistry of *G. incanum* seems to be available (Van Wyk *et al.*, 1997, p.137). These and other phytochemicals have been discussed in Section 2.2.

2.4. *Artemisia afra* (Jacq. ex Willd.)

2.4.1. The medicinal qualities and health supporting properties of *A. afra* and its use as a traditional medicinal plant

A. afra is one of the oldest, most well known and widely used traditional medicinal plants, not only in South Africa, but in many parts throughout Africa. It is popular amongst indigenous tribes of Africa up to this day, and early European colonists also recognised its healing qualities. This may have been

due to the plant's resemblance to European wormwood (*A. absinthium*), which is also known to have various medicinal properties. Jan van Riebeeck is said to have recorded the use of *A. afra* in his diary to treat an array of ailments ranging from jaundice to gout. (Mountain Herb Estate, 2006b; Roberts, 1999, pp.226-227; Van Wyk & Gericke, 2000, p.142; Van Wyk *et al.*, 1997, p.44; Watt & Breyer-Brandwijk, 1962, pp.199, 201).

Current literature indicates that *A. afra* is used to treat a diverse and great amount of ailments. These include coughs, colds, bronchitis, croup, whooping cough, sore throat, earache, gum- and mouth ulcers, toothache, indigestion and constipation, poor appetite, colic, heartburn, flatulence, menstrual- and childbirth related pains, neuralgia, inflammation, sprains and strains, haemorrhoids, measles, fever, rashes, bites and stings, pimples and boils, amongst others (Herbal Africa, n.d.; Jäger, 2003, p.596; Roberts, 1999, pp.226-227; Scott & Springfield, 2004b, p.3; Thring & Weitz, 2005, p.265; Van Wyk & Gericke, 2000, p.142; Van Wyk *et al.*, 1997, p.44; Vincent, Mabusela, Folk, Weitz, Rottinghaus, Mawhinney, Khan, Lee, Wenzel, Applequist, Gqaleni & Johnson, n.d.; Watt & Breyer-Brandwijk, 1962, pp.199, 201).

Furthermore, McGaw, Jäger and Van Staden (2000) found ethanol extracts of *A. afra* to be inhibitory to the growth of *Bacillus subtilis* and *S. aureus*, while Rabe and Van Staden (1997, p.84) found similar results when methanol extracts were used. Gundidza (as cited in Herbal Africa, n.d.) found the essential oil of *A. afra* to be inhibitory to the growth of *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus parasiticus*, *C. albicans*, *Alternaria alternate*, *Geotrichum candidum* and *Penicillium citrium*. *A. afra* is also used by individuals in the treatment of diabetes mellitus (Thring & Weitz, 2005, p.265; Van Wyk & Gericke, 2000, p.142; Vincent *et al.*, n.d.; Watt & Breyer-Brandwijk, 1962, pp.201) and is said to possess activity against *Plasmodium falciparum* (Scott & Springfield, 2004b, p.3; Vincent *et al.*, n.d.), *Mycobacterium tuberculosis* (Mukinda & Syce, in press; Scott & Springfield, 2004b, p.3; Vincent *et al.*, n.d.; Watt & Breyer-Brandwijk, 1962, pp.201) and Human Papilloma Virus (Vincent *et al.*, n.d.).

Van Wyk and Gericke (2000, p.142), Hutchings, Scott, Lewis and Cunningham (as cited in Mukinda & Syce, in press) as well as Scott and Springfield (2004b, p.3) all state *A. afra* to possess anti-histamine, narcotic and analgesic qualities. The Medical Research Council and SA Health Info (as cited in Mukinda & Syce, in press) and Guantai and Addae-Mensah (as cited in Herbal Africa, n.d.) claim *A. afra* to have a hypotensive effect and thus may be useful in the treatment of hypertension. Burits (as cited in Herbal Africa, n.d.) found *A. afra* essential oil to be an effective hydroxyl radical scavenging agent, while Stafford *et al.* (2005) found *A. afra* to have anti-convulsive properties and GABA_A – benzodiazepine receptor-binding activity.

Finally, SA Health Info (as cited in Mukinda & Syce, in press) report an aqueous extract of *A. afra* to be cytotoxic to HeLa cells *in vitro*, while a study conducted by Treurnicht (as cited in Scott *et al.*, 2004, p.210) found aqueous extracts of *A. afra* to be cytotoxic to HeLa, Vero, Jurkat E 6.1, AA-2 and CEM-SS cells.

The methods in which the plant is utilised for its medicinal qualities are as varied as the conditions for which it is used. Infusions or decoctions are often made, which is sweetened with sugar or honey and drunk as a tea or used as a mouthwash or gargle. An infusion may also be used as a wash for skin ailments. The Tswana are reported to roll fresh leaves and insert these into the nose to relief headache and congestion, while some tribes are said to smoke the leaves to relief respiratory conditions (Roberts, 1990, p.226). Some individuals place a fresh, rolled leaf onto a tooth to relief toothache (Watt & Breyer-Brandwijk, 1962, p.201). The Zulus are reported to use the plant as an enema for the treatment of intestinal worms (Roberts, 1990, p. 226). The Xhosa inhale the vapours after adding leaves to boiling water to relief colds and flu (Bhat & Jacobs, 1995, p.8). Females may also use the vapour as a steam in the genital area to relief menstrual and childbirth related pains (Watt & Breyer-Brandwijk, 1962, p.201). Furthermore, a poultice is sometimes made using heated leaves, which is then applied to strains, sprains and areas of inflammation (Scott & Springfield, 2004b, p.3). Roberts (1990, p.227) as well as Watt and Breyer-Brandwijk (1962, p.201) report that

brandy, sugar, ginger, thyme, rosemary, mint, chamomile, *Osmitopsis astericoides* or *Eucalyptus globulus* are added to preparations of *A. afra* and is then used as a tincture for the relief of respiratory- and gastrointestinal ailments.

There does not appear to be any precise traditional method regarding the preparation of an infusion of *A. afra*. Rather, these vary depending on the strength required and the condition which is to be treated. Roberts (1990, p. 226) states that one quarter cup of fresh leaves should be added to one cup of boiling water, left for 5 minutes and then strained. Scott and Springfield (2004b, p.4) states that 14 g fresh leaves should be infused in 1 litre boiling water and strained when cold.

From the above information it is clear that *A. afra* plays a very important role in the traditional indigenous medicinal knowledge of South Africa, and deserves further investigation.

2.4.2. Botanical classification and vernacular names

The botanical classification of *A. afra* is as follows:

Kingdom	: Plantae
Phylum	: Magnoliophyta
Class	: Magnoliopsida
Subclass	: Asteridae
Order	: Asterales
Family	: Asteraceae
Genus	: <i>Artemisia</i>
Species	: <i>A. afra</i>

Vernacular names: Wild wormwood, African wormwood [English]; Wilde als, Als, Alsem [Afrikaans]; Zengana [Southern Sotho]; Lengana [Sotho & Tswana]; Mhlonyane [Zulu]; Umhlonyane [Xhosa & Zulu] (Roberts, 1990; p.226; Van Wyk *et al.*, 1997, p.44; Van Wyk & Gericke, 2000, p.142)

2.4.3. Macroscopical morphology

A. afra is an erect perennial shrub which may reach a height of up to two meters, and may be easily identified by its characteristic, highly aromatic odour. The aerial parts of the plant are deciduous in areas experiencing cold winter months and die back, but rapidly regenerate from the base during spring. The finely divided feathery, ovate leaves are up to 8 cm long and 4 cm wide, and are silver-green in colour due to the presence of fine hairs. Each possess a pectinated midrib, and have a smooth or glandular punctated upper surface and canescent lower surface. Petioles may be up to 2 cm long and dilated towards the base, where a pair of simple or divided stipules are found. The ultimate segments are described as being linearly shaped with a smooth or toothed margin, and may be up to 1 cm long and 0.2 cm wide. The plant normally flowers between January and June, with the flowers being small and inconspicuous, and yellow in colour. These flowers are borne on the end of branches in globose capitula which are approximately 0.3 cm in diameter. The fruit is approximately 0.1 cm in length, slightly curved and three-angled, and have a silver to white coating (Komperlla, 2004, p.4; Scott & Springfield, 2004b, p.1; Van Wyk *et al.*, 1997, p.44).

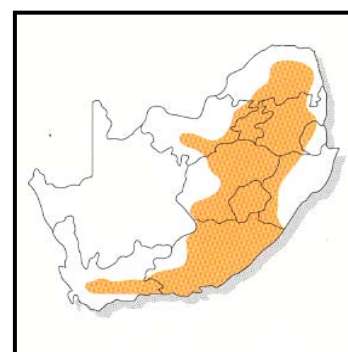


Figure 2.34: *A. afra*
(adapted from Mukinda, 2005, p.21).

2.4.4. Geographical distribution

A. afra is widespread throughout most of Africa, and is recognised and used by many African people for its medicinal qualities, including individuals from South West Africa (Namibia), Southern Rhodesia (Zimbabwe), Kenya, Tanganyika and Uganda (Watt & Breyer- Brandwijk, 1962, p.201). *A. afra* is widespread throughout South Africa, except the Northern Cape province, and may also be found in Lesotho and Swaziland and further North as far as Ethiopia in East Africa. The plant is usually found in mountainous habitats along streams and forest margins (Scott & Springfield, 2004b, p.2; Van Wyk *et al.*, 1997, p.44).

Figure 2.35: South African geographical distribution of *A. afra*, the orange area indicating the regional occurrence (taken from Van Wyk *et al.*, 1997, p.44).



2.4.5. Major chemical constituents

The known chemical constituents that have been identified in *A. afra* can be divided into two groups, those present in plant material, and those which are found in the essential oils. Microchemical tests conducted by Scott *et al.* (2004, p.209) indicated the presence of tannins and saponins in plant materials. Silbernager, Spreitzer and Buchbauer (as cited in Scott *et al.*, 2004, p.209) identified the triterpenes α - and β -amyrin and friedelin, as well as ceryl cerotate and n-nonacosane, which are alkanes, from the leaves of *A. afra*. Ten guaianolides and five glaucolides have also been detected during an analysis of the sesquiterpene lactones of *A. afra* by Jakupovic, Klenmeyer, Bohlmann and Graven (as cited in Scott *et al.*, 2004, p.209).

Furthermore, Wollenweber, Mann and Valant-Vetschera (as cited in Scott *et al.*, 2004, p.209) indicated the presence of two luteolin methyl ethers from the leaf exudate flavonoids, while Waithaka (as cited in Komperlla, 2004, p.5) indicated the presence of flavonoids such as luteolin and apegenin (both

flavones), kaempferol and quercetin (both flavonols) as well as hesperetin (a flavanone), which have been linked to the medicinal qualities of the *Artemisia* species. Examples of this include the *in vitro* GABA_A-benzodiazepine receptor activity of the flavonoids hispidulin and cirsilineol, which have been isolated from *Artemisia herba-alba* Asso traditionally used in Turkey (Stafford *et al.*, 2005), and the antimicrobial activity of flavonoids from *Artemisia annua* (Komperlla, 2004, p.6). Flavonoids isolated from *Artemisia copa* exhibited anti-inflammatory, analgesic and antitumour activities, while the anti-oxidant properties of *A. annua* are associated with the flavonols contained in the aerial parts of the plant (Mukinda, 2005, p.27).

The geographical variability of phytochemical compounds is a well known fact. From the results of various studies, Scott *et al.* (2004, p.209) concluded that the analysis of essential oils of *A. afra* leaves from different South African populations vary considerably in composition, with the major components being α - and β -thujone (Figure 2.20 and Figure 2.21, respectively), 1,8-cineole (also known as eucalyptol, Figure 2.22), camphor (Figure 2.24) and α -pinene (2.25). Van Wyk *et al.* (1997, p.44) stated borneol (Figure 2.23) to be another major component of the essential oil of *A. afra*, while other compounds present, but whose contribution to biological activity remains unknown, include terpenoids of the eudesmadien and germacratien types, coumarins and acetylenes. Goodson (as cited in Watt & Breyer-Brandwijk, 1962, p.202) further isolated triacontane (an alkane), scopoletin (a coumarin derivative), quebrachitol (methyl 1-inositol) and a wax ester, which he thought to be ceryl cerotate. Other minor compounds present in the essential oil of *A. afra* include δ -cadinene, α -terpineol, *e*-chrysanthenyl acetate, camphene (Figure 2.27), β -pinene, trans- β -ocimene and myrcene (Figure 2.28) (*Artemisia afra*, 2007).

Flavonoids, tannins and saponins, as well as the major chemical constituents which have been isolated from the essential oil of *A. afra*, being α - and β -thujone, 1, 8-cineole, camphor, α -pinene and borneol, have been discussed in Section 2.2.

2.5. Artemisia absinthium

2.5.1. The medicinal qualities and health supporting properties of A. absinthium and its use as a traditional medicinal plant

A. absinthium, also commonly known as wormwood, has been recognised since ancient times for its medicinal qualities. It was believed by the ancient people that wormwood could counteract the poisonous effects of hemlock and mushrooms, and could be used to treat the bite of the seadragon (Grieve, 2008). Wormwood has also been used as an antihelmintic for centuries, and reference to the extremely bitter plant was made in Egyptian papyri, early Syrian texts, and can also be found in many parts of the Bible, e.g. Deuteronomy 29:18, Proverbs 5:4, Jeremiah 9:15 and 23:15, Lamentations 3:15, Amos 5:7 and Revelation 8:11 (Quinlan, Quinlan & Nolan, 2002; Williamson, 2003, p.460).

Apart from its use as a vermifuge, wormwood is probably best known as the major constituent in the controversial alcoholic drink absinth. For a long time it was believed that the chronic consumption of absinth and more specifically the thujones present in the drink, gave rise to the syndrome absinthism. Thujones have been discussed in Section 2.2.4.1.1. Popular in the 19th century throughout Europe and enjoyed by famous individuals such as Arthur Rimbaud, Toulouse-Lautrec, Degas, Monet, Baudelaire, Picasso, Hemingway, Oscar Wilde and Vincent van Gogh, absinth was banned in many European countries and the USA for this reason. Other countries imposed limits on the allowable amount of thujone present in the drink. Absinthism is mainly characterised by addiction, acute visual and auditory hallucinations, gastrointestinal problems, insomnia, brain damage, epilepsy, convulsions, psychiatric illness and coma (Gambelunghe & Melai, 2002; Padosch *et al.*, 2006; Thujone, 2007).

Gambelunghe and Melai (2002), and Padosch *et al.* (2006), concluded that absinthism is probably not related to the thujone content of absinth, but rather to the extremely high alcohol content, which in some forms of absinth may be

as high as 50%. Padosch *et al.* (2006) stated that absinthism can not be exactly distinguished from the effects of chronic alcoholism. Adulterants which were often used in historical cheaper version of the liquor, such as copper sulfate and antimony chloride, are also believed to have contributed to the observed symptoms (Padosch *et al.*, 2006). Absinth is currently experiencing resurgence in popularity amongst young people, to whom it is being advertised as having spiritual elucidation and aphrodisiac stimulation properties, and has been legalised in many countries from which it was previously banned (Gambelunghe & Melai, 2002; Lachenmeier, Emmert, Kuballa & Sartor, 2006; Padosch *et al.*, 2006; Van Wyk & Gericke, 2000).

Today, wormwood is used in many cultures around the world in the treatment of various maladies. Usage of the plant as a vermifuge or antihelmintic is still popular (*Artemisia absinthium*, 2007; Gambelunghe & Melai, 2002; Grieve, 2008; Guarrera, 1999; Guarrera, 2005; Quinlan *et al.*, 2002; Uzun, Sariyar, Adsersen, Karacok, Ötük, Oktayoglu & Pirildar, 2004; Williamson, 2003, p.459). Other uses include the treatment of dyspepsia (Langone Medical Center, 2008), colds and rheumatism (Williamson, 2003, p. 453), esophageal reflux (Langone Medical Center, 2008), irritable bowel syndrome (Langone Medical Center, 2008), indigestion, flatulence and gastric pain (*Artemisia absinthium*, 2007; Gambelunghe & Melai, 2002; Grieve, 2008), inflammation and joint pain (Guarrera, 2005; Langone Medical Center, 2008), anorexia (Guarrera, 2005; Langone Medical Center, 2008) hypertension and cardiac disease (Eddouks, Maghrani, Lemhadri, Ouahidi & Jouad, 2002; Williamson, 2003, p.453), as well as diabetes mellitus (Andrade-Cetto & Heinrich, 2005; Eddouks *et al.*, 2002), insomnia, epilepsy and menstrual problems (Langone Medical Center, 2008). Wormwood is also used as a tonic (*Artemisia absinthium*, 2007; Grieve, 2008; Williamson, 2003, p.453), antiseptic (*Artemisia absinthium*, 2007; Langone Medical Center, 2008; Williamson, 2003, p.453), carminative and febrifuge (*Artemisia absinthium*, 2007; Williamson, 2003, p.453), antispasmodic (Williamson, 2003, p.453) and to relief pain during childbirth (Williamson, 2003, p.453).

A study conducted by Omer, Krebs, Omer and Noor (2007) showed wormwood to have a steroid sparing effect in patients with Crohn's disease, and could be used as a supplement in the treatment of the disorder. Furthermore, wormwood extract has been shown to exhibit antiviral activity towards Herpes Simplex virus 1 & 2, Epstein-Barr virus, Cytomegalovirus, Varicella-Zoster virus and Human Herpes virus 6 in monkey kidney cells (Vero-cells) and human epithelial type 2 (HEp-2) cells (Omer *et al.*, 2007). Other studies showed wormwood aqueous extract to be antipyretic in rabbits, exhibit choleric effects in humans, have anticholinesterase activity *in vitro* as well as having hepatoprotective properties against toxicity caused by paracetamol in rats (Williamson, 2003, p.459). The methanol extract of *A. absinthium* has also exhibited antimicrobial activity, ranging from 51.5% to 25.0% inhibition of growth, against strains of *Escherichia coli*, *Shigella sonnei*, *Shigella flexneri* and *Salmonella spp.*, with an aqueous extract showing weaker antimicrobial activity (Alanis, Calzada, Cervantes, Torres & Ceballos, 2005).

A wide array of methods employed in the preparation of wormwood extracts for medicinal use by humans have been reported, and it would appear that the mode of use is dependent on geographical location and culture it is being used in, as well as the ailment for which it is used. Flück and Jaspersen-Schib, (1941/1976, p.162) states that for the treatment of anorexia, poor digestion and colic, an infusion can be prepared by adding 1-2 teaspoons full of chopped plant material to one litre of cold water. Grieve (2008) states that wormwood tea, prepared by infusing 28 g of the plant material in approximately 500 ml water for 10 to 12 minutes and taken in wineglassful doses, will relieve melancholia and jaundice, in addition to being a good stomachic. The addition of a fixed alkaline salt to the infusion confers strong diuretic properties to the preparation. The people of central Italy consume an infusion prepared from the leaves of wormwood to stimulate appetite, while a decoction made from the aerial parts of the plant is used as an antiemetic. The aerial parts of the plant are also used, after prolonged decoction, to prepare a poultice which is applied to inflamed tendons. In Turkey, the aerial parts of the plant are infused and drunk as a tea, which is said to provide relief

from stomach disorders and flu. The infusion also acts as a tonic, vermifuge and appetite stimulant, and is believed to strengthen the immune system after flu. It is also used to ease childbirth as it causes uterine contractions (Uzun *et al.*, 2004).

The use of *A. absinthium* through the centuries by many cultures across the globe for the treatment of various ailments is an indication of the plant's well recognised medicinal properties. However, further scientific investigation into the therapeutic potential of the plant needs to be made which, judging by previous studies done and indication of the usage of the plant in the literature, may very well produce promising results.

2.5.2. Botanical classification and vernacular names

The botanical classification of *A. absinthium* is as follows:

Kingdom	: Plantae
Phylum	: Magnoliophyta
Class	: Magnoliopsida
Subclass	: Asteridae
Order	: Asterales
Family	: Asteraceae
Genus	: <i>Artemisia</i>
Species	: <i>A. absinthium</i>

Vernacular names: Wormwood; Absinthe; Absinthe Wormwood; Grand Wormwood; True Wormwood; Madderwort; Green Ginger; Absinthium [English]; Lapsent [Dominica]; Ascenzo, Szenzo, Ascenza or Ascenze [Italy]; Pelin Otu (Turkey), Ajenjo [Spain & Mexico](Andrade-Cetto & Heinrich, 2005; Gambelunghe & Melai, 2002; Guarrera, 1999; Guarrera, 2005; Quinlan *et al.*, 2002; Uzun *et al.*, 2004; Wormwood, n.d.)

2.5.3. Macroscopical morphology

A. absinthium is a herbaceous perennial growing to a height of 0.8-1.2 meters, and rarely exceeds 1.5 meters. The root system is hard, woody and rhizomatous. The stems appear silver-green in color and are straight, grooved and branched. Leaves are spirally arranged and covered in fine silky silver-white hair, with a grey-green appearance on top and white below. They may be obovate or lanceolate, entire or



Figure 2.36: *A. absinthium* (taken from Colorado Noxious Weeds ID Guide, 2006).

toothed and are pinnately divided, possessing tiny oil producing glands. Basal leaves may be bipinnate or tripinnate and up to 25 cm long with long petioles, while cauline leaves are less divided, 5-10 cm long and with shorter petioles. The uppermost leaves may be simple or sessile. Flowers are small, greenish-yellow and tubular without a pappus and clustered in globular bent-down capitulae. These are clustered on leafy and branched panicles. The fruit is described as a small achene. The plant carries flowers from summer to early autumn, and pollination is anemophilous (Artemisia absinthium, 2007; Williamson, 2003, p.459).

2.5.4. Geographical distribution

A. absinthium is not indigenous to South Africa, but can be found growing naturally throughout many regions of the world. Native to Europe, it was first introduced to the United States of America in 1841, and today it is naturalised across the USA and Canada. The plant is also found in North Africa and

Western Asia, but may grow as far East as Siberia. It primarily grows in disturbed sites in grasslands and pastures, as well as in uncultivated places, on walls, dry rocks and on roadsides (Carey, 1994; Flück and Jaspersen-Schib, 1941/1976, p.162; Grieve, 2008).

2.5.5. Major chemical constituents

With both *A. absinthium* and *A. afra* being of the same genus of *Artemisia*, similarities exist within the major chemical composition of both plants. Grieve (2008) states the major chemical constituents of the essential oil of *A. absinthium* to comprise of thujones, thujyl alcohol, cadinene, phellandrene and pinene, while absinthin, absinthic acid and tannins can be found within the plant material itself. Anabsinthin has also been found in plant materials (Gambelunghe & Melai, 2002). Kordali, Aslan, Çalmaşur and Cakir (2006) describes the essential oil as comprising of 1,8-cineole, camphor, borneol, terpinen-4-ol, p-cymen-8-ol, α -terpineol, β -selinene, geranyl isobutyrate, (Z)-sesquisabinenehydrate, caryophyllene oxide, chamazulene, nuciferol propionate and nuciferol butanoate. Lopes-Lutz, Alviano, Alviano and Kolodziejczyk (2008) investigated the chemical composition of the essential oil of *A. absinthium* and found the major constituents to be myrcene, trans-thujones and trans-sabinyl acetate. A detailed description of the chemical composition of *A. absinthium* is also given by Williamson (2003, p.459), including the volatile oil consisting of α - and β -thujones, thujyl alcohol, azulenes, bisabolene, cadinene, camphene, sabinene, trans-sabinylacetate, pinene and phellandrene. Sesquiterpene lactones to be found in the plant include artabsin, artabin, absinthin, anabsinthin, artemetin, arabsin and artemolin. Other compounds include various acetylenes, flavonoids, phenolic acids and lignans.

Thujones (Figure 2.20 and Figure 2.21), 1,8-cineole (Figure 2.22), camphor (Figure 2.24), borneol (Figure 2.23) and pinene (Figure 2.25), cadinene (Figure 2.26), camphene (Figure 2.27), myrcene (Figure 2.28), phellandrene (Figure 2.29 and Figure 2.30), and sabinene (Figure 2.31), which are the

major chemical constituents of *A. absinthium*, have been discussed in Section 2.2.4.1.

2.6. Cancer

It is well known that cancer is second only to cardiovascular disease as a natural cause of death, with an incidence of over 6 million cases reported annually across the globe (Srivastava, Negi, Kumar, Gupta & Khanuja, 2005). The World Cancer Report of 2003 (as cited in World Health Organisation, 2007a) stated that during the year 2003, of the 56 million fatalities that occurred worldwide during the year 2000, cancer was responsible for 12% of these, with 5.3 million males and 4.7 million females developing neoplasms, which claimed the lives of 6.2 million of these individuals. The World Health Organisation (WHO) (World Health Organisation, 2007b) reports that fatality due to cancer accounted for 7.6 million, or 13%, of the 58 million total fatalities worldwide during 2005. According to the WHO (World Health Organisation, 2007b), the main types of cancer responsible for the highest death rates are, in order of decreasing mortality rates, cancer of the lung (1.3 million deaths per annum), stomach (about 1 million deaths per annum), liver (662 000 deaths per annum), colon (655 000 deaths per annum) and breast (502 000 deaths per annum). In males, in order of decreasing frequency of occurrence, lung cancer, stomach cancer, liver cancer, colorectal cancer, oesophageal cancer and cancer of the prostate are the most commonly occurring types of cancer, while in females, in order of decreasing frequency of occurrence, breast cancer, lung cancer, stomach cancer, colorectal- and cervical cancer are the most commonly occurring types of cancer.

The WHO (World Health Organisation, 2007b) further states that during the year 2005, more than 70% of cancer related fatalities occurred in low- to middle income countries. Cancer related fatalities are projected to increase during the coming years, with 9 million cancer related deaths during 2015, and a further increase to 11.5 million cancer related deaths during 2030.

The literature often defines cancer simply as an uncontrolled proliferation of abnormal cells. Although correct, this very general definition does not do justice to the extremely complex and involved pathological state that is cancer. Cancer is not a simple molecular event, but a multifunctional and dynamic one, requiring changes that effect the neoplastic cell, the interaction between the neoplastic cell and its surrounding stroma, as well as the immune system (Carbone & Pass, 2004). Hanahan and Weinberg (as cited in Workman & Kaye, 2002) characterised cancer according to the following six hallmark traits:

- Cancer is self sufficient in proliferative growth signals.
- Cancer is insensitive to growth inhibitory signals.
- Cancer has the ability to evade apoptosis.
- Cancer has the potential to replicate in a limitless manner.
- The induction of angiogenesis is an important trait of cancers.
- Cancer has the potential to invade tissues and metastasise.

2.6.1. Aetiology and risk factors

Under normal circumstances, the somatic cells of an organism continue to divide and multiply until the organism has reached maturity, at which point the majority of cells enter a quiescent, non proliferative and metabolically active state which corresponds to G₀ of the cell cycle. Examples of such cells include neurons and myocytes. However, certain cells are required to remain in a proliferative, renewing state and do not enter senescence. Examples of such cells include intestinal epithelial cells, dermal cells and germ cells. The proliferation of cell populations is subject to the stringently controlled growth mechanisms of the cell cycle (O'Conner & Browder, 1998).

The growth mechanisms of the cell cycle maintain homeostasis within a cell under normal circumstances, and determine whether a cell will continue to proliferate, enter into a quiescent state, or undergo programmed cell death,

also known as apoptosis. Mutations of the intracellular signal pathways of a cell, which usually occur at the deoxyribonucleic acid (DNA) level, leaves such a cell independent of the growth control mechanisms of the cell cycle, enabling it to proliferate at an uncontrolled rate (Foster, in press). Mutations of cellular DNA may be due to the direct effect of environmental agents on the DNA as well as a cell's inherited genetic predisposition to certain mutations.

2.6.1.1. Environmental/external carcinogens and co-carcinogens

When discussing the environmental factors which may cause genetic mutations allowing a cell to overcome the various checkpoints of the cell cycle and continue to proliferate in an uncontrolled fashion, it is important to distinguish between carcinogens, co-carcinogens and promoters. Carcinogens, or initiators, are those factors which cause molecular alterations leading to the development of cancer. Co-carcinogens and promoters are factors which are not carcinogenic on their own, but which enhance the activity of carcinogens. Co-carcinogens may, however, at high amounts or in the presence of other co-carcinogens, become carcinogenic. A genetic predisposition may also lead to co-carcinogens having a carcinogenic effect (Carbone & Pass, 2004).

The environmental- or external factors which may act as carcinogens or co-carcinogens are classified as follows:

- Physical- examples are ultraviolet- (UV) and ionizing radiation.
- Chemical- examples include asbestos and tobacco smoke.
- Biological- examples are infection by Hepatitis B virus (HBV), Human Papilloma virus (HPV), *Helicobacter pylori* and *Aspergillus* fungae.

(World Health Organisation, 2007b)

A fourth group of external factors which may lead to the development of cancer may be classified as related to diet and exercise habits, and include

factors of both the physical and chemical groups. This fourth group is discussed separately.

2.6.1.1.1. Physical carcinogens and co-carcinogens

It is a well known fact that solar UV radiation causes mutations in the genetic makeup of cells via the formation of cyclobutane pyrimidine dimers, leaving the cells prone to becoming neoplastic (Migliore & Coppedè, 2002). UV radiation may give rise to any type of skin cancer. The risk of developing skin cancer for individuals suffering from the hereditary condition of xeroderma pigmentosum (XP), which is mainly characterised by the body's inability to repair UV induced DNA damage, is 2000 times greater than in healthy individuals. Individuals suffering from XP also run a much higher risk of developing various other neoplasms (Voet & Voet, 1995, p.1048). The importance of limiting exposure to solar radiation, using sunscreen and wearing protective clothing could not be emphasised enough where prevention of UV induced DNA damage is concerned.

Ionizing radiation is also known to cause certain types of cancer (World Health Organisation, 2007c). Radioactive decay of uranium in the crust of the earth leads to the formation of radon gas which, when inhaled, may lead to the development of lung cancer. Uranium miners and survivors of the Japanese nuclear holocaust have been shown to be at a higher risk of developing lung cancer, as well as cancer of the breast and thyroid, respectively. Excessive diagnostic radiology exposure, such as the performance of fluoroscopies to monitor iatrogenic pneumothoraces in women, has been shown to lead to the development of breast cancer. The use of thoratrast as a contrast agent in medical procedures has led to the development of tumours of the hepatobiliary tract and nasal sinuses. Furthermore, therapeutic irradiation of the breast, thyroid and skin leads to an increased incidence of tumour development in these respective sites (Neal & Hoskin, 1994, p.6).

2.6.1.1.2. Chemical carcinogens and co-carcinogens

The WHO (World Health Organisation, 2007a) reports that the consumption of tobacco products is the main avoidable risk factor for developing cancer. During the twentieth century, 100 million people died of illnesses related to tobacco use. Half of regular smokers die as a result of tobacco use, and a quarter of smokers die prematurely during their middle ages as a result of the habit. The relative risk of developing lung cancer amongst smokers is between twenty and thirty times that of non-smokers. In countries where tobacco consumption is high, 90% of lung cancer in both men and women is attributable to the use of tobacco products. Although lung cancer is the malignancy most often associated with tobacco use, smoking also increases the relative risk of developing a multitude of other malignancies. The relative risk of developing cancer of the oral cavity, pharynx, larynx and oesophagus (squamous cell carcinoma) as a result of smoking, is greater than six, while the relative risk for developing malignancy in the pancreas is between three and four. A relative risk factor of between two and three for the development of other malignancies as a result of tobacco smoking has also been described. These malignancies include cancer of the stomach, liver, cervix, nasal cavities and sinuses, kidneys, oesophageal adenocarcinoma and myeloid leukaemia (World Health Organisation, 2007a).

Under normal circumstances genetic damage caused by UV radiation is noted during the cell cycle, and the cell will attempt to correct this damage. If repair of the damaged DNA is not possible, the cell will undergo programmed cell death, also known as apoptosis, which is discussed later. Sugano, Minegishi, Kawamoto and Ito (2001) conducted a study on the effects of nicotine on UV induced apoptosis. They found that levels of nicotine normally obtained in the lungs of smokers inhibit UV induced apoptosis, allowing pre-neoplastic and neoplastic cells to proliferate in an uncontrolled fashion, which may ultimately lead to the development of cancer.

Cessation of smoking has been shown to have enormous benefits, with the best results obtainable if cessation occurs early in the third decade of life.

However, a risk reduction of 60% is possible if cessation occurs after the fifth decade of life (World Health Organisation, 2007c).

Apart from those present in tobacco smoke, exposure to a variety of chemicals often present in the occupational setting have also been linked to the development of various malignancies. As mentioned earlier, uranium miners are known to be at high risk of developing lung cancer. Dockers, plumbers, builders, engineers and miners are often exposed to asbestos, which is associated with lung carcinoma and mesothelioma of the pleura and peritoneum. Cancer of the renal pelvis, ureters and bladder is associated with exposure to β -naphthylamine and azo-dyes. Individuals in the rubber product and aniline dye industry are known to be exposed to these chemicals. Inhalation of hardwood dust by furniture workers is associated with adenocarcinoma of the nasal sinuses (Neal & Hoskin, 1994, p.4).

Occupational exposure to vinyl chloride is associated with hepatic angiosarcoma, while exposure to aromatic amines is associated with cancer of the bladder. Inhalation of arsenic is associated with the development of lung cancer, while ingestion of this compound leads to basal- and squamous cell carcinoma of the skin. Other metals such as cadmium, nickel and cobalt have been associated with an increased risk in the development of lung cancer (Migliore & Coppedè, 2002).

2.6.1.1.3. Biological carcinogens and co-carcinogens

The WHO (World Health Organisation, 2007a) reports that infectious agents are responsible for up to 23% of malignancies in developing countries, while this figure is decreased to only 8% in developed countries. This is mainly due to the availability of better healthcare services in developed countries as compared to those in developing countries.

External biological agents acting as carcinogens and co-carcinogens are mostly viral in nature. Viruses generally reproduce by integrating their genetic material with that of the host cell, which may lead to mutations in genes whose products normally act as regulators during the cell cycle. This may cause an infected cell to escape apoptotic signals of the cell cycle, allowing it to transform and enter into unchecked proliferation.

Ten percent of individuals infected with HBV develop chronic infection, which is known to lead to cirrhosis and hepatocellular carcinoma (Bishop, Duben-Engelkirk & Fody, 2000, p.364-365). Infection with certain strains of HPV, especially strains 16 and 18, is known to lead to the development of cervical and anogenital warts, occasionally leading to the development of cervical- and anogenital cancers (Neal & Hoskin, 1994, p.7). Infection with Epstein-Barr virus (EBV) is associated with the development of Burkitt's lymphoma in areas where malaria is endemic (Jensen, Wright & Robison, 1997, p.403). There also appears to be strong evidence that EBV might be the causative agent of nasopharyngeal carcinoma in Southeast Asia (Neal & Hoskin, 1994, p.7). Human T-cell lymphotropic virus type 1 (HTLV-1) has been strongly associated with adult T-cell lymphoma leukaemia in endemic areas of Japan and the Caribbean, where positive viral serology results were obtained in more than 95% of the cases (Neal & Hoskin, 1994, p.7). Kaposi's sarcoma-associated Herpes virus is thought to be the causative agent of Kaposi's sarcoma, a rare malignancy seen mostly in AIDS patients (Jensen, *et al.*, 1997, p.406).

Infection by a number of larger microbes is also associated with the development of neoplasms. Examples are *H. pylori* infection, which is thought to play an important role in the development of stomach cancers, schistosomiasis, which is thought to increase the risk of developing bladder cancer, and liver fluke infection, which is said to increase the risk of developing cholangiocarcinoma of the bile ducts (World Health Organisation, 2007c).

It should be mentioned at this point, however, that precautionary measures and early detection and intervention may be key to preventing the development of neoplasms associated with microbial infection. HBV vaccination has been shown to prevent hepatocellular carcinoma in high incidence countries (World Health Organisation, 2007a). The Pap-test, which is used to detect cervical dysplasia and neoplastic lesions of the cervix, has been the most effective preventative measure in cancer prevention up to date (Carbone & Pass, 2004). An effective HPV vaccine could become a reality within the next 3-5 years (World Health Organisation, 2007a).

2.6.1.1.4. Diet and exercise as factors in the development of malignancy

The 20th century has brought about a turnaround in human dietary consumption habits. Industrialisation has caused the migration of agrarian societies to urban centres, where industrialised societies were formed. This subsequently led to the industrialisation and commercialization of large scale non-organic food production, employing industrialised methods of food processing and distribution. These changes in food production methods have led to the turnaround in human dietary habits, with the consumption of animal fats and proteins increasing by more than three fold. Although the consumption of fruits and vegetables has also increased, most of it is not consumed fresh (Grandics, 2003). Japan demonstrates the lowest incidence of the major cancer types. This is possibly due to the Japanese custom of consuming food in a fresh or raw state. Even though the Japanese have adopted many aspects of the Western lifestyle and have the same risk factors for cancer development such as alcohol and tobacco consumption, obesity, which is another known risk factor for cancer development, is relatively low. This is attributable to Japanese dietary habits. Evidence suggests that dietary manipulations may be protective against 10-70% of cancer types (Grandics, 2003).

Diets rich in highly saturated fats and proteins may contribute to the development of colon cancer, while consumption of fibre in the form of fresh fruit and vegetables may be preventative (Grandics, 2003). Heterocyclic amines which form during the cooking of meat have been associated with the development of colorectal cancer (Migliore & Coppedè, 2002). Folate deficiency has been shown to cause nucleotide pool imbalance, DNA strand breaks, hypomethylation of DNA, increased gene expression, altered chromatin conformation and altered cellular proliferation. These have all been associated with carcinogenesis. Thus high dietary intake of folate may be protective in the prevention of malignancy. In the USA, breast cancer has been shown to be prevalent in areas where iodine consumption is low. Fibrocystic breast disease, which is a known risk factor for breast cancer development, may be prevented by proper iodine intake (Grandics, 2003).

Physical activity plays just as important a role as dietary habits when cancer prevention is considered. Diets rich in saturated lipids, proteins and refined carbohydrates, in conjunction with low- or no levels of physical activity, are known to contribute to the development of various disease states, including obesity, diabetes, cardiovascular disease and malignancy. The obesity epidemic prevalent in especially developed countries is commonly associated with poor dietary habits and little physical activity. This Western lifestyle is strongly linked to the development of various malignancies. Examples are colorectal cancer, endometrial carcinoma, oesophageal adenocarcinoma and cancers of the breast, gall bladder and kidneys (World Health Organisation, 2007a).

From the aforementioned, the maintenance of a healthy body by maintaining a healthy weight through regular physical activity and proper diet is paramount in the prevention of not only malignancy, but also diabetes and cardiovascular disease, two of the major health problems of our times.

2.6.1.2. Internal factors in carcinogenesis

As stated earlier, carcinogenesis is a complex pathology. It should not be classified as a single disease state, but rather a multifunctional and dynamic event involving a wide array of factors, including the internal cellular state, the cell's interaction with its environment and the immune system. The events leading up to a cell turning malignant often involve the mutagenic actions of carcinogens and co-carcinogens from the external environment, such as tobacco smoke, industrial chemicals and pollutants, and microbes. An individual's lifestyle may also contribute to the development of malignancy, as outlined in the previous section.

The following section will focus on what will be referred to as internal factors of carcinogenesis, i.e. those factors related to the individual's genetic makeup, immune system and endocrine function.

2.6.1.2.1. A genetic predisposition to cancer

Whether a cell proliferates, enters senescence or undergoes apoptosis is tightly regulated by the protein products of chromosomal DNA transcription and translation. Mutations in gene sequences that regulate these processes often lead to cellular transformation, immortalisation and uncontrolled growth. In fact, it would appear that all forms of cancer are due to genetic aberrations (Voet & Voet, 1995, p.1186), whether inherited or acquired due to the action of environmental carcinogens.

Cellular growth and proliferation is a function of the highly regulated cell cycle (Figure 2.37), a sequence of events occurring during the lifetime of a eukaryotic cell. The cell cycle is divided into the following stages:

1. G_1 phase: During this phase, which is normally the longest phase of the cell cycle, normal cellular growth occurs while the cell prepares for DNA synthesis. During the G_1 phase, the cell may enter into the quiescent G_0

phase, depending on the type of cell. Terminally differentiated cells such as myocytes and neurons usually assume this state permanently, while other non-terminally differentiated cells which have entered the G_0 phase will remain in such state in the case of insufficient nutrients or contact inhibition. Cells which do not enter the G_0 phase, or quiescent cells coming out of the G_0 phase, are compelled to enter the next phase of the cell cycle, during which DNA is copied. The cell cycle time of different cells may vary greatly depending on the type of cell, from 8 hours to 100 days and more, with the majority of this variation occurring during the G_1 phase. Cells in culture, however, normally complete the cycle within 16 hours to 24 hours.

2. S phase: Upon completion of the G_1 phase, the growing cell enters the next stage of the cell cycle, being the S phase, as a result of cytoplasmic factors stimulating DNA replication. Other factors that may stimulate a cell to enter the S phase and commence DNA replication include carcinogens and/or tumour viruses, surgical removal of tissue resulting in loss of contact inhibition and subsequent tissue regeneration, as well as mitogens binding to the cell surface receptors and inducing cellular division. Before the cell is able to continue to the third phase of the cycle, the newly replicated DNA is inspected by DNA-replicating enzymes such as DNA polymerase for any errors which might have occurred during replication. Once these errors have been rectified the cell moves into the third phase of the cycle, being the G_2 phase.
3. G_2 phase: During this phase the now tetraploid cell prepares for mitosis and cell division which occurs during the M-phase, the final stage of the cell cycle.
4. M phase: During this final stage of the cell cycle, the tetraploid parental cell undergoes mitosis and gives rise to two identical daughter cells, each entering the cell cycle again at the G_1/G_0 phase.

