Selective cytotoxicity of *Crotalaria agatiflora subspecies agatiflora* Schweinf

by

Karlien le Roux

Submitted in partial fulfilment of the requirements for the degree of:

Master of Science (Medicinal Plant Science)

In the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria
South Africa

Supervisor: Prof. Namrita Lall

16 February 2011

Declaration

I declare that this dissertation submitted at the University of Pretoria for the degree: MSc. Medicinal Plant Science has not been submitted by me for a degree at any other University or institution of higher education.

K. le Roux

ABSTRACT

Crotalaria species have been widely used in Chinese traditional medicine to treat several types of internal cancers. *Crotalaria agatiflora* is used as a medicinal plant in several African countries for the treatment of bacterial and viral infections as well as cancer. Researchers have found this species to relieve spasms and reduce blood pressure. The aims of the study were to investigate the potential anti-cancer as well as cancer preventative activity of *C. agatiflora* subspp. *agatiflora* and ultimately to evaluate the possible mechanism of action.

Water and ethanolic extracts were prepared after which the cytotoxcity of the samples were determined on four cancerous and one noncancerous cell lines, using XTT (Sodium 3' –[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate) colorimetric assay. Antioxidant activity was determined using DPPH (1,1-Diphenyl-2-picryl hydrazyl). Light microscopy (eosin and hematozylin staining), flow cytometry (Annexin-V and propidium iodide) and micro-Raman spectroscopy were used to evaluate the mechanism of action of the ethanol leaves extract of *C. agatiflora* and one of the isolated compounds. Both water extracts were relatively non-toxic against all cell lines tested. The ethanol extract, was found to be the most active extract against the proliferation of human leukemic U-937 cells (Inhibitory concentration of 50% of the cell population, IC_{50} = 73.9 µg/mL) and human oesophageal carcinoma (SNO) cells (IC_{50} = 111.5µg/mL). The ethanol extract exhibited an IC_{50} value of 352.4 µg/mL against non-cancerous African green monkey kidney (Vero) cells. The best selectivity index (SI = 4.8) of the ethanol extract was seen against U-937 cells and thus further investigations were focused on these cancerous cells. It was evident that the ethanol extract showed the highest antioxidant activity, with an IC_{50} (Inhibitory concentration of 50% of free radicals) value of 18.89 µg/mL, while the water extracts had similar IC_{50} values between 27 and 29 µg/mL respectively. A bioassay-guided fractionation led to the isolation of two bioactive compounds, namely madurensine and doronenine, from the alkaloidal fraction of the ethanolic extract of the leaves. It was found that doronenine was the most active having an IC_{50} of 87.7 µg/mL, while madurensine had an IC_{50} of 136.5 µg/mL against U-937 cells. Both compounds were relatively non-toxic having IC_{50} values higher than 2mg/mL on Vero cells. The SI values were higher than 100 for madurensine and higher than 30 for doronenine. Although doronenine was more active than madurensine, it was not further investigated due to madurensine having higher SI value and the small quantity of the sample. The crude extract treated U-937 cells showed definite signs of cell death during light microscopic investigation, while little apoptosis (10-20%) and necrosis (<2%) were detected with flow cytometry in cells treated cells. Raman spectroscopy confirmed the decrease in cell size and thus the apparent decrease in the concentration of proteins and lipids within several treated single analyzed U-937 cells. The ethanolic extract was the most active sample tested both for cytotoxicity and antioxidant activity. The mechanism of action was hypothesized as autophagy, but should be confirmed with further analysis. *Crotalaria agatiflora* subspp. *agatiflora* could be further investigated as a chemo-preventative drug in the future.