(Voet & Voet, 1995, p.1038)

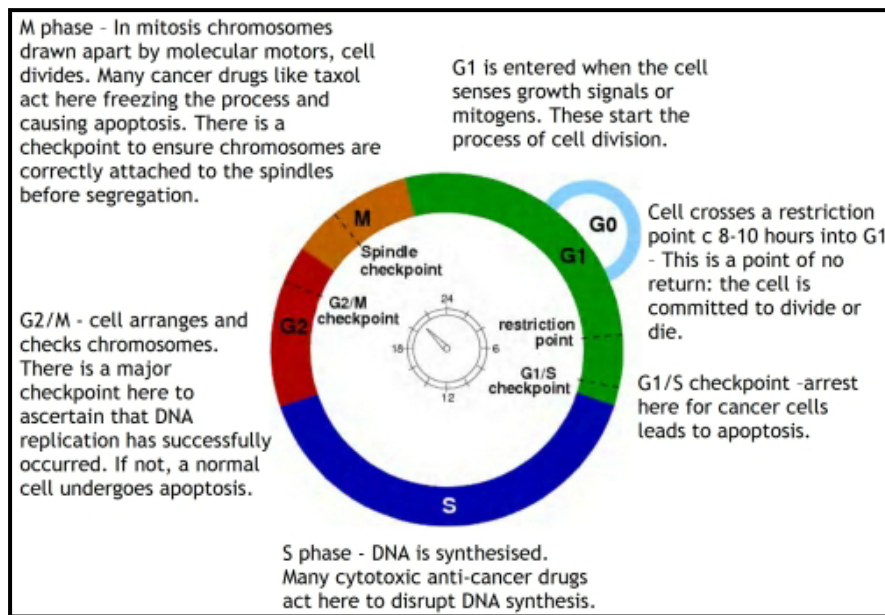


Figure 2.37: A graphical representation of the cell cycle (taken from Physiomics, 2008).

Cyclins and cyclin dependent kinases (CDK's) are two molecules which play key roles in the regulation of the cell cycle. Cyclins, which are regulatory molecules, combine with and activate CDK's to form active heterodimers, which phosphorylate proteins guiding the cell to the next level of the cell cycle. The type of protein activated is dependent on the combination of the type of cyclin and its CDK. While CDK's are constitutively expressed, cyclins are synthesised and broken down in the cell according to various cellular signals. A variety of cyclin-CDK complexes are active in the regulation of the cell cycle. G₁ cyclin-CKD's are active during the G₁ phase of the cell cycle, and regulate the expression of transcription factors which promote S phase cyclin expression, the synthesis of DNA replication enzymes as well as the ubiquitination of S phase inhibitory molecules. A pre-replication complex formed during the G₁ phase is further activated by S cyclin-CDK which forms during the S phase. Activation of this complex prevents new complexes of the same type from forming, ensuring that only one copy of the genome is made during the S phase. Additionally, mitotic cyclin-CDK complexes which form during the S phase and G₂ phase activate a crucial complex, anaphase promoting complex (APC), further downstream. APC is responsible for the

breakdown of structural proteins of the chromosomal kinetochore, enabling mitosis as well as the degradation of mitotic cyclins (Cell Cycle, 2008).

Two families of tumour suppressor genes, tumour suppressor genes being discussed later in this section, which play a crucial role in cell cycle regulation and prevention in the formation of malignancy by preventing cell cycle progression, are the *cip/kip* and *INK4a/ARF* families. The *cip/kip* family includes *p53*, which is activated by DNA damage and in turn activates the *p21* gene, as well as transforming growth factor β (*TGF β*) which activates the *p27* gene. Activated *p21* and *p27* in turn binds to and inactivates cyclin-CDK's during the G₁ phase of the cell cycle, inhibiting further cellular growth and replication. The *INK4a/ARF* family of genes include *p16INK4a*, which bind to *CDK4* and halts the cell cycle during the G₁ phase and *p14arf*, which prevents the degradation of *p53* (Cell Cycle, 2008). *P53* is discussed in more detail later in this section.

External carcinogens as discussed in Section 2.6.1.1., however, occasionally cause mutations in the genes coding for the proteins which regulate the cell cycle, resulting in cells with abnormal growth patterns being allowed to proliferate unchecked, and cancer to develop.

Two types of genes that play important roles in the cell cycle and control of cellular growth and proliferation are proto-oncogenes and tumour suppressor genes. Proto-oncogenes are normal genes whose translational protein products stimulate cell division, while tumour suppressor genes generally inhibit cellular growth and transformation by regulating the expression of proto-oncogenes. Normal activity of these genes take place during somatic growth, regeneration and repair, occurring during phase G₁/G₀ of the cell cycle. A highly controlled homeostatic balance is maintained between the up- and down-regulation of these two groups of genes. Any alteration in this balance favouring the expression of proto-oncogenes result in cellular proliferation and potential cancer (Macfarlane, Reid & Callander, 2000, p. 151).

Oncogenes are the result of mutations occurring in normal proto-oncogenes. The activation (transcription and translation) of oncogenes lead to the formation of neoplasms in various ways:

- The transcription and translation of oncogenes may give rise to protein products, including growth factors, growth factor receptors and intracellular signal transducers, whose activities are anomalous relative to the corresponding products of proto-oncogene activation. This may potentially lead to malignancy.
- Malignant transformation may arise due to an oncogene product being degraded at a slower rate than the corresponding normal proto-oncogene product, leading to an inappropriately high intracellular concentration of the anomalous protein.
- Chromosomal rearrangement may lead to the inappropriate transcription of an oncogene under a foreign regulatory sequence. The translocation of a proto-oncogene to a highly active regulatory sequence may also lead to an inappropriately high concentration or untimely expression of the protein product, which is a major factor in cellular transformation.
- The multiple replication of an oncogene (gene-amplification), either as sequentially repeated chromosomal copies or extra-chromosomal particles, may give rise to malignancy.
- Insertion of a viral genome into the host genome may lead to a proto-oncogene being brought under the transcriptional control of a viral regulatory sequence, leading to the inappropriate expression of the gene product.
- The loss or inactivation of a tumour suppressor gene that normally regulates the expression of a proto-oncogene or oncogene, may lead to the uncontrolled transcription of these genes, resulting in inappropriately high concentrations of their products and uncontrolled cellular proliferation.

(Voet & Voet, 1995, pp.1186-1187)

Examples of proto-oncogenes whose mutation or up-regulation may lead to the development of cellular proliferation and potential malignancy, include *Ras*, *Raf*, *Myc*, *Neu*, *Src* and *Sis* (Neal & Hoskin, 1994, p.2). Anti-apoptotic genes of the *Bcl-2* family of genes, which include genes coding for anti-apoptotic survival proteins as well as genes coding for pro-apoptotic death proteins, include *Bcl-2*, *Bcl-x_L*, *Mcl-2*, *Bfl-1*, *Bcl-w*, *A1* and *Ced-9* (Bowen, Bowen & Jones, 1998, p.73). The *Bcl-2* family is discussed in more detail later.

Normal cells contain one of a pair of tumour suppressor genes on each homologous chromosome. Individuals from families in which cancer is prevalent often inherit only a single copy of this gene, have a normal phenotype and act as carriers. However, deletion or mutation of the single inherited tumour suppressor gene may lead to cancer in such a person. For cancer to develop sporadically in an individual not genetically predisposed to it, both copies of a tumour suppressor gene must be mutated or deleted. The most commonly altered gene in human malignancy has been shown to be *p53*, a tumour suppressor gene which is altered in 50% of cancers (Voet & Voet, 1995, p.1188). In fact, it is considered that alteration or loss of *p53* is crucial to the development of most, if not all, malignancies (Lohrum & Vousden, 2000). *P53* may be activated by many types of stress, including UV- and gamma irradiation, chemical-induced genetic damage, hypoxia, oncogene activation and loss of normal growth- and survival signals. This leads to the cell entering cell cycle arrest in the G₁ phase, allowing damaged DNA to be repaired before the cell enters the S-phase of the cell cycle. Irreparable DNA damage leads to *p53*-directed apoptosis of the cell. Both these responses prevent the replication of cells undergoing oncogenic changes, and thus possible tumour formation (Ryan, Phillips & Vousden, 2001).

Rb (retinoblastoma gene) is another well known tumour suppressor gene that plays a vital role in regulating cell cycle progression. Retinoblastoma is a malignancy affecting mostly infants and children, and is the result of the deletion of the *Rb* gene from chromosome 13 of retinoblasts. This leads to

uninhibited cellular proliferation of retinoblasts, and subsequent retinoblastoma. Offspring of individuals who survive retinoblastoma also have a high incidence of the disease as well as other forms of malignancy (Voet & Voet, 1995, p.1187). *Rb* protein normally binds and inactivates the *E2F* transcription factors, which normally function to regulate the expression of genes required for cell cycle progression into the G₁ phase. Loss of *Rb* leads to cellular proliferation, but also apoptosis, through the exogenous expression of *E2F* which, through a complex pathway, leads to apoptogenic *Bax*-mediated cytochrome C release. In this way a tumour suppressor molecule which directly contributes to cellular proliferation also transactivates components of the apoptosis mechanism, indicating the complexity of cell cycle regulation and cancer (Makin & Dive, 2003).

2.6.1.2.2. Telomeres

The Hayflick limit refers to the finite replicative potential of mammalian cells. Upon reaching this limit, cells enter senescence, a state of replication cessation and changes associated with ageing. The ability of a cell to escape senescence and proliferate in an uncontrolled fashion is a prerequisite for malignancy. Thus senescence is seen as a means of inhibiting tumourigenesis (Neumeister, Albanese, Balent, Greally & Pestell, 2002).

This state of replicative senescence is thought to be due to the shortening of telomeres during each cycle of DNA replication. Telomeres are nucleoprotein structures capping the ends of chromosomes. These non-coding DNA sequences consist of up to 25 000 base pairs and protect chromosomes against degradation, rearrangement and interchromosomal fusion. The normal lagging strand method used by DNA polymerase to transcribe DNA makes transcription of the ends of linear DNA impossible, leading to the loss of between 50 and 200 base pairs of telomeric DNA per round of DNA replication. This erosion of telomeres is thought to trigger cellular senescence and form the basis of ageing (Neumeister *et al.*, 2002).

Telomerase is an enzyme with reverse transcriptase activity, present in self-renewing cell populations of the body such as germ cells, epidermal basal cells, lymphocytes and haematopoietic cells, but down-regulated in most other somatic cells. Its function is to replicate telomeres and thus to counteract the progressive shortening of telomeres that normally occur during DNA replication. Although normally absent or present in very low levels in normal somatic cells, 70% of immortalised somatic cell populations and between 90% and 95% of cancer cells express telomerase activity, leading to the stabilisation of telomeric lengths and subsequent escape from senescence. A strong correlation appears to exist between the maintenance of telomere length and cellular immortalisation and tumour formation (Liu, Lai, Andrews & Tollefsbol, 2004). This may explain the absence of telomerase function in somatic cells. The entering of cells into the senescent phase as a result of telomere shortening and absence of telomerase contributes to the prevention of immortalisation of these cells and eventual malignancy (Voet & Voet, 1995, p.1045). Thus it would appear that ageing is the price multicellular organisms pay in exchange for protection against malignancy.

2.6.1.2.3. Immune factors in malignancy

The favourable prognostic indicator of lymphocytic infiltration in tumours such as seminomas and melanomas, spontaneous regression in hypernephroma and melanoma, as well as the objective responses observed during cytokine stimulation of T-cells, are thought to be evidence of the immune system's ability to mount a response in the presence of cancer cells. Immunosuppression leads to the development of certain types of malignancy which, in some cases, are virally mediated (Neal & Hoskin, 1994, p.8). Nowhere is this more evident than in the case of AIDS. Up to 40% of individuals with AIDS will develop a malignancy, most notably Kaposi's sarcoma, which is thought to be caused by Kaposi's sarcoma associated Herpesvirus (Jensen *et al.*, 1997, p.406), and non-Hodgkin's lymphoma (Neal & Hoskin, 1994, p.8).

An increase in the incidence of Kaposi's sarcoma, non-Hodgkin's lymphoma and skin cancer is also observed in transplant recipients receiving steroids, azothioprine or cyclosporin (Neal & Hoskin, 1994, p.8).

2.6.1.2.4. Endocrine factors in malignancy

Over-stimulation of cellular hormone receptors by endogenous- or exogenous hormones may lead to hyperplasia, resulting in adenomatous change and occasionally cancer. An example of this is the predisposition of the endometrium to the development of well differentiated endometrial adenocarcinoma, through excessive stimulation from endogenous steroids, such as oestrogen secreting ovarian granulosa cell tumours, or stimulation from exogenous steroids, as in hormone therapy. A further example is the rise in thyroid stimulating hormone (TSH), subsequently leading to goitre and possible follicular carcinoma in cases of chronic severe iodine deficiency (Neal & Hoskin, 1994, p.8).

2.6.2. Cell death: necrosis versus apoptosis

In order to maintain the homeostatic balance of the body, around 10 billion cells die on any given day in order to counter the amount of new cells arising through mitosis. Programmed cell death, or apoptosis, is a vital link in this life-death cycle. It sculpts the body during development and gives rise to organs, fingers and toes. Both the immune- and nervous systems arise from an overproduction of cells. Those cells that respectively fail to produce antigen specificities or form functional synaptic connections are removed via apoptosis. Furthermore, apoptosis is necessary for the elimination of pathogen infected cells from the body, as well as activated- or auto aggressive immune cells (Heemels, 2000, p.769). Another important function of apoptosis is the elimination of cells that have acquired genetic mutations which can not be repaired during the cell cycle. Failure of these cells to be eliminated through apoptosis may lead to their immortalisation and possible malignancy (Evan & Littlewood, 1998).

Apoptotic cells undergo morphological cellular changes which distinguish them from necrotic cells. These changes occur on cytoplasmic-, plasma membrane- and nuclear levels. Apoptotic cells round up, condense and lose contact with neighbouring cells. This is accompanied by loss of microvilli and desmosomes. Dilation of the endoplasmic reticulum and outer nuclear envelope is observed in the cytoplasm. Vacuoles and vesicles are formed due to swelling of the reticulum cisternae. The cell has a characteristic spongy appearance due to the fusion of these vacuoles and vesicles with the plasma membrane. Other cytoplasmic organelles generally remain intact. Compaction of the cytoplasm due to loss of water and shrinkage leads to an increase in cell density. Convolution of the plasma membrane leads to budding or blebbing and the formation of several membrane-bound spheres known as apoptotic bodies, which are rapidly phagocytosed by neighbouring cells and macrophages. This inhibits the launch of an inflammatory response in the tissue. On a nuclear level, chromatin condensation and margination is usually observed. Convolution of the nuclear membrane may lead to the budding off of several fragments and the forming of apoptotic bodies. Apoptotic cells or apoptotic bodies that are shed into lumina lose their membrane integrity and undergo apoptotic changes known as secondary necrosis (Bowen *et al.*, 1998, pp.17-18).

Necrosis is the term denoting passive cell death as a result of lethal trauma and pathology. Unlike apoptotic cells, necrotic cells swell instead of shrink. Calcium and sodium loss as a result of plasma membrane damage leads to a loss of water balance, acidosis and shock. The drop in pH causes chromatin precipitation and a subsequent pyknotic nucleus. In the mitochondria, distension of both the inner- and outer compartments is observed, as well as dense deposits of lipoprotein. Swelling and bursting of both the endoplasmic reticulum and lysosomes is further observed. The latter of these two organelles contain digestive enzymes which contribute to further autolysis of the cell and its final disintegration. This releases cellular debris which elicits the inflammatory response that normally accompanies necrosis (Bowen *et al.*, 1998, p.23).

2.6.2.1. Pathways in apoptosis

In general, initiation of apoptosis occurs through the activation of the extrinsic pathway (death receptor pathway), intrinsic pathway (mitochondrial pathway), or both (Perik, De Vries, Gietema, Van der Graaf, Sleijfer, Suurmeijer & Van Veldhuisen, 2005). The common denominator in both these pathways is a family of evolutionary highly conserved cysteine proteases known as the caspases. It could be said caspases are the central executioners of apoptosis, as elimination of these enzymes significantly slows down and may even inhibit apoptosis (Hengartner, 2000, p.770).

Caspases function by cleaving their respective target proteins at one or more locations, always after an aspartate residue. Cleavage by a caspase inactivates a protein in most cases, however the cleaving off of a negative regulatory domain or the cleaving of a regulatory subunit may also activate some target proteins. Caspases themselves occur as enzymatically inert zymogens that need to be activated in order to carry out their catalytic activities. Three models of caspase activation have been proposed. These are:

- Autocatalytic activation through cleavage of pro-caspase molecules by previously activated caspases. This suggests a caspase activation cascade which is known to be utilised by cells to activate caspases 3, 6 and 7. However, this cascade does not account for the activation of the initiator caspase.
- The induction by proximity model proposes the cleavage and activation of pro-caspase molecules that are in close proximity to each other. During the extrinsic pathway of apoptosis, which is discussed later, death receptor molecules on the plasma membrane recruit several molecules of pro-caspase 8. The high localized concentration of zymogen is thought to initiate induction by proximity, leading to the formation of active caspase 8, which may then continue in the activation of pro-caspases further along the caspase cascade.

- A third means of caspase activation, particularly activation of caspase 9, which plays an important role in the intrinsic apoptotic pathway discussed later, is via the association with a regulatory subunit. Activation of caspase 9 requires the association of pro-caspase 9 with two protein co-factors; *Apaf-1* and cytochrome C. This haloenzyme complex is known as the apoptosome, and may well contain various other proteins.

(Hengartner, 2000, p.771)

It appears that all three above models may be utilised by the cell to facilitate caspase activation and apoptosis. Effector caspases are normally activated proteolytically by a caspase further up the caspase cascade, while initiator caspases are activated through interaction between different proteins, such as the case with caspase 8 and caspase 9 activation.

Although the extrinsic- and intrinsic pathways of apoptosis are initiated through different mechanisms, both these pathways seem to converge at the activation of pro-caspase 3, which in turn activates pro-caspases further along the caspase pathway that ultimately leads to apoptosis of the cell.

2.6.2.1.1. Extrinsic apoptotic pathway

Although much more complex, the basic principle of the activation of the extrinsic pathway involves the binding of death signal ligands such as apoptosis related cytokines to their respective receptors, such as the *Fas* receptor (FASR), tumour necrosis factor receptor 1 (*TNFR1*) and *CD95* on the plasma membrane. The binding of a ligand to example *FASR* causes its death domain to bind to that of the cytosolic adaptor molecule *Fas* associated death domain protein (*FADD*) (Bowen *et al.*, 1998, p.98). *FADD* then continues to recruit pro-caspase 8, resulting in the formation of a death inducing signalling complex (*DISC*). Activation of pro-caspase 8 occurs at this point through induced proximity. However, caspase 8 activation can be blocked at this point through the recruitment of the degenerate caspase homologue, *c-FLIP* (Hengartner, 2000, p.773). Activated caspase 8 may

catalytically activate caspase 1, which in turn activates caspase 3, or it may directly cleave nuclear poly-(ADP-ribose) polymerase (*PARP*), an enzyme related to the repair of DNA strand breaks (Bowen *et al.*, 1998, p.95). Caspase 8 may also cleave and activate *Bid*, a pro-apoptotic member of the *Bcl-2* protein family which acts as a mediator between the extrinsic- and intrinsic apoptotic pathway. Activated *Bid* migrates to the mitochondria, where it facilitates the release of cytochrome C, which is vital to the successful completion of apoptosis via the intrinsic pathway (Hengartner, 2000, p.773).

2.6.2.1.2. Intrinsic apoptotic pathway

The *Bcl-2* protein family is a group of related proteins that have important functions in the intrinsic apoptotic pathway. Members of this family are ordered into three groups based on their structural similarities and functions. Group 1 include anti-apoptotic proteins such as *Bcl-2* and *Bcl-X_L*, while group 2 include pro-apoptotic proteins such as *Bax*, *Bcl-X_S*, *Bak* and *Bad*. Group 3 includes a large and diverse group of proteins which may be structural homologues of proteins from both groups 1 and 2. Examples of proteins in this group include *Bik* and *Bid* (Hengartner, 2000, p.774).

The intrinsic pathway of apoptosis is activated by DNA damage caused by factors such as carcinogenic chemicals and irradiation. DNA damage causes the activation of the tumour suppressor gene *p53*, which is mutated in 50% of malignancies (Voet & Voet, 1995, p.1188). Activation of *p53* in turn leads to the transcription of various apoptotic genes, including *PUMA*, *Bax*, *NOXA*, *Bid*, *PIG3*, *CD95*, *DR5* and *p53AIP1* (Yu, 2006). *Bax*, a pro-apoptotic *Bcl-2* protein, migrates to the mitochondrial surface along with other pro-apoptotic *Bcl-2* proteins. Here they compete with anti-apoptotic *Bcl-2* proteins such as *Bcl-2* and *Bcl-X_L* for the regulation of the release of cytochrome C from the mitochondria.

The mechanism of exit of cytochrome C from the mitochondria is not known, although Hengartner (2000, pp.772-774) has suggested three scenarios. These include:

- The insertion of *Bcl-2* proteins into the outer mitochondrial membrane where, after conformational change, they form pores to facilitate the exit of cytochrome C.
- The recruitment of other mitochondrial outer membrane proteins such as the voltage dependant anion channel (VDAC) by pro-apoptotic *Bcl-2* proteins, to aid in the formation of a large pore channel.
- Alteration of mitochondrial physiology by apoptotic signals, resulting in rupture of the mitochondrial outer membrane and leakage of cytochrome C to the cytoplasm.

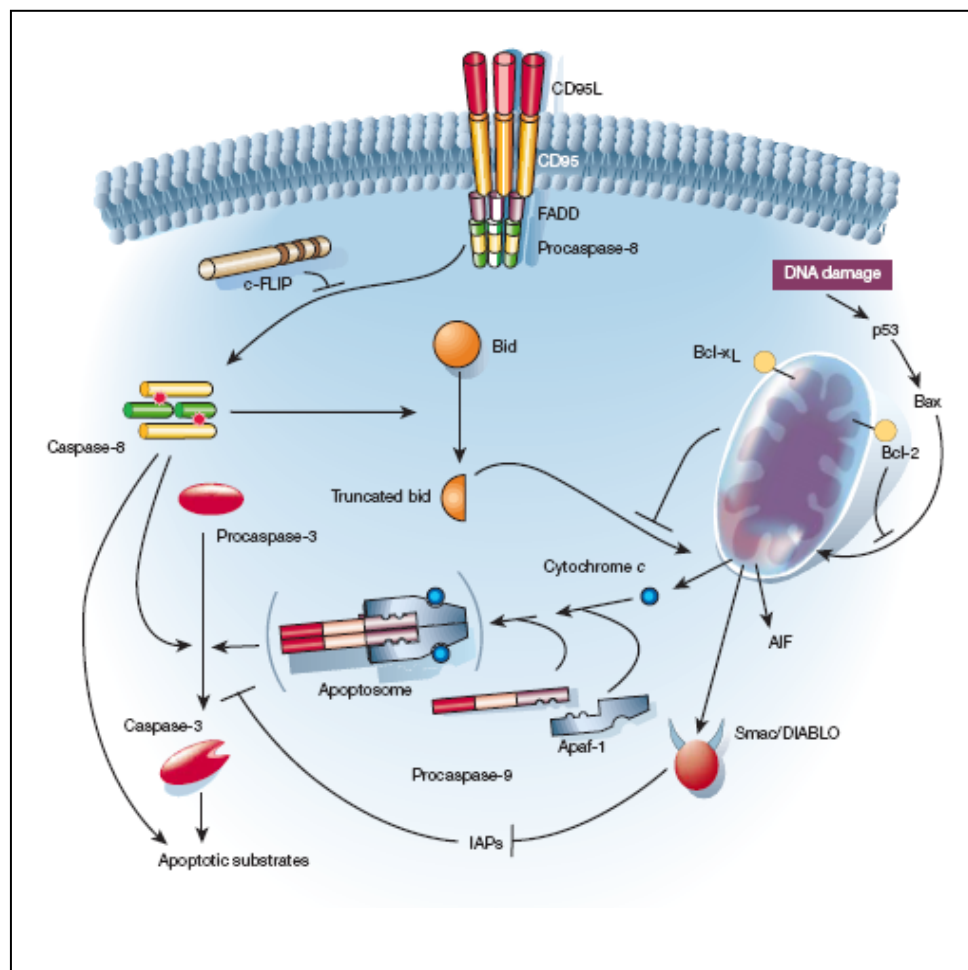


Figure 2.38: The basic mechanisms of the extrinsic- and intrinsic- apoptotic cellular pathways (taken from Hengartner, 2000, p. 773).

After release from the mitochondria, cytochrome C and *Apaf-1* bind pro-caspase 9, forming the apoptosome protein complex. The apoptosome is thought to be responsible for the conversion of pro-caspase 3 to its active form, which may then carry on activating other pro-caspases further down the caspase cascade (Hengartner, 2000, p.773).

The release of cytochrome C from the mitochondria is accompanied by the simultaneous release of other mitochondrial proteins, including apoptotic inducing factor (*AIF*), *Smac/DIABLO* and various pro-caspases, including pro-caspases 2, 3 and 9. Mammalian DNA codes for inhibitor of apoptosis (*IAP*) proteins, which function to inhibit the activation of pro-caspase 3 in the intrinsic pathway. *Smac/DIABLO* functions as an inhibitor of *IAP*'s, thus its release from the mitochondria prevent *IAP*'s from interfering with the apoptotic mechanism (Hengartner, 2000, p.774).

As mentioned earlier, caspase 8 is responsible for the cleavage and subsequent inactivation of the DNA repair protein *PARP*. Various other caspases possess similar functions, including:

- The cleavage of *PARP* by caspases 1 to 7 and caspase 9.
- Cleavage of DNA dependant kinase by caspase 3.
- Attack of nuclear ribonucleo-proteins by caspases 3, 6 and 7.
- Cleavage of nuclear lamins by caspase 6, which facilitates the packaging of condensed chromatin into apoptotic bodies.
- Cleavage of the cytoskeletal protein fodrin by caspase 3, which may account for the blebbing characteristically observed in apoptotic cells.

(Bowen *et al.*, 1998, p.96)

2.6.3. Apoptosis as a target for cancer therapy

A cell that has accumulated sufficient genetic damage to escape cell cycle checkpoints and apoptosis has the potential to transform and proliferate in an uncontrolled manner and ultimately lead to the development of cancer. Thus

a chemotherapeutic agent that has the potential to reactivate the apoptotic pathway in cancer cells may be an effective anticancer agent.

Current chemotherapy is toxic to healthy cells and often fails in destroying malignant cells. Thornberry and Lazebnik (1998) proposed an alternative strategy of designing chemotherapeutic drugs that activates target caspases directly. One way of achieving this is the activation of death receptor complexes of the extrinsic apoptotic pathway which in turn activates the caspase cascade. Death receptor complexes, however, occur on normal cells as well as healthy cells. Thus this approach should include a strategy for targeting death receptor complexes of cancer cells only.

Furthermore, it has been observed that certain oncoproteins that deregulate the cell cycle are responsible for the activation of the caspases and subsequent apoptosis. Thus this oncogenic transformation acts as a pro-apoptotic signal present only in transformed cells. Uncoupling of this signal with caspase activation leads to the survival of the transformed cell and cancer. Knowledge about the mechanism of recoupling this signal may be another target for cancer therapy (Thornberry & Lazebnik, 1998).

2.6.4. Phytotherapy in cancer treatment and plant-derived anticancer agents

The search for anticancer agents from natural sources can be dated back to, at least as far as, the Ebers papyrus of 1550 BC. This search has only been pursued in more earnest during the 1960's, initiated by research into the anticancer potential of podophyllotoxin and its derivatives (Srivastava *et al.*, 2005).

Plant products have been used by many individuals globally either to prevent or cure cancer, or to alleviate the accompanying symptoms. In South Africa, the best example of a plant traditionally used to treat internal cancer would

probably be *Sutherlandia frutescens*, better known as the cancer bush. Another well known South African plant which has a long history of traditional use as a medicine is the African potato, a common name for an indigenous *Hypoxis* species. The African potato has shown remarkable effects in prostate cancer, and may be used to reduce the side-effects of radiation- and chemotherapy. Furthermore, the well known illegal narcotic dagga (*Cannabis sativa*) has been used as a folk remedy in the form of a tea for various ailments, including cancer. Dagga is also used medicinally to treat nausea caused by chemotherapy (Fairall, 1999, pp.72-74). Scott *et al.* (2004, p.193) report that *Elytropappus rhinocerotis* is traditionally used in South Africa to treat cancer, while Thring and Weitz (2006, p.266-267) report the traditional use of *Leonotis leonurus*, better known as *wilde dagga*, in the treatment of cancer, as well as the use of *Agathosma betulina* in the prevention of cancer. Ginseng, usually in combination with other herbs, is traditionally used by the Chinese in the treatment of cancer. Other herbal preparations used by individuals from different cultures include iscador, lapacho, cat's claw and chapparal (Fairall, 1999, pp.74, 76-77).

Sarath, So, Won and Gollapudi (2007) conducted a study on the anticancer properties of *Artemisia princeps* var. *orientalis*, and found that smoke and water soluble extracts of the plant induced apoptosis in MCF-7 (human breast adenocarcinoma) cells via the mitochondrial pathway. Another example of an *Artemisia* species exhibiting promising results in cancer studies is the traditional Chinese medicinal plant, *A. annua*. Various articles report on the anticancer- and antimalarial effects of artemisinin (Figure 2.39), a sesquiterpene lactone isolated from the plant, as well as its synthetic derivatives, artesunate, artemether and dihydroartemisinin. Artemisinin contains an endoperoxide bridge as the active moiety of the molecule. Upon reaction with free iron, peroxides form highly reactive, oxygen based free radicals. The *Plasmodium* parasite, the causative agent of malaria, infects iron rich red blood cells. While the body normally stores iron in a bound state, excess iron accumulates in the parasite. This accumulation allows excess iron to spill from the parasite and become free. When artemisinin comes into contact with this free iron, free radicals are released, and the parasite is

destroyed along with the infected cell (Rowan, 2004; Li, Lam, Roos, Zdzienicka, Kaina & Efferth, 2008).

Based on this knowledge, Singh and Lai (2004) conducted research on the effects of dihydroartemisinin on Molt-4 (human acute lymphoblastic leukaemia) cells. The rapid generation time of cancer cells imply that they need relatively more iron in order for DNA replication to be carried out. Subsequently, malignant cells express relatively more transferrin receptors on their outer membranes than do their normal counterparts. Lai stated that leukaemia cells may have a concentration of iron 1000 times more than normal cells (as cited in Denver Naturopathic Clinic, 2005). Singh and Lai (2004) found that addition of dihydroartemisinin to Molt-4 cells induced apoptosis and significantly reduced the number of viable cells *in vitro*. Addition of dihydroartemisinin and halotransferrin, which enhances iron supply to cells, had even more significant results. Similar results were observed when HTB 27 (human breast cancer) cells were treated with combinations of halotransferrin followed by artemisinin. Artemisinin exhibited high selectivity, being toxic to HTB 27 cells, but having only a marginal impact on normal breast cells (Singh & Lai, 2001).

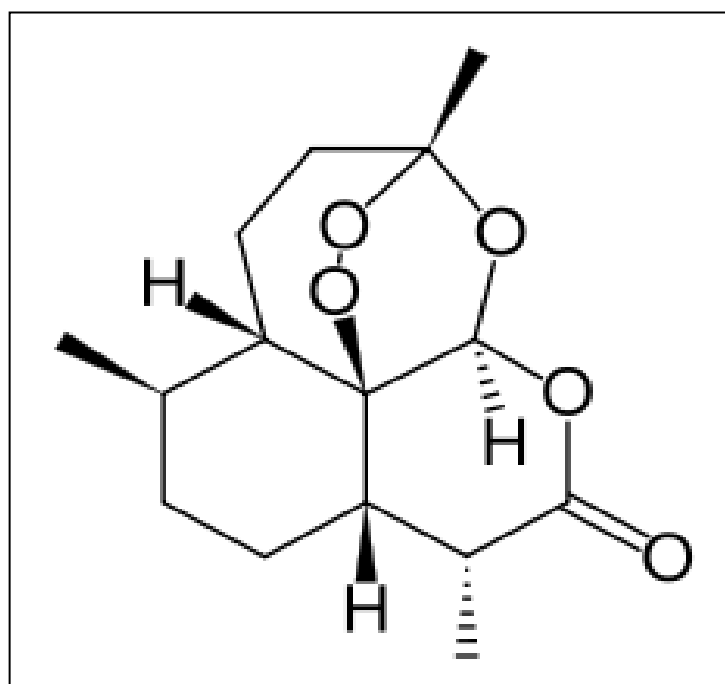


Figure 2.39: Structure of artemisinin (taken from Artemisinin, 2008).

Zheng (1994) investigated the cytotoxic effects of compounds isolated from *A. annua* on various human tumour cells lines, and found artemisinin and quercetagenin 6,7,3',4'-tetramethyl ether to express cytotoxicity towards P-388 (human lymphocytic leukaemia) cells, A-549 (human lung carcinoma) cells, HT-29 (human colonic adenocarcinoma) cells, MCF-7 cells and KB (human oral tumor) cells. Chen, Zhou and Fang (2003) found artesunate and dihydroartemisinin to exhibit varying levels of toxicity towards HeLa cells, JAR (human uterus chorion cancer) cells, RD (human embryo transversal cancer) cells and HO-8910 (human ovarian cancer) cells. A study conducted by Efferth (2007) further indicated that cancer cells expressing genes which confer resistance to current chemotherapeutic drugs were not resistant to artesunate. Artesunate and dihydroartemisinin have also been shown to be potent inhibitors of angiogenesis, a process which is vital to neoplastic formation, growth and metastasis (Chen *et al.*, 2003).

With *A. afra* and *A. absinthium* being of the same species as *A. annua*, and artemisinin and its derivatives possessing anticancer activities as discussed above, one might assume that *A. afra* and *A. absinthium* might contain these compounds and express anticancer activity as well. According to Klayman *et al.* (as cited in Van der Kooy, Verpoorte & Meyer, 2008, p.186), however, no other species of *Artemisia* possess artemisinin. Van der Kooy *et al.* (2008, pp.186-189) conducted a study in which they screened *A. afra* leaves collected from four different plants for the presence of artemisinin, concluding that *A. afra* does in fact not contain any trace of the compound or its derivatives. Artemisinin not only possesses anticancer activity, but is also currently used in the treatment of malaria (Christen & Vuthey, as cited in Van der Kooy *et al.*, 2008, p.186). If *A. absinthium* and *A. afra*, the latter having been traditionally used to treat malaria (Scott & Springfield, 2004b, p.3), does in fact not contain artemisinin and additionally expresses toxicity towards malignant cells, it could strongly suggest the presence of one or more unknown compounds other than artemisinin present in *A. afra* and *A. absinthium* with both anticancer- and antimalarial activity that needs to be identified.

An increasing interest in novel plant-derived drugs have been witnessed in recent years, largely due to the fact that conventional medicines are often ineffective and cause side effects, the inaccessibility by a large proportion of the global population to conventional drugs, and the apparent safety of traditional medicine. Smith, Pharms and Boon (as cited in Bungu, 2005, p.13) report that as many as 86% of cancer patients use some form of traditional medicine. Ernst (as cited in Bungu, 2005, p.13) found that 25% of Canadian breast cancer patients make use of traditional medicine. By the year 1992, the National Cancer Institute (NCI) had screened 33 000 plants for anticancer activity (Rates, 2001). Various chemical compounds isolated from plants have lead to the development of effective anticancer agents used today in the clinical setting. Plant-derived anticancer drugs are divided into four classes:

- Vinca alkaloids – Vinblastine and Vincristine were isolated from *Catharanthus roseus*, and have been used clinically for over 40 years.
- Epipodophyllotoxins – Podophyllotoxin was originally isolated from *Podophyllum peltatum* and found to be too toxic. However etoposide and teniposide, two analogues, are used in the treatment of lymphomas, acute leukaemia, testicular cancer, small cell lung cancer, ovarian-, bladder- and brain cancers.
- Taxanes – Taxol was first isolated from *Taxus brevifolia* and is used in the clinical setting for the treatment of refractive ovarian cancer as well as metastatic breast- and lung cancer and Kaposi's sarcoma. Analogues include Paclitaxel and Taxotere.
- Camptothecin – This anticancer agent was first isolated from *Camptotheca acuminata*. Analogues include Topotecan, Irinotecan and Rubitecan. These drugs show activity against cancers of the colon, breast, liver, prostate and pancreas.

(Balunas & Kinghorn, 2005; Srivastava *et al.*, 2005)

A fifth group of anticancer molecules are the combretastatins, particularly combretastatin A-4, which was isolated from the South African tree *Combretum caffrum*. Combretastatin A-4 demonstrates activity against lung

cancer, colon cancer and leukaemia, and is said to be the most cytotoxic phytochemical isolated thus far (Srivastava *et al.*, 2005).

Although nature has provided mankind with a number of effective chemotherapeutic drugs which are utilised in the fight against cancer, an anticancer drug that is safe, economic to develop and site-specific is still elusive (Srivastava *et al.*, 2005). Many plant species still remain to be screened for their anticancer properties, and might hold the key to the development of this elusive molecule.

2.7. Infection and the role of microbial drug resistance in infectious pathology

History has borne witness to humanity's suffering as a result of various pandemics, including cholera, plague, typhoid, influenza, and tuberculosis, to name but a few. Other infections which, by today's standards, would seem trivial, such as skin, ear and throat infections, often lead to deafness, disfigurement and death. The average life expectancy for 19th century Europeans and North Americans is said to have been around 50 years. The likelihood of dying prematurely as a result of infectious disease was as high as 40% and women routinely succumbed to child birth related infections which are easily treatable today (Brundtland, 2000).

In 550 A.D., the first major documented plague pandemic, also known as the Black Death, claimed the lives of 100 million people. The next major pandemic occurred in the 14th century, which wiped out 25% of the European population (Jensen *et al.*, 1997, p.340). This, in conjunction with successive outbreaks of smallpox and typhus during the 14th and 15th centuries diminished the European population by 50% (Brundtland, 2000). It was, however, not only Europeans who suffered plague pandemics. It is estimated that between 1896 and 1948, 12.5 million people succumbed to plague on the Indian subcontinent alone. Cholera has also greatly contributed to infection

related mortalities over the centuries. In 1783, it claimed the lives of 20 000 pilgrims on the way to the Indian holy site of Hardawar, while nearly half of all individuals participating in the Hajj to Mecca and Medina during 1831 were infected with cholera. It rapidly spread to Istanbul and Alexandria, and from there radiated outward as far as Hungary, devastating populations and economies (Brundtland, 2000).

Peurperal sepsis, a streptococcal infection, was responsible for the death of more than 70% of post partum mothers lying in at various hospitals across Europe during the early 1800's, with one Italian infirmary having reported not a single mother to have survived childbirth for a period of a year. This death rate only declined once health workers adopted proper hand washing procedures. The global influenza pandemic of 1918, which is said to have claimed the lives of more than 30 million people, devastated entire economies, and is thought to have had a higher mortality rate than the First World War. Another example of an epidemic which may very well have altered the course of European history is the louse-borne typhus infection which struck the army of Napoleon Bonaparte on its way to Moscow, and reduced it from 655 000 to 93 000. Those who returned home spread the infection amongst family and friends, leading to the death of a further 2 million people (Brundtland, 2000).

During the Spanish invasion of the Americas in 1495, it was not their firepower or horses which lead to their conquests of the natives, but the introduction of smallpox, influenza and measles by the Spaniards to these previously unexposed communities. Over a time span of only ten years, Mexico's population declined by 74% as a result of epidemics (Brundtland, 2000). As if in some ironic retaliation by the Native Americans, Spaniards returning home carried with them the beginnings of a new epidemic which, until then, was unknown in Europe, and characterised by genital ulcers, rash, dementia and death. Today the condition is known as syphilis (Brundtland, 2000).

Many of the maladies of previous centuries still pester mankind today, especially in impoverished communities of developing nations. Malaria, acute respiratory infections, diarrhoea and tuberculosis have lead to the deaths of many people over the centuries, and continue to do so today in various developing nations where sanitation is poor, and adequate healthcare and drugs is inaccessible or simply too expensive. The advent of HIV and AIDS during the last 25 years has struck a further blow to these communities.

2.7.1. The discovery of antimicrobial agents

In 1928 Gerhard Domagk demonstrated that Prontosil can successfully be used to treat hemolytic streptococcal infections, including puerperal fever.

The discovery of penicillin by Alexander Fleming in 1928 revolutionised medical science, and has since saved the lives of millions. In 1938 A.J. Evans developed the drug sulphanilamide, which also proved to be successful in treating streptococcal and pneumococcal infections. In 1948 Selman Waksman developed the tuberculosis drug streptomycin, and when isoniazid was later developed, the world thought that finally tuberculosis might be something of the past. However, the development of resistance to these drugs necessitated the development of yet another tuberculosis drug. This came in the form of rifampicin, developed by Piero Sensi. Until recently, a combination of these three drugs proved to be successful in treating tuberculosis. Subsequent discoveries include tetracycline, quinolones, antifungals, antiparasitics and antivirals, which saved the lives of millions of people, prevented disabilities such as deafness, blindness and disfigurement as a result of infection, and allowed invasive surgical procedures to be performed which, in the pre-antibiotic era, would have proven fatal due to post-operative infections (Brundtland, 2000).

A radical change in the way mankind viewed, understood and subsequently treated infectious disease only occurred during the 20th century. The discovery and development of antimicrobial agents and vaccines has lead to the drastic decline in occurrence of infections which often had fatal outcomes

as little as 100 years ago, for example measles, typhoid fever, rubella, diphtheria, tetanus, yellow fever, pertussis and polio, while some previously fatal afflictions have now been eradicated, e.g. smallpox. Apart from the smallpox vaccine, quinine and penicillin, the discovery of most modern day antimicrobial agents and vaccines was not an accidental occurrence, but the result of many decades of intensive research and vast amounts of money (Brundtland, 2000).

The last 30 years have seen the development of acyclovir, which is used to treat herpes related infections, the anti-retroviral drug zidovudine (AZT), used in the treatment of HIV, and the development of the first protease inhibitor used in the clinical setting. Currently, the number of antimicrobial drugs used to combat infection and infection-related disorders amounts to more than 150, with the cost of researching and developing each new drug amounting to about US\$500 million per drug (Brundtland, 2000). The discovery of antimicrobial agents and subsequent development of various agents used in the clinical setting has certainly greatly improved the life expectancy of the current generation and individuals privileged enough to have access to quality drugs, compared to that of generations of the previous century. However, humanity is currently faced with another crisis, one which, if not dealt with swiftly and efficiently, could very well see future generations plummeted into a post-antibiotic era which may be similar to pre-antibiotic times. Infectious diseases which were until recently curable are becoming harder to treat, such as typhus and gonorrhoea, while tuberculosis and malaria continue to exhibit an increasing amount of resistance to drugs which were formerly effective (Brundtland, 2000).

2.7.2. Microbial resistance

The development of antimicrobial resistance amongst microbes does not appear to be a phenomenon of the last decade. Only a few years after penicillin was introduced to the market, a penicillin-resistant strain of *S. aureus* was discovered, followed in the next 20 to 25 years by resistant strains

of *Gonorrhoea* spp., *Shigella* spp. and *Salmonella* spp. Since then the problem of microbial resistance has grown to one with tremendous economic, social and political implications. Multi-drug resistant tuberculosis (MDR-TB) strains are no longer exclusively associated with individuals infected with HIV, poor living conditions and poverty. It has infiltrated Europe, Asia and Africa, and may be found amongst the general population and healthcare workers. Penicillin-resistant pneumococci are spreading rapidly, while resistant strains of the malaria parasite kill millions of individuals on an annual basis. As recently as a decade ago all strains of cholera collected around New Delhi, India, were shown to be susceptible to the first line drugs furazolidone, ampicillin, co-trimoxazole and nalidixic acid. Today, however, these drugs have no effect on the previously susceptible organisms (Brundtland, 2000).

In Southeast Asia, 98% of all gonorrhoea cases are multi-drug resistant, while 60% of all cases of visceral leishmaniasis in India are reported to be resistant to first line antimicrobials. In the industrialised world, as much as 60% of nosocomial infections are caused by drug resistant organisms, the most recently identified being Vancomycin Resistant Enterococcus (VRE) and Methicillin Resistant *S. aureus* (MRSA). The development of microbial resistance may be attributed mainly to overuse of antibiotics by developed nations and the under-use of effective antibiotics by developing nations, as a result of ignorance, poverty and poor healthcare services (Brundtland, 2000). It has now become necessary to develop and implement second- and third line drugs which are considerably more expensive to fight off infectious disease such as MDR-TB, which exacerbates the problem of poor individuals in developing nations often not being able to access these drugs.

2.7.2.1. Major problematic organisms expressing antimicrobial resistance

Although humanity has been plagued by a myriad of infectious diseases throughout the ages, ranging from bacterial and fungal infections to viral and parasite related ailments, some of which have been brought under control or

even eradicated, a number of infectious diseases continue to devastate many populations around the globe. In her infectious disease report of 2000, Dr Gro Harlem Brundtland, Director General of the WHO, included a list of infectious related pathologies that are becoming increasingly more difficult to treat as resistance to current antimicrobials develops in the causative organisms. The list includes:

- **Pneumonia** – Including *Streptococcus pneumoniae* and *Haemophilus influenzae* as the primary pathogens.
- **Diarrhoeal diseases** – Including *Shigella dysenteriae* and *Salmonella typhi* as the primary pathogens.
- **Tuberculosis** – Including *Mycobacterium tuberculosis* and the development of MDR-TB and XDR-TB as the main causes of concern.
- **Malaria** – The development of resistance in *P. falciparum* against chloroquine and other 2nd and 3rd generation antimalarial drugs is increasingly becoming a concern.
- **AIDS** – The major obstacles in combating the disease remain ignorance, a lack of education, poor health service delivery in developing nations and the inaccessibility of antiretroviral drugs (ARV's) and diagnostic tests to individuals in rural areas.
- **Hepatitis viruses** – Including Hepatitis B virus (HBV) and hepatitis C virus (HCV) as the primary causes of concern.
- **Gonorrhoea** – The development of resistance to penicillin and ciprofloxacin in *Neisseria gonorrhoea*, as well as the appearance of MDR strains is particularly worrying.
- **Nosocomial infections** – MRSA and VRE are increasingly becoming bigger health risks in hospitals. Vancomycin, the only drug available to successfully treat MRSA, is itself becoming less effective in the face of the emergence of Vancomycin Intermediate *Staphylococcus aureus* (VISA).

(Brundtland, 2000)

2.7.2.2. Factors contributing to the development of resistance

The development of antimicrobial resistance is the result of a combination of various factors, and occurs through a process of natural selection by which susceptible organisms are killed by a specific drug, while resistant ones survive and subsequently pass on their genes either by replication or conjugation. The latter is a natural process entailing the exchange of DNA-containing plasmids between bacteria (Brundtland, 2000).

Antimicrobial drugs are categorised into several general groups according to their mode of action:

- Drugs that selectively target and inhibit microbial cell wall synthesis.
- Drugs that inhibit prokaryotic translation and protein synthesis.
- Drugs that disrupt the cytoplasmic membrane.
- Drugs selectively inhibiting prokaryotic metabolic pathways.
- Drugs which inhibit nucleic acid synthesis.
- Drugs preventing microbial recognition of- or attaching to- its host.

(Bauman, 2007, p.289)

Antimicrobial-resistant microbes have developed and employ various strategies which circumvent the above listed mechanisms of antimicrobial action. Many bacteria possess porin proteins which act as channels into the cell for a variety of substances, as well as cytoplasmic penicillin-binding proteins (PBP's) which are vital for cell wall synthesis (Hopley & Van Schalkwyk, 2006). Beta lactams such as penicillin function by entering the cell via the porin and binding to PBP's, hampering cell wall synthesis and thus destroying the organism. Bacteria may, however, avoid this process by employing one of the following strategies:

- Some bacterial cells lose their porin proteins, a process which would, under normal circumstances, be disastrous for the cell as it would lose its competitive edge. In the presence of penicillin, however, the cell survives as penicillin does not enter it.

- Bacteria may possess specialised “pumps” which are used to pump out penicillin and other beta lactams as soon as they enter the cell.
- The beta lactam binding site on PBP's may be altered, resulting in the inability of the beta lactam to bind and subsequently inactivate PBP's.
- Enzymes capable of inactivating a wide variety of antimicrobials are employed by some bacteria, including beta lactamases capable of inactivating beta lactams, as well as enzymes which inactivate aminoglycosides, chloramphenicol etc. Several substances which are able to inhibit beta lactamases have been used, including clavulinic acid.

(Hopley & Van Schalkwyk, 2006)

Beta lactamases may be classified into the following groups:

1. Cephalosporinases, not inhibited by clavulanic acid.
2. Penicillinases, inhibited by clavulanic acid.
3. Penicillinases, not inhibited by clavulanic acid.
4. Metallo-beta-lactamases

(Hopley & Van Schalkwyk, 2006)

A wide variety of Gram negative bacteria possess group 1 enzymes. *Amp C* cephalosporinases are common and occur in the plasmids of organisms such as *Klebsiella pneumoniae* and *E. coli*. They are resistant to 1st, 2nd, and 3rd generation cephalosporins, as well as penicillins, cephamycins, monobactams and combinations of beta lactams and beta lactamase inhibitors. Group two enzymes include TEM-1 beta lactamases and extended spectrum beta lactamases. They show resistance to a wide variety of cephalosporins and aztreonam. Group 3 enzymes are capable of hydrolysing carbapenems such as imipenem, and are found in *Aeromonas*, *Bacteroides* and *P. aeruginosa*, while group 4 enzymes are uncommon.

A fifth means of antimicrobial resistance involves the antimicrobial trimethoprim and its role in the inhibition of folic acid synthesis by bacteria. Bacteria synthesise their own folic acid with the aid of dihydrofolate reductase.

Trimethoprim inhibits this enzyme and subsequent folate synthesis, which is crucial for DNA formation. However, some organisms acquire plasmid-borne enzymes capable of bypassing the dihydrofolate reductase step, escaping the inhibiting effects of trimethoprim.

Fungi employ similar mechanisms in the development of antifungal resistance, which is observed at an increasing rate, especially in the intensive care units of hospitals (Hopley & Van Schalkwyk, 2006). Various factors have contributed to the stance of antimicrobial resistance observed today. These include:

- **Poverty and drug access** – Poverty remains one of the major forces indirectly driving the alarming development and spread of antimicrobial resistance currently observed. Many individuals in developing nations can simply not afford, or do not have access to effective drugs, while those who can afford single doses start on treatment and continue to use the medication only until they feel better. The infecting organism is not necessarily eliminated, however, which greatly contributes to the development of resistance. Treatment with 2nd and 3rd line drugs is relatively more expensive than 1st line drugs, is more toxic to the patient and is becoming less effective due to the rapid rate at which microbes mutate and develop resistance to new drugs.
- **Misdiagnosis** – Often a result of poor healthcare systems in developing nations, overworked and ill-informed physicians and healthcare workers are unable to give adequate attention to all patients. A lack of diagnostic laboratory services and tests further increases the pressure on physicians to make an accurate diagnosis. This may lead to healthcare workers often having to “guess” the diagnosis based on the symptoms, subsequently leading to the wrongful prescription of medication.
- **Counterfeit drugs** – Being a US\$21 billion industry and comprising 5% of all antibiotics sold worldwide, counterfeit drugs lead to the death of those whose lives might have been saved had they been administered the proper medication. The abuse, misuse and administration of antimicrobial

drugs at lower levels than treatment guidelines dictate, contribute to the phenomenon of natural selection amongst microbes, leading to the elimination of only those in a population susceptible to a specific drug, and allowing resistant ones to flourish. As many as 51% of the counterfeiting cases uncovered by the WHO between 1992 and 1994 revealed counterfeit drugs to contain no active ingredients. A further 17% contained the wrong active ingredient, while 11% contained active ingredients lower than the dictated dose. Furthermore, some of these drugs showed to contain fatal poisons.

- **Corruption** – In countries where physicians earn less than what the global trend dictates, unethical pharmaceutical companies offer these healthcare workers commission on prescribing more expensive, broad-spectrum antimicrobials to their patients, while cheaper, narrow-spectrum drugs would have been adequate. This leads to a smaller, more expensive pool of drugs used to treat infectious disease, and greatly contributes to the development of drug resistance amongst microbes.
- **Media and advertising** – Pressure on physicians from their patients requesting specific drugs seen advertised in the media has lead to many physicians prescribing drugs which would otherwise not have been necessary. As many as 70% of American physicians questioned admitted to succumbing to patient pressure and prescribing drugs they would have otherwise avoided. Patient ignorance and misinformation creates a further problem. In the Philippines many parents believe isoniazid to be a “lung vitamin”, with which they dose their children. The sub-therapeutic administration of this anti-tuberculosis drug directly contributes to the development of resistant organisms.
- **Lack of education** – The shortage of qualified healthcare workers in developing nations leads to many patients having to rely on their own judgement regarding the use of antimicrobials. Additionally, many drug dispensers in developing nations are under-qualified and dispense drugs without a physician prescription or consulting with the patient. In developed countries, the topic of antimicrobial resistance enjoys little

attention in medical schools, and is rather dealt with as a subject of expertise.

- **Role of hospitals** – A study of 10 training hospitals worldwide showed that between 40% and 91% of antibiotics prescribed were inappropriate, and that healthcare workers disregarded basic hygiene procedures such as hand washing and the changing of gloves in between patient visits. In a separate study of clinics in Tanzania, researchers demonstrated that 40% of reusable needles which were supposed to be sterile were contaminated. Inadequate training and monitoring of proper hygiene procedures by healthcare workers can have dire consequences for both clinician and patient.
- **Use of antimicrobials as growth promoters in food production** – Only 50% of antimicrobials produced are consumed by humans. The other half is used in the treatment of sick livestock, as growth promoters in these animals and to rid and protect cultivated foods from infecting organisms. This low-level prophylactic use of antimicrobials greatly contributes to the development of resistance amongst organisms in livestock, and creates the possibility of a resistant strain jumping the species to humans, such as VRE.
- **Globalisation and travel** – The current rate of international travel means that microbes originating from Africa or Asia may arrive in North America in less than a day. The possibility of unknowingly aiding in the spread of resistant microbes across continents is all too real, as was shown when two Canadian cases of MRSA were traced back to India.

(Brundtland, 2000)

In an effort to curb the current trend in the development of antimicrobial resistance amongst microbes, Brundtland (2000) has proposed the following guidelines and suggestions to be implemented by governments:

- Adoption of WHO strategies and policies
- Education of health workers and the public on the proper use of medications.

- The containment of resistance in hospitals and the prevention of the spread of resistant organisms to the public.
- A reduction in the use of antimicrobials in livestock as growth promoters and substitutes for high-quality animal hygiene practices.
- Increased efforts for the research and development of new drugs and vaccines.
- The forging of alliances between governments, international organisations, non-government organisations (NGO's) and the private- and public sectors to increase access to antimicrobials.
- An increase in the availability of essential drugs.
- Increasing the accessibility of effective quality drugs to the poor.

The employment of more effective treatment strategies, immunisation- and vaccination programmes, better nutrition, enhanced vector control and initiatives aimed at the impoverished have greatly contributed towards controlling the spread of drug resistance amongst microbes. However, individuals, governments, NGOs, large pharmaceutical companies and private- and public organisations need to invest time, money, effort, flexibility, cooperation, philanthropy and personal commitment if the growing problem of antimicrobial resistance is to be halted. If quality antimicrobials are not used wiser and made more accessible, current effective drugs may soon be rendered useless against the increasing population of drug-resistant microbes (Brundtland, 2000).

2.7.3. Phytomedicinal sources of antimicrobial agents

The medicinal qualities of plants have been known to and exploited by man for centuries. Historic texts are rich in information regarding the medicinal applications of plants. The previously mentioned Ebers papyrus contains information on nearly a thousand different medicines, most of which are of plant origin. Of the hundreds of plants mentioned in the Bible, 25 to 30 have been identified as medicinal, while ancient Chinese scripts refer to thousands

of phytomedicinal remedies. Hippocrates, who is widely considered to be the father of modern medicine, mentioned about 400 drugs, 91% of these originating from plants (Lev, 2007).

Al-Bakri and Afifi (2007) claim that the vast majority of modern drugs are derived from traditional herbal remedies. Indeed, more than 50% of drugs currently used are products of or derived from higher plants. Seventy eight percent of drugs of natural origin approved by the United States Food and Drug Administration between 1983 and 1994 were of antibacterial type (Du Toit, Elgorashi, Malan, Mulholland, Drewes & Van Staden, 2007). Higher plants produce thousands of chemical compounds with a wide array of functions, including acting as pollinator attractants and defending the plant against insects, herbivores and microbes. Many phytochemicals acting as antimicrobial agents in the plant's defence are likewise active against human pathogenic organisms, and various studies report on the antimicrobial activities of crude plant extracts (Rojas, Bustamante, Bauer, Fernández, Albán & Lock, 2003). Although the majority of current antimicrobials are derived from soil-borne microbes, higher plants have also proven to be a valuable source of antibiotics, such as the isoquinoline alkaloid emetine obtained from the underground parts of *Cephaelis ipecacuanha*, as well as the antiparasitic agent quinine, obtained from the bark of the Cinchona tree. The bacteriostatic and antifungal properties of lichens, the antibiotic action of the garlic (*Allium sativum*) compound alliin, and the antimicrobial action of berberines in goldenseal (*Hydrastis Canadensis*) have also been described. Other examples of plants with the potential of producing novel antimicrobial agents include *Garcinia cola*, *Aframomum melegueta*, *Xylopiya aethiopica*, *Cryptolepis sanguinolenta*, *Chasmanthera dependens*, *Nauclea latifolia* and *Araliopsis tabouensis*, all of which exhibit various forms of antibacterial-, antifungal- and antiviral activities (Janick, 1999, 457-461).

The alarming increase in the incidence of new and reoccurring infectious diseases, as well as the continued evolving of antimicrobial resistance amongst a wide array of pathogenic microbes, highlights the dire need for novel, effective antimicrobial agents. Of the 250 000 to 500 000 known plant

species, very few have been investigated for their pharmacological qualities, with an estimated 5000 having been investigated for their medicinal properties (Rates, 2001). The emergence of multiple-drug-resistant organisms necessitates the exploration of all available avenues by man in the search for novel antimicrobial agents, including plants and traditional phytomedicines. Plant-derived drugs not only have fewer negative side-effects in general, but are also cheaper and more accessible than modern Western drugs to the impoverished in developing nations, who appear to be the worst off concerning infectious diseases. *G. incanum*, *A. afra* and *A. absinthium* have traditionally been used in the treatment of a wide variety of maladies, as mentioned in Sections 2.3.1, 2.4.1. and 2.5.1., respectively. Most of these ailments are of an infectious nature, and the traditional use of these plants in the treatment and relief of infectious disease is an indication of their antimicrobial qualities.

CHAPTER 3

INTRODUCTION TO THE PRESENT STUDY

3.1. Aims and objectives

The present study had two main aims, and it was attempted throughout the study to reach these in as scientific a manner as possible. The main aims of the study were:

1. To scientifically determine the antimicrobial properties of two plants, being *Geranium incanum* and *Artemisia afra*, these traditionally having been used in South Africa for their medicinal properties, and comparing the antimicrobial properties of *A. afra* to those of its European counterpart, *Artemisia absinthium*, and
2. To scientifically determine the anticancer properties of *G. incanum* and *A. afra*, and comparing the anticancer properties of *A. afra* to those of *A. absinthium*.

Through the use of well established scientific methods and research materials, the present study aimed to fulfill the following objectives:

1. Collection and identification of plant materials used in the study.
2. Preparation of both traditional and non-traditional plant extracts using various extractants and solvents.
3. Determination of the inhibitory qualities of plant extracts against selected strains of microbes representing both Gram positive and Gram negative organisms as well as yeasts, using an adapted agar dilution method.
4. Determination of the IC₅₀ values of the various plant extracts after exposure of three cancer cell lines to these extracts, using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay.

5. Determination of the mode of cell death induced (apoptosis or necrosis) in any one of the three cancer cell lines expressing the highest level of sensitivity to a specific extract through the use of fluorescence staining and fluorescence microscopy.
6. Determination of the degree of sensitivity of peripheral blood mononuclear cells (PBMC's) to the plant extract which show the highest degree of toxicity towards any one of the three plant extracts screened, using the Cell Titer-Blue® assay.
7. Determination of the percentage of apoptotic cells present in a sample of cancer cells from the line most susceptible to a specific extract following exposure to that extract, comparing these results to the percentage apoptotic cells present in the same cell line after exposure to cisplatin, and determining which phase of the cell cycle is most affected by the specific extract using the Beckman Coulter FC 500 cytometer and Coulter® DNA Prep™ Reagents Kit.

3.2. Collection, preparation and extraction of plant materials

The three plants *G. incanum*, *A. afra* and *A. absinthium* have long been used both in South Africa and beyond its borders for their medicinal properties, as outlined in the previous chapter, and were selected based on this literature.

For the purposes of antimicrobial screening, plant materials were collected and extracted over three seasons, having been winter, spring and summer. It is a well established fact that the phytochemical profile of plants and their potency as medicinal plants are influenced by various factors such as seasonal changes, geographical distribution, climatic variations and other environmental factors (Fennell *et al.*, 2004), and for this reason the extraction yields obtained for antimicrobial screening from the three plants were compared according to the season in which they were prepared. Due to time limitations, the same could not

be done for extracts used in anticancer studies, with one set of extracts having been prepared for anticancer studies during the summer.

Furthermore, the medicinal properties of plants are dependent on the extractant used, with variation in the biological activities of plants having been observed for different extractants used. The literature reports on various extractants used to extract biologically active compounds from plants, including water, ethanol, methanol, hexane, chloroform, petroleum ether, methylene dichloride, ethyl acetate and methanol-chloroform-water mixture (Eloff, 1998; Eloff, 1999; George, Laing & Drewes, 2001; Lin, Opoku, Geheeb-Keller, Hutchings, Terblanche, Jäger & Van Staden, 1999; Martini & Eloff, 1998; Shale, Stirk & Van Staden, 1999), with relative variation in the amount of active compounds extracted when different extractants are used. George *et al.* (2001) has stated it essential for preparation of medicinal plant extracts according to traditional methods to be taken into account when the extractant is chosen. Therefore, it was decided to employ water, which is reported to be routinely used by traditional healers as extractant (Lin *et al.*, 1999; Shale *et al.*, 1999), as well as two other extractants, including methanol and acetone. Infusions of *G. incanum* and *A. afra* were prepared according to the traditional methods described by Scott & Springfield (2004a, p.4) and Scott & Springfield (2004b, p.4), respectively. As the medicinal properties of *A. afra* were to be compared to those of *A. absinthium*, an infusion of the latter was prepared in the same manner as for *A. afra*. Aqueous-, methanol- and acetone extracts of all three plants were further prepared according to a method adapted from Eloff (1998) and Lin *et al.* (1999).

3.3. Screening of plant extracts for antimicrobial activity

An agar dilution method adapted from Alanis *et al.* (2005), Kumar, Chauhan, Padh & Rajani (2006), Tshikalange, Meyer & Hussein (2004) and Weckesser, Engel, Simon-Haarhaus, Wittmer, Pelz & Schempp. (in press), was employed in the screening the plant extracts for antimicrobial activity towards various

microbial strains of *C. albicans*, *E. coli*, *P. aeruginosa*, *Enterococcus faecalis*, *S. aureus* and *Bacillus cereus*. Screening was performed in triplicate, with a new set of extracts having been prepared for each round of screening, at an extract concentration of 100 mg/ml agar. A Mast Diagnostics® (UK) multipoint inoculator was used to inoculate agar plates containing the extracts, and plates were visually inspected after 48 hours incubation. Where a specific strain of any one of the test organisms was found to be resistant to an extract for two out of the three rounds of screening, that specific strain was reported as being resistant to inhibition of growth by the relevant extract. Likewise, where a specific strain of any one of the test organisms was found to be inhibited by an extract for two out of the three rounds of screening, that specific strain was reported as being susceptible to growth inhibition by the relevant extract. Three control plates were simultaneously run and compared to test plates following incubation in order to establish the validity of the results.

3.4. Screening of plant extracts for anticancer activity

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, a quantitative means of determining mammalian cell survival and proliferation *in vitro*, was adapted from Mosmann (1983, pp.55-63) and used to screen for the anticancer activities of the various plant extracts towards MCF-7 (human breast adenocarcinoma) cells, HT-29 (human colonic adenocarcinoma) cells and HeLa (human cervical cancer) cells, at initial extract concentrations ranging between 500 µg/ml and 10 µg/ml. Variation in the degree of toxicity expressed by the various extracts in the three cancer cell lines was observed, depending on the extractant used, with some extracts, especially those for which acetone was used as the extract, showing higher degrees of toxicity in the malignant cells.

Where extracts appeared to significantly inhibit cell growth or induce cell death, screening of the specific extract on the respective cell line was repeated using lower concentrations of the extract. Cells cultured in cell growth media lacking

extract served as an extract blank and negative control for each of the extracts screened against each of the three cancer cell lines, while cisplatin, a known anticancer agent, was serially diluted and served as a positive control. Screening of various dilutions of each extract against the three malignant cell lines was performed in triplicate, and cell viability, as well as inhibition of cell growth/cell death was expressed as a mean percentage \pm standard deviation of the mean negative control absorbance. Dose response curves were constructed from this data, and IC₅₀ values for each of the extracts on each of the three cell lines was determined through regression analysis.

It was decided to investigate the mode of cell death induced in the cell line most susceptible to inhibition by any one of the twelve plant extracts prepared, i.e. the extract expressing the highest degree of toxicity at the lowest concentration for any one of the three cell lines, as determined from the results obtained during the MTT assay. Fluorescence microscopy, using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodide (PI) as fluorescent dyes, was employed for this purpose. Due to structural differences between cells undergoing apoptosis as apposed to necrosis, DAPI is able to penetrate and stain apoptotic and living cells, while PI is excluded. PI, however is capable of entering and staining the nuclei of necrotic cells (Darzynkiewicz *et al.*, as cited in Bungu, 2005, p.50).

Cells were separately stained with DAPI and PI using a combined, modified method of Moongkarndi, Kosem, Kaslungka, Luanratana, Pongpan & Neungton (2004), Bungu (2005, p.51) and Botha (2003, p.80), and observed under a fluorescence microscope for staining patterns and hallmarks specific for apoptosis or necrosis. As a positive control, the same type of cells were exposed to cisplatin, which induces apoptosis in malignant cells, for 48 hours, and separately stained with DAPI and PI, followed by observation through a fluorescence microscope. Cells undergoing apoptosis as a result of exposure to cisplatin were compared to cells treated with the extract to establish the

possibility of apoptosis induced by the extract. As a negative control, cells cultured in media lacking any extract or cisplatin were separately stained with DAPI and PI and observed through a fluorescence microscope. Photographs were taken of both cells exposed to cisplatin and cells exposed to the extract, and examined for correlations.

The toxicity of the extract tested during fluorescence microscopy on normal, non-malignant human cells was determined using the CellTiter-Blue® assay as outlined in the Promega Technical Bulletin (2006), where it is described as a fluorometric method for the estimation of the amount of viable cells present in a sample, based upon the principle of viable cells being able to reduce the indicator dye resazurin, which possesses very little intrinsic fluorescence, to the highly fluorescent resorufin, with an excitation wavelength of 544 nm and emission wavelength of 590 nm. Although the MTT and CellTiter-Blue® assays are in principle similar, it was decided to use the latter in the screening of peripheral blood mononuclear cells (PBMC's) as apposed to the former assay. During the MTT assay, formazen crystals are retained inside the viable malignant cells adhering to the bottom of the microtitre plate well, which is then dissolved using DMSO following removal of the growth medium by vacuum, and the absorbance measured spectrophotometrically. During the CellTiter-Blue® assay fluorescent resorufin, formed from the conversion of resazurin by viable cells in suspension, precipitates into the surrounding growth medium, eliminating removal of growth medium (as this would also remove the resazurin formed) and dissolving with DMSO as in the MTT assay. It would have been possible to use the CellTiter-Blue® assay instead of the MTT assay in the general screening for toxicity of plant extracts in the three cancer cell lines, although this would essentially have been financially less viable and unnecessary, as both assays are similar.

PBMC's were isolated from a healthy, consenting adult male and exposed to various concentrations of the extract, which ranged between 500 µg/ml and 2.65

µg/ml, for 48 hours, followed by the addition of the assay reagent and measurement of the fluorescence using a Flouroskan Ascent® fluorometer. Three controls, including a control consisting of PBMC's cultured in growth media only, a negative control consisting of growth media lacking PBMC's or extract, and a test compound control consisting of various concentrations of the extract tested, having ranged between 500 µg/ml and 2.65 µg/ml, in growth media lacking PBMC's, were run simultaneously. The purpose of this third control was to exclude any fluorescence emitted by the extract itself or fluorescence produced as a result of interaction between the chemistry of the extract and assay reagent.

Screening was performed in triplicate, and fluorescence and possible interference of the extract itself was excluded by subtracting the mean values of any fluorescence emitted by each of the extract dilutions from the third control from the fluorescence readings obtained for the PBMC's treated with the respective dilutions of the extract. The percentage of PBMC viability, as well as the percentage cell death was determined by expressing the final fluorescence values for PBMC's treated with the extract as a mean percentage \pm standard deviation of the mean fluorescence readings recorded for the vehicle control. This data was used to construct a dose response curve of the extract for PBMC's, and compared to that constructed for the same extract on malignant cells. Regression analysis was also done in order to determine the IC₅₀ of the extract for PBMC's.

Finally, the effect of the extract screened against PBMC's on the cell cycle of the malignant cell line used during fluorescence microscopy was investigated by exposing the cells to the IC₅₀ of the extract as determined during the MTT assay, lysing and staining of the cells with a lysis buffer and PI, respectively, which were both included in the Coulter® DNA Prep™ Reagents Kit, and analysing the cell cycles of the cells using the Beckman Coulter FC 500 cytometer. As a positive control, the same cell line was exposed to cisplatin, while untreated cells were

used as a negative control. Both cisplatin treated cells and untreated cells were lysed and stained as was the case with the cells exposed to the extract, their cell cycles were analysed and the percentage apoptotic cells present in the cell sample exposed to the extract was compared to the percentage apoptotic cells present in the sample exposed to cisplatin as well as in the untreated cell sample, respectively. Screening was performed in triplicate.

CHAPTER 4

METHODS AND MATERIALS

4.1. Introduction

The current chapter discusses the materials and methods used in the harvesting of *G. incanum*, *A. afra* and *A. absinthium* plant materials, the preparation of infusions, aqueous-, methanol- and acetone extracts of the three respective plants, as well as the performance of antimicrobial activity screening of the respective infusions and extracts. Maintenance of cell cultures and anticancer activity screening of the respective infusions and extracts, as well as assays performed to determine whether apoptosis or necrosis were induced in selected cell lines, are also discussed.

4.2. Antimicrobial activity studies

4.2.1. Collection and preparation of plant materials

Antimicrobial screening assays were done in triplicate. Fresh plant materials were harvested and fresh extracts prepared for each round of antimicrobial screening. Due to large extraction volumes, freeze drying of infusions and aqueous extracts were lengthy, which resulted in plant materials being collected under different seasonal and environmental conditions between the triplicate antimicrobial screenings. Furthermore, due to an inconsistency in the availability of *G. incanum*, fresh plant material was collected from three different locations for each round of antimicrobial screening.

The harvesting and preparation of fresh plant materials occurred during June 2007, September 2007 and January 2008 for each round of antimicrobial

screening, respectively. For the initial round of antimicrobial screening, *G. incanum* plant material was obtained from the garden of Mrs. E. Baxter of the Nelson Mandela Metropolitan University (NMMU). In September 2007, *G. incanum* plant material was harvested from the horticultural service department of the NMMU, while fresh plant material of *G. incanum* was collected from the garden of Dr. N. Smith of the NMMU in January, 2008. *G. incanum* plants were identified and authenticated by Mr. Clayton Weatherall-Thomas from the Botany department of the NMMU, and a voucher specimen was submitted to the NMMU Ria Olivier herbarium. *A. afra* plants were obtained from New Plant Nurseries in George, Western Cape, in June 2007, and nursed at the horticultural service department of the NMMU, from where fresh plant material was collected in June 2007, September 2007 and January 2008, respectively. *A. afra* plants were identified and authenticated by Mrs. Cornelia Garner from the Botany department of the NMMU, and a voucher specimen was submitted to the NMMU Ria Olivier herbarium. Fresh plant material of *A. absinthium* was obtained from the garden of Dr. M van de Venter of the NMMU during aforementioned months for the initial-, second- and third rounds of antimicrobial screening, respectively. *A. absinthium* plants were identified and authenticated by Mrs. Estelle Brink from the Botany department of Rhodes University, Grahamstown, and a voucher specimen was submitted to the Selmar Schonland herbarium at Rhodes University, Grahamstown.

Fresh leaves were separated from the stems of the respective plants and separately rinsed in distilled water in order to remove dust, soil and insects. The washed leaves were allowed to dry at room temperature, separately weighed and used immediately for the preparation of infusions, while the leaves used in the preparation of aqueous-, methanol- and acetone extracts were first dried overnight in an oven at 40°C.



Figure 4.1: Dried *G. incanum* plant material.



Figure 4.2: Dried *A. afra* plant material.



Figure 4.3: Dried *A. absinthium* plant material.

4.2.2. Extraction of plant materials

4.2.2.1. Preparation of infusions

Infusions of *G. incanum* were prepared according to the traditional method described by Scott & Springfield (2004a, p.4). A quarter teacup full of fresh leaves (approximately 6 g) was added to a teacup full (180 ml) of boiling distilled water and left to infuse for ten minutes. The infusion was filtered using Whatman No 1 filter paper to remove insoluble plant material and the filtrate frozen at -20°C in clean, pre-weighed 50 ml centrifuge tubes (Eppendorf®, Germany). Samples were freeze dried (VirTis® freeze-dryer, USA) and weighed in order to determine the percentage yield.

Infusions of *A. afra* were also prepared according to a traditional method described by Scott & Springfield (2004b, p.4). Approximately 14 g of fresh leaves were added to 1 litre of boiling distilled water and left to infuse until cool. Samples were then dried as described above. *A. absinthium* was included in this study to compare its biochemical effects to those of *A. afra*. For this reason, the infusion and extracts of *A. absinthium* were prepared in the same manner as that of *A. afra*.

As mentioned previously, fresh extracts were prepared for each round of antimicrobial screening, which was done in triplicate. Thus the above procedures for the preparation of infusions of the three test plants, including the amount of fresh leaves and extraction medium used, were also done in triplicate for each of the respective rounds of antimicrobial screening. However, due to the lengthy time required for the freeze drying of the large volumes of *A. afra* and *A. absinthium* infusions and aqueous extracts, as well as time constraints, the above method was slightly modified upon the third round of infusion preparation. For the third round, infusions were not freeze dried immediately, but were instead concentrated in a laminar air flow cabinet (Labscheme Allchem®, South Africa)

by evaporation in a hot water bath for 48 hours at 40°C to 50 ml. This significantly reduced the volume of infusions and aqueous extracts of *A. afra* and *A. absinthium*, and subsequently the time required for freeze drying. Freeze dried extracts were stored at room temperature in the dark until required.

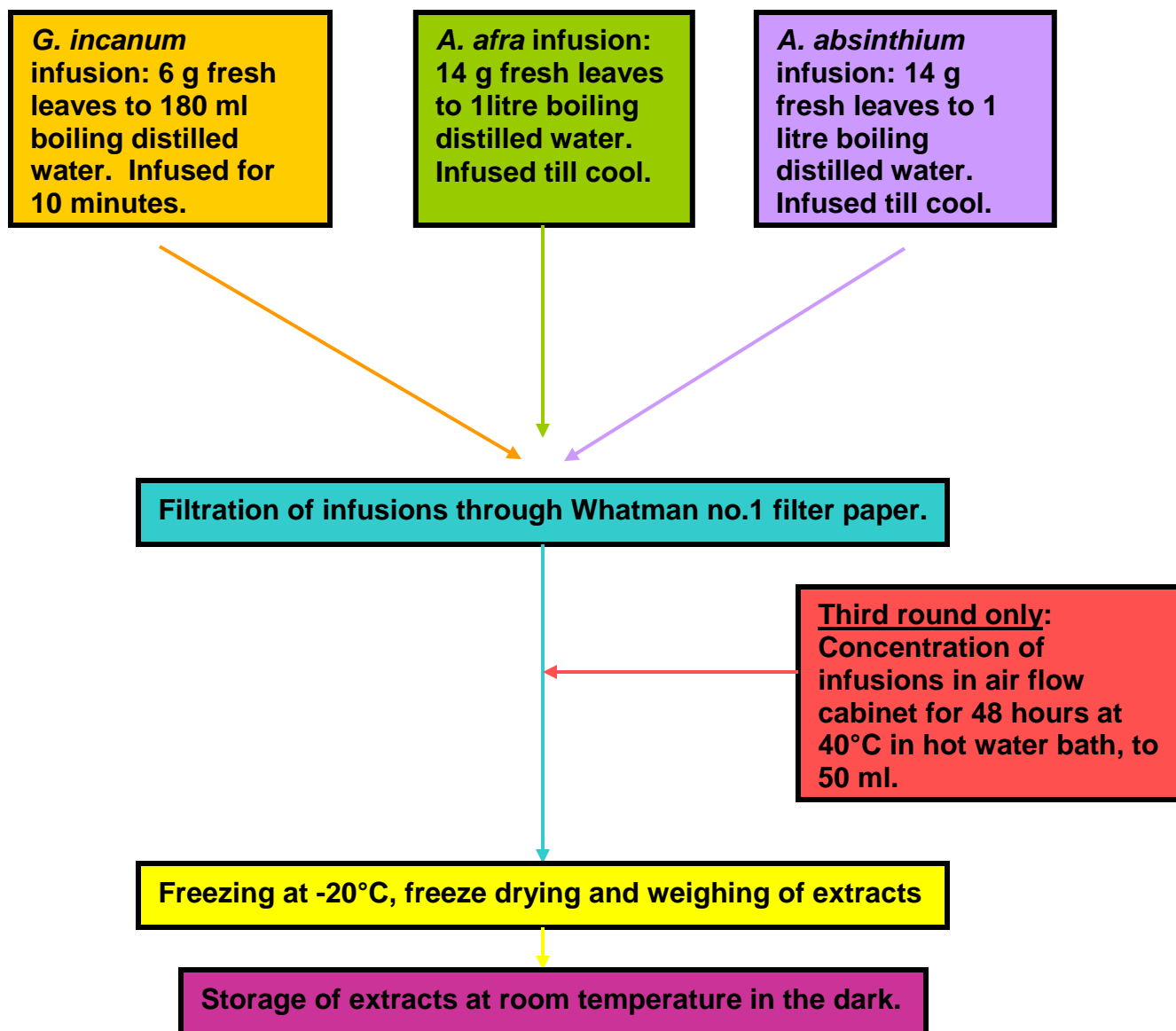


Figure 4.4: Schematic representation of the preparation of infusions for antimicrobial screening.

4.2.2.2. Preparation of aqueous-, methanol- and acetone extracts

An extraction method adapted from Eloff (1998) and Lin *et al.* (1999) was used to prepare the aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium*, respectively. The remaining fresh leaves of the three test plants were separately weighed, dried over night in an oven at 40°C and weighed again the following morning in order to determine the percentage yield of dry plant material. The dried leaves of each of the three respective plants were then divided into three equal amounts from which aqueous-, methanol- and acetone extracts were prepared.

For the preparation of aqueous extracts, 4.47 g of dry *G. incanum* leaves, 12.15 g of dry *A. afra* leaves, and 12.46 g of dry *A. absinthium* leaves were separately and equally divided into 50 ml centrifuge tubes. The centrifuge tubes were then filled to 40 ml with distilled water at room temperature (RT), shaken vigorously for 3 minutes and centrifuged in an Eppendorf® 5810 centrifuge (Germany) at 1811 x g for 15 minutes. After centrifugation, the supernatants were decanted separately into clean, pre-weighed 50 ml centrifuge tubes. This process was repeated two more times for each of the three test plants. The respective aqueous extracts were then frozen at -20°C in pre-weighed centrifuge tubes and freeze dried, followed by storage in the dark at room temperature until required. This procedure was followed for the initial- and second rounds of preparation of aqueous extracts. However, it was decided to further adapt this method in order to decrease the amount of time required for freeze drying of the large volumes of aqueous extracts as described in Section 4.2.2.1.

The preparation of methanol- and acetone extracts was performed in the same manner as aqueous extracts, with the only exception having been that the supernatants were decanted into clean, pre-weighed glass beakers which were then placed in a hot water bath at 40°C over night in a laminar air flow cabinet to allow the methanol and acetone to evaporate, as apposed to freeze drying as

was done for aqueous extracts. After evaporation of the extractants, the glass beakers were weighed in order to determine the amount of crude methanol- and acetone extracts and percentage yield obtained, followed by storage at 4°C until required.

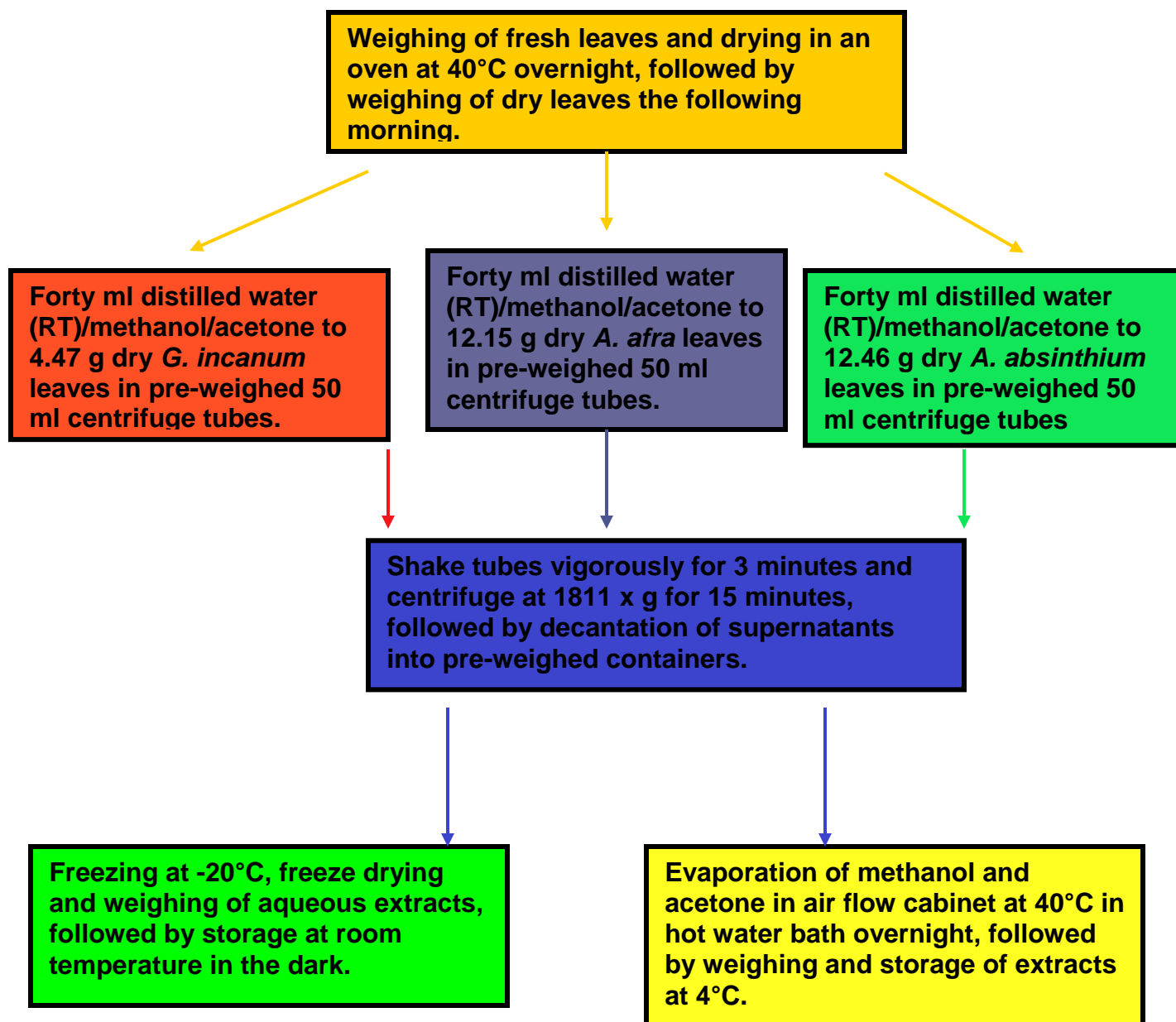


Figure 4.5: Schematic representation of the preparation of aqueous-, methanol- and acetone extracts for antimicrobial screening.

4.2.3. Antimicrobial screening

4.2.3.1. Culturing and maintenance of microbial test strains

A Pro-Lab Diagnostics Microbank™ kit (Toronto, Canada) was supplied by the Department of Biomedical Technology of the NMMU, and utilised in the culturing and maintenance of the bacterial test strains. The kit contained 5 different families of microbes, including *S. aureus*, *C. albicans*, *E. coli*, *E. faecalis* and *P. aeruginosa*. Ten strains of each of the 5 species of the aforementioned microbes were included in the kit. The first strain of each of the 5 families was from the American Type Culture Collection (ATCC), including *C. albicans* (ATCC 66027), *E. coli* (ATCC 35218), *P. aeruginosa* (ATCC 27853), *E. faecalis* (ATCC 29212) and *S. aureus* (ATCC 43300). The remaining nine strains of each of the five respective microbial families were clinical isolates. Each strain, except for the seventh strain of *P. aeruginosa*, which was not available at that time and subsequently excluded from the first round of antimicrobial screening, was inoculated onto porous colored beads contained in cryovials. Furthermore, 4 strains of *B. cereus* inoculated in agar deeps, including *B. cereus* (ATCC 10876) and 3 clinical isolates, were supplied by the Department of Biomedical Technology of the NMMU and used as a sixth microbial species for screening.