TABLE OF CONTENTS

CHAPTER 3

CHAPTER 4

LIST OF FIGURES

LIST OF TABLES

Abbreviations:

^{13}C: Carbon

^{1}H: Hydrogen (Proton)

A: Adenine

AChE: Acetylcholinesterase

AIDS: Acquired Immune Deficiency Syndrome

ALT: Alternative Lengthening of Telomeres

ATCC: American Type Culture Collection

ATCLI: Acetylthiocholine iodide

ATP: Adenosine Triphosphate

Bp: Base pair

C: Cytosine

CANSA: Cancer Association of South Africa

CARISA: Cancer Research Initiative of South Africa

CC: Colum Chromatography

CERG: Cancer Epidemiology Research Group

CSIR: Council for Scientific and Industrial Research of South Africa

DCM: Dichloromethane

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acids

DPPH: 1,1-Diphenyl-2-picryl-hydrazyl

DTNB: 5,5-dithiobis-2-nitrobenzoic acid

EDTA: Ethylenediaminetetraacetic acid

EtOH: Ethanol

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration of the United States of America

G: Guanine

G_0-phase: Gap phase 0 where no mitosis is taking place within cell cycle

GABA: Gamma-aminobutyric acid

H_2O_2: Peroxide

HCl: Hydrochloric acid

HeLa: Human cervical adenocarcinoma (ATCC: CCL-2)

HIV: Human Immunodeficiency Virus

HOCl: Hypochlorous Acid

IC_{50}: Inhibitory concentration of 50% of tested population

IL-2: Interleukin-2

MCF-7: Human breast adenocarcinoma (ATCC: HTB-22)

MeOH: Methanol

M-phase: Mitosis phase

MRC: Medical Research Council of South Africa

NADH / NADPH: Nicotinamide adenine denucleotide / phosphate (reduced form)

NCI: National Cancer Institute

NF-κβ: Necrosis Factor

NMR: Nuclear Magnetic Resonance

NO: Nitroxide

NSAID: Non-steroidal Anti-Inflammatory Drugs

p53: Tumour suppressor protein gene

PROMEC: Programme on Mycotoxins and Experimental Carcinogenesis

PRU: Schweikerdt Herbarium, University of Pretoria, South Africa

PS: Phosphatidylserine

RNA: Ribonucleic Acids

ROS: Reactive Oxygen Species

RSA: Republic of South Africa

SERM: Selective Estrogen Receptor Modulator

SI: Selectivity Index

SNO: Human oesophageal epithelial cancer cells

SOD: Superoxide Dismutase

S-phase: Synthesis phase

T: Thymine

TB: Tuberculosis

TLC: Thin Layer chromatography

U: Uracil

U-937: Human histiocytic lymphoma cells representing leukemic-like cells (ATCC: CRL-1593.2)

UCT: University of Cape Town

USA: United States of America

UV: Ultra Violet

Vero: Normal healthy kidney cells harvested from adult African green monkey (ATCC: CCL-81)

WHO: World Health Organisation

XTT: Sodium 3' – [1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate

CHAPTER 1:

PHYTOTHERAPY AND CANCER

1. Introduction

This chapter focuses on traditional medicine practice and usage of plants for the treatment of cancer in South Africa. The epidemiology of cancer is briefly discussed. Synopsis of previous research with regard to medicinal plants for cancer treatment has been mentioned. The ethnobotanical and phytochemical information of the selected plant have been indicated.

1.1 Traditional medicine

Since the beginning of civilization mankind has relied on nature as an essential resource for survival. Plants have been and are still being utilized for building shelters, clothing, food, flavours and fragrances as well as medicine. The interest in natural products as a source of medicine is growing. Natural products and their chemically synthesized derivatives represent more than 50% of the clinically used drugs currently on the market. Higher plants contribute approximately 25% of the estimated naturally derived medicines (Gurib-Fakin, 2007).

During the industrial revolution, organic chemistry was developed which introduced a new market for synthetic medicine products. Pure compounds were easily manufactured and compounds could be manipulated to potentially more active and less toxic derivatives. A vast amount of naturally derived compounds cannot at present be synthesized economically because of the complex structures of those compounds. Thus many plants are still being harvested in the wild or from cultivations to obtain the bioactive compounds. There are several reasons for the growing interest in plants. These reasons include side effects of synthetic medicines, the abusiveness and the risk of addiction. Ineffectiveness of conventional medicines as well as the price of these medicines is under scrutiny (Rates, 2001).

Worldwide traditional medicines are still used especially by poorer communities, in particular Africa and India, where community medical centres are absent and culture involves taking medicines from traditional healers. Traditional medicines are usually in the form of crude drugs such as tinctures, teas, powders and other herbal formulations. The very specific plants, application and preparation for medicinal use are usually taught to the next generations verbally (Balunas and Kinghorn, 2005).

1.2 Traditional medicine in South Africa

South Africa flourishes in high biodiversity as well as cultural diversity. Approximately 30,000 species of higher plants are found in South Africa of which many of them are endemic. These species are coordinated in particular habitats which consist of variable climate areas and habitat types. South

Africa has 11 official languages of which nine are indigenous. Thus South Africa is rich in different cultures, which support a kaleidoscope of traditional customs (Light *et al.*, 2005).

Traditional medicine plays an important role in the primary healthcare of people living in rural areas. Although the government only supplies and grants western medicine, most people still insist on consulting traditional healers. There are several reasons why traditional medicine is still so popular. These reasons include the inaccessibility of western medicine in rural areas, the high costs of these medicines as well as the cultural importance of traditional medicine. Since South Africa has such diverse vegetation and cultures, plants are commonly used for medicines. It is estimated that 10%, that would say approximately 3,000 plant species are being used for medicinal purposes at present. Approximately 500 species are traded in large quantities at informal medicinal trading markets (Light *et al.,* 2005). Prescriptions and the use of traditional medicine are presently not regulated in South Africa. Without regulation the use of traditional medicine has high risks of being used incorrectly, which can be fatal when toxic plants are utilized (Fennell *et al.*, 2004). Thus traditional medicine should be investigated to ensure that those plants are in fact effective for the disease it is prescribed for. It is important to question the toxicity and the effectiveness of medicinally used plants. If the plants are effective and relatively nontoxic, the effective dose, lethal dose and half-life of the plant extract or active compounds should be determined scientifically.

Through scientific evaluation, plants can be used for the benefit of humans and animals under strict regulations. Of course potential business enterprises can be developed and thus uplift rural communities of Southern Africa.

1.3 Plants' ability to produce secondary compounds used in traditional medicine

Plants are able to produce primary and secondary plant metabolites. Primary metabolites are defined as compounds produced by plants for basic functioning of the plant. Secondary metabolites are defined as compounds which are produced to protect the plant. These metabolites can be broadly classified as forming part of the immune system of plants. It is then also secondary metabolites which are most commonly of interest for medicinal properties.

Briefly, primary compounds include glycerides, starches and pectins. Glycerides are storage products in seeds, nuts and fruits. Pectins are used to cement cells together and form part of the primary cell walls. Secondary compounds include waxes, pigments, tannins, resins, gums, latex, volatile oils, saponins, hormones and alkaloids. Waxes protect plants against extensive water loss and provide protection against pathogens and insects. Pigments attract pollinators and protect the plant from harmful ultra violet damage. Tannins are also involved in assuring that plants don't lose too much

water and form part of the defence system. Gums and latex are used for plant wound sealing and defence. Volatile oils and hormones are used to attract pollinators and for defence. Saponins and alkaloids are produced by the plants solely for the purpose of defence (Cotton, 1996).

Ethnobotanical use of glycerides includes media for lubricants, cosmetics, cooking and the metal salts for soaps. Pectins can be used as gelling agents in cosmetics and in the food industry. Commonly pigments are used for dyes, body paints, pigments and medicine. Tannins are used as sealants, illumination, adhesives and preservatives. Volatile oils are now commonly used as flavours and fragrances, food preservatives and medicine. Plant derived hormones are used as birth control agents and pest control agents. Saponins are important for production of fish poisons and soaps, while alkaloids are used for pest control and medicines (Cotton, 1996).