For the culturing of the test organisms, one bead from each of the 49 respective cryovials was placed into 10 ml sterilised nutrient broth (Merck-Biolab, South Africa), while a small amount of each of the 4 strains of *B. cereus* was removed from the agar deeps and respectively inoculated into 10 ml sterile nutrient broths. The broths were then incubated at 37°C for 48 hours until turbid. In order to determine the purity of the cultures, the nutrient broths containing *S. aureus* and *E. faecalis* were inoculated on blood agar plates, while nutrient broths containing strains of *C. albicans* were inoculated on Sabouraud Dextrose agar (Merck-Biolab, South Africa). The remaining strains of *E. coli*, *P. aeruginosa* and *B. cereus* were respectively inoculated on nutrient agar plates (Merck-Biolab, South

Africa). The subcultured plates were then incubated at 37°C for 24 hours and examined for contamination.

4.2.3.2. Agar dilution method

An agar dilution method adapted from Alanis *et al.* (2005), Kumar *et al.* (2006), Tshikalange *et al.* (2004) and Weckesser *et al.* (in press), was employed in the screening of the microbes for sensitivity towards the infusions, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium*, respectively. The first round of antimicrobial screening was done in August, 2007. The 12 crude extracts of the 3 plants were dissolved in dimethylsulfoxide (DMSO) (Merck-Saarchem, South Africa) and added to 12 separate aliquots of 25 ml molten Mueller Hinton (MH) agar (Merck-Biolab, South Africa) in order to obtain a final concentration of 100 mg extract/ml agar. Lin *et al.* (1999) found that the growth media used appeared to play a decisive role in the determination of antimicrobial activity. When MH agar was used as growth medium, some extracts showed an increased capacity to inhibit the growth of *S. aureus* as apposed to when blood agar was used as growth medium. Similar results were reported when potato dextrose agar and SS agar were used to evaluate the inhibitory activity of extracts towards *C. albicans* and *Salmonella-Shigella* spp., respectively (Lin *et al.*, 1999). In both cases, MH agar was found to be a more effective growth medium to use for the assessment of antimicrobial activity.

Due to variation in the solubility of the various extracts in DMSO, different amounts of DMSO were used to solubilise the different extracts. Subsequently, different amounts of the respective extracts in DMSO had to be added to the agar in order to maintain a final concentration of 100 mg extract/ml agar. Variation also existed between the amounts of extracts retrieved during the three rounds of plant extractions, as is indicated in Section 5.2.1. Thus the amount of DMSO used to dissolve any specific extract also varied during the three

respective rounds. The maximum amount of DMSO used to dissolve any specific extract equaled 3.5 ml. This was used, both in the first and second rounds of extract preparation, to dissolve the aqueous extract of *A. absinthium*. The minimum amount of DMSO used to dissolve any specific extract equaled 1 ml. This was used during all three rounds of extract preparation to dissolve the infusion extract of *G. incanum*.

After addition of the dissolved extracts, the 12 aliquots of agar containing the respective plant extracts were poured into Petri plates and allowed to set. A Mast Diagnostics® (UK) multipoint inoculator (Figure 4.6) was used to inoculate dilutions equivalent to 1 McFarland standard of each of the pure strains of the six families of test microbes onto the MH agar plates. In order to establish whether any microbial susceptibility was due to the action of the respective extract being tested or merely a result of the DMSO used to dissolve the extract, 3 control plates were included in the screening. The first control plate consisted of MH agar containing DMSO equivalent to the highest volume of DMSO-dissolved extract added to a plate for a specific round of screening. For example, during the first round of screening, 3.5 ml DMSO was used to dissolve the aqueous extract of *A. absinthium*, and 702 µl of this extract in DMSO was added to 24.30 ml MH agar to obtain a concentration of 100 mg/ml. Thus, for the control, 702 µl of pure DMSO was added to 24.30ml MH agar.

The second control plate consisted of MH agar containing DMSO equivalent to the average volume of DMSO-dissolved extract added to a plate for a specific round of screening. For example, during the first round of screening, 2.26 ml DMSO was used to dissolve the acetone extract of *A. absinthium*, and 446 µl of this extract in DMSO was added to 24.55 ml MH agar to obtain a concentration of 100 mg/ml. Thus, for the control, 446 µl of pure DMSO was added to 24.55 ml MH agar.

The third control plate contained no DMSO. Due to the fact that the amount of DMSO added to controls 1 and 2 was dependent on the highest and average amounts of extracts in DMSO added to agar, and the fact that the amount of any specific extract added to agar varied during the three rounds of screening in order to maintain a concentration of 100mg/ml, the amounts of DMSO added to controls 1 and 2 also varied during the three rounds of antimicrobial screening. However, control 1 always contained DMSO equivalent to the highest amount of extract in DMSO added to a plate, while control 2 always contained DMSO equivalent to the average amount of extract in DMSO added to a plate. The control plates were inoculated with the various strains of the 6 species of microbes being tested, and incubated together with the test plates for 48 hours at 37°C, followed by examination for inhibition of microbial growth.



Figure 4.6: The Mast Diagnostics® multipoint inoculator.

The second- and third rounds of antimicrobial screening were done as described above, with some exceptions, during January 2008. During the second round of screening, strains 5 and 6 of *P. aeruginosa* were found to be non-viable upon

subculture, and were subsequently excluded from the second and third rounds of screening. Strain 7 of *P. aeruginosa* was available during the second and third rounds of screening, but was found to be contaminated with a *Proteus* organism. Thus any results pertaining to this strain were not taken into consideration. Finally, *P. aeruginosa* (ATCC 27853) was found to be infected with a bacteriophage during the third round of antimicrobial screening. Results obtained during the third round of antimicrobial screening pertaining to this particular strain of *P. aeruginosa* were not taken into consideration. Only those extracts showing activity, at least twice, out of the three sensitivity trials, against a specific microbe, were considered to have activity and the respective microbe was reported as being sensitive.

4.3. Anticancer studies

4.3.1. Collection and preparation of plant materials

Unlike the antimicrobial screening, only one set of infusions and extracts were prepared for use in anticancer studies as this required significantly less extract than the antimicrobial activity screening.

Fresh plant material was harvested and prepared for extract preparation during January 2008. *G. incanum* plant material was obtained from the garden of Dr N. Smith, while fresh *A. afra* plant material was collected from the horticultural services of the NMMU. Dr. M. van de Venter once again supplied fresh *A. absinthium* plant material from her garden. The fresh leaves were separated from the stems of the respective plants and separately rinsed in distilled water in order to remove dust, soil and insects. The fresh leaves were then allowed to dry at room temperature and separately weighed. Leaves used in the preparation of infusions were extracted immediately, while the remaining leaves used in the preparation of aqueous-, methanol- and acetone extracts were dried in an oven at 40°C over night.

4.3.2. Extraction of plant materials

4.3.2.1. Preparation of infusions

The preparation of extracts for use in anticancer studies was slightly adapted from the method used during extract preparation for antimicrobial screening with regards to the amount of plant material used. It was estimated that 60 mg of each of the crude infusion-, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium*, respectively, would be required for the completion of anticancer studies. The amount of plant material used and the crude extract yields obtained during the preparation of extracts for antimicrobial screening was used to approximate the amount of plant material needed to obtain 60 mg of each of the respective extracts of the three plants for use in anticancer studies.

Infusions of *G. incanum* were prepared according to an adapted traditional method described by Scott & Springfield (2004a, p.4). Approximately 2.04 g of fresh leaves was added to 60.96 ml of boiling distilled water and left to infuse for ten minutes. The infusion was then filtered using Whatman No 1 filter paper to remove residual plant material, the infused leaves were discarded, and the filtrate was taken to dryness in a laminar air flow cabinet (Labscheme Allchem®, South Africa) by evaporation in a hot water bath for 48 hours at 40°C. The crude extract was then re-suspended in approximately 2 ml of distilled water and frozen at -20°C in clean, pre-weighed 2 ml microtubes (Axygen® Scientific, USA), followed by freeze drying and weighing of the microtubes in order to determine the amount of crude extract and to calculate the percentage yield.

The infusion of *A. afra* was prepared according to an adapted traditional method described by Scott & Springfield (2004b, p.4). Approximately 5.09 g of fresh leaves were added to 363.71ml of boiling distilled water and left to infuse until cool. The infusion was then filtered using Whatman No 1 filter paper to remove

residual plant material, the infused leaves were discarded, and the filtrate was taken to dryness in a laminar air flow cabinet by evaporation in a hot water bath for 48 hours at 40°C. The crude extract was then re-suspended in approximately 2 ml of distilled water and frozen at -20°C in clean, pre-weighed 2 ml microtubes, followed by freeze drying and weighing of the microtubes in order to determine the amount of crude extract and percentage yield obtained from the fresh plant material of *A. afra*. The infusion of *A. absinthium* was prepared in exactly the same manner, except that 5.03 g fresh leaves were added to 358.93 ml boiling distilled water for infusion.

4.3.2.2. Preparation of aqueous-, methanol- and acetone extracts

Water, methanol and acetone were once again used as extractants for the preparation of extracts of the three respective plants, which were prepared as described in Section 4.2.2.2., with a few exceptions. The remaining fresh leaves of the three test plants were separately weighed, dried over night in an oven at 40°C and weighed again the following morning in order to determine the percentage yield of dry plant material.

One gram of dried *G. incanum* plant material was used in the preparation of aqueous- and methanol extracts, while 7.01 g dried plant material was used for the preparation of the acetone extract. For the preparation of the aqueous- and methanol extracts of *A. afra*, 1 g of dried plant material was used, respectively, while 1.5 g of dried plant material was used in the preparation of the acetone extract. One gram of dried *A. absinthium* plant material was used for the preparation of the aqueous-, methanol- and acetone extracts, respectively.

The aqueous extracts of the three plants were prepared in the same manner as was done during extract preparation for antimicrobial screening (Section 4.2.2.2.), except for different amounts of dried plant material used, and extracts

having been re-suspended in approximately 2 ml distilled water following evaporation in a hot water bath for 48 hours at 40°C and freezing of the extracts in pre-weighed 2 ml microtubes instead of 50 ml centrifuge tubes.

The methanol- and acetone extracts of the respective plants were prepared in the same manner as the aqueous extracts, except that after centrifugation the respective supernatants were separately combined in clean, pre-weighed glass beakers and placed in a hot water bath at 40°C over night in a laminar air flow cabinet, allowing the extractants to evaporate. The glass beakers were then weighed in order to determine the amount of crude methanol- and acetone extracts and percentage yields obtained from the dried leaves of the three respective plants.

All crude extracts were re-dissolved in DMSO to a concentration of 2 mg/10 µl. Re-dissolved methanol- and acetone extracts were removed from the glass beakers and separately placed in clean, 2 ml microtubes. These, together with the microtubes containing the respective re-dissolved aqueous extracts, were stored as stock solutions at -20°C in the dark until the day of testing.

4.3.3. Maintenance of cell cultures

The infusions, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium* were screened for anticancer activity against three cell lines, namely MCF-7, HT-29, and HeLa, which were obtained from the Department of Biochemistry and Microbiology at the NMMU. Cells were routinely maintained by culturing in 10cm Cellstar® DNase- and RNase- free tissue culture dishes (Greiner Bio-one™, Germany) without antibiotics in BioWhittaker RPMI-1640 medium (Lonza™, Switzerland), supplemented with 10% Gibco® heat inactivated fetal bovine serum (FBS) (Invitrogen™, UK). Cells were incubated in a ThermoForma 310 (Labotec™, USA) direct heat humidified incubator with 5% CO₂ at 37°C, and subcultured when 80% confluency was reached, which was

approximately every 72 hours. Subculturing entailed the removal of old growth medium by vacuum suction, followed by washing the cells twice with phosphate buffered saline (PBS) lacking calcium and magnesium. After removal of the PBS, 1 ml trypsin (10% in PBS containing ethylene diaminetetra-acetic acid [EDTA, 0.2 g/l]) was added to the cells and allowed to completely cover the monolayer. The trypsin was then removed by vacuum suction, and the cells incubated at 37°C for approximately 10 minutes until complete dislodgement of the cells from the surface of the culture dish had occurred. Cells were then diluted to the required concentration, usually 1:5 to 1:8, by the addition of RPMI-1640 growth medium supplemented with 10% FBS, and subcultured in sterile cell culture dishes (Freshney, 2000, pp.1-7).

4.3.4. Determination of plant extract toxicity towards cancer cells by means of the MTT assay

Mosmann (1983, pp.55-63) first described the MTT assay as a quantitative means of determining mammalian cell survival and proliferation *in vitro*. Dehydrogenase enzymes of viable cells convert 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, a yellow tetrazoleum salt, to purple formazan crystals. The amount of viable cells present following exposure to a test substance, in this case the plant extracts, is directly proportional to the amount of formazan formed, which can be measured spectrophotometrically.

A method adapted from the MTT assay as described by Mosmann (1983, pp.55-63), was utilised during this study to test for cell viability and cytotoxicity *in vitro* following exposure to the various plant extracts. Cells were washed twice with PBS, trypsinised (10% trypsin in PBS with EDTA [0.2 g/l]), harvested and counted using a hemocytometer and the amount of viable cells present visualized using trypan blue. The cell membrane of necrotic cells loses its

integrity and ability to exclude certain substances such as trypan blue. Microscopically, necrotic cells stained with trypan blue will appear blue, while viable cells remain unstained. Cultured cells from a plate were used when the cell viability as counted with trypan blue exclusion was more than 90%. Cells were seeded in Costar® polystyrene 96 well plates (Corning Incorporated, USA), each well containing 200 µL RPMI-1640 media supplemented with 10% heat inactivated FBS, using a Multipette Plus (Eppendorf®, Germany), at a density of 5000 cells per 200 µL. The tissue culture plates were incubated for 24 hours in relative humidity at 37°C with 5% CO₂, followed by inspection of the wells for microbial contamination and removal of the media by vacuum. Stock solutions (2 mg extract/10 µL DMSO) of the plant extracts of *G. incanum*, *A. afra* and *A. absinthium* were serially diluted with RPMI-1640 media supplemented with 10% heat inactivated FBS, starting at 500 µg/ml (0.25% DMSO) diluted to 375 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 25 µg/ml and 10 µg/ml. Each set of dilutions of the various extracts were individually tested on all three cell lines, with wells containing 200 µL of the respective diluted extracts. RPMI-1640 media supplemented with 10% heat inactivated FBS lacking any extract was added to the top and bottom peripheral wells of each plate, as extract blanks and negative controls. All three cell lines were also treated with serial dilutions of cisplatin, a known anticancer agent which induces apoptosis. Cisplatin was kindly donated by Professor J. du Preez of the Chemistry department of the NMMU. The dilutions of cisplatin used ranged from a concentration of 30 µg/ml in RPMI-1640 media supplemented with 10% heat inactivated FBS (0.25% DMSO) to 22.5 µg/ml, 15 µg/ml, 7.5 µg/ml, 3.75 µg/ml and 1.5 µg/ml. Since the extracts were dissolved in DMSO to a final concentration of 0.25% DMSO, this concentration of DMSO not being toxic to malignant cells (M. van de Venter, personal communication, January, 2008), a DMSO control was not included during screening. Tissue culture plates were incubated for 48 hours in relative humidity at 37°C with 5% CO₂ following addition of the diluted extracts and cisplatin.

After 48 hours the medium was removed and 200 μ L of fresh RPMI-1640 medium and 20 μ L of MTT (4 mg/ml in distilled water) was added to each well and the plate incubated a further 4 hours at 37°C. Thereafter the medium was removed and 100 μ L of DMSO was added to each well in order to solubilise the formazan crystals produced by viable cells. Following incubation at room temperature for 15 minutes, the optical densities of the formazan solution in each well was measured at 570 nm using a BioTek PowerWave XS™ photometer (Progen Scientific®, UK) (Figure 4.7).



Figure 4.7: The BioTek PowerWave XS™ photometer.

Screening of all the extracts on the three respective cell lines was done in triplicate, with cell viability, as well as inhibition of cell growth/cell death having been expressed as a mean percentage \pm standard deviation (SD) of the mean negative control absorbance. This data was analysed in GraphPad Prism® (San Diego, USA) and used to construct dose response curves of the various extracts on the respective cell lines. Where extracts appeared to significantly inhibit cell growth or induce cell death, screening of the specific extract on the respective cell line was repeated using lower concentrations of the extract. GraphPad Prism® was used to perform regression analysis in order to determine IC₅₀ values, the concentration of each extract, as well as cisplatin, at which 50% of the cells are inhibited. When the coefficient of correlation (r^2) was not ≥ 0.95 for any of the regression analyses, the results were considered insignificant and screening of the specific extract on the relevant cell line was repeated.

4.3.5. Apoptotic and necrotic assay – fluorescence microscopy

As discussed in Section 2.6.2., the two modes of cell death, being apoptosis and necrosis, are characterised by two different sets of morphological as well as biochemical changes in the cell. By observing and identifying these two sets of cellular changes, it is possible to discern apoptotic cells from necrotic ones. Darzynkiewicz *et al.* (as cited in Bungu, 2005, p.50) describes various methods and assays having been developed for the identification of apoptotic parameters, including the activation of apoptotic caspases, as discussed in Section 2.6.2.1., the release of cytochrome c and AIF into the cytoplasm, as discussed in Section 2.6.2.1.2., the formation of apoptotic bodies in individual cells and/or cell populations, and alterations in the integrity of the plasma membrane.

As the induction of apoptosis in malignant cells is the mechanism of action of many anticancer agents, it would be desirable for a plant extract expressing toxicity towards malignant cells to induce apoptosis in these cells rather than necrosis. During the current study, apoptotic body formation, nuclear chromatin condensation and loss of membrane integrity in necrotic cells were used as parameters for the detection of apoptotic- and necrotic cells. This was accomplished by separately exposing malignant cells treated with a plant extract to two fluorescent dyes, being 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodide (PI), and observing any morphological changes under a fluorescence microscope.

4.3.5.1. DAPI and PI exclusion staining

The principle of this assay exploits the phenomenon of apoptotic cells maintaining the integrity and functions of their plasma membranes, unlike necrotic cells, in which case integrity of the plasma membrane is compromised. While the intact cell membrane of live- and apoptotic cells selectively exclude various substances, including cationic dyes such as PI and trypan blue, others are transported into the cell, such as DAPI. In necrotic cells, having lost their cell membrane integrity, cationic dyes such as PI and trypan blue are able to penetrate into the cytosol of the cell, staining cellular structures.

DAPI is a fluorescent indole dye which is capable of penetrating the cell membrane of viable- and apoptotic cells, where it forms fluorescent complexes with natural double stranded DNA in the nucleus, which appears blue during fluorescence microscopy. According to Darzynkiewicz *et al.* (as cited in Bungu, 2005, p.50), one of the trademarks of apoptotic cells is nuclear chromatin condensation. Apoptotic cells generally fluoresce brighter than non-apoptotic cells when stained with a nuclear fluorescence dye such as DAPI. DAPI has an excitation wavelength of 340 nm and an emission wavelength of 488 nm in aqueous solution. When dying or dead cells are exposed to PI, which has an excitation wavelength of 488nm and an emission wavelength of 560-680nm, the dye enters the cell and forms fluorescent complexes with double stranded nucleic acids, rendering a red color to the nucleus during fluorescence microscopy (DAPI, 2007; Bungu, 2005, p.50).

DAPI and PI staining of cells was performed using a combined, modified method of Moongkarndi *et al.* (2004), Bungu (2005, p. 51) and Botha (2003, p. 80). For the purpose of this study, it was decided to investigate the mechanism of toxicity of the plant extract exhibiting the highest degree of toxicity to a specific cell line (Table 5.9). Cells were counted using trypan blue exclusion and seeded on sterile 13mm glass coverslips in Cellstar® DNAase- and RNAase- free 6-well

tissue culture plates (Greiner Bio-one™, Germany), each well containing 5 ml RPMI-1640 media supplemented with 10% heat inactivated FBS, at a density of 5000 cells per 200 μ L. The tissue culture plates were incubated for 24 hours in relative humidity at 37°C with 5% CO₂. The stock solution of the extract (2 mg extract/10 μ L DMSO) was diluted with RPMI-1640 media supplemented with 10% heat inactivated FBS, to the extract's IC₅₀ value for the particular extract as indicated in Section 5.3.2., and 5 ml of this dilution was added to the first and second wells. As a negative control, 5 ml RPMI-1640 media supplemented with 10% heat inactivated FBS lacking any extract was added to the third and fourth wells, while 5 ml RPMI-1640 media supplemented with 10% heat inactivated FBS containing cisplatin at a concentration of 10 μ M (0.25% DMSO) was added to each of the remaining two wells of the first plate. This served as a positive control for the induction of apoptosis. To two wells of the second tissue culture plate, 5 ml RPMI-1640 media supplemented with 10% heat inactivated FBS lacking any extract was added, which was used to establish the accuracy of the method by permeabilising and staining the cells in these two wells, as discussed in the following paragraph. The tissue culture plates were then incubated for 48 hours in relative humidity at 37°C with 5% CO₂.

After removal of the spent medium, wells were washed once with PBS lacking EDTA, calcium and magnesium, followed by the addition of 1 ml of ice cold 3:1 methanol:acetic acid (Carnoy's fixative) to each of the 6 wells of the first plate, and 1 ml ice cold permeabilising agent (1:1 methanol:acetone) to the remaining two wells of the second plate. The fixative and permeabilising agent was removed after ten minutes incubation at room temperature. All wells were once again washed with PBS and 1 μ g/ml DAPI (made up in PBS lacking EDTA, calcium or magnesium) was added to one of each of the wells containing cells treated with plant extract, cisplatin, untreated cells and permeabilised cells. To the remaining wells, 5 μ g/ml PI was added. Cells were then incubated for 30 minutes in relative humidity at 37°C with 5% CO₂, followed by the removal of the DAPI and PI solutions. The coverslips were removed from the tissue culture

plate using a forceps and mounted upside down in PBS on microscope slides. An Olympus BX-60 fluorescent microscope (Olympus™, Japan) was used to examine each of the eight slides for cellular staining and the presence of apoptotic and necrotic morphological parameters, and photographs were taken using an Olympus camera.

4.3.6. Determination of plant extract toxicity in peripheral blood mononuclear cells

The aim of modern cancer chemotherapy is to selectively destroy malignant cells while having as little effect as possible on the patient's normal cell populations and general homeostatic balance. Unfortunately, however, more often than not the chemotherapeutic agent being administered not only leads to the destruction of malignant cells, but also adversely affects various healthy cell populations in varying degrees, especially those types which replicate and divide faster, such as the haemopoietic stem cells, cells of the digestive tract, cells of the reproductive system as well as hair follicles (MedicineNet, 2004). As stated earlier, the mechanism of action of many clinical anticancer agents is the induction of apoptosis in malignant cells. A plant extract or compound isolated from such an extract exhibiting anticancer activity is not only required to induce apoptosis in malignant cells as opposed to necrosis; it also needs to do so selectively in malignant cells without affecting normal healthy cells.

The degree of toxicity expressed by extracts of *G. incanum*, *A. afra* and *A. absinthium* on MCF-7 cells, HT-29 cells and HeLa cells, as well as the mechanism of cell death induced by the most toxic of these extracts in a specific cell line (apoptosis vs. necrosis) have been investigated and discussed in Sections 4.3.4. and 4.3.5., respectively. Investigation into the possible expression of toxicity of the latter extract in normal, healthy human cells, having

been peripheral blood mononuclear cells (PBMC's) for the purpose of this study, was judged the next logical step, and is discussed in Sections 4.3.6.1. and 4.3.6.2.

4.3.6.1. Isolation and preparation of PBMC's

The isolation of PBMC's was performed according to a Ficoll™ (Amersham, USA) density gradient centrifugation protocol adapted from the Immune Tolerance Network (n.d.). In order to obtain PBMC's, venous blood was collected from a healthy, consenting adult male into 2 x 8 ml BD Vacutainer® CPT mononuclear cell preparation tubes (Becton Dickinson and Company, USA) containing a density gradient polymer gel, sodium heparin and Ficoll™ medium, and processed within half an hour of sample collection. Both tubes were gently inverted 10 times, followed by centrifugation of the blood collection tubes at 1800 x g for 30 minutes in an Eppendorf® 5810 centrifuge (Germany) at room temperature. Plasma and PBMC's were then aspirated and transferred to clean, 15 ml Eppendorf® centrifuge tubes, which were gently inverted 10 times and centrifuged again at 300 x g for 15 minutes. Following centrifugation, the plasma was aspirated and discarded, except for approximately 1 ml, which was used to re-suspend the cell pellet. Ten millilitre of RPMI-1640 media supplemented with 10% heat inactivated FBS was then added to the re-suspended cell pellet, and the suspension was centrifuged at 300 x g for 15 minutes, followed by aspiration of the supernatant and re-suspension of the cell pellet in approximately 4 ml RPMI-1640 media supplemented with 10% heat inactivated FBS.

4.3.6.2. The CellTiter-Blue® cell viability assay

The CellTiter-Blue® Cell Viability Assay (Promega, USA) is a flourometric method for the estimation of the amount of viable cells present in a sample, and

is based upon the principle of viable cells being able to reduce the indicator dye resazurin, which is dark blue in color and possessing very little intrinsic fluorescence, to the highly fluorescent resorufin, which is pink in color. The amount of fluorescence present after completion of the assay is directly proportional to the number of viable cells present in the sample.

The CellTiter-Blue® assay was performed according to the method as outlined in the Promega Technical Bulletin (2006). For the purpose of the assay, screening of the extract against PBMC's was done in triplicate, while three different controls were simultaneously run, each control having been performed in duplicate. Following the re-suspension of the PBMC pellet in 4 ml RPMI-1640 medium supplemented with 10% heat inactivated FBS, viable cells were counted using trypan blue exclusion and seeded in the first 33 wells of a Costar® polystyrene 96 well plate (Corning Incorporated, USA). Eleven serial dilutions of the extract were made in RPMI-1640 media supplemented with 10% heat inactivated FBS and screened against PBMC's, ranging between 500µg/ml (0.25% DMSO) and 2.65µg/ml (0.25% DMSO). The respective dilutions were added to the 33 wells containing the seeded cells to a final volume of 100 µl per well at a cell density of 100 000 cells per well. The first control consisted of 22 wells containing cells at a density of 100 000 cells per well in 100 µl RPMI-1640 media supplemented with 10% heat inactivated FBS only. The fluorescence obtained from cells treated with the extract was expressed as a percentage of the fluorescence of these untreated cells in order to determine the percentage cell viability of cells exposed to the extract. As a second control, 22 wells were filled with 100 µl of RPMI-1640 medium supplemented with 10% heat inactivated FBS only, which served as a negative control for the determination of possible background fluorescence. As a third control, the eleven respective extract dilutions mentioned above were added to 22 wells at a final volume of 100 µl, which served to rule out fluorescence of the extract itself or possible interference of the extract with the chemistry of the assay reagent. The tissue culture plates were then incubated for 48 hours in relative humidity at 37°C with 5% CO₂.

All wells were visually inspected for bacterial contamination after 48 hours, followed by the addition of 20 μ l of the CellTiter-Blue® reagent to each well and further incubation for 4 hours in relative humidity at 37°C with 5% CO₂. After 4 hours of incubation, a Flouroskan Ascent® fluorometer (Thermo Labsystems, Finland) (Figure 4.8) was used to record the fluorescence at an excitation wavelength of 544 nm and an emission wavelength of 590 nm.



Figure 4.8: The Flouroskan Ascent® fluorometer.

Fluorescence and possible interference of the extract itself was excluded by subtracting the mean values of any fluorescence emitted by each of the eleven extract dilutions from the third control from the fluorescence readings obtained from the three triplicate sets of wells containing PBMC's treated with the respective eleven dilutions of the extract. The percentage of PBMC viability, as well as the percentage cell death was determined by expressing the final fluorescence values of the wells containing PBMC's treated with the extract as a mean percentage \pm standard deviation (SD) of the mean fluorescence readings recorded for the wells containing untreated cells (control 1). This data was

recorded in GraphPad Prism® (San Diego, USA) and used to construct a dose response curve of the extract for PBMC's. GraphPad Prism® was also used to perform regression analysis in order to determine the IC₅₀ of the extract for PBMC's. When the coefficient of correlation (r^2) was not ≥ 0.95 , the results were considered insignificant and the assay repeated to confirm the results.

4.3.7. Analysis of the effects of the most toxic extract on the cell cycle of the most sensitive cells by means of flow cytometry

As stated under Section 2.6.1.2.1., the development of malignancy is usually the result of aberrations in the regulatory mechanisms of the cell cycle, allowing for uncontrolled proliferation of undifferentiated cells. The final step in the current investigation was to determine whether the most toxic extract, as indicated in Table 5.9, could in fact alter the cell cycle of the most sensitive cell line of the three lines tested, in such a manner as to decrease or completely halt proliferation of these cells. This was done by analyzing the cell cycle of the cells following treatment with the extract, lysis of the cells and staining of the DNA with PI using flow cytometric analysis, a quantitative technique with the ability to rapidly measure large amounts of cells. The assay is based on the principle that PI is able to bind to double stranded DNA, which emits fluorescence. By measuring the fluorescence, which is directly proportional to the amount of DNA present, it is possible to establish during which phase of the cell cycle a specific cell is, proportional to its DNA content (Beckman Coulter Technical Bulletin, 2003).

Cell cycle analysis was performed in triplicate. Cells were seeded in three sets of three Cellstar® DNAase- and RNAase- free 10 cm tissue culture plates, each at a density of 3×10^6 cells/ml in RPMI-1640 medium supplemented with 10% heat inactivated FBS, and incubated for 24 hours in relative humidity at 37°C with 5% CO₂. Following 24 hour incubation, the growth medium was removed by vacuum from each of the tissue culture dishes, and each dish was washed twice

with PBS lacking EDTA, calcium and magnesium. To three of the dishes cisplatin was added at a concentration of 10 μ M (0.25% DMSO) in RPMI-1640 media supplemented with 10% heat inactivated FBS. This served as a positive control. To the second set of three plates, fresh RPMI-1640 medium supplemented with 10% heat inactivated FBS lacking cisplatin or extract was added, which served as a negative control. To the last three plates, the most toxic extract was added to a final concentration of its IC₅₀ (0.25% DMSO) for the most sensitive cell line (Table 5.9) in RPMI-1640 medium supplemented with 10% heat inactivated FBS. After a further 24 hour period of incubation in relative humidity at 37°C with 5% CO₂, the medium, extract and cisplatin was removed from the plates by vacuum, and each plate was rinsed twice with PBS containing EDTA (0.2 g/l). Cells were trypsinised by the addition of 1 ml trypsin (10% in PBS containing EDTA [0.2 g/l]) to each plate, which was allowed to cover the plate surface before removal by vacuum and incubation of the plates in relative humidity at 37°C with 5% CO₂ for 15 minutes to facilitate trypsinisation. Following trypsinisation, 1 ml RPMI-1640 media supplemented with 10% heat inactivated FBS was added to each of the respective plates in order to create cell suspensions. The suspensions were aspirated and placed into separate 5 ml Beckman Coulter™ FC 500 cytometer sample tubes and centrifuged at 500 x g for 5 minutes, followed by discarding 900 μ l of the respective supernatants. To each sample tube, 100 μ l of lysis buffer, which was included in the Coulter® DNA Prep™ Reagents Kit used for this study, was added. Trout red blood cells (NPE Systems, USA) were used as a DNA reference calibrator. Two drops of trout red blood cells were added to a separate clean cytometer sample tube together with 100 μ l lysis buffer, which was left together with the other cell samples to incubate for 5 minutes at room temperature. Following incubation, 1 ml PI, also included in the Coulter® DNA Prep™ Reagents Kit, was added to each of the sample tubes and incubated for 15 minutes in relative humidity at 37°C with 5% CO₂ in the dark. Samples were subsequently loaded into a Beckman Coulter™ FC 500 cytometer for analysis, and the percentages of cells present in each respective phase of the cell cycle of cells treated with the extract was compared to the

percentages of cells occurring in the corresponding phases of the cell cycles for cells treated with cisplatin as well as cells which remained untreated.

CHAPTER 5

RESULTS

5.1. Introduction

The current chapter discusses the results obtained during the present study, including the percentage yields obtained after fresh leaves were dried, the infusion- and extract yields obtained from the respective plants, as well as descriptions of the infusions and crude extracts obtained after freeze drying and evaporation. The results obtained during antimicrobial screening studies, as well as results obtained during anticancer screening studies, are also given.

5.2. Antimicrobial activity studies

5.2.1. Percentage yields of dried plant material obtained, and the percentage yields of infusions and crude extracts obtained from *G. incanum*, *A. afra* and *A. absinthium*

Infusions as well as aqueous-, methanol- and acetone extracts were prepared as discussed in Sections 4.2.2.1. and 4.2.2.2., respectively. The percentage yields of plant material obtained after drying, used in the preparation of aqueous-, methanol- and acetone extracts for antimicrobial screening, is shown in Table 5.1.

Table 5.1: Percentage yields of plant material obtained after drying, used in the preparation of aqueous-, methanol- and acetone extracts for antimicrobial screening.

<u>Plant</u>	<u>Weight of fresh plant material before drying (gram)</u>	<u>Weight of dried plant material (gram)</u>	<u>Percentage yield of dried plant material (%)</u>
<i>G. incanum</i>	52.6	13.4	25.5
<i>A. afra</i>	169.2	36.4	21.5
<i>A. absinthium</i>	196.9	37.4	19.0

Weighing of dried *G. incanum* plant material indicated a reduction in weight to the amount of 39.2 g. Water constituted approximately 74.5% of the weight of fresh *G. incanum* plant material. Weighing of dried *A. afra* plant material indicated a reduction in weight of 132.7 g. Water constituted approximately 78.5% of the weight of fresh *A. afra* plant material. The weight of *A. absinthium* plant material was reduced by 159.5 g during the drying of fresh plant material, indicating a water content of approximately 81.0% in fresh *A. absinthium* plant material.

After freeze drying of infusions and aqueous extracts, and evaporation of methanol- and acetone extracts, respectively, the pre-weighed containers containing the crude extracts of *G. incanum*, *A. afra* and *A. absinthium* were weighed again in order to determine the weight of crude extracts obtained. Tables 5.2, 5.3 and 5.4 indicate the percentage yields of extracts obtained for each of the three respective rounds of infusion and extract preparation for antimicrobial screening. The weights of the fresh and dried plant materials used as well as the weights of extracts after freeze drying (infusions and aqueous extracts) and evaporation (methanol- and acetone extracts) are indicated in Tables 1, 2 and 3 in Appendix I, respectively.

Table 5.2: Percentage yields of extracts obtained after the initial round of extract preparation for antimicrobial screening, June 2007 (winter).

<u>Extract</u>	<i>Geranium incanum</i>	<i>Artemisia afra</i>	<i>Artemisia absinthium</i>
	Yield % (w/w)	Yield % (w/w)	Yield % (w/w)
Infusion (Fresh leaves used)	5.4	2.9	2.4
Aqueous extract (Dry leaves used)	8.6	9.9	9.0
Methanol extract (Dry leaves used)	9.0	11.9	10.0
Acetone extract (Dry leaves used)	1.3	6.9	8.1

Table 5.3: Percentage yields of extracts obtained after the second round of extract preparation for antimicrobial screening, September 2007 (spring).

<u>Extract</u>	<i>Geranium incanum</i>	<i>Artemisia afra</i>	<i>Artemisia absinthium</i>
	Yield % (w/w)	Yield % (w/w)	Yield % (w/w)
Infusion (Fresh leaves used)	1.7	3.7	4.6
Aqueous extract (Dry leaves used)	5.6	5.1	7.6
Methanol extract (Dry leaves used)	6.3	7.6	7.7
Acetone extract (Dry leaves used)	0.9	5.5	6.0

Table 5.4: Percentage yields of extracts obtained after the third round of extract preparation for antimicrobial screening, January 2008 (summer).

<u>Extract</u>	<i>Geranium incanum</i>	<i>Artemisia afra</i>	<i>Artemisia absinthium</i>
	Yield % (w/w)	Yield % (w/w)	Yield % (w/w)
Infusion (Fresh leaves used)	7.2	10.1	6.3
Aqueous extract (Dry leaves used)	13.2	7.7	10.4
Methanol extract (Dry leaves used)	3.2	7.4	9.9
Acetone extract (Dry leaves used)	5.8	6.8	6.4

The previous three tables clearly indicate variation in the extract yields obtained after each of the three respective rounds of plant extractions. Generally, the second round of extractions (September 2007, spring) appears to have produced the lowest yields in all but four of the twelve respective infusion and extract preparations, including:

- The methanol extracts of *G. incanum* and *A. afra*, in which case the lowest yields were obtained during the third round of extraction (January 2008, summer).
- The infusions of *A. afra* and *A. absinthium*, in which case the lowest yields were obtained during the first round of extraction (June 2007, winter).

The first round of extractions (June 2007, winter) produced the highest yields in 6 of the 12 extracts prepared, including the aqueous extract of *A. afra*, the methanol extracts of *G. incanum*, *A. afra* and *A. absinthium* as well as the acetone extracts of *A. afra* and *A. absinthium*, respectively. The remaining six highest yields were obtained during the third round of extraction (January 2008, summer), including the infusions of *G. incanum*, *A. afra* and *A. absinthium*, the aqueous extracts of *G. incanum* and *A. absinthium* as well as the acetone extract of *G. incanum*.

The greatest variation in extract yield obtained occurred between the second (September 2007, spring) and third (January 2008, summer) aqueous extracts of *G. incanum*, with 0.3g (57.5%) less extract having been obtained during the second round (September 2007, spring).

The least amount of variation in extract yield obtained occurred between the first (June 2007, winter) and third (January 2008, summer) methanol extracts of *A. absinthium*, with only 0.03g (0.9%) less extract having been obtained during the third round (January 2008, summer).

5.2.2. Description of crude infusions and extracts obtained after freeze drying and evaporation, used in antimicrobial screening

A marked variability in the physical appearances of the crude infusions and aqueous extracts obtained after freeze drying, as well as the crude methanol- and acetone extracts obtained after evaporation for *G. incanum*, *A. afra* and *A. absinthium*, respectively, was observed. Crude extracts and infusions differed in volume, texture, color and degree of dryness. Table 5.5 gives brief descriptions of the physical appearances of crude infusions and extracts of *G. incanum*, *A. afra* and *A. absinthium*, respectively.

Table 5.5: Descriptions of the physical appearances of crude infusions and extracts of *G. incanum*, *A. afra* and *A. absinthium*.

<u>Infusion/Extract</u>	<u>Physical appearance</u>
<u>G. incanum</u>	
Infusion	Completely dehydrated, fluffy and very light brown in color
Aqueous extract	Completely dehydrated, fluffy and slightly darker than infusion in color
Methanol extract	Smooth, solid, glassy and speckled appearance, being a light green-brown in color
Acetone extract	Smooth, solid, glassy and wispy in appearance, being yellow-green to black in appearance
<u>A. afra</u>	
Infusion	Very viscous, sticky consistency, being toffee brown in color
Aqueous extract	Very viscous, sticky consistency, slightly crumbly, being darker than infusion in color
Methanol extract	Very viscous, semi dehydrated appearance, being dark green in color
Acetone extract	Smooth, solid and glassy appearance, being a light toffee-brown in color
<u>A. absinthium</u>	
Infusion	Dry and sticky consistency, being dark brown in color
Aqueous extract	Very viscous, sticky consistency, being dark brown in color
Methanol extract	Very viscous, semi dehydrated appearance, being dark green in color
Acetone extract	Very viscous, sticky consistency, being golden-green in color



Figure 5.1: *G. incanum* infusion (left) and aqueous extract (right) after freeze drying.



Figure 5.2: *G. incanum* methanol- (left) and acetone extract (right) after evaporation of extractants.



Figure 5.3: *A. afra* infusion (left) and aqueous extract (right) after freeze drying.



Figure 5.4: *A. afra* methanol- (left) and acetone extract (right) after evaporation of extractants.



Figure 5.5: *A. absinthium* infusion (left) and aqueous extract (right) after freeze drying.



Figure 5.6: *A. absinthium* methanol- (left) and acetone extract (right) after evaporation of extractants.

All crude extracts were re-dissolved in DMSO after having been taken to dryness, either *in vacuo* as was the case with infusions and aqueous extracts, or through evaporation of the extractant in a laminar air flow cabinet using a hot water bath at 40°C over night. Due to variation in the solvating capability of DMSO on the various extracts, different amounts of DMSO had to be used, depending on the solvability of the respective extracts in DMSO. As indicated in Section 5.2.1., variation also existed between the amounts of extracts retrieved during the three rounds of plant extractions. Thus the amount of DMSO used to dissolve any specific extract also varied during the three respective rounds. The aqueous extract of *A. absinthium* proved to be the least soluble in DMSO, while the infusion extract of *G. incanum* was the most soluble in DMSO.



Figure 5.7: DMSO- dissolved extracts of *G. incanum*.
 From left to right: infusion extract, aqueous extract, methanol extract, acetone extract.

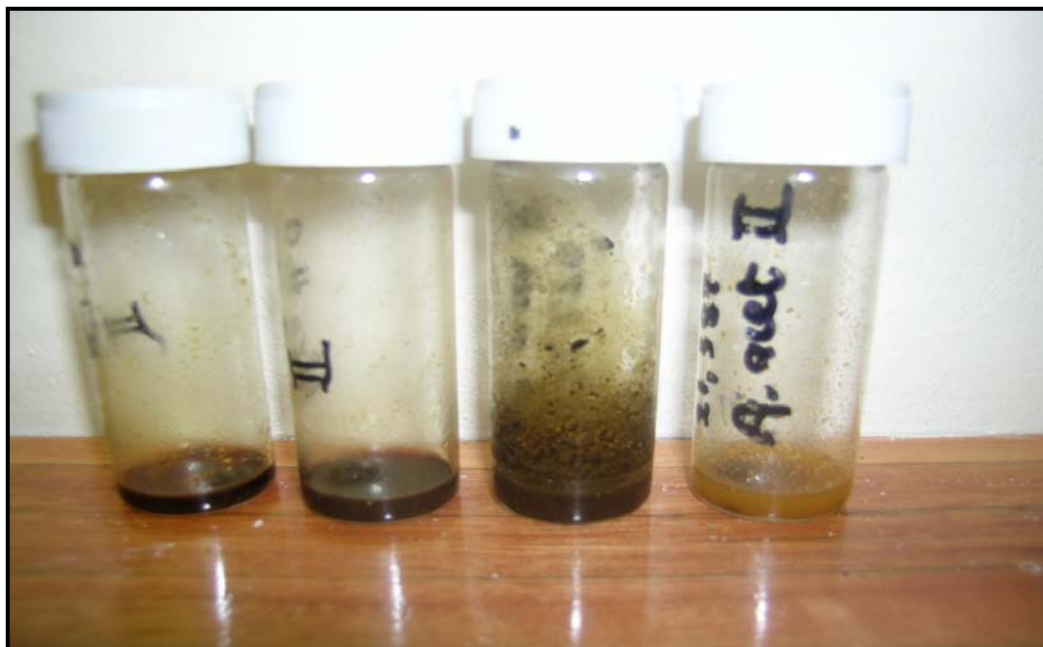


Figure 5.8: DMSO- dissolved extracts of *A. afra*.
 From left to right: Infusion extract, aqueous extract, methanol extract, acetone extract.

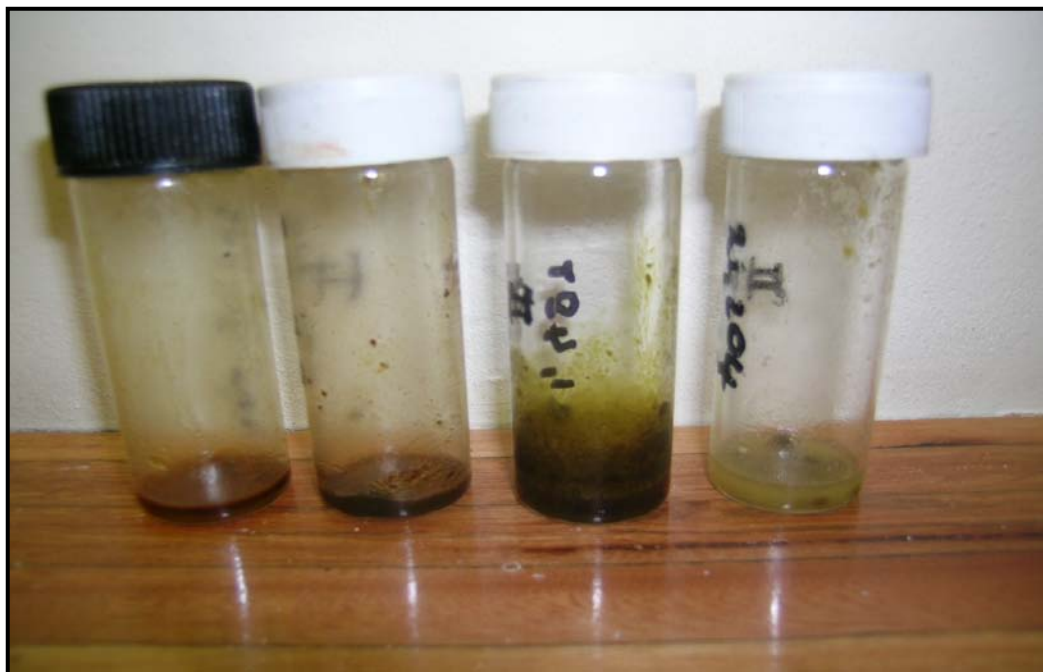


Figure 5.9: DMSO-dissolved extracts of *A. absinthium*.
From left to right: Infusion extract, aqueous extract, methanol extract, acetone extract.

5.2.3. Inhibition of the growth of various strains of *C. albicans*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus* and *B. cereus* by infusions, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium*

Infusions, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium*, respectively, were screened for inhibition of growth of ATCC strains and various clinical isolates of *Candida albicans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Bacillus cereus*, using the agar dilution method. All extracts were screened at a final concentration of 100 mg extract/ml agar. Three control plates were concomitantly run with each round of screening as discussed in Section 4.2.3.2. Table 5.6 indicates the final results obtained in the screening of infusions,

aqueous-, methanol- and acetone extracts for growth inhibition of various strains of *C. albicans*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus* and *B. cereus*. Where a specific strain of any one of the test organisms was found to be resistant to an extract for two out of the three rounds of screening, that specific strain was reported as being resistant to inhibition of growth by the relevant extract. Likewise, where a specific strain of any one of the test organisms was found to be inhibited by an extract for two out of the three rounds of screening, that specific strain was considered to be susceptible to growth inhibition.

During the initial round of antimicrobial screening (August, 2007), strain 7 of *P. aeruginosa* was unavailable, and subsequently omitted from this round. During the second round of screening (January, 2008), strains 5 and 6 of *P. aeruginosa* were found to be non-viable upon subculture, and were subsequently excluded from the second and third rounds of screening. Strain 7 of *P. aeruginosa* was available during the second and third rounds of screening, but was found to be contaminated with a *Proteus* organism. Thus any results pertaining to this strain were not taken into consideration. Finally, *P. aeruginosa* (ATCC 27853) was found to be infected with a bacteriophage during the third round of antimicrobial screening (January, 2008). It was, however, found to be resistant to inhibition of growth by any one of the 12 extracts during the first and second rounds of screening, and was subsequently reported in the final results as such.

Table 5.6: Susceptibility to growth inhibition of various strains of *C. albicans*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus* and *B. cereus* to infusions, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium*.

<u>Microbial strain</u>	<u><i>G. incanum</i></u>				<u><i>A. afra</i></u>				<u><i>A. absinthium</i></u>				<u>Controls</u>		
	Infusion	Aqueous	Methanol	Acetone	Infusion	Aqueous	Methanol	Acetone	Infusion	Aqueous	Methanol	Acetone	Control 1	Control 2	Control 3
<u><i>C. albicans</i></u>															
ATCC 66027	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-
Clinical isolate 2	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
Clinical isolate 3	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
Clinical isolate 4	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
Clinical isolate 5	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
Clinical isolate 6	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
Clinical isolate 7	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
Clinical isolate 8	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
Clinical isolate 9	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
Clinical isolate 10	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
<u><i>E. coli</i></u>															
ATCC 35218	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

<u>Microbial strain</u>	<u>G. incanum</u>				<u>A. afra</u>				<u>A. absinthium</u>				<u>Controls</u>		
	Infusion	Aqueous	Methanol	Acetone	Infusion	Aqueous	Methanol	Acetone	Infusion	Aqueous	Methanol	Acetone	Control 1	Control 2	Control 3
<u>E. coli</u>															
Clinical isolate 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<u>P. aeruginosa</u>															
ATCC 27853	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<u>E. faecalis</u>															
ATCC 29212	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clinical isolate 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

<u>Microbial strain</u>	<u><i>G. incanum</i></u>				<u><i>A. afra</i></u>				<u><i>A. absinthium</i></u>				<u>Controls</u>		
	Infusion	Aqueous	Methanol	Acetone	Infusion	Aqueous	Methanol	Acetone	Infusion	Aqueous	Methanol	Acetone	Control 1	Control 2	Control 3
<u><i>S. aureus</i></u>															
ATCC 43300	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 2	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 3	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 4	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 5	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 6	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 7	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 8	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 9	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 10	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
<u><i>B. cereus</i></u>															
ATCC 10876	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 2	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 3	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-
Clinical isolate 4	+	+	+	-	+	-	+	+	-	-	+	+	-	-	-

+: Inhibition of growth; - : No growth inhibition

Growth inhibition by plant extracts was observed mainly in strains of *C. albicans*, *S. aureus* and *B. cereus*, as reflected in Table 5.6. Strains of *S. aureus* and *B. cereus* were the most susceptible to inhibition, and had similar inhibition patterns. *C. albicans* consistently exhibited sensitivity to growth inhibition by the *G. incanum* extracts, but was not sensitive to other extracts, with the exception of the *A. afra* acetone extract.

All ten strains of *C. albicans* were sensitive to the infusion, aqueous-, methanol- and acetone extracts of *G. incanum*. *C. albicans* ATCC strain 66027 was inhibited by the methanol extract of *A. afra*, while all ten strains were inhibited by the *A. afra* acetone extract. No inhibition of *C. albicans* was observed for the infusion or aqueous extract of *A. afra* or for any of the *A. absinthium* extracts.

All ten strains of *S. aureus* were sensitive to growth inhibition by eight of the twelve extracts tested, including the infusions of *G. incanum* and *A. afra*, the aqueous extract of *G. incanum*, the methanol extracts of *G. incanum*, *A. afra* and *A. absinthium* and the acetone extracts of *A. afra* and *A. absinthium*. None of the *S. aureus* strains tested exhibited any sensitivity to the acetone extract of *G. incanum*, the aqueous extracts of *A. afra* and *A. absinthium* or the infusion of *A. absinthium*.

The four strains of *B. cereus* exhibited sensitivity to the same extracts as the strains of *S. aureus*. All four *B. cereus* strains were sensitive to the infusions of *G. incanum* and *A. afra*, the aqueous extract of *G. incanum*, the methanol extracts of *G. incanum*, *A. afra* and *A. absinthium* and the acetone extracts of *A. afra* and *A. absinthium*. None of the *B. cereus* strains exhibited any sensitivity to the acetone extract of *G. incanum*, the aqueous extracts of *A. afra* and *A. absinthium* or the infusion of *A. absinthium*.

All strains of the test organisms grew on both Control 1 and Control 2 during all three rounds of screening and were not inhibited by the addition of DMSO. Furthermore, all organisms grew on Control 3, which did not contain any DMSO or extract, as expected. It was noted during the second round of testing (January, 2008) that all *P. aeruginosa* colonies were significantly larger than colonies of other organisms on the agar plate containing the *A. afra* acetone extract. The same observation was made during the second round of testing of the effect of the aqueous extract of *A. absinthium* on *S. aureus* and *B. cereus*.

strains. Bacterial colonies of all the strains of *S. aureus* and *B. cereus* appeared larger than surrounding colonies on the agar plate containing the *A. absinthium* aqueous extract. During the third round of screening (January, 2008), *P. aeruginosa* colonies on agar plates containing infusions of *A. afra* and *A. absinthium* as well as those on the agar plate containing the *A. absinthium* aqueous extract appeared larger than the surrounding colonies. These results were not observed consistently during all three rounds of screening, and were thus not considered significant. However, the possibility of stimulation of growth of the various organisms by the corresponding extracts as mentioned above can not be excluded.

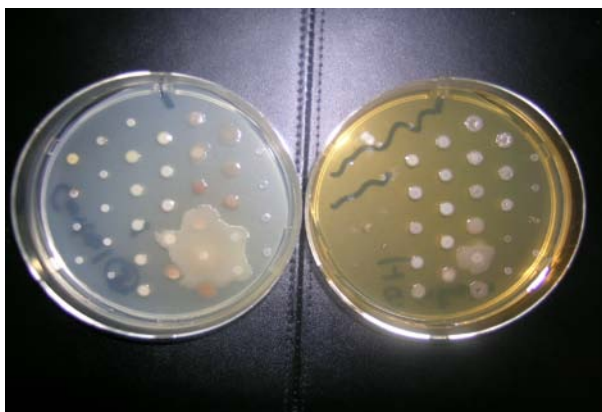


Figure 5.10: Inhibition of *C. albicans* by *G. incanum* infusion (Left: negative control, no DMSO; Right: extract).



Figure 5.11: Inhibition of *C. albicans* by *G. incanum* aqueous extract (Left: negative control, no DMSO; Right: extract).



Figure 5.12: Inhibition of *C. albicans* by *G. incanum* methanol extract (Left: negative control, no DMSO; Right: extract).



Figure 5.13: Inhibition of *C. albicans* by *A. afra* acetone extract (Left: negative control, no DMSO; Right: extract).



Figure 5.14: Inhibition of *S. aureus* and *B. cereus* by *G. incanum* infusion (Left: negative control, no DMSO; Right: extract).

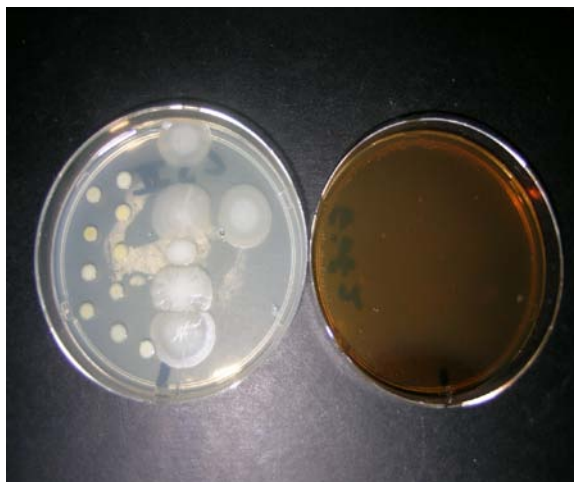


Figure 5.15: Inhibition of *S. aureus* and *B. cereus* by *G. incanum* aqueous extract (Left: negative control, no DMSO; Right: extract).



Figure 5.16: Inhibition of *S. aureus* and *B. cereus* by *G. incanum* methanol extract (Left: negative control, no DMSO; Right: extract).



Figure 5.17: Inhibition of *S. aureus* and *B. cereus* by *A. afra* infusion (Left: negative control, no DMSO; Right: extract).

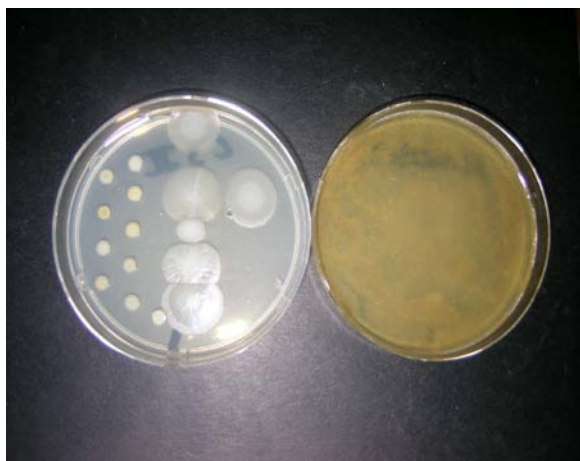


Figure 5.18: Inhibition of *S. aureus* and *B. cereus* by *A. afra* methanol extract (Left: negative control, no DMSO; Right: extract).



Figure 5.19: Inhibition of *S. aureus* and *B. cereus* by *A. afra* acetone extract (Left: negative control, no DMSO; Right: extract).



Figure 5.20: Inhibition of *S. aureus* and *B. cereus* by *A. absinthium* acetone extract (Left: negative control, no DMSO; Right: extract).

5.3. Anticancer activity studies

5.3.1. Percentage yields of dried plant material obtained, and the percentage yields of infusions and crude extracts obtained from *G. incanum*, *A. afra* and *A. absinthium*

The preparation of extracts for use in anticancer studies was slightly adapted from the method used during extract preparation for antimicrobial screening with regards to the amount of plant material used. It was estimated that 60 mg of each of the crude infusion-, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium*, respectively, would be required for the completion of anticancer studies. The amount of plant material used and the crude extract yields obtained during the preparation of extracts for antimicrobial screening was used to approximate the amount of plant material needed to obtain 60 mg of each of the respective extracts of the three plants for use in anticancer studies.

Preparation of infusions, as well as aqueous-, methanol- and acetone extracts for anticancer studies was done as described in Sections 4.3.2.1. and 4.3.2.2., respectively. Table 5.7 reflects the weights of fresh plant material of *G. incanum*, *A. afra* and *A. absinthium*, harvested for anticancer studies, as well as the weights of plant material after drying and the percentage yields of dried plant material obtained.

Table 5.7: Percentage yields of plant material obtained after drying, used in the preparation of aqueous-, methanol- and acetone- extracts for anticancer studies, January 2008 (summer).

<u>Plant</u>	<u>Weight of fresh plant material before drying (gram)</u>	<u>Weight of dried plant material (gram)</u>	<u>Percentage yield of dried plant material (%)</u>
<i>G. incanum</i>	52.1	16.7	32.2
<i>A. afra</i>	101.3	26.1	25.8
<i>A. absinthium</i>	160.6	40.1	25.0

Weighing of dried *G. incanum* plant material indicated a reduction in weight to the amount of 35.3 g. Water constituted approximately 67.9% of the weight of fresh *G. incanum* plant material. Weighing of dried *A. afra* plant material indicated a reduction in weight of 75.2 g. Water constituted approximately 74.2% of the weight of fresh *A. afra* plant material. The weight of *A. absinthium* plant material was reduced by 120.4 g during the drying of fresh plant material, indicating a water content of approximately 75.0% in fresh *A. absinthium* plant material. After freeze drying of infusions and aqueous extracts, and evaporation of methanol- and acetone extracts, respectively, the pre-weighed microtubes and glass beakers containing the crude extracts of *G. incanum*, *A. afra* and *A. absinthium* were weighed again in order to determine the weight of crude extracts obtained. Table 5.8 reflects the percentage yields of extracts obtained during the preparation of extracts used in anticancer studies. The weights of the fresh and dried plant materials used as well as the weights of extracts after freeze drying (infusions and aqueous extracts) and evaporation (methanol- and acetone extracts) are indicated in Table 4 in Appendix I.

Table 5.8: Percentage yields of extracts obtained after the extract preparation for anticancer studies, January 2008.

<u>Extract</u>	<i>Geranium incanum</i>	<i>Artemisia afra</i>	<i>Artemisia absinthium</i>
	Yield % (w/w)	Yield % (w/w)	Yield % (w/w)
Infusion (Fresh leaves used)	8.4	3.8	2.4
Aqueous extract (Dry leaves used)	15.0	11.5	41.9
Methanol extract (Dry leaves used)	19.5	15.5	28.6
Acetone extract (Dry leaves used)	4.8	8.0	11.6

The results presented in Table 5.8. clearly indicate variation in the percentage yields of *G. incanum* extracts obtained during preparation for antimicrobial screening compared to the percentage yields of *G. incanum* extracts obtained during preparation for anticancer studies. The greatest variation exists in the percentage yield of methanol extract obtained during the third round of extract preparation for antimicrobial screening (January 2008, summer) compared to that obtained during extract preparation for anticancer studies (January 2008, summer), with an increase of 83.9% extract having been obtained during the latter. Similar results were observed for the methanol extracts prepared during the second (September 2007, spring) and first rounds (June 2007, winter) of *G. incanum* extract preparation for antimicrobial screening as compared to the methanol extract prepared for anticancer studies (January 2008, summer). An increase of 67.7% of methanol extract obtained was observed during preparation for anticancer studies compared to the second round of extract preparation for antimicrobial studies (September 2007, spring), while an increase of 53.7% in *G. incanum* methanol extract obtained was observed for extract preparation for anticancer studies compared to the initial round of extract preparation for antimicrobial screening (June 2007, winter).

The least variation was observed between the third round of acetone extract preparation for antimicrobial screening (January 2008, summer) compared to

extract preparation for anticancer studies (January 2008, summer), with 17.4% less extract having been obtained during the latter.

Variation also existed in the percentage yields of *A. afra* extracts obtained during preparation for antimicrobial screening compared to the percentage yields of *A. afra* extracts obtained during preparation for anticancer studies. Interestingly, this variation once again occurred mainly between the methanol extracts of *A. afra*, as was the case with *G. incanum*. Increases of 23.3%, 50.7% and 52.5% of *A. afra* methanol extract obtained during extract preparation for anticancer studies was observed, compared to amounts obtained for initial (June 2007, winter), second (September 2007, spring) and third rounds (January 2008, summer) of antimicrobial screening extract preparation, respectively. The least variation was observed during the second round of *A. afra* infusion preparation (September 2007, spring) for antimicrobial screening compared to *A. afra* infusion preparation for anticancer studies (January 2008, summer), with an increase of only 1.6% extract obtained during the latter.

Overall, the greatest variation in the amount of extracts obtained was observed for the aqueous- and methanol extracts of *A. absinthium*. Increases of 78.6%, 81.9% and 75.1% of *A. absinthium* aqueous extract, as well as increases of 65.1%, 73.2% and 65.2% of *A. absinthium* methanol extract obtained during extract preparation for anticancer studies was observed (January 2008, summer), compared to amounts obtained for initial (June 2007, winter), second (September 2007, spring) and third rounds (January 2008, summer) of antimicrobial screening extract preparation, respectively. The least variation was observed for the first round of *A. absinthium* infusion preparation for antimicrobial screening (June 2007, winter) compared to *A. afra* infusion preparation for anticancer studies (January 2008, summer), with a decrease of only 2.9% extract obtained during the latter.

Figure 5.21 is a graphical representation of the variation in extract yields obtained during the first (June 2007, winter), second (September 2007, spring) and third rounds (January 2008, summer) of extract preparation for antimicrobial screening, referred to as the first, second and third rounds respectively, as well as extract preparation for anticancer studies, referred to as the fourth round of extract preparation (January 2008, summer).

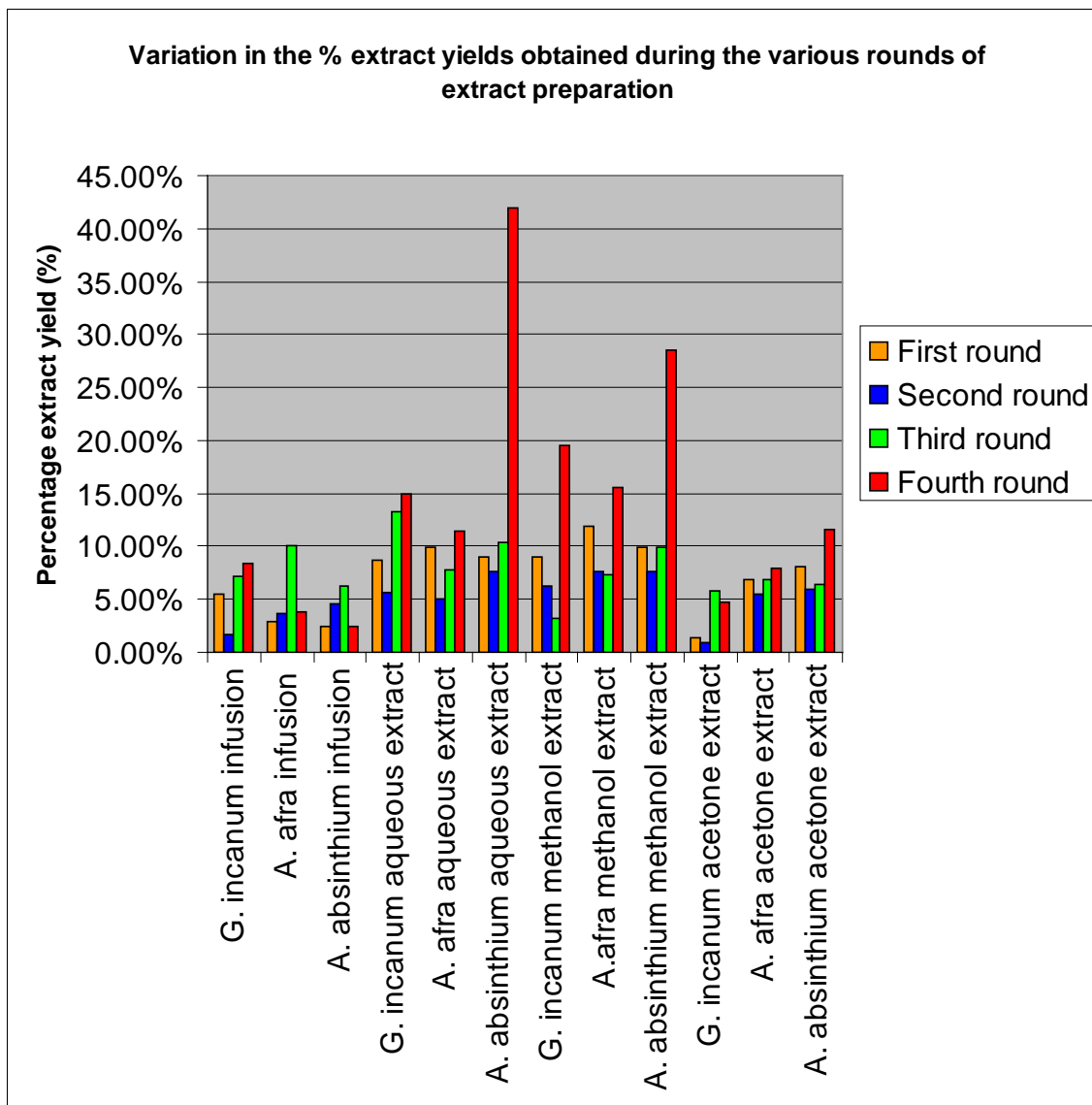


Figure 5.21: Variation in the percentage extract yields obtained during the three rounds of extract preparation for antimicrobial screening, referred to as the first, second and third rounds, respectively, and extract preparation for anticancer studies, referred to as the fourth round.

5.3.2. Cytotoxicity of various extracts in MCF-7, HT-29 and HeLa cell lines

For the purpose of the current study, infusions, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium* were prepared and screened against MCF-7 cells, HT-29 cells and HeLa cells at concentrations ranging from 500 µg/ml (0.25% DMSO) to as little as 0.781 µg/ml (0.25% DMSO) for toxicity or inhibition of growth towards the aforementioned cell lines. The MTT assay, as described in Section 4.3.4., was used to determine the percentage cell viability after cells were treated with the various extracts for 48 hours. All three cell lines were exposed to cisplatin, constituting the positive control. Screening of all the extracts on the three respective cell lines was done in triplicate, with cell viability, as well as inhibition of cell growth/cell death being expressed as a mean percentage \pm standard deviation (SD) of the mean negative control absorbance. This data was analysed using GraphPad Prism® (San Diego, USA) and used to construct dose response curves of the various extracts on the respective cell lines. Where extracts appeared to significantly inhibit cell growth or induce cell death, screening of the specific extract on the respective cell line was repeated using lower concentrations of the extract. GraphPad Prism® was used to perform regression analysis for each of the extracts as well as the positive control on the three cell lines in order to determine IC₅₀ values, the concentration of each extract as well as cisplatin, at which 50% of the cells were inhibited. When the coefficient of correlation (r^2) was not ≥ 0.95 for any of the regression analyses, the results were considered insignificant and screening of the specific extract on the relevant cell line was repeated.

Three different sets of graphs were constructed with the data obtained from the aforementioned procedures. Firstly, graphs comparing the serially diluted infusions, aqueous-, methanol- and acetone extracts of each of the respective three plants against each of the three respective cell lines were constructed in order to visually determine which extract of each of the respective plants

exhibited the strongest inhibitory activity against each of the three cell lines. A second set of graphs individually comparing only one serially diluted extract of each of the three respective plants on all three of the cell lines was constructed in order to visually determine which of the three cell lines was most susceptible to inhibition by a specific extract. Thirdly, a graph comparing serially diluted cisplatin against all three cell lines was constructed in order to visually determine which cell line was most susceptible to inhibition by cisplatin.

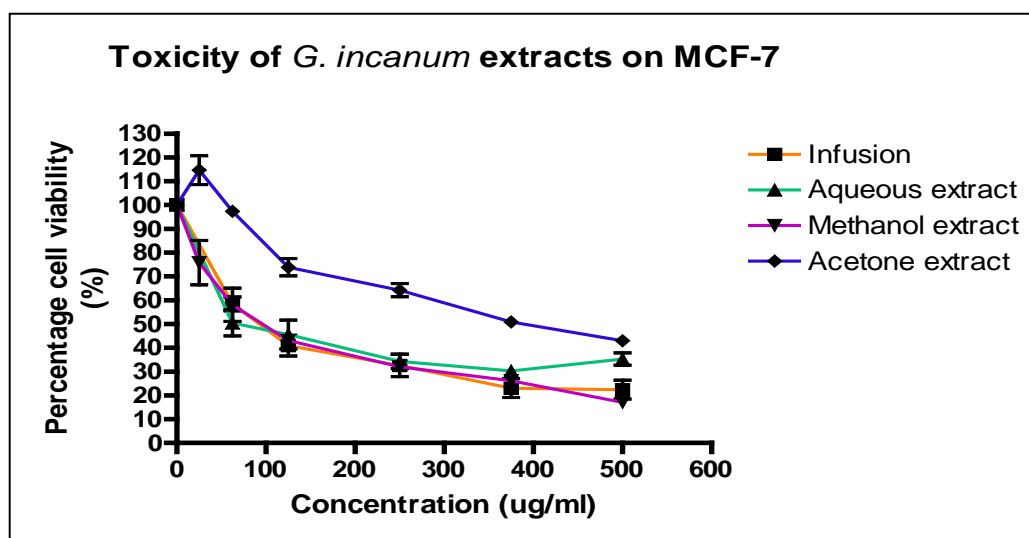


Figure 5.22: Comparison of inhibitory activity of *G. incanum* infusion, aqueous-, methanol- and acetone- extracts in MCF-7 cells.

Figure 5.22 indicates *G. incanum* acetone extract to be considerably less toxic towards MCF-7 cells than the infusion, aqueous- or methanol extracts of the same plant (Figure 5.22, blue plot versus orange-, green- and purple plots, respectively). The latter three extracts exhibited a similar degree of toxicity in MCF-7 cells, however, in order to determine their respective IC_{50} values, regression analysis was done, and the results are presented in Table 5.9.

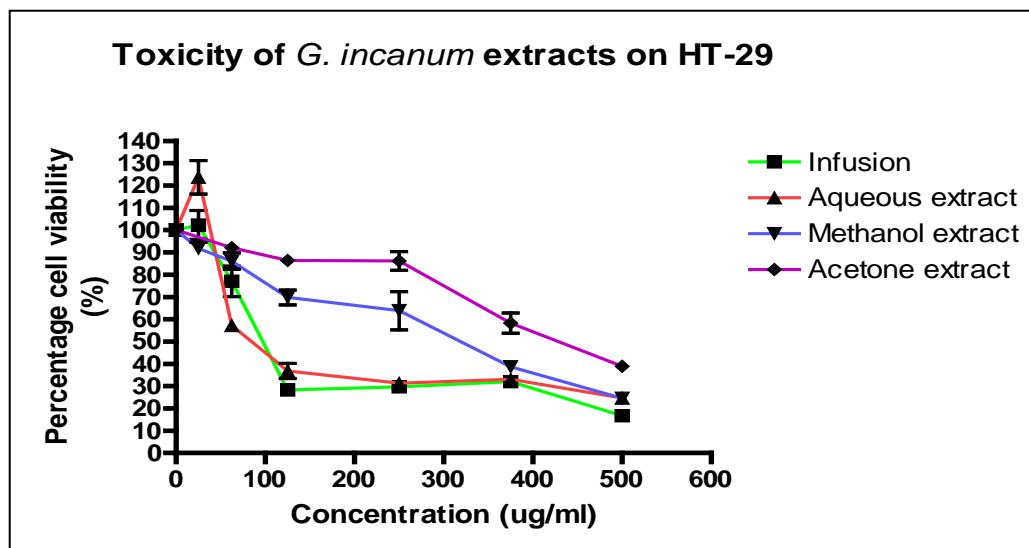


Figure 5.23: Comparison of inhibitory activity of *G. incanum* infusion, aqueous-, methanol- and acetone extracts in HT-29 cells.

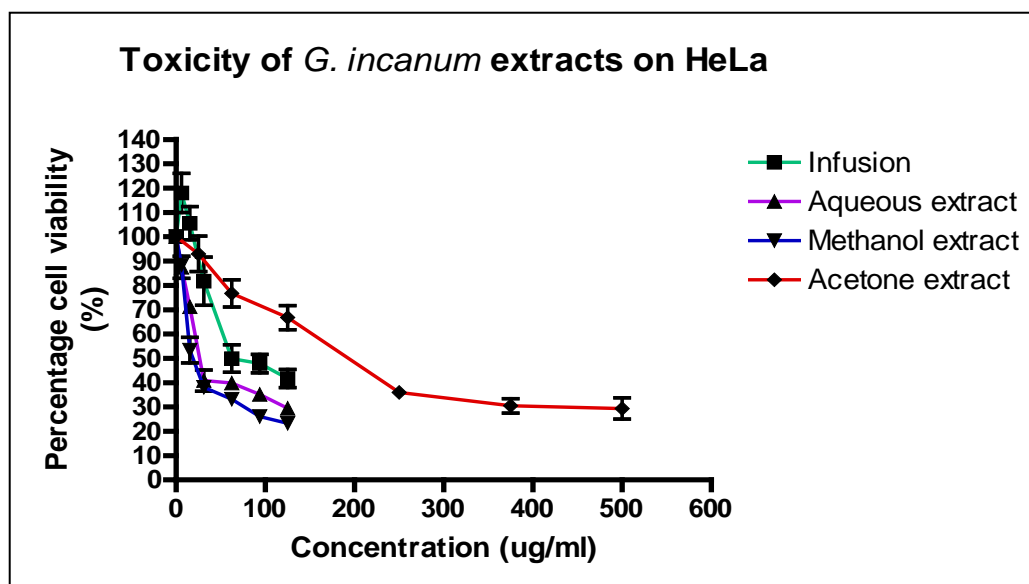


Figure 5.24: Comparison of inhibitory activity of *G. incanum* infusion, aqueous-, methanol- and acetone extracts in HeLa cells.

Figures 5.23 and 5.24 allow the comparison of the toxicity of the four *G. incanum* extracts on HT-29 cells and HeLa cells, respectively. The infusion and aqueous extract of *G. incanum* appeared to be more toxic in HT-29 cells, with the aqueous extract showing the highest degree of toxicity (Figure 5.23, orange plot), as

compared to the methanol- and acetone extracts (Figure 5.23, blue- and purple plots, respectively), which both had higher IC₅₀ values and thus less toxicity (Table 5.9). With regard to toxicity in HeLa cells, the methanol extract of *G. incanum* showed the highest degree of toxicity (Figure 5.24, blue plot), followed by the aqueous extract (Figure 5.24, purple plot). The infusion was slightly less toxic to HeLa cells (Figure 5.24, green plot), with HeLa cells being the least susceptible to toxicity by the acetone extract (Figure 5.24, red plot). IC₅₀ values were calculated using nonlinear regression analysis, and the results are presented in Table 5.9, showing this trend.

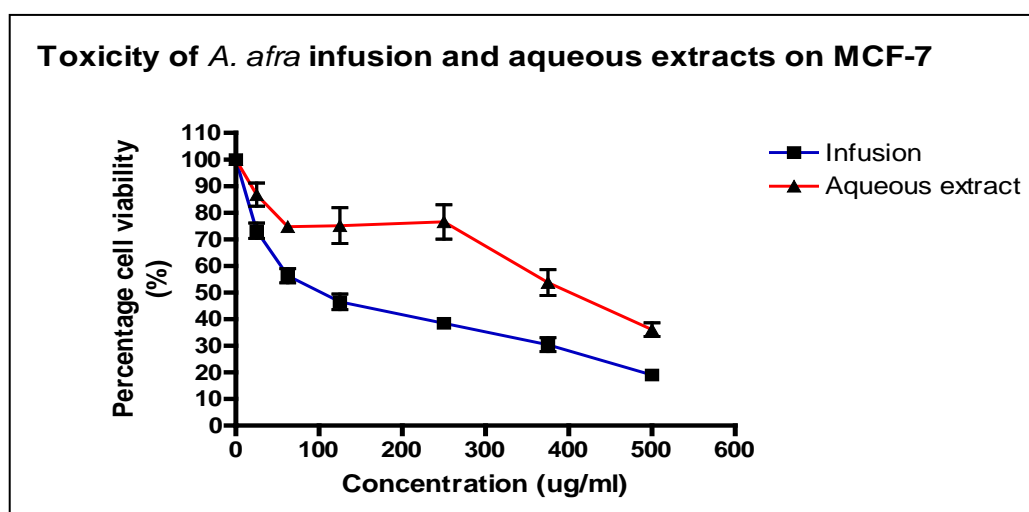


Figure 5.25: Comparison of inhibitory activity of *A. afra* infusion and aqueous extract in MCF-7 cells.

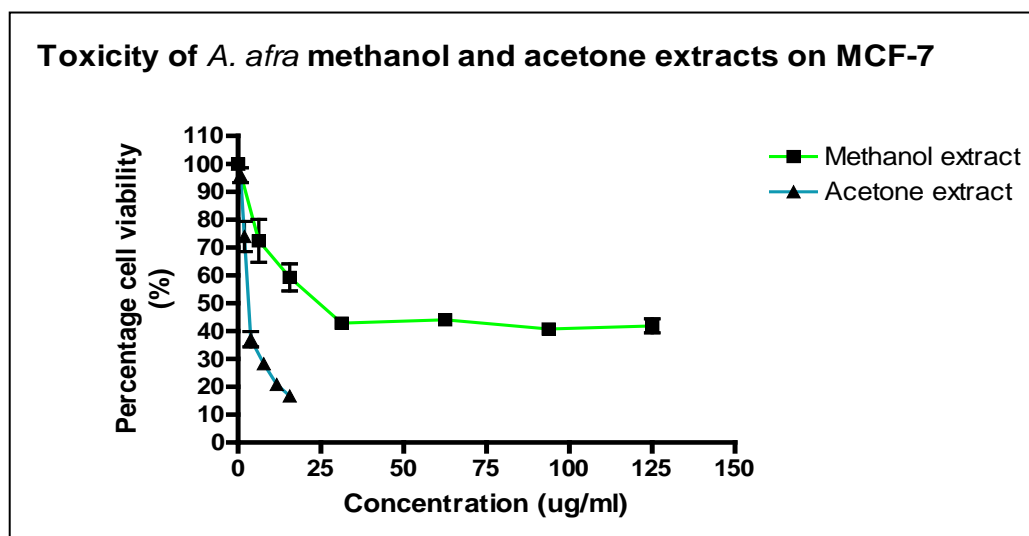


Figure 5.26: Comparison of inhibitory activity of *A. afra* methanol- and acetone extracts in MCF-7 cells.

Figures 5.25 and 5.26 respectively show the toxicities of *A. afra* infusion (Figure 5.25, blue plot) and aqueous extract (Figure 5.25, red plot), as well as the toxicities of *A. afra* methanol- and acetone extracts in MCF-7 cells (Figure 5.26, green- and blue plots, respectively). The two graphs were not combined to allow the various plots to be distinguishable from each other. Compared to the infusion and aqueous extract (Figure 5.25), the methanol- and acetone extracts of *A. afra* were significantly more toxic to MCF-7 cells (Figure 5.26), with IC_{50} values of the methanol- and acetone extracts being between 0 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$, while the IC_{50} values of the infusion and aqueous extract were between 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ (Table 5.9).

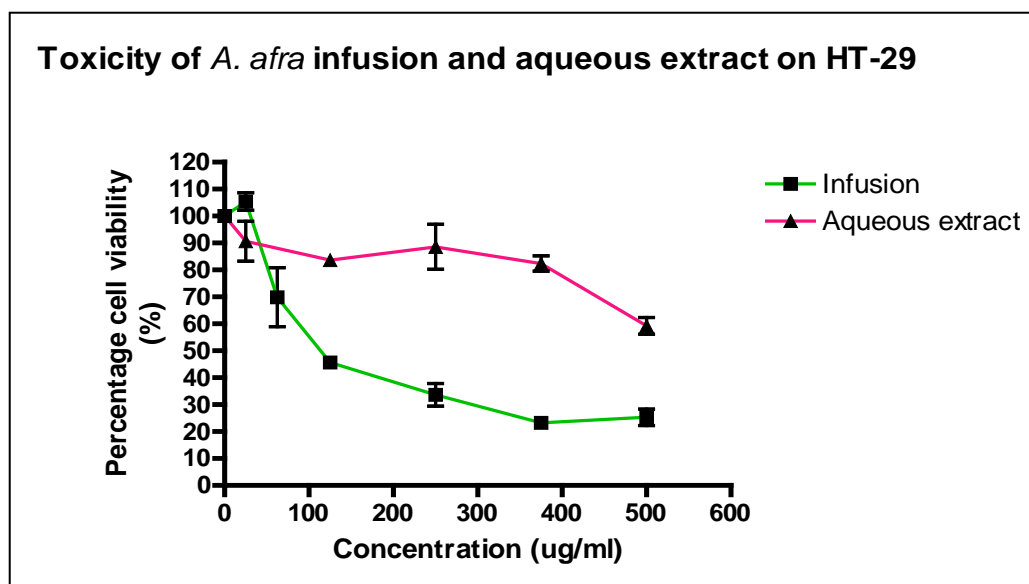


Figure 5.27: Comparison of inhibitory activity of *A. afra* infusion and aqueous extract in HT-29 cells.

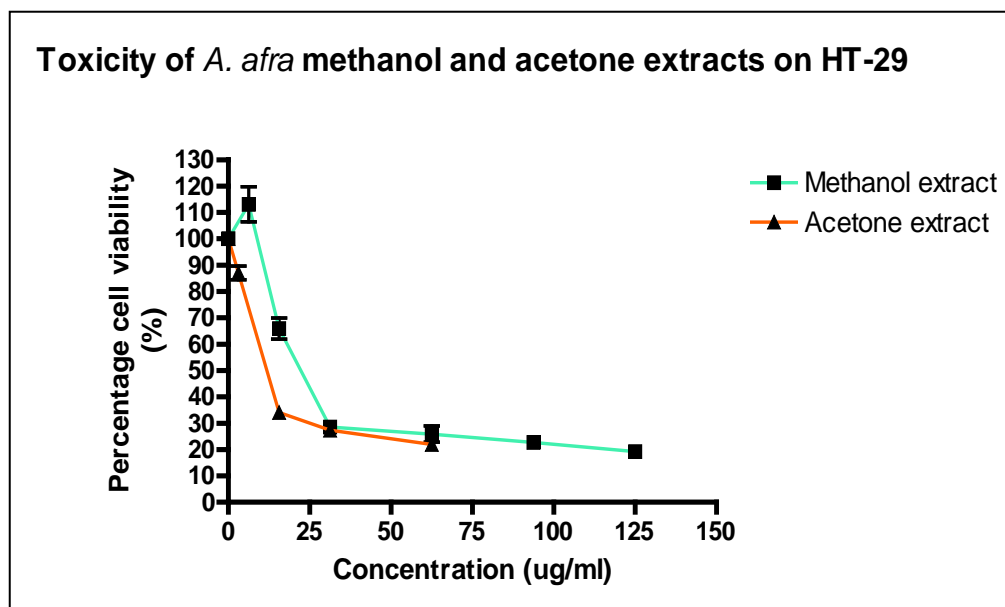


Figure 5.28: Comparison of inhibitory activity of *A. afra* methanol- and acetone extracts in HT-29 cells.