These compounds have various functions within plants, and for whatever reasons for the presence of these compounds within plants, they provide a valuable resource used to find new drug molecules (Gurib-Fakin, 2007).

2. Cancer

A brief summary has been given for carcinogenesis, hallmarks of cancer and chemoprevention. Conventional cancer treatments have been discussed with particular attention to chemotherapy. Lastly traditional medicine specifically used in Africa for the treatment of cancer has been examined.

2.1 Carcinogenesis

Cancer is classified as a disease involving active changes within the genome. The basis has been the discovery of mutations that produce oncogenes with dominant gain of function or tumour suppressor genes with recessive loss of function (Hanahan and Weinberg, 2000). Carcinogenesis is defined as a process during which healthy cells responsive to homeostatic feedback mechanisms are changed to cells capable of independent growth and invasion, thus leading to cancer. Carcinogenesis is a multiple step process, which can be subdivided into three stages (Figure 1.1). These stages are initiation (days), promotion (several years) and progression (1-5 years) (Bertram, 2001).

Initiation is said to be irreversible and caused by chemical or physical carcinogens. Damage is caused directly to deoxyribonucleic acids (DNA). Presently it is evident that random mutations in genes, controlling proliferation and apoptosis independently, are responsible for cancer. Most mutations causing cancer are not inherited, but arise from spontaneous events within cells. Changes within genomic material include; point mutations (amino acid substitutions), frame-shift mutations

(inappropriate expression of genes), or epigenetic modifications of DNA (gene silencing). In developing cancerous cells two types of mutations occur. These mutations either cause increased activity of proteins they code for (oncogenes) or mutations which inactivates tumour suppressor genes. The replication of DNA and successive cell division are required to convert chemical damage to inheritable change in DNA. A 'thriving' cancer cell is required to have mutations in at least five genes. The probability of cells having five mutations simultaneously is highly unlikely. The parent cell gets mutated in one gene and as the cell divides the progeny can get another mutation incorporated within the population of cells. Progressively the population of cells acquire more adaptations for better abnormal survival capabilities. Thus over generations of new cells, mutations can lead to independent cancerous cells. In many cases cancer is found in humans without noticeable exposure to environmental carcinogens. Many cancers occur in organs for which no environmental or genetic causes have been identified. Thus it can be predicted that spontaneous DNA damage can give rise to carcinogenic mutations (Bertram, 2001).

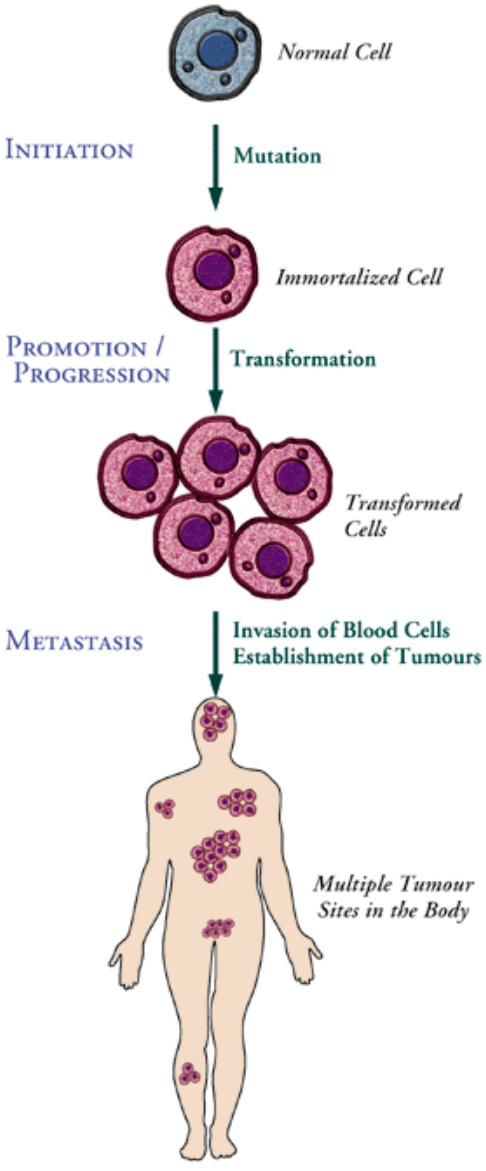


Figure 1.1: Diagram of carcinogenesis (Positive Health, 2008)