Figures 5.27 and 5.28 respectively show the toxicities of *A. afra* infusion (Figure 5.27, green plot) and aqueous extract (Figure 5.27, pink plot), as well as the toxicities of *A. afra* methanol- and acetone extracts in HT-29 cells (Figure 5.28, turquoise- and orange plots, respectively). The two graphs were not combined to allow the various plots to be distinguishable from each other. Compared to the infusion and aqueous extract (Figure 5.27), the methanol- and acetone extracts of *A. afra* were significantly more toxic (Figure 5.28), as was the case with MCF-7 cells. IC_{50} values of the methanol- and acetone extracts ranged between 0 μ g/ml and 25 μ g/ml, while the IC_{50} values of the infusion and aqueous extract ranged between 50 μ g/ml and 100 μ g/ml (Table 5.9).

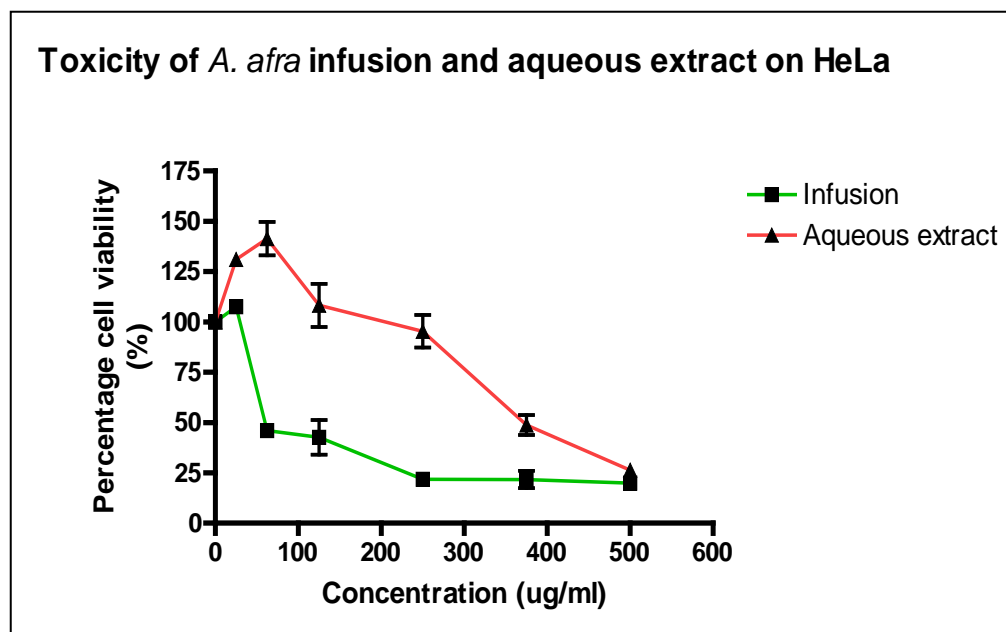


Figure 5.29: Comparison of inhibitory activity of *A. afra* infusion and aqueous extract in HeLa cells.

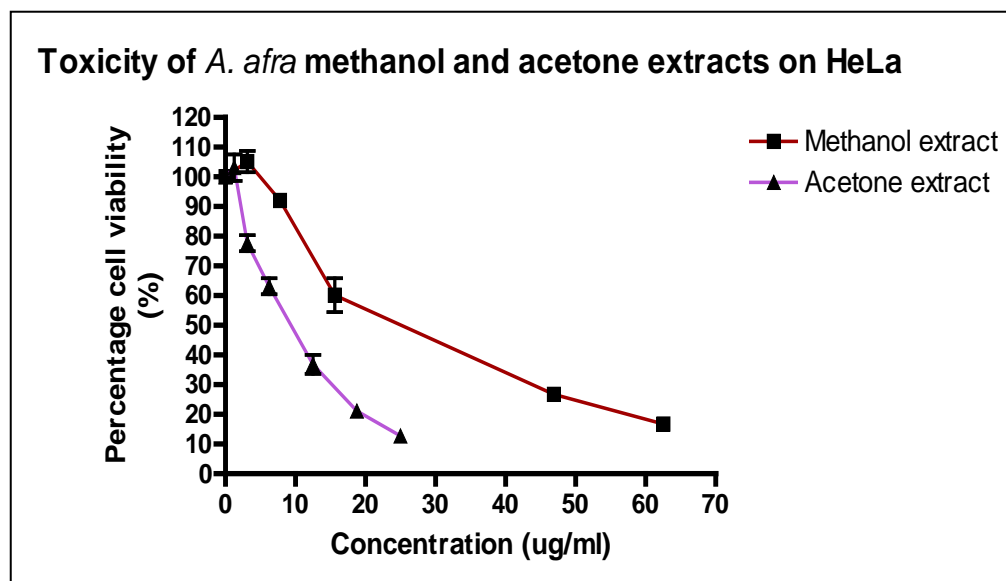


Figure 5.30: Comparison of inhibitory activity of *A. afra* methanol- and acetone extracts in HeLa cells.

Figures 5.29 and 5.30 respectively show the toxicities of *A. afra* infusion (Figure 5.29, green plot) and aqueous extract (Figure 5.30, red plot), as well as the toxicities of *A. afra* methanol- and acetone extracts in HeLa cells (Figure 5.30, maroon- and purple plots, respectively). The *A. afra* methanol- and acetone extracts showed a significantly higher degree of toxicity in HeLa cells (Figure 5.30), as was the case with MCF-7 cells (Figure 5.26) and HT-29 cells (Figure 5.28). Of the four *A. afra* extracts, the acetone extract appeared to have the highest degree of toxicity in HeLa cells, with the lowest IC₅₀, followed by the methanol extract (Table 5.9). The infusion showed the third highest degree of toxicity in HeLa cells, with the aqueous extract having been the least toxic of the four (Table 5.9). In general, it would appear that the methanol- and acetone extracts of *A. afra* are more toxic in all three cell lines tested as compared to the infusion and aqueous extract, with the acetone extract being the most toxic. MCF-7 cells appear to be the most susceptible to toxicity by the acetone extract, having had the lowest IC₅₀ of the three cell lines tested (Table 5.9).

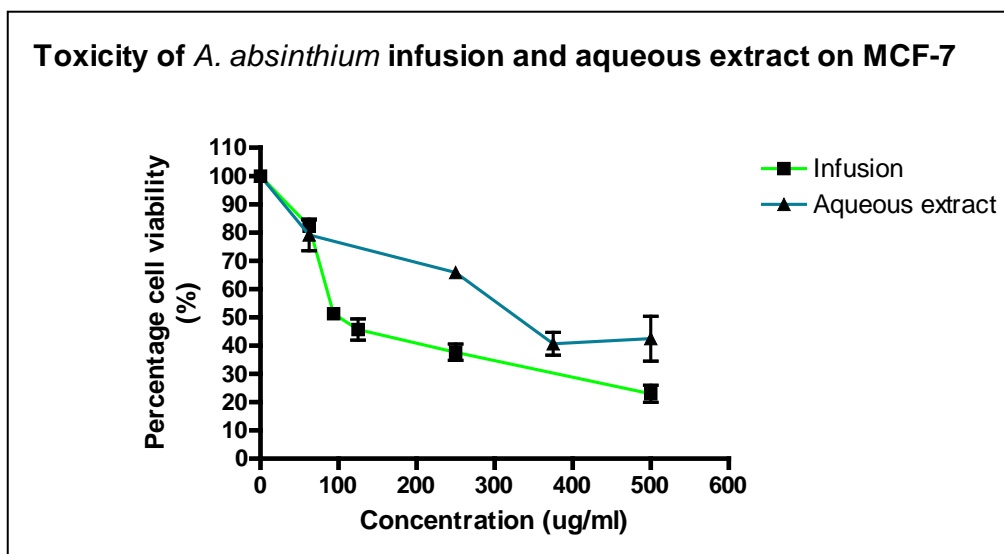


Figure 5.31: Comparison of inhibitory activity of *A. absinthium* infusion and aqueous extract in MCF-7 cells.

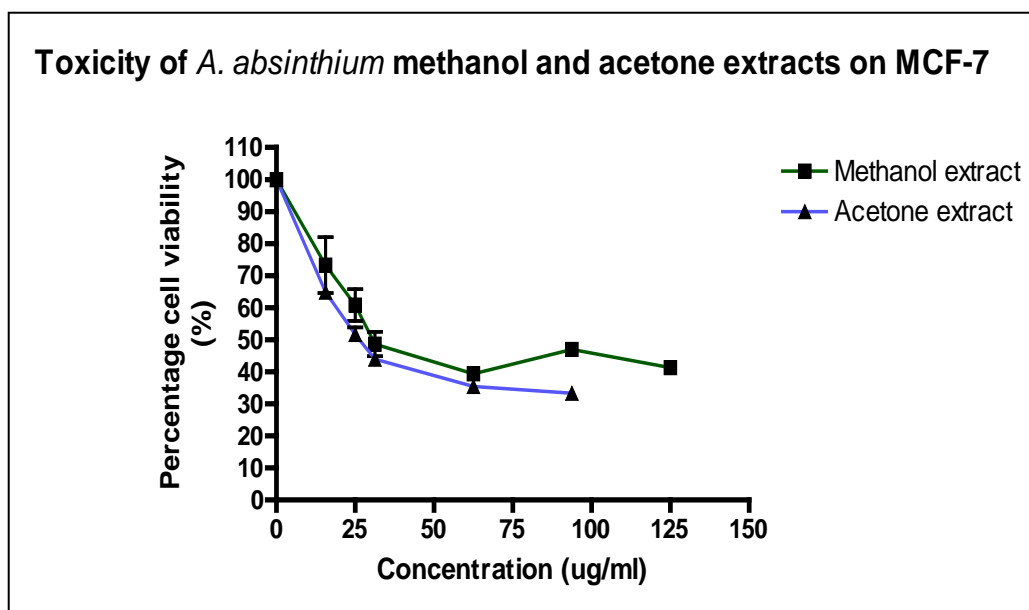


Figure 5.32: Comparison of inhibitory activity of *A. absinthium* methanol- and acetone- extracts in MCF-7 cells.

Figures 5.31 and 5.32 respectively show the toxicities of *A. absinthium* infusion (Figure 5.31, green plot) and aqueous extract (Figure 5.31, blue plot), as well as the toxicities of *A. absinthium* methanol- and acetone extracts in MCF-7 cells (Figure 5.32, green- and blue plots, respectively). The *A. absinthium* methanol- and acetone extracts showed a degree of toxicity towards MCF-7 cells, with IC_{50} values occurring between 0 μ g/ml and 25 μ g/ml (Table 5.9), which was more significant than that shown by the infusion and aqueous extract, which had IC_{50} values between 25 μ g/ml and 250 μ g/ml (Table 5.9).

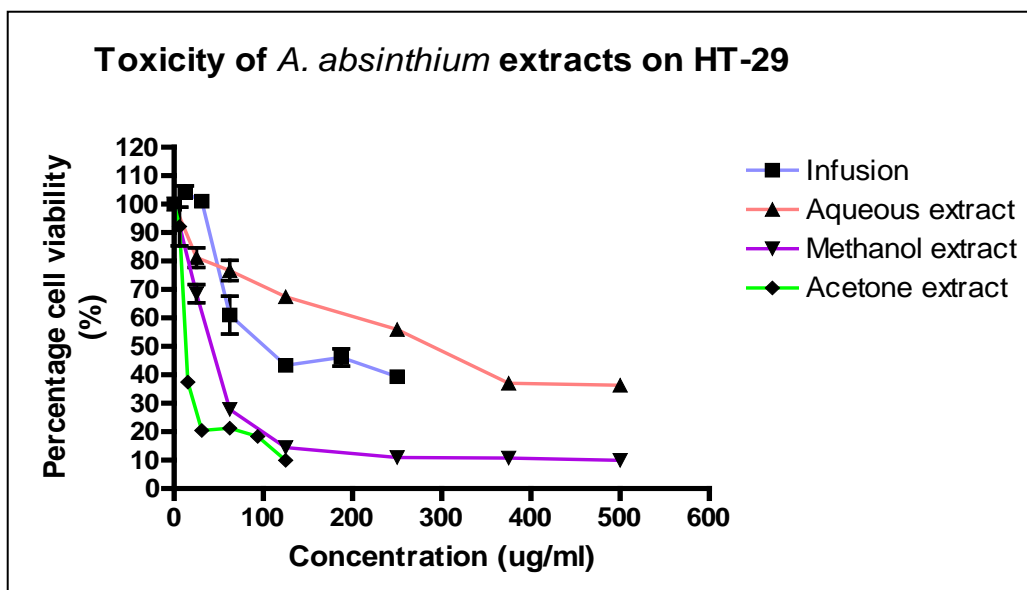


Figure 5.33: Comparison of inhibitory activity of *A. absinthium* infusion, aqueous-, methanol- and acetone- extracts in HT-29 cells.

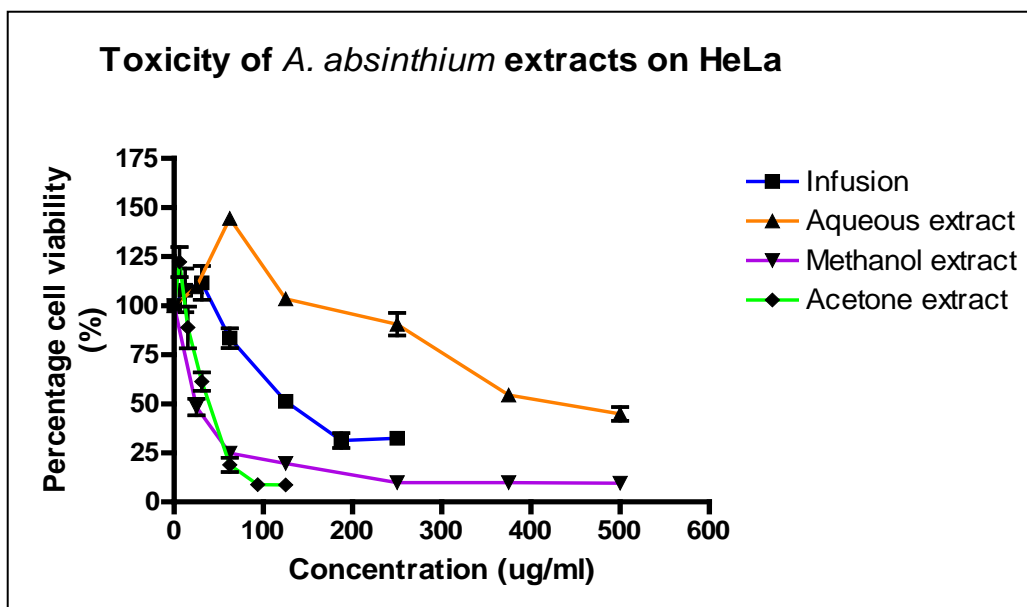


Figure 5.34: Comparison of inhibitory activity of *A. absinthium* infusion, aqueous-, methanol- and acetone- extracts in HeLa cells.

Figures 5.33 and 5.34 respectively show toxicity of the four *A. absinthium* extracts on HT-29 cells and HeLa cells. The methanol- and acetone extracts showed the highest degree of toxicity in both HT-29 cells (Figure 5.33, purple- and green plots, respectively) and HeLa cells (Figure 5.34, purple- and green plots, respectively), as was the case with the *A. afra* methanol- and acetone extracts in all three cell lines (Figures 5.26, 5.28 and 5.30), as well as the *A.*

absinthium methanol- and acetone extracts in MCF-7 cells (Figure 5.32, green- and blue plots, respectively). Thus it would appear that all three cell lines are more susceptible to the methanol- and acetone extracts of *A. afra* and *A. absinthium* as compared to their respective infusions and aqueous extracts. With regard to the *A. absinthium* extracts screened against HT-29 cells, the acetone extract showed the highest degree of toxicity with the lowest IC₅₀, followed by the methanol extract with a higher IC₅₀ (Table 5.9). The aqueous extract showed a higher degree of toxicity in HT-29 cells as compared to the infusion, which appeared to have been the least toxic (Table 5.9). With regard to the effects of *A. absinthium* extracts in HeLa cells, the methanol extract showed the highest degree of toxicity with the lowest IC₅₀, followed by the acetone extract with a slightly higher IC₅₀ (Table 5.9). The infusion showed a higher degree of toxicity in HT-29 cells as compared to the aqueous extract, which appeared to have been the least toxic with the highest IC₅₀ value (Table 5.9). Figures 5.35 to 5.46 are comparisons of the serially diluted individual extracts of each of the three plants against all three cell lines, which were prepared in order to visually determine which of the three cell lines was most susceptible to inhibition by a specific extract.

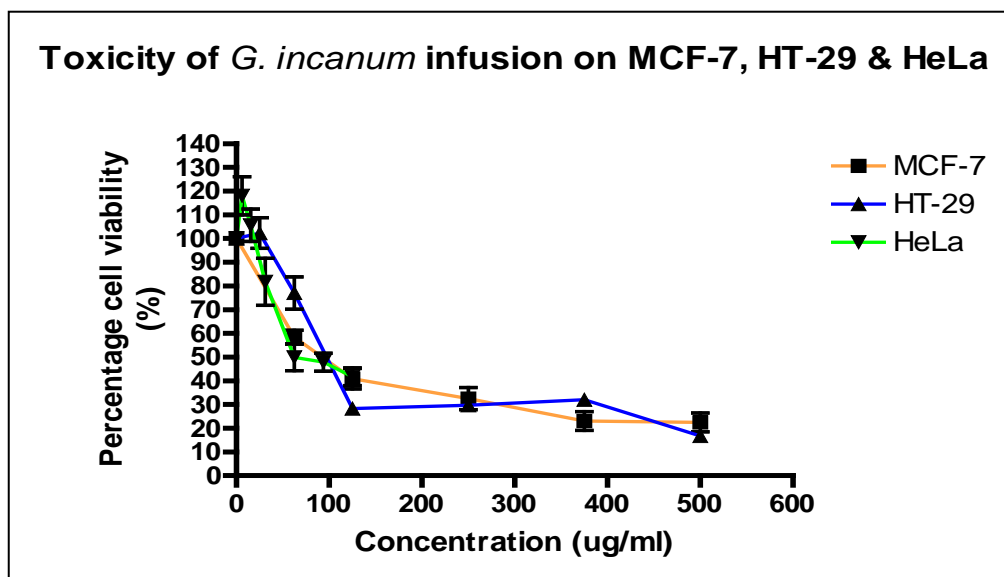


Figure 5.35: Comparison of the toxicity of *G. incanum* infusion in MCF-7, HT-29 and HeLa cells.

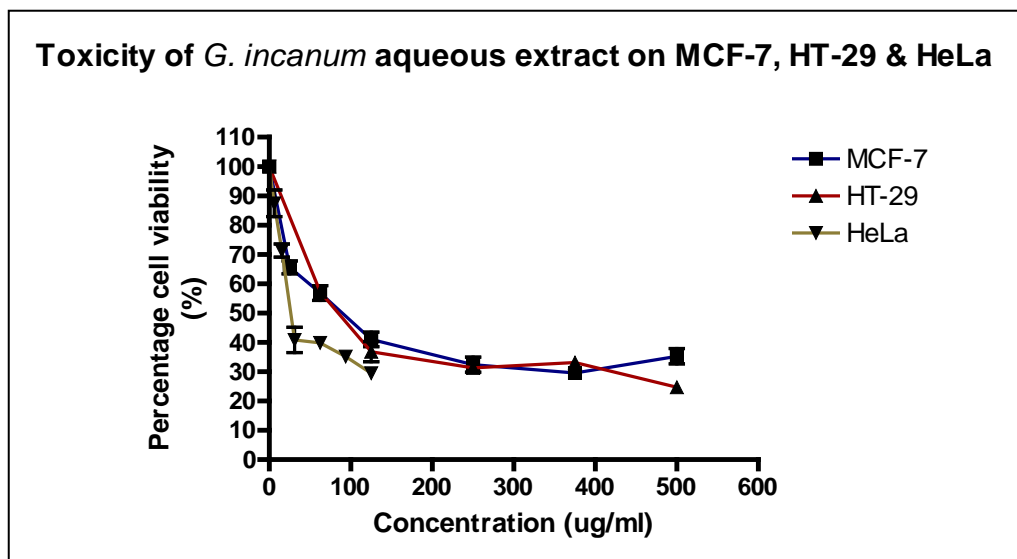


Figure 5.36: Comparison of the toxicity of *G. incanum* aqueous extract in MCF-7, HT-29 and HeLa cells.

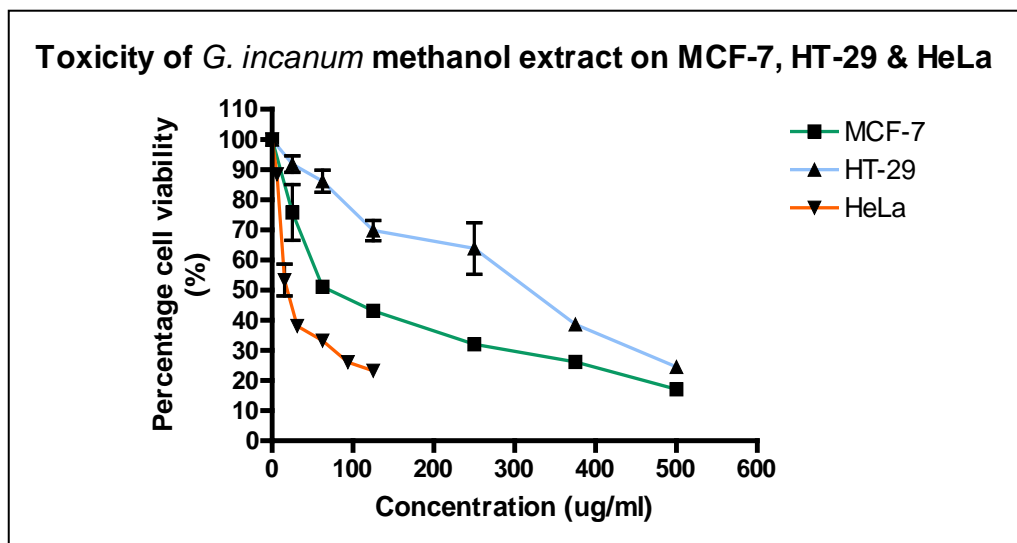


Figure 5.37: Comparison of the toxicity of *G. incanum* methanol extract in MCF-7, HT-29 and HeLa cells.

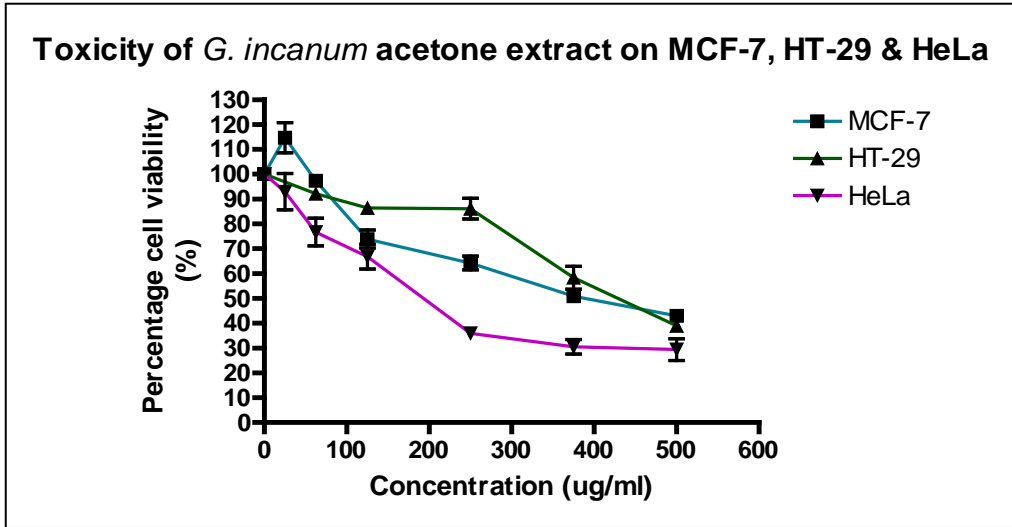


Figure 5.38: Comparison of the toxicity of *G. incanum* acetone extract in MCF-7, HT-29 and HeLa cells.

Figures 5.35 to 5.38 show the susceptibility of MCF-7 cells, HT-29 cells and HeLa for the infusion, aqueous-, methanol- and acetone extract of *G. incanum*, respectively. In each of the four cases the HeLa cell line consistently showed the highest sensitivity for each of the four *G. incanum* extracts (Figure 5.35, green plot; Figure 5.36, brown plot; Figure 5.37, orange plot; Figure 5.38, purple plot), having the lowest IC₅₀ values as compared to those for MCF-7 cells and HeLa cells (Table 5.9). MCF-7 cells appeared to have had the second highest degree of sensitivity for each of the four *G. incanum* extracts (Figure 5.35, orange plot; Figure 5.36, blue plot; Figure 5.37, green plot; Figure 5.38, blue plot), while HT-29 cells appeared to have been the least sensitive of the three cell lines for any of the four *G. incanum* extracts (Figure 5.35, blue plot; Figure 5.36, maroon plot; Figure 5.37, blue plot; Figure 5.38, green plot).

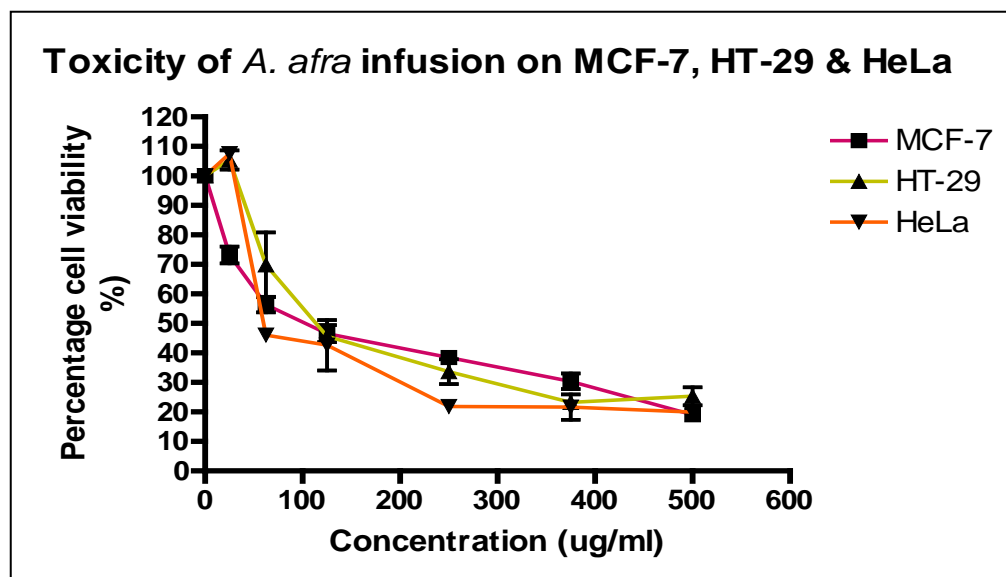


Figure 5.39: Comparison of the toxicity of *A. afra* infusion in MCF-7, HT-29 and HeLa cells.

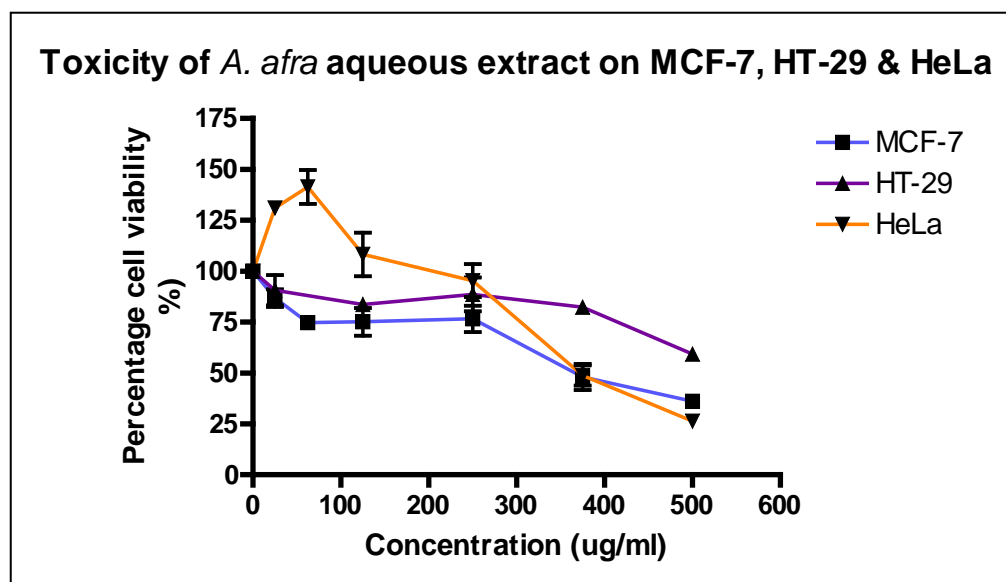


Figure 5.40: Comparison of the toxicity of *A. afra* aqueous extract in MCF-7, HT-29 and HeLa cells.

Toxicity of *A. afra* methanol extract on MCF-7, HT-29 & HeLa

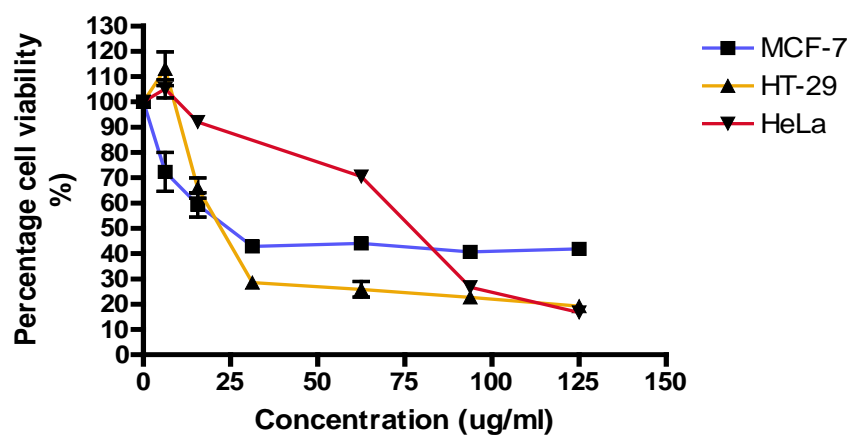


Figure 5.41: Comparison of the toxicity of *A. afra* methanol extract in MCF-7, HT-29 and HeLa cells.

Toxicity of *A. afra* acetone extract on MCF-7, HT-29 & HeLa

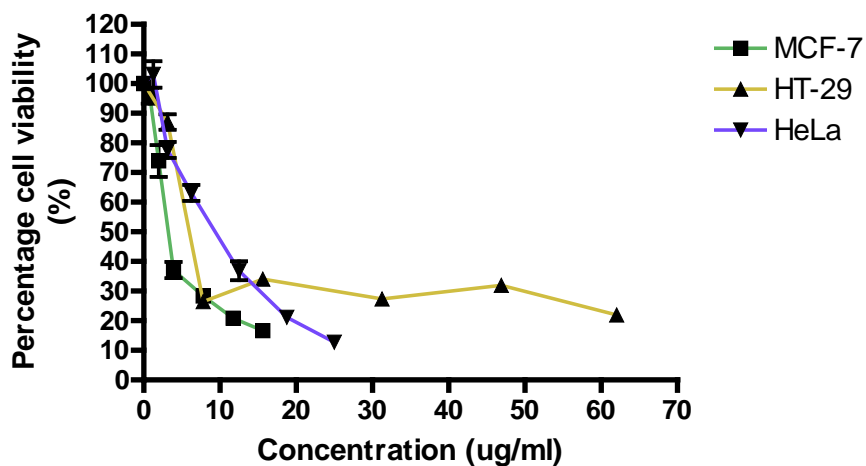


Figure 5.42: Comparison of the toxicity of *A. afra* acetone extract in MCF-7, HT-29 and HeLa cells.

Figures 5.39 to 5.42 show the susceptibility of MCF-7 cells, HT-29 cells and HeLa cells towards the infusion, aqueous-, methanol- and acetone extract of *A. afra*, respectively.

Unlike the case with *G. incanum* extracts (Figure 5.35, orange plot; Figure 5.36, blue plot; Figure 5.37, green plot; Figure 5.38, blue plot), MCF-7 cells appeared to show the highest sensitivity towards each of the four *A. afra* extracts (Figure 5.39, pink plot; Figure 5.40, blue plot; Figure 5.41, blue plot; Figure 5.42, green plot), having the lowest IC₅₀ values as compared to those for HT-29 cells and HeLa cells (Table 5.9). In the case of the *A. afra* infusion, HeLa cells (Figure 5.39, orange plot) were more susceptible to growth inhibition as compared to HT-29 cells (Figure 5.39, yellow plot), with the infusion showing a lower IC₅₀ for HeLa cells compared to HT-29 cells (Table 5.9). In the case of the *A. afra* aqueous extract, HT-29 cells (Figure 5.40, purple plot) were significantly more susceptible to growth inhibition compared to HeLa cells (Figure 5.40, orange plot), with the aqueous extract showing an IC₅₀ for HeLa cells approximately double that showed for HT-29 cells (Table 5.9).

HT-29 cells also showed a lower IC₅₀ when treated with the *A. afra* methanol extract (Figure 5.41, yellow plot) as compared to HeLa cells (Figure 5.41, purple plot), indicating HT-29 cells to be more sensitive to growth inhibition by the methanol extract than HeLa cells (Table 5.9). Finally, HeLa cells showed an IC₅₀ after treatment with the *A. afra* acetone extract (Figure 5.42, purple plot) which was only slightly lower than the IC₅₀ showed for HT-29 cells (Figure 5.42, yellow plot), indicating HeLa cells to be slightly more sensitive to growth inhibition by the acetone extract than HT-29 cells (Table 5.9).

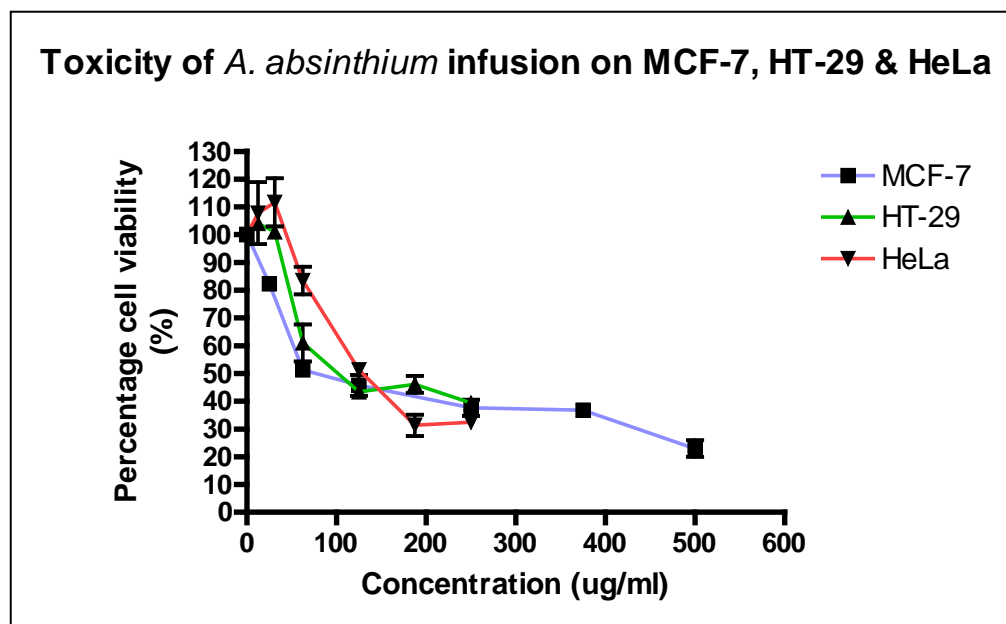


Figure 5.43: Comparison of the toxicity of *A. absinthium* infusion in MCF-7, HT-29 and HeLa cells.

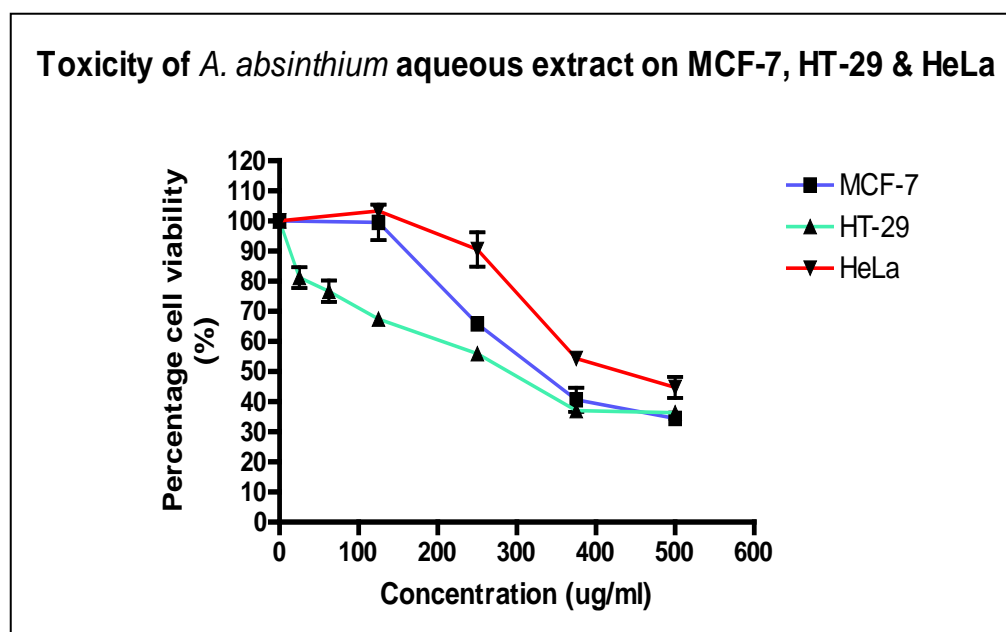


Figure 5.44: Comparison of the toxicity of *A. absinthium* aqueous extract in MCF-7, HT-29 and HeLa cells.

Toxicity of *A. absinthium* methanol extract on MCF-7, HT-29 & HeLa

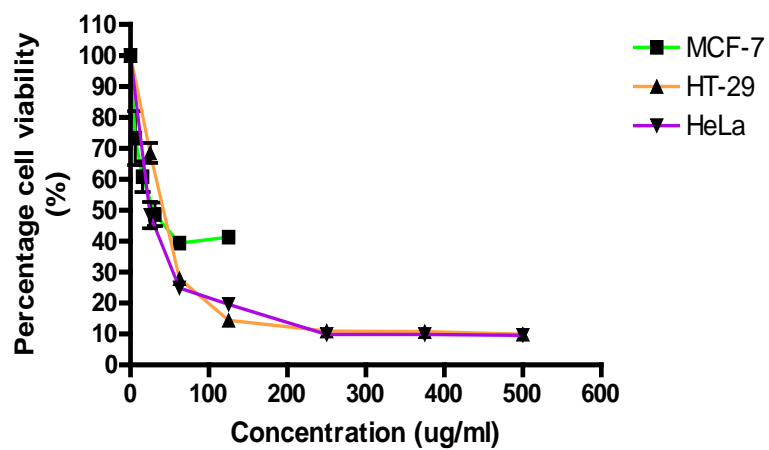


Figure 5.45: Comparison of the toxicity of *A. absinthium* methanol extract in MCF-7, HT-29 and HeLa cells.

Toxicity of *A. absinthium* acetone extract on MCF-7, HT-29 & HeLa

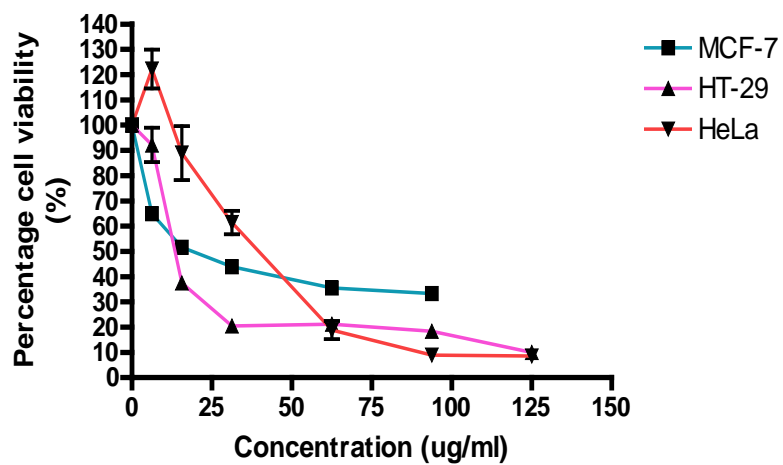


Figure 5.46: Comparison of the toxicity of *A. absinthium* acetone extract in MCF-7, HT-29 and HeLa cells.

Figures 5.43 to 5.46 show the susceptibility of MCF-7 cells, HT-29 cells and HeLa cells towards the infusion, aqueous-, methanol- and acetone extract of *A. absinthium*, respectively.

MCF-7 cells appeared to exhibit the highest sensitivity towards three of the four *A. absinthium* extracts, being the infusion (Figure 5.43, red plot), methanol- (Figure 5.45, green plot) and acetone extracts (Figure 5.46, blue plot). In the case of the aqueous extract, HT-29 cells (Figure 5.44, turquoise plot) showed the highest degree of sensitivity as compared to MCF-7 cells (Figure 5.44, blue plot) and HeLa cells (Figure 5.44, red plot). HT-29 cells showed the second highest degree of sensitivity towards the infusion of *A. absinthium* (Figure 5.43, green plot), with an IC_{50} lower than that for HeLa cells, indicating the latter to be the least sensitive to the infusion (Table 5.9). MCF-7 cells showed the second highest degree of sensitivity towards the aqueous extract of *A. absinthium* (Figure 5.44, blue plot), with an IC_{50} lower than that for HeLa cells, indicating HeLa cells to be the least sensitive to inhibition by the aqueous extract (Table 5.9).

With respect to the methanol extract of *A. absinthium*, HeLa cells (Figure 5.45, purple plot) showed the second highest degree of sensitivity with an IC_{50} lower than that for HT-29 cells (Figure 5.45, orange plot), which appeared to be the least sensitive of the three cell lines to growth inhibition by the *A. absinthium* methanol extract (Table 5.9). Finally, in the case of the *A. absinthium* acetone extract, HT-29 cells (Figure 5.46, pink plot) showed the second highest degree of sensitivity with an IC_{50} lower than that for HeLa cells (Figure 5.46, orange plot), which appeared to be the least sensitive of the three cell lines to growth inhibition by the *A. absinthium* acetone extract (Table 5.9).