Spontaneous DNA mutations can occur as a direct consequence of errors during replication or indirectly as a consequence of chemical exposure, leading to errors while DNA polymerase is interpreting faulty DNA during replication. Fortunately approximately 97% of DNA is non-coding and thus the probability for one of these mutations occurring in oncogenes or tumour suppressor genes is small. Oncogenes and tumour suppressor genes are usually mutated in cancer cells. Mutations occur during replication because apurinic sites can result in random base insertions. Uridines (U) can base pair with adenine (A) leading to a guaninie – thymine (G-T) mutation, while deamination of methylcytosine will lead ultimately to a cytosine – thymine (C-T) transition which is frequent in human cancers. In addition to these spontaneous changes, DNA damage occurs as the result of chemical attack, in large part by products of oxidative metabolism and probably the most frequent potentially mutagenic event. Carcinogens can cause two different types of changes in the genome namely chemical or physical. These carcinogens are collectively called environmental carcinogens (Bertram, 2001).

The chemical reaction present in most environmental carcinogens can be characterized as electrophilic attack upon a tissue nucleophile. The most significant tissue nucleophiles to be damaged are guanines. The chemical changes induced interfere with base-pair recognition during replication. Examples of these carcinogens include: polycyclic hydrocarbons (coal tar), 2-naphthylamine (rubber and chemical industries), dimethylnitrosamine (chemical solvent) and N-nitrosamine (several consumer items including beer, tobacco smoke and cosmetics). Physical carcinogens include ionizing radiation, both particulate and photon, and ultraviolet radiation. Ionizing radiation causes direct damage to DNA. The effected DNA can break in one or both of the DNA strands. Ionizing radiation can also cause water to split (radiolysis) to yield free radicals. Ultraviolet irradiation is absorbed by DNA, which causes chemical reactions. These reactions can cause cyclobutane-linked thymine dimers, which are covalent cross linked neighbouring thymidines. Approximately 90% of skin cancers are caused by ultraviolet exposure (Bertram, 2001).

Promotion involves epigenetic mechanisms. This process is reversible, slow and causes an accumulation of pre-malignant cells which divides abnormally. Progression is irreversible. This last process leads to the final phase of carcinogenesis. During this process tumours grow and become more prone to being invasive and metastatic. During the passage between pre-malignant and malignant, tumour suppressor genes are inactivated and proto-oncogenes activated (Bertram, 2001).

2.2 Cancer cell characteristics

More than one hundred distinct types of cancers have been discovered and subtypes have been classified in different organs. Seven important changes must take place before healthy cells become cancerous. The seven characteristics of cancerous cells are: self-sufficiency in growth signals,

insensitivity of anti-growth signals, evading apoptosis, limitless replication potential, sustained angiogenesis, genomic instability and metastasis (Hanahan and Weinberg., 2000). For the present study only two of these hallmarks are relevant and have been discussed below.

2.2.1 Evading apoptosis

Data processing indicates that an apoptotic program is present, but masked in nearly all cell types. Apoptosis has numerous key characteristics: cellular membrane integrity is lost, the cytoplasmic and nuclear skeletons are broken down, cytosol leaks out of the cell, chromosomes are degraded and the nucleus fragmented. This whole progress completes in approximately 30-120 minutes. In the end the shrivelled cell corpse is engulfed by nearby cells and disappears, typically within 24h. Machinery involved in apoptosis is divided into two classes; sensors and effectors. Sensors monitor extracellular and intracellular environments. Resisting apoptosis can be gained by cancerous cells through different strategies. One of the most general strategies involves the mutation within the p53 tumour suppressor gene. Inactivation of p53 protein is observed in more than half of human cancers. When this protein is inactive a vital component of DNA damage sensory that induces apoptosis is lost. Other signals such as hypoxia and hyper-expression of oncogenes are also directed through p53 to the apoptotic machinery. These two signals are impaired at eliciting apoptosis when p53 function is lost (Hanahan and Weinberg, 2000).