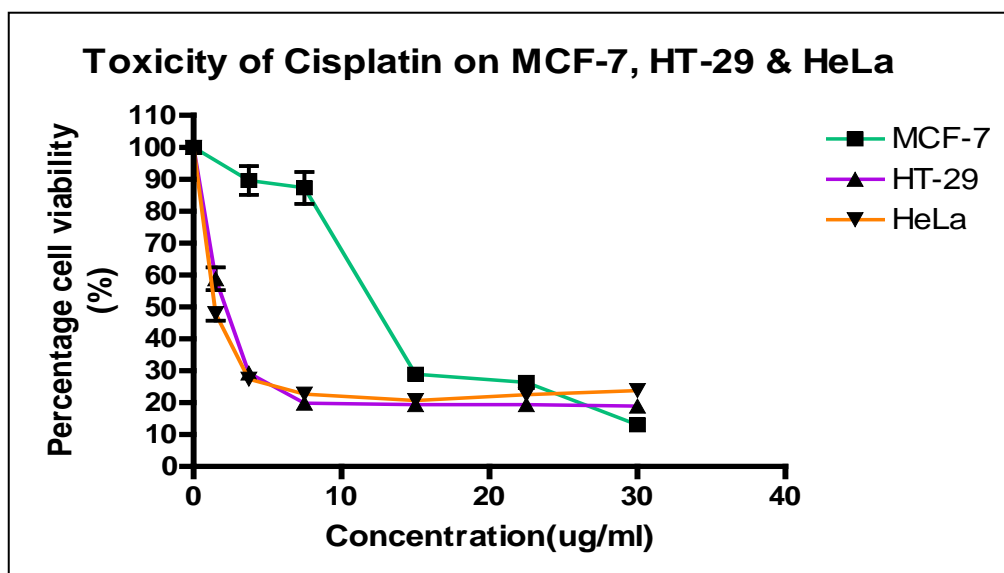


Figure 5.47: Toxicity of Cisplatin on MCF-7, HT-29 and HeLa cells.

Figure 5.47 shows the comparison of the toxicity of serially diluted cisplatin, a known anticancer agent, in MCF-7 cells, HT-29 cells and HeLa cells. Cisplatin showed a similar degree of toxicity in both HT-29 cells (Figure 5.47, purple plot) and HeLa cells (Figure 5.47, orange plot), with IC_{50} values ranging between 0 µg/ml and 10 µg/ml (Figure 5.47). MCF-7 cells, on the other hand, appeared to be slightly less susceptible to cisplatin toxicity (Figure 5.47, turquoise plot), with an IC_{50} occurring in the range of 10 µg/ml to 20 µg/ml (Figure 5.47).

Table 5.9 is a summary of the various IC_{50} values calculated using nonlinear regression analysis of the toxicity of the infusions, aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium* in MCF-7 cells, HT-29 cells and HeLa cells. The IC_{50} for the extract of each of the respective plants which exhibited the strongest inhibitory activity against a specific cell line is indicated with a * symbol, while the IC_{50} for the cell line most susceptible to each one of the four extracts of each plant is indicated with a # symbol.

Table 5.9: IC₅₀ values as determined by nonlinear regression analysis of the MCF-7 cells, HT-29 cells and HeLa cells after respective treatment with the infusions, aqueous-, methanol- and acetone- extracts of *G. incanum*, *A. afra* and *A. absinthium* for 48 hours. The standard deviation, r² value and 95% confidence interval for each IC₅₀ is also indicated.

Plant extract	Cells					
	MCF-7		HT-29		HeLa	
	IC ₅₀ ± Standard Deviation (µg/ml)	r ² ; 95% confidence interval (µg/ml)	IC ₅₀ ± Standard Deviation (µg/ml)	r ² ; 95% confidence interval (µg/ml)	IC ₅₀ ± Standard Deviation (µg/ml)	r ² ; 95% confidence interval (µg/ml)
<i>G. incanum</i> infusion	66.25±1.03	r ² =0.99; 60.95 to 72.02	77.97±1.06	r ² =0.96; 69.12 to 87.95	#26.74±1.07	r ² =0.96; 23.03 to 31.05
<i>G. incanum</i> aqueous extract	27.11±1.11	r ² =0.96; 20.82 to 35.29	*53.38±1.06	r ² =0.95; 46.75 to 60.96	#17.87±1.07	r ² =0.96; 15.55 to 20.53
<i>G. incanum</i> methanol extract	*19.16±1.10	r ² =0.95; 15.25 to 24.07	147.70±1.08	r ² =0.95; 123.9 to 176.10	*#13.71±1.06	r ² =0.96; 12.04 to 15.61
<i>G. incanum</i> acetone extract	183.30±1.08	r ² =0.96; 155.50 to 216.00	198.50±1.11	r ² =0.95; 156.70 to 251.50	#127.80±1.06	r ² =0.96; 111.40 to 146.60
<i>A. afra</i> infusion	54.81±1.11	r ² =0.95; 43.64 to 68.84	90.47±1.11	r ² =0.98; 71.56 to 114.40	#38.31±1.10	r ² =0.97; 29.80 to 49.24
<i>A. afra</i> aqueous extract	#87.69±1.09	r ² =0.95; 72.04 to 106.70	136.6±1.09	r ² =0.95; 109.70 to 170.10	272.80±1.04	r ² =0.95; 249.40 to 298.50
<i>A. afra</i> methanol extract	#8.36±1.08	r ² =0.97; 7.08 to 9.88	15.80±1.04	r ² =0.97; 14.43 to 17.30	16.68±1.06	r ² =0.97; 14.60 to 19.04
<i>A. afra</i> acetone extract	*#2.65±1.05	r ² =0.97; 2.39 to 2.94	*6.98±1.06	r ² =0.99; 6.07 to 8.02	*6.54±1.06	r ² =0.96; 5.79 to 7.38
<i>A. absinthium</i> infusion	#37.33±1.04	r ² =0.98; 34.03 to 40.95	54.65±1.05	r ² =0.97; 49.01 to 60.93	95.98±1.06	r ² =0.95; 84.83 to 108.60

Plant extract	Cells					
	MCF-7		HT-29		HeLa	
	IC ₅₀ ± Standard Deviation (µg/ml)	r ² ; 95% confidence interval (µg/ml)	IC ₅₀ ± Standard Deviation (µg/ml)	r ² ; 95% confidence interval (µg/ml)	IC ₅₀ ± Standard Deviation (µg/ml)	r ² ; 95% confidence interval (µg/ml)
A. <i>absinthium</i> aqueous extract	244.90± 1.03	r ² =0.98; 230.20 to 260.60	#37.82± 1.36	r ² =0.97; 18.31 to 78.14	285.30± 1.03	r ² =0.97; 265.80 to 306.20
A. <i>absinthium</i> methanol extract	#14.31± 1.05	r ² =0.97; 12.74 to 16.08	33.16± 1.02	r ² =0.99; 31.50 to 34.92	*20.96± 1.07	r ² =0.96; 18.10 to 24.28
A. <i>absinthium</i> acetone extract	*#6.11± 1.10	r ² =0.95; 5.00 to 7.45	*12.29± 1.05	r ² =0.97; 10.96 to 13.78	26.86± 1.07	r ² =0.96; 22.99 to 31.38

*: IC₅₀ for the extract of a specific plant exhibiting the strongest inhibitory activity against a specific cell line.

#: IC₅₀ for the cell line most susceptible to each individual extract of the three test plants.

Figure 5.48 is a graphical representation of the IC₅₀ values and their respective SD values as indicated in Table 5.9.

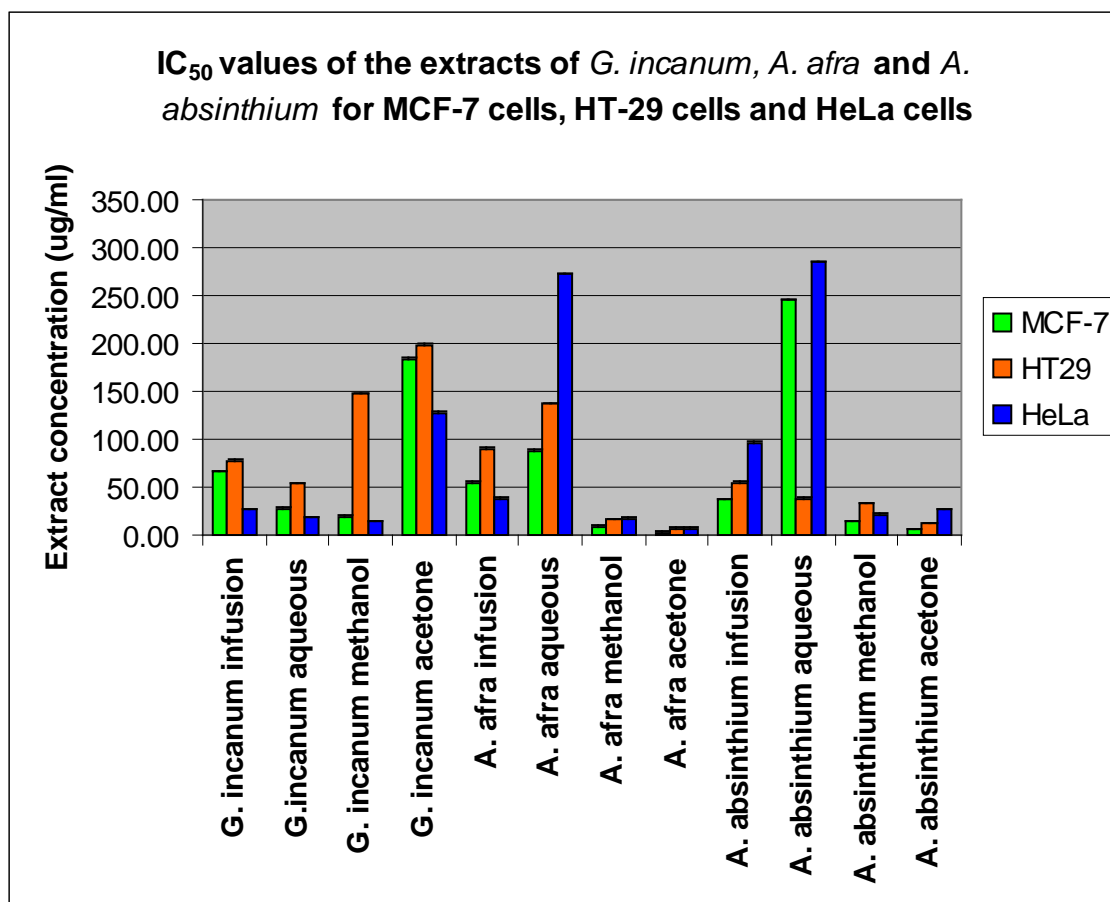


Figure 5.48: Graphical representation of the differences between IC₅₀ values of the different plant extracts for MCF-7 cells, HT-29 cells and HeLa cells.

Of the four *G. incanum* extracts used, the methanol extract appeared to have had the highest degree of toxicity in MCF-7 cells (Figure 5.48, *G. incanum* methanol green plot), with the lowest IC₅₀ at 19.16 ± 1.10 µg/ml (Table 5.9). The aqueous extract showed the second highest degree of toxicity towards MCF-7 cells (Figure 5.48, *G. incanum* aqueous green plot), with an IC₅₀ of 27.11 ± 1.11 µg/ml (Table 5.9), followed by the infusion (Figure 5.48, *G. incanum* infusion green plot). It would seem that the acetone extract showed the lowest degree of toxicity towards MCF-7 cells (Figure 5.48, *G. incanum* acetone green plot), with an IC₅₀ of 183.30 ± 1.08 µg/ml, which is considerably higher than that obtained for the previous three extracts (Table 5.9).

The degree and order of toxicity of the four *G. incanum* extracts changed considerably in the HT-29 cells, compared to MCF-7 cells (Figure 5.48). The aqueous extract showed the highest degree of toxicity in HT-29 cells (Figure 5.48, *G. incanum* aqueous orange plot), with an IC_{50} of $53.38 \pm 1.06 \mu\text{g/ml}$ (Table 5.9), followed by the infusion (Figure 5.48, *G. incanum* infusion orange plot), with an IC_{50} of $77.97 \pm 1.06 \mu\text{g/ml}$ (Table 5.9). The methanol extract showed the third highest degree of toxicity (Figure 5.48, *G. incanum* methanol orange plot), with an IC_{50} of $147.70 \pm 1.08 \mu\text{g/ml}$ (Table 5.9), followed by the lowest degree of toxicity showed by the acetone extract (Figure 5.48, *G. incanum* acetone orange plot), as was the case with MCF-7 cells, at an IC_{50} of $198.50 \pm 1.11 \mu\text{g/ml}$ (Table 5.9).

The same toxicity pattern was observed in the case of HeLa cells as was seen with MCF-7 cells (Figure 5.48). The methanol extract of *G. incanum* showed the highest degree of toxicity towards HeLa cells (Figure 5.48, *G. incanum* methanol blue plot), with an IC_{50} of $13.71 \pm 1.06 \mu\text{g/ml}$ (Table 5.9), followed by the aqueous extract (Figure 5.48, *G. incanum* aqueous blue plot), with an IC_{50} of $17.87 \pm 1.07 \mu\text{g/ml}$ (Figure 5.48). The infusion showed the third highest degree of toxicity in HeLa cells (Figure 5.48, *G. incanum* infusion blue plot), followed by the acetone extract (Figure 5.48, *G. incanum* acetone blue plot) with the lowest IC_{50} (Table 5.9). The methanol extract of *G. incanum* had the highest degree of toxicity in two of the three cell lines, being MCF-7 cells and HeLa cells (Figure 5.48, *G. incanum* methanol green- and blue plots, respectively), with the lowest overall IC_{50} occurring for HeLa cells treated with the methanol extract (Table 5.9). The aqueous extract showed the second highest degree of toxicity in two of the three cell lines, once again MCF-7 cells and HeLa cells (Figure 5.48, *G. incanum* aqueous green- and blue plots, respectively). The infusion showed the third highest degree of toxicity in the case of MCF-7 cells and HeLa cells (Figure 5.48, *G. incanum* infusion green- and blue plots, respectively), while being the second most toxic extract tested against HT-29 cells (Figure 5.48, *G. incanum* infusion orange plot). The acetone extract of *G. incanum* appeared to show the lowest

degree of toxicity in all three cell lines, as compared to the other three extracts (Figure 5.48). Of the MCF-7 cells, HT-29 cells and HeLa cells screened, the latter showed the highest degree of susceptibility to toxicity induced by the *G. incanum* infusion, aqueous-, methanol- and acetone extracts (Figure 5.48).

Of the four *A. afra* extracts used, the acetone extract appeared to have had the highest degree of toxicity in MCF-7 cells, HT-29 cells and HeLa cells. In the case of MCF-7 cells (Figure 5.48, *A. afra* acetone green plot), the acetone extract showed an IC_{50} of $2.65 \pm 1.05 \mu\text{g/ml}$ (Table 5.9). In the case of HT-29 cells (Figure 5.48, *A. afra* acetone orange plot) the acetone extract had an IC_{50} of $6.98 \pm 1.06 \mu\text{g/ml}$ (Table 5.9), and an IC_{50} of $6.54 \pm 1.06 \mu\text{g/ml}$ (Table 5.9) was calculated in the case of HeLa cells (Figure 5.48, *A. afra* acetone blue plot). The methanol extract showed the second highest degree of toxicity towards all three cell lines, with an IC_{50} of $8.36 \pm 1.08 \mu\text{g/ml}$ for MCF-7 cells, $15.80 \pm 1.04 \mu\text{g/ml}$ for HT-29 cells and $16.68 \pm 1.06 \mu\text{g/ml}$ for HeLa cells (Table 5.9), followed by the infusion and aqueous extracts showing the third and fourth highest degrees of toxicity, in that order (Figure 5.48). In summary, the acetone extract produced the highest degree of toxicity in all three cell lines screened, followed by the methanol extract, infusion and aqueous extract, in that order. Of the MCF-7 cells, HT-29 cells and HeLa cells screened, HeLa cells showed the highest degree of susceptibility to toxicity induced by the *A. afra* infusion, while MCF-7 cells showed the highest degree of susceptibility to toxicity by the aqueous-, methanol- and acetone extracts.

Of the four *A. absinthium* extracts screened, the acetone extract appeared to have had the highest degree of toxicity in MCF-7 cells (Figure 5.48, *A. absinthium* acetone green plot), with the lowest IC_{50} at $6.11 \pm 1.10 \mu\text{g/ml}$ (Table 5.9). The methanol extract showed the second highest degree of toxicity in MCF-7 cells (Figure 5.48, *A. absinthium* methanol green plot), with an IC_{50} of $14.31 \pm 1.05 \mu\text{g/ml}$ (Table 5.9), followed by the infusion (Figure 5.48, *A. absinthium* infusion green plot) with an IC_{50} of $37.33 \pm 1.04 \mu\text{g/ml}$ (Table 5.9).

The aqueous extract showed the lowest degree of toxicity in MCF-7 cells (Figure 5.48, *A. absinthium* aqueous green plot), with an IC_{50} of $244.90 \pm 1.03 \mu\text{g/ml}$ (Table 5.9), which is considerably higher than that obtained for the previous three extracts.

As was the case with *G. incanum* extracts, the degree and order of toxicity shown by the four *A. absinthium* extracts varied in HT-29 cells, as compared to MCF-7 cells. The acetone- and methanol extracts once again showed the highest and second highest degrees of toxicity in HT-29 cells (Figure 5.48, *A. absinthium* methanol and acetone orange plots, respectively), with IC_{50} values of $12.29 \pm 1.05 \mu\text{g/ml}$ and $33.26 \pm 1.02 \mu\text{g/ml}$, respectively (Table 5.9). However, unlike as was the case with MCF-7 cells, the aqueous extract of *A. absinthium* showed the third highest degree of toxicity in HT-29 cells (Figure 5.48, *A. absinthium* aqueous orange plot), with an IC_{50} of $37.82 \pm 1.36 \mu\text{g/ml}$ (Table 5.9), followed by the infusion being the least toxic in HT-29 cells (Figure 5.48, *A. absinthium* infusion orange plot), with an IC_{50} of $54.65 \pm 1.05 \mu\text{g/ml}$ (Table 5.9).

Finally, in the case of HeLa cells, the *A. absinthium* methanol extract showed the highest degree of toxicity of the four *A. absinthium* extracts tested (Figure 5.48, *A. absinthium* methanol blue plot), with an IC_{50} of $20.96 \pm 1.07 \mu\text{g/ml}$ (Table 5.9), followed by the acetone extract showing the second highest degree of toxicity (Figure 5.48, *A. absinthium* acetone blue plot), with an IC_{50} of $26.86 \pm 1.07 \mu\text{g/ml}$ (Table 5.9). The infusion and aqueous extract respectively showed the third and fourth highest degrees of toxicity in HeLa cells (Figure 5.48, *A. absinthium* infusion and aqueous blue plots, respectively). In summary, the acetone extract of *A. absinthium* showed the highest degree of toxicity in two of the three cell lines, being MCF-7 cells and HT-29 cells (Table 5.9). The methanol extract showed the second highest degree of toxicity in two of the three cell lines, once again MCF-7 cells and HT-29 cells (Table 5.9), while showing the highest degree of toxicity in one cell line, being HeLa cells (Table 5.9). MCF-7 cells were the most susceptible to three of the four *A. absinthium* extracts screened, being the

infusion, methanol- and acetone- extracts, while HT-29 cells appeared to be the most susceptible to toxicity induced by the aqueous extract (Table 5.9).

5.3.3. Determination of the mode of cell death induced by *A. afra* acetone extract in MCF-7 cells by means of fluorescence microscopy

As previously mentioned, the induction of apoptosis in malignant cells is the mechanism of action of many anticancer agents. Thus it would be desirable for a plant extract showing toxicity in malignant cells to induce apoptosis in these cells rather than necrosis.

For the purpose of this study, it was decided to investigate the mechanism of cell death, i.e. apoptosis versus necrosis, of that plant extract which showed the highest degree of toxicity towards a specific cell line, in this case the toxicity of the *A. afra* acetone extract in MCF-7 cells, by exposing MCF-7 cells for 48 hours to the IC₅₀ of the *A. afra* acetone extract for MCF-7 cells (2.65 ± 1.05 µg/ml), as discussed in Section 4.3.5.1. MCF-7 cells treated with cisplatin at a concentration of 10 µM for 48 hours, and MCF-7 cells grown in RPMI-1640 media lacking extract or cisplatin, served as positive- and negative controls, respectively, while MCF-7 permeabilised with 1:1 methanol:acetone dilution was used to establish method validity and accuracy. Two fluorescent dyes, DAPI and PI, were used separately to stain MCF-7 cells treated with the *A. afra* acetone extract, as well as the MCF-7 cells of the positive control, negative control and method validation control, as described in Section 4.3.5.1. Fixed and permeabilised cells were finally observed under a fluorescent microscope for staining patterns morphological characteristics consistent with apoptosis or necrosis, as mentioned in Section 2.6.2.

Figure 5.49 shows the morphological changes observed in MCF-7 cells following 48 hour treatment with cisplatin and the *A. afra* acetone extract, respectively,

followed by staining with DAPI. As mentioned in Section 2.6.2., chromatin condensation (Figure 5.49; Aa, arrow 3) and nuclear fragmentation (Figure 5.49; Ab, arrow 4) leading to the formation of apoptotic bodies, are hallmarks of cells undergoing apoptosis. Variation in the intensity of fluorescence between apoptotic cells and non-apoptotic cells due to chromatin condensation in the former, has also been observed (Figure 5.49; A[a-c] and B[a-c], arrow 2) for cells treated with cisplatin as well as the *A. afra* acetone extract. Non-apoptotic cells are indicated in Figure 5.49 (A[a-c] and B[a-c], arrow 1). Untreated MCF-7 cells were also stained with DAPI and observed during fluorescence microscopy for any morphological changes indicative of apoptosis, however, neither apoptotic cells nor nuclear fragmentation was observed, as was expected. Similarly, MCF-7 cells were also stained with PI in order to rule out the possibility of cell death through induction of necrosis. Staining with PI was not observed for cells treated with cisplatin or *A. afra* acetone extract, indicating that neither of the two induced significant necrosis during the treatment period. Furthermore, necrotic cells were not observed amongst the MCF-7 cells cultured in media lacking any cisplatin or extract. Permeabilised cells stained with DAPI as well as PI, however, indicating that the method was accurate.

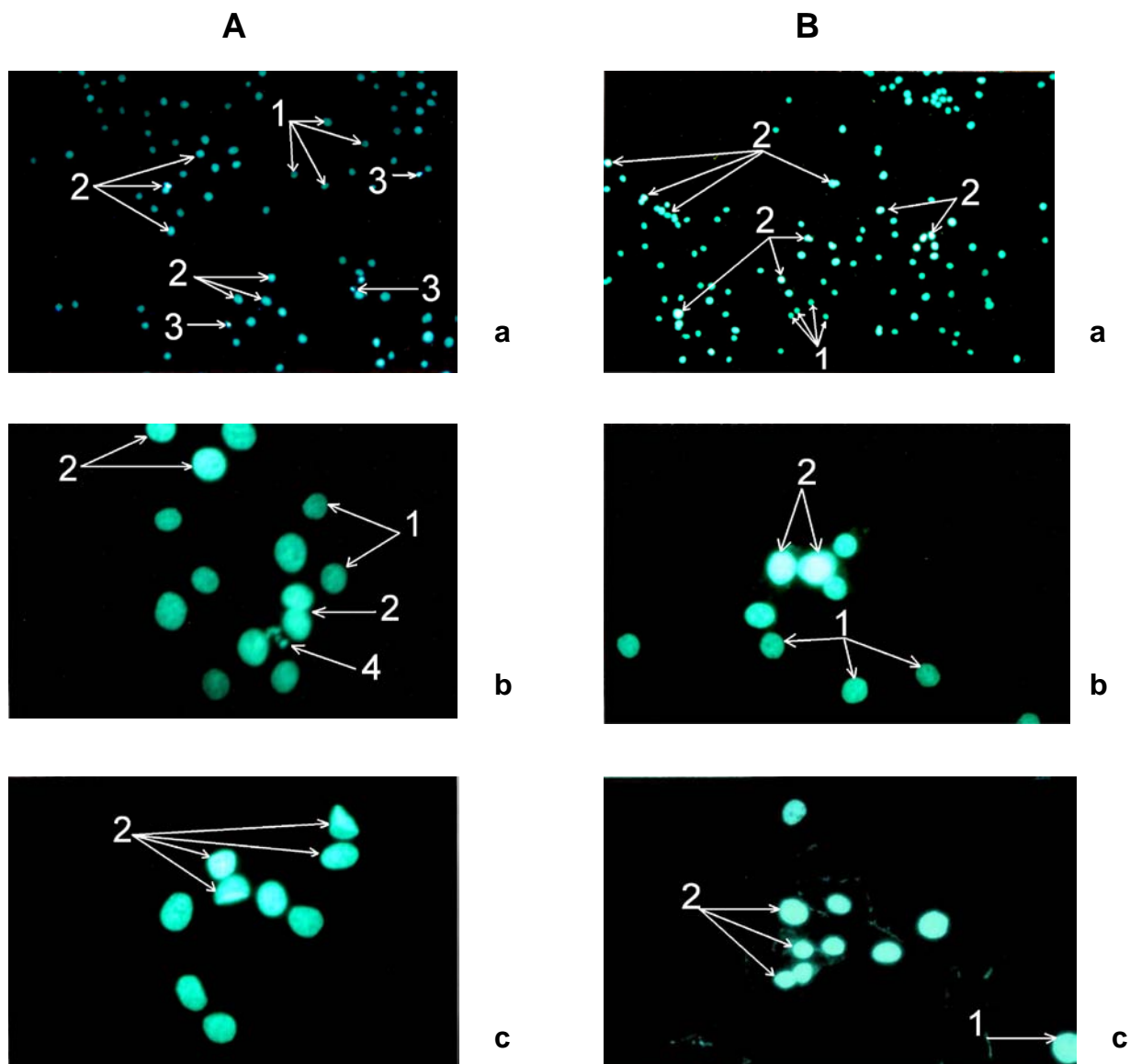


Figure 5.49: Morphological changes in MCF-7 cells after 48 hours exposure to cisplatin **[A(a-c)]** and *A. afra* acetone extract **[B(a-c)]**, followed by DAPI staining. 1→: normal cells; 2→: apoptotic cells; 3→: chromatin condensation; 4→: nuclear fragmentation.

Results obtained from the MTT assay (Section 5.3.2.) performed as described in Section 4.3.4. showed the *A. afra* acetone extract to be toxic in MCF-7 cells. The reasonable correlation in the DAPI staining pattern of MCF-7 cells treated with cisplatin, which is known to induce apoptosis in cancer cells, and the *A. afra*

acetone extract (Figure 5.49), respectively, suggests the *A. afra* acetone extract to induce apoptosis in MCF-7 cells, rather than necrosis. The observation of certain cellular morphological characteristics in MCF-7 cells treated with the *A. afra* acetone extract, for example variation in the intensity of fluorescence between apoptotic cells and non-apoptotic cells due to chromatin condensation in the former (Figure 5.49; A[a-c] and B[a-c], arrow 2), and the absence of PI staining of MCF-7 cells treated with the plant extract, further supports this notion.

5.3.4. Determination of the degree of toxicity of the *A. afra* acetone extract in PBMC's, compared to MCF-7 cells, utilising the CellTiter-Blue® assay

A number of anticancer drugs currently employed in the clinical setting have been derived from botanical sources, and examples of these have been given in Section 2.6.4. Nonetheless, an anticancer drug that is safe, economic to develop and site-specific is still elusive (Srivastava *et al.*, 2005). Thus far it has been shown that the *A. afra* acetone extract inhibits the growth of *C. albicans*, *S. aureus* and *B. cereus*, as well as showing toxicity in HT-29 cells, HeLa cells and MCF-7 cells, and inducing apoptosis in the latter. However, as stated earlier, a plant extract or compound isolated from such an extract showing anticancer activity is not only required to induce apoptosis in malignant cells as opposed to necrosis, it also needs to do so selectively in malignant cells without affecting normal healthy cells.

In order to determine the degree of toxicity of the *A. afra* acetone extract in normal human cells, PBMC's were isolated from a healthy adult male and treated at various concentrations of the extract for 48 hours, as described in Section 4.3.6. The CellTiter-Blue® assay, a flourometric method for the estimation of the amount of viable cells present in a sample after exposure to a test compound, was used to determine the percentage cell viability and percentage cell death of

PBMC's following exposure to the *A. afra* acetone extract. Screening of the extract, which totaled eleven dilutions and ranged in concentration between 500 µg/ml and 2.65 µg/ml, was performed in triplicate, while three different controls were simultaneously run in duplicate, the first control having consisted of PBMC's in media only, the second having consisted of media without extract or cells and acting as a background fluorescence control, and the third control having consisted of the eleven extract dilutions in media lacking PBMC's, which acted as a test compound control to rule out any fluorescence of the extract itself which might have interfered with the assay.

It should be mentioned here that during analysis of the raw fluorometric data obtained from the assay, it was noted that fluorescence values for the test compound control, which consisted of medium and serial dilutions of the extract and lacking PBMC's, were slightly increased at an extract concentration of 500 µg/ml and gradually decreased with a decrease in extract concentration, to the final concentration of 2.65 µg/ml, as compared to the values obtained for the background fluorescence control, which remained consistent. This was indicative of either the extract itself possessing fluorescent properties, or the extract interfering with the chemistry of the assay reagent.

As the aim of the assay was to measure the amount of fluorescence emitted after exposure of the PBMC's to the extract, with the amount of fluorescence being directly proportional to the amount of living PBMC's present, any fluorescence due to the extract or any sources other than the reduction of resazurin to resorufin by PBMC's would produce elevated fluorometric readings. Thus, in order to exclude fluorescence of the extract itself, the mean fluorescence values of each of the duplicate sets of the eleven extract dilutions in the test compound control were subtracted from each of the three corresponding extract dilution fluorescence readings obtained from the three triplicate sets of PBMC's treated with the respective extract dilutions. The percentage of PBMC viability, as well as the percentage cell death was determined by expressing the final

fluorescence values of the extract dilutions containing PBMC's as a mean percentage \pm standard deviation (SD) of the mean fluorescence readings recorded for the untreated cell control representing 100% cell viability.

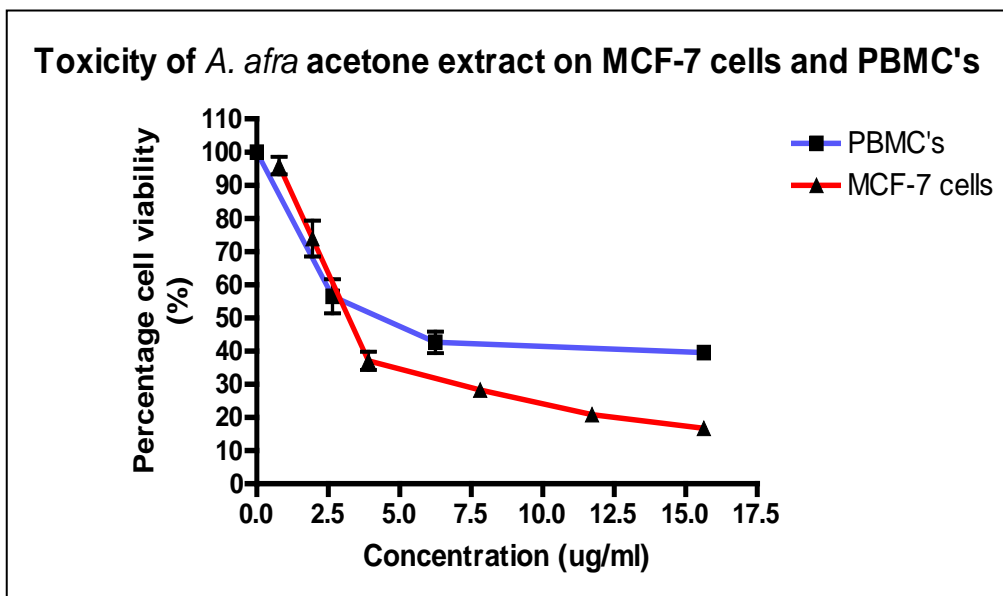


Figure 5.50: Comparison of the toxicity of *A. afra* acetone extract in MCF-7 cells and PBMC's.

Figure 5.50 allows for the comparison of the toxicity shown by the *A. afra* acetone extract towards MCF-7 cells and PBMC's, respectively. Both PBMC's (Figure 5.50, blue plot) and MCF-7 cells (Figure 5.50, red plot) showed a similar degree of sensitivity for the extract up to an extract concentration of approximately 2.65 $\mu\text{g/ml}$. PBMC's, however, appeared to be less sensitive to extract concentrations ranging between 2.65 $\mu\text{g/ml}$ and 15 $\mu\text{g/ml}$, compared to MCF-7 cells, which were more sensitive and showed lower levels of cell viability in this extract concentration range. Regression analysis showed an IC_{50} of 4.54 ± 1.07 $\mu\text{g/ml}$ with a 95% confidence interval between 3.96 $\mu\text{g/ml}$ and 5.21 $\mu\text{g/ml}$ ($r^2 = 0.98$) for PBMC's treated with the *A. afra* acetone extract, compared to MCF-7 cells, which had an IC_{50} of 2.65 ± 1.05 $\mu\text{g/ml}$ with a 95% confidence interval between 2.39 $\mu\text{g/ml}$ and 2.94 $\mu\text{g/ml}$ ($r^2 = 0.97$) (Table 5.9). Thus, although MCF-7 were more sensitive to the extract at higher concentrations as compared to

PBMC's, it was only marginally so, indicating the extract to be toxic in both malignant cells, in this case MCF-7 cells, and normal human cells, in this case PBMC's.

5.3.5. Flow cytometry analysis of the effects of *A. afra* acetone extract on the cell cycle of MCF-7 cells

The effect of the *A. afra* acetone extract on the cell cycle of MCF-7 cells was investigated in order to determine during which phase of the cell cycle the extract exerts its effects. MCF-7 cells separately treated with 2.65 µg/ml of the extract, 10 µM of cisplatin (positive control), as well as untreated cells (negative control), were lysed and stained with PI as described in Section 4.3.7.

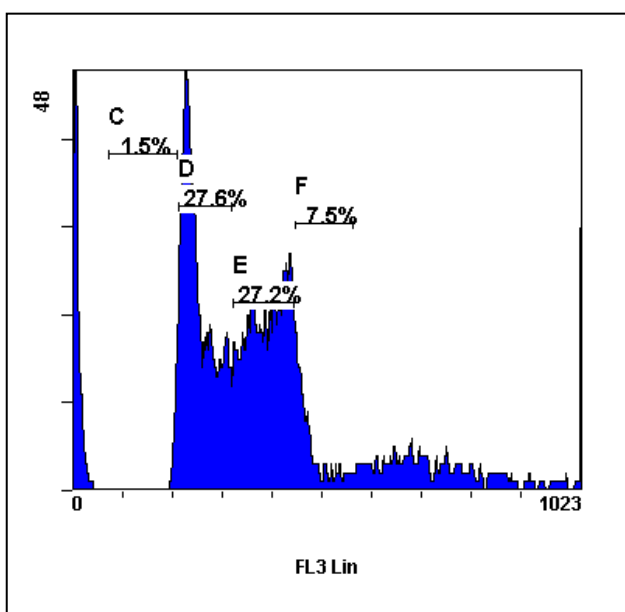


Figure 5.51: Percentages untreated MCF-7 cells (negative control) present during the different stages of the cell cycle. C: apoptotic cells; D: G₁/G₀ phase; E: S phase; F: G₂/M phase.

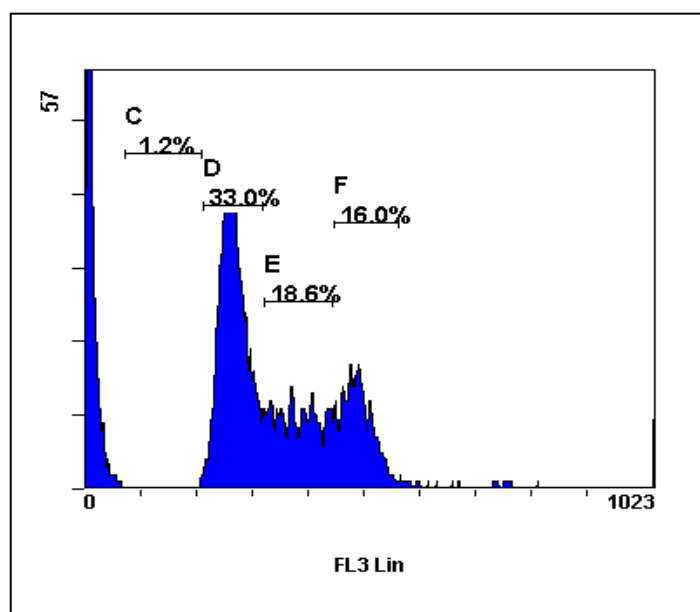


Figure 5.52: Percentages MCF-7 cells exposed to cisplatin (positive control) present during the different stages of the cell cycle. C: apoptotic cells; D: G₁/G₀ phase; E: S phase; F: G₂/M phase.

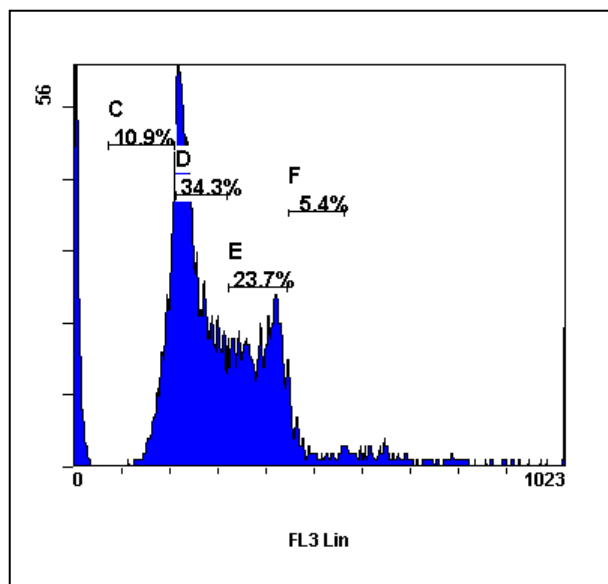


Figure 5.53: Percentages MCF-7 cells exposed to the *A. afra* acetone extract present during the different stages of the cell cycle. C: apoptotic cells; D: G_1/G_0 phase; E: S phase; F: G_2/M phase.

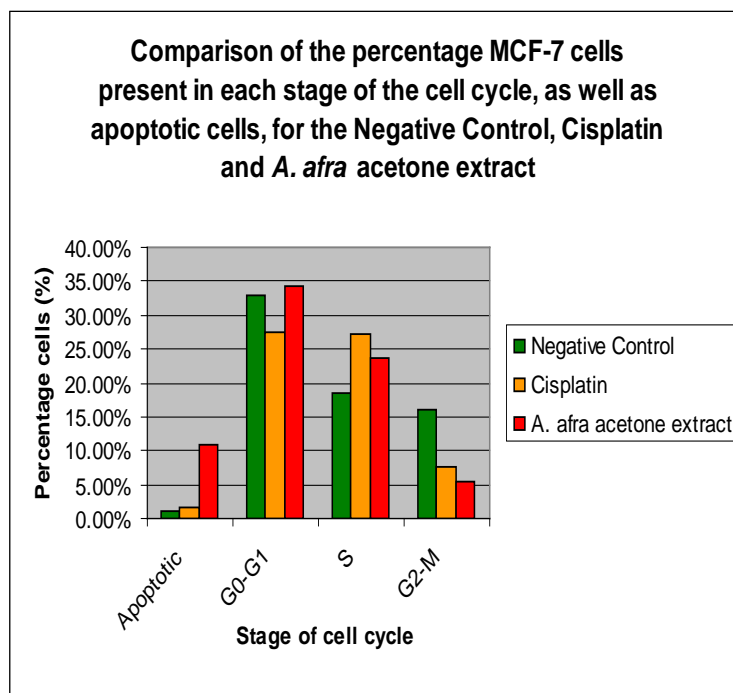


Figure 5.54: Variation in the percentage MCF-7 cells present in each phase of the cell cycle between untreated cells, cells exposed to cisplatin and cells exposed to the *A. afra* acetone extract.

Figures 5.51 to 5.53 indicate the percentages of MCF-7 cells present in the G_1/G_0 phase of the cell cycle (D), the S phase of the cycle (E) and the G_2/M phase (F) for untreated cells, cells exposed to cisplatin and cells exposed to the *A. afra* acetone extract, respectively. The percentages of apoptotic cells present in each of the cell samples are also indicated (C), with Figure 5.54 being a summary of Figures 5.51, 5.52 and 5.53. Considerably more apoptotic cells were present in the cell sample exposed to the extract (10.9%) (Figure 5.53) compared to that present in the sample exposed to cisplatin (1.5%) (Figure 5.52) and untreated cells (1.2%) (Figure 5.51). Of the 3 cell samples, cells exposed to the extract showed the highest percentage of cells present in the G_1/G_0 phase of the cycle (34.3%) (Figure 5.53), followed by untreated cells (33%) (Figure 5.51) and cells exposed to cisplatin (27.6%) (Figure 5.52). The cell sample exposed to cisplatin contained the highest percentage of cells present in the S phase (27.2%) (Figure 5.52), while cells exposed to the extract showed the second highest percentage

of cells in the same phase (23.7%) (Figure 5.53), followed by untreated cells (18.6%) (Figure 5.51). Finally, the untreated cell sample contained the highest percentage of cells in the G₂/M phase of the cell cycle (16%) (Figure 5.51), followed by cells exposed to cisplatin (7.5%) (Figure 5.52) and cells exposed to the extract (5.4%) (Figure 5.53). The fact that considerably more apoptotic cells were present in the sample exposed to the extract as compared to that present in the sample exposed to cisplatin, could signify *A. afra* acetone extract to be more active in MCF-7 cells in an anticancer capacity than cisplatin. The finding of the extract's toxicity in normal PBMC's (Section 5.3.4.), however, indicates that *A. afra* may be unsuitable for the treatment of breast cancer due to non-selective toxicity.

CHAPTER 6

DISCUSSION & CONCLUSION

Mankind has been turning to Nature and, more specifically, its botanical resources to fulfill its most basic needs, including food, shelter, clothing and medicines for thousands of years, and will most probably continue doing so indefinitely. As mentioned in the first chapter, the human race has recognised and has been using plants for their medicinal properties for at least the last 60 millennia.

An increasing interest in traditional medicines has been witnessed in recent years, largely due to the fact that conventional medicines are often ineffective and cause serious side effects. Furthermore, the inaccessibility to conventional drugs by a large proportion of the global population and the belief that natural products are safe, also contribute to the popularity of traditional medicine. However, the emergence of new diseases, the development of drug resistance and the existence of pathologies for which no effective treatment has been developed as yet, has now necessitated the exploration of natural resources, especially botanical in kind, for the discovery of novel chemotherapeutic agents more than ever before (Cragg, Newman & Snader, 1997, p.57-58).

With over 30 000 flowering plant species, which constitutes more or less one tenth of the global higher plant species (Stafford *et al.*, 2005), and of which 3000 to 4000 of these plant species are used for their medicinal properties by approximately 27 million South Africans (Fennell *et al.*, 2004; Mulholland & Drewes, 2004), South Africa's rich botanical biodiversity and resources possesses the potential to partially fulfill the need for novel chemotherapeutic agents in the ongoing fight against ailments for which no safe and effective treatment currently exists.

The present study sought to fulfill two main aims, which included:

1. Scientifically determining the antimicrobial properties of the two medicinal plants *G. incanum* and *A. afra*, and comparing the antimicrobial properties of *A. afra* to those of its European counterpart, *A. absinthium*, and
2. Scientifically determining the anticancer properties of *G. incanum* and *A. afra*, and comparing the anticancer properties of *A. afra* to those of *A. absinthium*.

Using the correct extractant plays an important role in the retrieval and isolation of biologically active compounds from plant materials. The literature reports on various extractants used to extract biologically active compounds from plants. There appears to be relative variation in the amount of active compounds extracted when different extractants are used. Water is reported to be routinely used by traditional healers in the extraction process (Lin *et al.*, 1999; Shale *et al.*, 1999). Other solvents which have been used in various studies, with varying degrees of success mostly surpassing that of water, include ethanol, methanol, hexane, chloroform, petroleum ether, methylene dichloride, ethyl acetate and methanol-chloroform-water mixture (Eloff, 1998; Eloff, 1999; George *et al.*, 2001; Lin *et al.*, 1999; Martini & Eloff, 1998; Shale *et al.*, 1999). Shale *et al.* (1999) found methanol and water extracts to possess higher antimicrobial activity than hexane extracts, while Lin *et al.* (1999) found methanol extracts to show the most potent antimicrobial activity. In his study, *Which extractant should be used for the screening and isolation of antimicrobial components from plants*, Eloff (1998) reports that acetone was the best extractant, followed by methanol-chloroform-water mixture, methylene dichloride, methanol, ethanol and finally water. However, this does not necessarily indicate water to be an ineffective extractant for the preparation of traditional medicines. Shale *et al.* (1999) report that a dosage of traditional medicine for adults may be up to 4 cups a day. Considering this dosage, water may very well be an effective extractant to use in the preparation of a traditional remedy. A four cup dosage of e.g. methanol- or

hexane extracts would be more concentrated and potentially dangerous. In addition, methanol and hexane may extract higher concentrations of other potentially toxic compounds. Ultimately, the choice of solvent should depend on the purpose for which the extract is to be used. If all the chemical constituents of an extract are to be evaluated, solvents with increasing dielectric constants should be used. If the extract is to be used in antimicrobial screening, it is important that the solvent is not toxic to and does not inhibit the bioassay itself. However, if the aim is simply to isolate chemical compounds without any bioassays, toxicity of the solvent is irrelevant, as the solvent can be removed from the extract before isolation of compounds (George *et al.*, 2001).

Regarding the use of fresh vs. dried plant material being used in the preparation of extracts, Eloff (1998) stated that dried plant material is preferred by most scientists for the following reasons:

- Fewer problems occur with the large scale extraction of dried plant material.
- Differences in the water content of fresh plant material may affect the solubility or separation of compounds by liquid-liquid extraction.
- Secondary plant metabolites need to be stable, especially if employed in antimicrobial screening.
- Dried plant material is often used by traditional healers.

The above information having been considered, it was decided to use distilled water, methanol and acetone as extractants and dried plant material as opposed to fresh plant material in the preparation of extracts of *G. incanum*, *A. afra* and *A. absinthium*.

Screening of *G. incanum*, *A. afra* and *A. absinthium* for possible antimicrobial activity was performed in triplicate, and for each respective round of screening, a new set of extracts, including infusions, aqueous-, methanol- and acetone extracts of each plant was made during June 2007, September 2007 and

January 2008, respectively. Since the anticancer studies performed required significantly less crude extract than the antimicrobial activity screening, only one set of infusions and extracts of the respective test plants was prepared for use in anticancer studies, the plant materials having been harvested and extracts prepared during January 2008.

It was interesting to note variation in the percentage yield of crude extracts obtained amongst the three rounds of extract preparation for antimicrobial screening. Generally, the second round of extractions, which was performed during September 2007 (spring), appears to have produced the lowest yields in all but four of the twelve respective infusion and extract preparations, as compared to yields obtained during the first (June 2007, winter) and thirds rounds (January 2008, summer). On comparing the type of extractant used and yields obtained relative to the month of extract preparation, the use of boiling water as an extractant in the form of an infusion produced the highest yield in extract obtained for all three plants during January, 2008 (summer). A similar pattern was observed when cold water was used as extractant in the form of a decoction. The highest yields of aqueous extract for *G. incanum* and *A. absinthium* were obtained during January 2008 (summer), although the *A. afra* aqueous extract produced the highest yield during June, 2007 (winter).

A pattern also existed between the percentage crude extract obtained when methanol was used as the extractant and the month during which extraction was performed. In all three of the plants, methanol as extractant produced the highest percentage crude extract yield during June, 2007 (winter). The use of acetone as extractant produced similar results, with the highest percentage extract yield for *A. afra* and *A. absinthium* having been obtained during June 2007 (winter). However, the highest percentage extract yield obtained for *G. incanum* with acetone as extractant occurred during January, 2008 (summer). In general, it would appear that water, having been used in the form of either an infusion or decoction, produced the highest percentage extract yields during

summer (January, 2008), while both methanol and acetone produced the highest percentage extract yields during winter (June, 2007). One possible explanation for this might be the fact that plants do not consistently produce the same chemicals in the same quantities. According to Prance (as cited in Buwa and Van Staden, 2007), various factors including internal biochemical factors, plant part extracted and external environmental factors such as climate, location, season and growth conditions all influence the effectiveness of medicinal plants. This may, for example, explain why the aqueous extract of *A. afra* produced a higher percentage extract yield during June 2007 compared to other times of the year when cold water was used as extractant (Figure 5.21). Furthermore, it was also noted that when cold water was used as an extractant, for example, it produced the highest percentage extract yield for *A. afra* during June 2007, compared to *G. incanum* and *A. absinthium*, for which the highest respective yields were obtained during January 2008, with cold water as the extractant. The finding of a particular extract, for example cold water, extracting different amount of phytochemicals from different plant species during different times of the year emphasises the importance of using the most appropriate extractant for a specific plant at the most appropriate time of year in order to obtain a maximum percentage extract yield.

Previous studies have also shown variation in the amount of bioactive phytochemicals produced by plants depending on the age of the plant, the nature of the soil and processing of plant material (Buwa and Van Staden, 2007). Except for *G. incanum*, both *A. afra* and *A. absinthium* were harvested from the same location for each round of extract preparation for antimicrobial screening. It is not known, however, if plants were exposed to fertiliser or other chemicals during this time, which could also have possibly influenced the amount of bioactive compounds produced by the plant. The fact that harvesting and extraction occurred during three seasons for each round of extraction, could very well have influenced the quantity of phytochemicals produced by the plants.

Another possible explanation for the variation in the amount of plant extract yielded for each respective plant relative to the time at which it was extracted, could be the actual extractant used. The literature reports on various extractants used with relative variation in the amount of active compounds extracted, as discussed above. The possibility that one extractant could extract different quantities of phytochemicals produced by a plant depending on the season can thus not be overlooked.

In order to investigate the antimicrobial properties of *G. incanum*, *A. afra* and *A. absinthium*, various ATCC strains and clinical isolates of *C. albicans*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. aureus* and *B. cereus* were treated with the infusions, aqueous-, methanol- and acetone extracts of the three respective plants, using an agar dilution method as described in Section 4.2.3.2. Extracts were tested at a concentration of 100 mg/ml. It should be mentioned here that the solubility of the extracts in DMSO varied, and was not a factor of the type of extractant used. For example, the *G. incanum* infusion and aqueous extract were quite soluble, while the aqueous extracts of both *A. afra* and *A. absinthium* were considerably less so. The same occurred when methanol and acetone were used as extractants. It would appear that the solubility of the extract rather depended on the plant extracted and the method employed in taking the extracts to dryness, i.e. freeze drying vs. evaporation of the extract.

Growth inhibition by plant extracts was observed mainly in strains of *C. albicans*, *S. aureus* and *B. cereus*, while inhibition of *E. coli*, *P. aeruginosa* and *E. faecalis* was not observed. Strains of *S. aureus* and *B. cereus* were the most susceptible to inhibition, and had similar inhibition patterns. All of the *C. albicans* strains screened consistently showed sensitivity to growth inhibition by all the *G. incanum* extracts. A study conducted by Scott *et al.* (2004, p.198) found that an aqueous extract of *G. incanum* showed no growth inhibition of *C. albicans*, unlike the findings of this study. A possible explanation for this could once again be the variation in biologically active compounds produced by plants as a function of

internal and external factors. The use of different scientific methods in the determining of microbial susceptibility producing a variation in results should also not be overlooked. Scott *et al.* (2004, p.187) made use of a disc diffusion assay to screen for antimicrobial activity, while an agar dilution assay was used during the present study (Section 4.2.3.2.). A third possible explanation for this discrepancy could be the use of different strains of *C. albicans* by Scott *et al.* (2004, p.198) showing different susceptibility patterns compared to the strains used in this study. *C. albicans* ATCC 10231 was tested for sensitivity to *G. incanum* in the study by Scott *et al.* (2004, p.195), while *C. albicans* ATCC 66027 and nine other clinical isolates were used in the present study (Section 4.2.3.1.).

C. albicans was not sensitive to any of the other plant extracts tested, with the exception of the *A. afra* acetone extract, the acetone possibly having extracted active compounds from the plant to which *C. albicans* was sensitive, while water and methanol might not have been able to extract the same compounds. The *C. albicans* ATCC strain was also noted to be inhibited by the *A. afra* methanol extract. Gundidza (as cited in Herbal Africa, n.d.) found *C. albicans* to be sensitive to inhibition by the essential oils of *A. afra*. This seems to support the present findings as essential oil might very well have been present in the acetone- and methanol extracts of the plant. The absence of inhibition of any of the *C. albicans* strains by any of the *A. absinthium* extracts in the present study also seems to be partially supported by a study conducted by Uzun *et al.* (2004). This study found an absence of inhibition of *C. albicans* growth when treated with ethanol- and petroleum ether extracts of *A. absinthium*.

None of the *E. coli*, *P. aeruginosa* or *E. faecalis* strains showed sensitivity for any extract of the three respective plants. Scott *et al.* (2004, p.198) reported similar results regarding the sensitivity of *P. aeruginosa* to an aqueous extract of *G. incanum*, while Uzun *et al.* (2004) reported the absence of growth inhibition of *E. coli* and *P. aeruginosa* following exposure to ethanol- and petroleum ether extracts of *A. absinthium*. The failure of methanol- and acetone extracts of *A.*

absinthium to inhibit growth of *E. coli* and *Pseudomonas fluorescens* has been reported by Endoğrul (2002, p.271). However, Williamson (2003, p.459) found *E. coli* to be inhibited by a methanol extract of *A. absinthium*, while Wilson (2004, p.51) found various strains of *P. aeruginosa* and *Pseudomonas species* to be inhibited by decoctions, aqueous-, methanol- and acetone extracts of *A. afra*. Variation in biologically active compounds produced by plants as a function of internal and external factors, the use of different scientific methods in the determination of microbial susceptibility as well as the use of different strains of *E. coli* and *P. aeruginosa* in these respective studies, could once again be possible explanations for the variation in findings amongst different studies.

All ten strains of *S. aureus* were sensitive to growth inhibition by eight of the twelve extracts tested. The infusion, aqueous- and methanol extracts of *G. incanum* inhibited growth of all the *S. aureus* strains, which is in part supported by the findings of Scott *et al.* (2004, p.192) that included inhibition of *S. aureus* by an aqueous extract of *G. incanum*. Of the *A. afra* extracts screened, the infusion, methanol- and acetone extracts, with the exception of the aqueous extract, inhibited the growth of all the *S. aureus* strains. An aqueous extract of *A. afra* was also ineffective in the inhibition of *S. aureus* when screened by McGaw *et al.* (2000), while Rabe and Van Staden (1997, p.84) found a methanol extract of *A. afra* to effectively inhibit *S. aureus*. This, in part, supports the aforementioned findings. It was expected that both the infusion and aqueous extract would have the same inhibition patterns since water was used as extractant in both cases. However, it could be possible that, since boiling water was used to prepare the infusion as apposed to cold water used for the aqueous extract, the boiling water was able to lyse plant cells and release bioactive compounds more efficiently than cold water.

Of the *A. absinthium* extracts screened, only the methanol- and acetone extracts showed any inhibitory activity for *S. aureus*. A study conducted by Uzun *et al.* (2004) found absence of growth inhibition of *S. aureus* when treated with

ethanol- and petroleum ether extracts of *A. absinthium*. It is quite possible that methanol and acetone are able to extract compounds inhibitory to the growth of *S. aureus* which ethanol and petroleum ether can not. This does not, however, explain the lack of inhibition of *S. aureus* following exposure to methanol- and acetone extracts of *A. absinthium* as reported by Endoğrul (2002, p.271). Once again, the internal and external factors influencing the production of bioactive compounds by the plant must also be taken into account, as an extract might be able to extract active compounds from one plant of a species which is inhibitory to *S. aureus* growth, while the same compounds may not be present in another plant of the same species subjected to different intrinsic and extrinsic influences.

The four strains of *B. cereus* showed sensitivity to the same extracts as the strains of *S. aureus*. The infusion, aqueous- and methanol extracts of *G. incanum* inhibited growth of all four strains of *B. cereus*. Literature appears to be rather limited in research conducted on the antimicrobial properties of *G. incanum*, and no previous research could be found to support these results. The infusion, methanol- and acetone extracts of *A. afra* inhibited growth of all four *B. cereus* strains. McGaw *et al.* (2000) has reported the inhibition of *Bacillus subtilis* by an ethanol extract of *A. afra*, while Mangena and Muyima (1999, p.294) found the essential oil of *A. afra* to be only slightly inhibitory to the growth of *B. cereus*. Only the methanol- and acetone extracts of *A. absinthium* inhibited all four strains of *B. cereus*, thus it could be deduced that water is not an effective extractant of phytochemicals inhibiting the growth of *B. cereus*, and that acetone or methanol should rather be used instead. None of the *B. cereus* strains showed any sensitivity to the acetone extract of *G. incanum*, the aqueous extracts of *A. afra* and *A. absinthium* or the infusion of *A. absinthium*.

Evidence indicating the use of *G. incanum*, *A. afra* or *A. absinthium* in the treatment of specific microbial infections could not be found in the literature. In general, all three plants are employed in the treatment of diseases in which microbial contamination could be a complication, or in the treatment of diseases

which could have been caused by any number of microbes. A select number of the above findings might, however, support the use of the three plants in the treatment of certain ailments to an extent. It must be stated here that traditionally the plants are extracted using water in some form or the other, instead of acetone or methanol, which might be toxic. For this reason only the inhibition of an organism by the infusions and/or aqueous extracts of the plants can be interpreted as possibly supporting the traditional use of the plants for their medicinal properties. Inhibition of *C. albicans* by the infusion and aqueous extract of *G. incanum* supports the traditional use of the plant in the treatment of urinary tract infections in females, of which *C. albicans* is occasionally the causative agent (Jensen *et al.*, 1997, p.489). Inhibition of *B. cereus* by the infusion and aqueous extract of *G. incanum* might also support the traditional use of the plant in treating diarrhea/gastroenteritis, provided the condition is caused by a *B. cereus* infection, which might occasionally be the case (Jensen *et al.*, 1997, p.273). The traditional use of *A. afra* in the treatment of pimples and boils, of which *S. aureus* is often a causative agent, is supported by the inhibition of the organism by the infusion of the plant.

In general, amongst the micro-organisms tested, the Gram positive organisms, with the exception of *E. faecalis*, showed a higher degree of sensitivity to inhibition by the plant extracts compared to the Gram negative organisms. A possible explanation for this might be the presence of a lipopolysaccharide layer covering the peptidoglycan layer of the cell wall in Gram negative bacteria, which may render the cell considerably less permeable and consequently less susceptible to antimicrobial compounds as opposed to Gram positive organisms, which do not possess a lipopolysaccharide layer (Kumar *et al.*, 2006).

Although literature reporting on the traditional use of *G. incanum*, *A. afra* and *A. absinthium* being used in the treatment of cancer could not be found, various extracts of these three plants showed anticancer activity to one or more cancer cell line in variable degrees. General anticancer activity of the infusions,

aqueous-, methanol- and acetone extracts of *G. incanum*, *A. afra* and *A. absinthium* were screened for at concentrations ranging between 500 µg/ml and 10 µg/ml using the MTT assay, MCF-7 cells, HT-29 cells and HeLa cells (Section 4.3.4.). Cisplatin, a known anticancer agent which induces apoptosis in malignant cells, was used throughout as a positive control. Cisplatin ($\text{Cl}_2\text{H}_6\text{N}_2\text{Pt}$) acts by cross linking two nucleotide bases on a DNA molecule following the removal of two chloride ions. The damaged DNA attracts *HMG-1* and other DNA repair proteins which bind irreversibly to the distorted DNA molecule, making repair impossible and initiating apoptosis in the cell (Cisplatin, 2008). Cisplatin is clinically used in the treatment of a wide array of neoplasms, including carcinomas, lymphomas, sarcomas and germ cell tumours, and was selected as a positive control for this study based on this information.

Of the four *G. incanum* extracts tested against MCF-7 cells, the methanol extract showed the strongest inhibitory/cytotoxic activity with an IC_{50} of 19.16 ± 1.10 µg/ml, followed by the aqueous extract, infusion and acetone extract, in order of decreasing activity. HT-29 cells showed the highest degree of susceptibility to the aqueous extract of *G. incanum* with an IC_{50} of 53.38 ± 1.06 µg/ml, followed by the infusion, methanol- and acetone extracts, in order of decreasing activity. The same toxicity pattern was observed in the case of HeLa cells as was seen with MCF-7 cells, with HeLa cells having shown the highest degree of susceptibility to the methanol extract of *G. incanum* with an IC_{50} of 13.71 ± 1.06 µg/ml, followed by the aqueous extract, infusion and acetone extract, in order of decreasing activity. The finding that the aqueous extract showed the highest degree of toxicity in HT-29 cells as apposed to the methanol extract, as was the case with MCF-7 cells and HeLa cells, could be explained by the possibility that water in the form of an aqueous extract was able to extract a phytochemical or phytochemicals synergistically being more toxic towards HT-29 cells than to MCF-7 cells or HeLa cells. It should also be taken into account that the metabolism of HT-29 cells could differ from that of MCF-7 cells and HeLa cells, and that an aqueous extract of *G. incanum* could possibly contain compounds

which activate the apoptotic- or cell death pathway signals in HT-29 cells at sites different to and more susceptible to influence by phytochemical compounds present in the aqueous extract, as compared to MCF-7 cells and HT-29 cells, in which case the methanol extract could have contained compounds affecting different points in the apoptotic pathway more susceptible to influence as compared to compounds present in the aqueous extract. Cytotoxicity shown by an aqueous extract of *G. incanum* in HeLa cells has been reported previously (Treurnicht, as cited in Scott *et al.*, 2004, p.208), though it was not indicated whether this was compared to the cytotoxic effects of other extracts of the plant.

An interesting pattern was observed in the screening of the *A. afra* extracts for toxicity in all three cell lines. The acetone extract produced the highest degree of toxicity in all three cell lines screened, followed by the methanol extract, infusion and aqueous extract. In the case of MCF-7 cells, the acetone extract showed an IC_{50} of $2.65 \pm 1.05 \mu\text{g/ml}$. In the case of HT-29 cells, the acetone extract showed an IC_{50} of $6.98 \pm 1.06 \mu\text{g/ml}$, while an IC_{50} of $6.54 \pm 1.06 \mu\text{g/ml}$ was obtained for the acetone extract in HeLa cells. Marginal variation was observed between the IC_{50} values of the three cell lines after exposure to the methanol extract, however, this variation increased significantly for cell lines treated with the infusion and aqueous extract, respectively. This might be due to water not being able to extract the same compounds which showed such high levels of toxicity compared to when acetone and methanol are used as extractants. The consistent pattern of toxicity amongst the three cell lines with regard to the different extracts is strongly indicative of a common biochemical mechanism of action of a compound(s) present in the respective extracts, amongst each of the three cell lines. Thus the active compound(s) in e.g. the acetone extract of *A. afra* probably affect the same or similar site(s) in all three cell lines, indicating the possibility that the compound(s) could be used in the treatment of various malignancies as opposed to only one or two types. Inhibition of growth of HeLa cells by an aqueous extract of *A. afra* has been reported previously (SA Health Info, as cited in Mukinda & Syce, in press; Treurnicht, as cited in Scott *et al.*,

2004, p.210), and supports the inhibition of HeLa cells by the aqueous extract as found in this study.

Unlike the case of susceptibility of MCF-7 cells, HT-29 cells and HeLa cells for the extracts of *A. afra*, no pattern in the toxicity of *A. absinthium* extracts in the three cell lines could be distinguished. In the case of MCF-7 cells, the acetone extract of *A. absinthium* showed the highest degree of toxicity with an IC_{50} of 6.11 ± 1.10 $\mu\text{g/ml}$, followed by the methanol extract, infusion and aqueous extract, in order of decreasing activity. As was the case with *G. incanum* extracts, the degree and order of toxicity shown by the four *A. absinthium* extracts varied with regard to HT-29 cells, compared to MCF-7 cells. The acetone extract showed the highest degree of toxicity in HT-29 cells, with an IC_{50} of 12.29 ± 1.05 $\mu\text{g/ml}$, followed by the methanol extract, aqueous extract and infusion, in order of decreasing activity. In the case of HeLa cells, the methanol extract showed the highest degree of toxicity with an IC_{50} of 20.96 ± 1.07 $\mu\text{g/ml}$, followed by the acetone extract, infusion and aqueous extract, in order of decreasing activity.

It was noticed that the acetone- and methanol extracts, respectively, showed the highest and second highest degrees of toxicity in two of the three cell lines, with the exception of the methanol extract having showed the highest degree of toxicity, followed by the acetone extract, in HeLa cells. This might suggest that methanol and acetone either extract the active compound(s) in larger quantities or more effective combinations as opposed to water, or that they are able to extract compounds which are considerably more toxic to malignant cells as opposed to the compounds extracted by water.

It was interesting to note that a pattern of susceptibility existed in the cell lines most susceptible to each of the four *G. incanum* extracts. Of the MCF-7 cells, HT-29 cells and HeLa cells, the latter was consistently the most sensitive to toxicity of each of the *G. incanum* extracts, followed by the MCF-7 cells and HT-29 cells, in order of decreasing sensitivity. The results possibly indicate that, if

used to treat malignancy, *G. incanum* would probably give more favorable results in the treatment of cervical cancer as opposed to breast cancer or colon cancer.

Concerning the cell line most susceptible to any one of the four *A. afra* extracts screened, it was found that HeLa cells showed the highest degree of susceptibility to the *A. afra* infusion compared to MCF-7 cells and HT-29 cells, while MCF-7 cells showed the highest degree of susceptibility to the aqueous-, methanol- and acetone extracts. Of the three cell lines, HT-29 cells were the least sensitive to any of the four *A. afra* extracts, as was the case with all four of the *G. incanum* extracts. With regard to cisplatin, however, it was noted that not HT-29 cells but rather MCF-7 cells appeared to be the least sensitive cisplatin, while HT-29 cells and HeLa cells showed a similar sensitivity pattern (Figure 5.47).

With regard to the four *A. absinthium* extracts screened, MCF-7 cells were the most susceptible to three of the four extracts, namely the infusion, methanol- and acetone extracts, while HT-29 cells appeared to be the most susceptible to the aqueous extract. The fact that, apart from the aqueous extract of *A. absinthium*, HT-29 cells were consistently the least sensitive to all the other extracts screened, could indicate that the aqueous extract of *A. absinthium* probability contained compounds more toxic to HT-29 cells than to MCF-7 cells and HeLa cells, compared to the other extracts.

Another interesting observation was that certain extracts appeared to stimulate rather than inhibit the growth of the malignant cells against which they were screened, at lower concentrations of the respective extracts. Based on the MTT assay, the *G. incanum* acetone extract stimulated the growth of MCF-7 cells at concentrations of 25 µg/ml and less (Figure 5.22), while the aqueous extract stimulated the growth of HT-29 cells at the same extract concentrations (Figure 5.23). The *A. afra* infusion and methanol extract both promoted the growth of HT-29 cells at the respective concentrations of 25 µg/ml and less (Figure 5.27)

and 6.25 µg/ml and less (Figure 5.28), while the *A. afra* infusion, aqueous extract and methanol extract all promoted the growth of HeLa cells at the respective concentrations of 25 µg/ml and less (Figure 5.29), 125 µg/ml and less (Figure 5.29) and 3.125 µg/ml and less (Figure 5.30). The *A. absinthium* infusion stimulated the growth of HT-29 cells at a concentration of 31.25 µg/ml and less (Figure 5.33), while the infusion, aqueous extract and acetone extract all promoted the growth of HeLa cells at the respective concentrations equal to or less than 31.25 µg/ml, 125 µg/ml and 6.25 µg/ml (Figure 5.34). The implication of these results is that, although none of the above extracts are reported for traditional use in the treatment of malignancy, growth of breast cancer, colon cancer and cervical cancer could be promoted rather than inhibited should the above extracts be used, at the low concentrations, stimulating malignant growth. As previously mentioned, traditional healers aim to restore balance and a general sense of well being of a patient on both a mental and physiological level. For this reason a combination of plants are usually prescribed to patients by traditional healers. This is because it is believed that the synergistic or additive effects of the correct combination of plants will be more successful in treating a patient than would be a single drug (Gurib-Fakim, 2006). The possibility therefore exists that a person taking a traditional preparation containing one or more of the above extracts at a concentration which stimulates malignant cell growth could unknowingly contribute to growth of a known or underlying malignancy. The above results are those of *in vitro* testing, however, and *in vivo* testing needs to be conducted to determine the promotion of malignant cell growth by these extracts at their respective concentrations.

For the purpose of this study, it was decided to investigate the mechanism of toxicity of the plant extract showing the highest degree of toxicity, namely that of the *A. afra* acetone extract in MCF-7 cells (IC₅₀ of 2.65 µg/ml). MCF-7 cells were separately treated with cisplatin at a concentration of 10 µM which served as a positive control, as well as with the *A. afra* acetone extract at a concentration of 2.65 µg/ml. The negative control consisted of untreated MCF-7 cells. Treated

and untreated cells were stained with DAPI and PI as discussed in Section 4.3.5.1. and observed during fluorescence microscopy for the presence of apoptotic and necrotic cells.

As previously mentioned, chromatin condensation and nuclear fragmentation leading to the formation of apoptotic bodies are both hallmarks of cells undergoing apoptosis. Both these hallmarks were observed for cells treated with cisplatin. Cells exposed to cisplatin did not stain with PI, indicating that necrotic cells were not present. Untreated MCF-7 cells did not stain with either DAPI or PI, respectively, indicating that neither apoptosis nor necrosis occurred amongst these cells, which was expected.

MCF-7 cells exposed to the *A. afra* acetone extract and stained with DAPI showed signs of chromatin condensation and fluoresced considerably brighter (Figure 5.49; B[a-c], arrow 2) than non-apoptotic cells which were also present (Figure 5.49; B[a-c], arrow 1). Staining with PI was not observed for cells treated with the *A. afra* acetone extract, indicating that the decrease in cell viability as a result of exposure to the extract observed during the MTT assay was not due to necrosis being induced in the cells. It is possible, however, that the extract could be toxic to MCF-7 at higher concentrations and induce necrosis as apposed to apoptosis. Optimal dosage concentrations are still to be established.

The reasonable correlation in the DAPI staining pattern of MCF-7 cells, treated with cisplatin and the *A. afra* acetone extract, respectively, the observation of apoptotic cells fluorescing brighter than non apoptotic cells amongst the MCF-7 cells treated with the plant extract, and the absence of PI staining of MCF-7 cells treated with the plant extract, are all indicative of apoptosis being the mode of cell death induced in MCF-7 cells treated with the *A. afra* acetone extract, as apposed to necrosis.

The toxicity of the *A. afra* acetone extract in normal, differentiated human cells was investigated by exposing PBMC's to different concentrations of the extract, which varied between 500 µg/ml and 2.65 µg/ml, for 48 hours using the CellTiter-Blue® assay as described in Section 4.3.6.

Both PBMC's and MCF-7 cells showed a similar degree of sensitivity for the extract up to an extract concentration of approximately 2.65µg/ml. PBMC's appeared to be less sensitive to extract concentrations ranging between 2.65 µg/ml and 15 µg/ml as compared to MCF-7 cells. MCF-7 cells were more sensitive and expressed lower levels of cell viability in this extract concentration range, however. Regression analysis indicated an IC₅₀ of 4.54 ± 1.07 µg/ml with a 95% confidence interval between 3.96 µg/ml and 5.21 µg/ml ($r^2 = 0.98$) for PBMC's treated with the *A. afra* acetone extract, compared to MCF-7 cells, which had an IC₅₀ of 2.65 ± 1.05 µg/ml with a 95% confidence interval between 2.39 µg/ml and 2.94 µg/ml ($r^2 = 0.97$). Thus, although MCF-7 cells were more sensitive to the extract at higher concentrations as compared to PBMC's, it was only marginally so, indicating the extract to be toxic in both malignant cells, in this case MCF-7 cells, and normal human cells, in this case PBMC's. Mukinda (2005, p.122) conducted a study on the toxicity of an aqueous extract of *A. afra* administered via both the oral route and intraperitoneal route in rats and mice, and concluded that acute doses of *A. afra* aqueous extract are relatively non-toxic in mice, while chronic doses are safe when administered to rats.

In the final part of this study, the effect of the *A. afra* acetone extract on the cell cycle of MCF-7 cells was analysed and compared to that of cisplatin, as well as untreated cells. MCF-7 cells exposed to the extract showed more apoptosis (10.9%) compared to cells exposed to cisplatin (1.5%) and untreated cells (1.2%). Cells in the sample exposed to the extract also showed the highest percentage of cells in the G₁/G₀ phase of the cell cycle (34.3%), compared to cells exposed to cisplatin (27.6%) and untreated cells (33%). A small difference was seen in the percentage of cells present in the S phase for cells exposed to

cisplatin (27.2%), compared to cells exposed to the extract (23.7%), while untreated cells showed the lowest percentage of cells in the S phase (18.6%). Untreated cells showed the highest percentage cells in the G₂/M phase (16%), which was significantly more than for cells exposed to cisplatin (7.5%) and cells exposed to the extract (5.4%). The G₂/M phase of the cell cycle is characterised by cellular growth and proliferation through mitosis, This is inhibited by cisplatin and the *A. afra* acetone extract through the induction of apoptosis in MCF-7 cells (Section 5.3.3.). It was to be expected that the untreated cell sample would have relatively more cells undergoing mitosis compared to cells exposed to cisplatin and the extract, respectively. The correlation in the percentage cells in the S phase of the cell cycle of cells exposed to cisplatin (27.2%) and cells exposed to the extract (23.7%), could indicate that the extract exerts its inhibitory effect(s) during or just prior to the S phase of the cell cycle. This is further supported by the relatively small percentage of cells present in the G₂/M phase for cells, respectively, exposed to cisplatin and the extract, compared to untreated cells. The fact that considerably more apoptotic cells were present in the sample exposed to the extract compared to that present in the sample exposed to cisplatin, could signify *A. afra* acetone extract to be more active in MCF-7 cells in an anticancer capacity than cisplatin.

The aims of this study included investigating the antimicrobial properties of *G. incanum* and *A. afra*, and comparing the antimicrobial activity of the latter to that of *A. absinthium*, as well as investigating the anticancer properties of *G. incanum* and *A. afra*, and comparing the anticancer activity of the latter to that of *A. absinthium*. Results obtained from antimicrobial screening indicated that all three plants possess antimicrobial properties for certain microbes, with activity being largely dependent on the extract used. In general, Gram positive organisms were more susceptible to inhibition than Gram negative organisms, with the exception of *E. faecalis*. *A. afra* and *A. absinthium* showed relative similarity in their inhibition patterns of certain microbes, especially in the case of the methanol- and acetone extracts.

All of the extracts of *G. incanum*, *A. afra* and *A. absinthium* possessed anticancer activity in MCF-7 cells, HT-29 cells and HeLa cells to varying extents, which varied with the extract and cell line screened. *A. afra* and *A. absinthium* showed similar inhibitory patterns, with the methanol- and acetone extracts being the most potent inhibitors of each of the respective cell lines in general, compared to the infusions and aqueous extracts. The acetone extract of *A. afra* proved to be the most effective inhibitor with the lowest IC₅₀ having been expressed in MCF-7 cells. Fluorescence microscopy indicates that the acetone extract of *A. afra* induces apoptosis in MCF-7 cells as apposed to necrosis, and the results are comparable to those obtained from cells exposed to cisplatin.

The *A. afra* acetone extract proved to be toxic to human PBMC's, with PBMC's only marginally less susceptible to toxicity by the extract compared to MCF-7 cells at the same extract concentrations. Cell cycle analysis of MCF-7 cells exposed to the IC₅₀ of the *A. afra* acetone extract indicated that the extract induced apoptosis in considerably more cells compared to cells exposed to cisplatin. A correlation existed in the percentage MCF-7 cells in the S phase of the cell cycle of cells exposed to cisplatin and cells exposed to the extract. This, in conjunction with the relatively small percentage of cells present in the G₂/M phase of the cell cycle for cells exposed to cisplatin and the extract compared to untreated cells in which cellular growth and mitosis could continue unhampered during the G₂/M phase, suggests that the *A. afra* acetone extract probably exerts its inhibitory effect(s) during or just prior to the S phase of the cell cycle.

It is thus concluded that *G. incanum*, *A. afra* and *A. absinthium* do possess antimicrobial and anticancer properties for at least certain microbes and malignant cell lines, respectively, and that this activity is largely dependent on the extract used. It is further concluded that *A. afra* and *A. absinthium* are similar in their inhibitory patterns against, at least, the micro-organisms and malignant cells screened during this study, and that this is once again a factor of the extract

used. However, further studies are required in order to gain more clarity as to the specificity and biochemical mechanisms responsible for the antimicrobial and anticancer properties of these three plants. As mentioned previously, the phytochemical profile of a plant may vary from one specimen to another, and is largely dependent on internal- and external influences. A standardised protocol for plant collection and preparation needs to be established in order to rule out any discrepancies in the activity of the plants due to internal- and external factors and different analytical assays used. The variation in antimicrobial and anticancer activities between different extracts of a specific plant as was found in this study strongly suggests that different extractants extract bioactive compounds from plants in different ratios and combinations with possible antagonistic or synergistic effects, which could greatly affect its medicinal value. It is, therefore, of utmost importance that the extractant which produces the most effective combination of bioactive compounds for each of the three plants be identified, and that extraction procedures are standardised. It is also possible that different antimicrobial and anticancer screening methods could produce different results, which should be taken into account when the current results are compared to previous findings. The possible loss of volatile components present in the extracts during freeze drying and evaporation might have produced results different from those obtained in studies where other methods of extract drying were employed.

The bioactive compounds responsible for antimicrobial and anticancer activities of the three plants need to be identified and isolated, and it should be taken into account that it might possibly be the synergistic activity of two or more compounds responsible for the medicinal properties of the three plants. It should also be taken into account that results obtained from *in vitro* assays may not necessarily be reproducible *in vivo* due to the metabolic processes of the test subject. It is, therefore, suggested that the extracts which showed anticancer and antimicrobial activity during this study be tested *in vivo*, and that their toxicity patterns and mutation-inducing capacities be established.

With regards to the antimicrobial activities of the respective plant extracts, minimum inhibitory concentrations (MIC's) of the various extracts which proved to have antimicrobial activity for the respective susceptible microbes need to be determined, the optimal dose concentrations established and the antimicrobial activity of the extracts for other pathogenic micro-organisms should be investigated. With regard to the anticancer activity of the respective extracts, the optimal dosages of the extracts need to be determined, time controlled cell cycle analysis needs to be performed in order to establish the amount of time necessary of optimal inhibition of malignant cellular proliferation, the anticancer activities of the respective extracts in malignant cells other than those which were screened for in this study needs to be investigated, and the toxicity profiles of the extracts for human cells other than PBMC's should be determined.

To date, considerable progress has been made in the identification and isolation of bioactive phytochemicals which has proven to be of immeasurable value to mankind in its continuing fight against pathogens and disease. The emergence of multiple drug resistant micro-organisms, the ever growing and threatening menace of diseases such as HIV/AIDS and malaria, unsanitary conditions and poverty in many countries across the globe and the unhealthy sedentary lifestyles adopted by many individuals in especially the First World countries have now, however, made it more important than ever to develop alternative, cheap yet effective and safe medicinal compounds for human consumption. If done in a sustainable manner, mankind may be able to continue taking advantage of the wealth of medicinal compounds nature has to offer when our own devices fail.

“The reasonable man adapts himself to the world; the unreasonable one persists in trying to adapt the world to himself. Therefore, all progress depends on the unreasonable man” (Bernard Shaw).

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APPENDIX 1

Tables 1, 2 and 3 indicate the weights of plant materials used (fresh and dried), the weights of infusions and crude extracts after freeze drying and evaporation, as well as the percentage yields of extracts obtained for each of the three respective rounds of infusion and extract preparation for antimicrobial screening.

Table 1: Weights of plant material used, weights of infusions and crude extracts after freeze drying and evaporation, and the percentage yields of extracts obtained after the initial round of extract preparation for antimicrobial screening, June 2007

<u>Extract</u>	<i>Geranium incanum</i>			<i>Artemisia afra</i>				<i>Artemisia absinthium</i>			
	Weight of leaves used (gram)	Weight of extract (gram)	Yield % (w/w)	Weight of leaves used (gram)	Weight of extract (gram)	Yield % (w/w)		Weight of leaves used (gram)	Weight of extract (gram)	Yield % (w/w)	
Infusion (Fresh leaves used)	6.012	0.326	5.42	14.000	0.402	2.87		14.000	0.342	2.44	
Aqueous extract (Dry leaves used)	4.473	0.386	8.63	12.147	1.196	9.85		12.463	1.117	8.96	
Methanol extract (Dry leaves used)	4.473	0.404	9.03	12.147	1.444	11.89		12.463	1.243	9.97	
Acetone extract (Dry leaves used)	4.473	0.059	1.32	12.147	0.839	6.91		12.463	1.003	8.05	

Table 2: Weights of plant material used, weights of infusions and crude extracts after freeze drying and evaporation, and the percentage yields of extracts obtained after the second round of extract preparation for antimicrobial screening, September 2007.

<u>Extract</u>	<i>Geranium incanum</i>				<i>Artemisia afra</i>				<i>Artemisia absinthium</i>			
	Weight of leaves used (gram)	Weight of extract (gram)	Yield (w/w)	%	Weight of leaves used (gram)	Weight of extract (gram)	Yield (w/w)	%	Weight of leaves used (gram)	Weight of extract (gram)	Yield (w/w)	%
Infusion (Fresh leaves used)	6.012	0.101	1.68		14.000	0.522	3.73		14.000	0.639	4.56	
Aqueous extract (Dry leaves used)	4.473	0.251	5.61		12.147	0.619	5.10		12.463	0.944	7.57	
Methanol extract (Dry leaves used)	4.473	0.282	6.30		12.147	0.928	7.64		12.463	0.956	7.67	
Acetone extract (Dry leaves used)	4.473	0.040	0.89		12.147	0.666	5.48		12.463	0.743	5.96	

Table 3: Weights of plant material used, weights of infusions and crude extracts after freeze drying and evaporation, and the percentage yields of extracts obtained after the third round of extract preparation for antimicrobial screening, January 2008.

<u>Extract</u>	<i>Geranium incanum</i>			<i>Artemisia afra</i>			<i>Artemisia absinthium</i>		
	Weight of leaves used (gram)	Weight of extract (gram)	Yield % (w/w)	Weight of leaves used (gram)	Weight of extract (gram)	Yield % (w/w)	Weight of leaves used (gram)	Weight of extract (gram)	Yield % (w/w)
Infusion (Fresh leaves used)	6.012	0.434	7.22	14.000	1.414	10.10	14.000	0.878	6.27
Aqueous extract (Dry leaves used)	4.473	0.591	13.21	12.147	0.937	7.71	12.463	1.299	10.42
Methanol extract (Dry leaves used)	4.473	0.141	3.15	12.147	0.894	7.36	12.463	1.239	9.94
Acetone extract (Dry leaves used)	4.473	0.257	5.75	12.147	0.829	6.83	12.463	0.797	6.40

Table 4 indicates the weights of plant materials used (fresh and dried), the weights of infusions and crude extracts after freeze drying and evaporation, as well as the percentage yields of extracts obtained during the preparation of extracts used in anticancer studies.

Table 4: Weights of plant material used, weights of infusions and crude extracts after freeze drying and evaporation, and the percentage yields of extracts obtained after the extract preparation for anticancer studies, January 2008

<u>Extract</u>	<i>Geranium incanum</i>				<i>Artemisia afra</i>				<i>Artemisia absinthium</i>			
	Weight of leaves used (gram)	Weight of extract (gram)	Yield (w/w)	%	Weight of leaves used (gram)	Weight of extract (gram)	Yield (w/w)	%	Weight of leaves used (gram)	Weight of extract (gram)	Yield (w/w)	%
Infusion (Fresh leaves used)	2.036	0.171	8.40		5.092	0.193	3.79		5.025	0.119	2.37	
Aqueous extract (Dry leaves used)	1.000	0.150	15.00		1.000	0.115	11.50		1.000	0.419	41.90	
Methanol extract (Dry leaves used)	1.000	0.195	19.50		1.000	0.155	15.50		1.000	0.286	28.60	
Acetone extract (Dry leaves used)	7.010	0.333	4.75		1.500	0.120	8.00		1.000	0.116	11.60	