2.2.2 Limitless replication potential

Most, if not all mammalian cells carry built-in, cell-autonomous programs that limit multiplication. This program operates independently of the cell-to-cell signalling pathways. This build-in program must be disrupted in order for development of tumours. Once healthy cell populations have progressed through a specific number of proliferation steps, they stop growing. This process is called senescence. When cells are able to grow indefinitely it is termed immortalization. Counting of the cell generations are measured by the ends of the chromosomes, called telomeres. Telomeres are composed of thousands' repeats of a short 6 base pair (bp) sequence element. Replicating generations are counted by the 50-100 bp loss of telomeric DNA from the ends of every chromosome during each cell cycle. Advanced shortening of the telomeres are caused by the incapability of DNA polymerase to replicate the 3' ends of chromosomal DNA during each S-phase. During successive cycles of replication, the continuous shortening of telomeres, causes the chromosomal DNA ends to be unprotected. Telomere maintenance is unmistakably present in all types of malignant cells. Approximately 85-90% of malignant cells up regulate expression of telomerase. Telomerase is the enzyme that specifically adds hexanucleotide repeats onto the ends of telomeric DNA. Other mechanisms include alternative lengthening of telomeres (ALT), which maintain telomeres through recombination based interchromosomal exchanges of sequence information. While telomere maintenance is clearly a key component of the capability for unlimited replication, the circumvention of

cellular senescence is still under investigation. The above-cited observations might argue that senescence, much like apoptosis, reflects a protective mechanism that can be activated by shortened telomeres or conflicting growth signals that forces abnormal cells irreversibly into a G_0- phase (Hanahan and Weinberg., 2000).

2.3 Chemoprevention

Between 19% (WHO, 2008) and 35% cancer-related mortalities are associated with nutritional factors. These nutritional factors include the presence of suspected carcinogens in the diet or the absence of compounds that are described as cancer preventative. Dietary guidelines have been established to reduce the risk of cancer (Russo, 2007).

Chemoprevention includes the use of natural or pharmacological agents to suppress; arrest or reverse carcinogenesis in the initiation stage. Chemoprevention refers to molecules that not only prevents cancer but also cures cancer. The National Cancer Institute (NCI) divided chemo-preventative agents into five classes. These five classes have showed promising results in clinics. These classes include; selective estrogen receptor modulators (SERMs), non-steroidal anti-inflammatory drugs (NSAIDs), calcium compounds, glucocoticoids and retinoids. In addition to these five classes, the NCI also has numerous reports on naturally occurring compounds having anti-cancer activity, which was identified in 40 edible plants. These chemo-preventative effects are primarily based on cell culture and animal model studies. Only a few of these compounds qualified for clinical trials. A high-quality chemo-preventative agent should have general requirements, which are also required from any other new drug. The agent needs to prevent cancer in high risk healthy individuals, must be able to prevent cancer formation in individuals that had already developed pre-malignant lesions and prevention of secondary cancer in patients previously treated for primary cancers. Adequate clinical data should be collected as evidence for the reduction of cancer. Studies usually start from epidemiological and basic laboratory data and ends with stepwise trials (Russo, 2007).

2.4 Traditionally used plants for treatment and prevention of cancer

Ethnobotanical information forms the foundation of many ethnopharmacological research projects. Insufficient literature regarding the history and ethnobotany of African traditional medicine is common. This is an exception when considering the vast amount of literature found on Indian Ayurvedic and Chinese traditional medicine (Light *et al.*, 2005).

Reports on plants that are traditionally used in South Africa for the treatment of cancer are infrequent. It is postulated that the lack of traditional knowledge is most certainly associated with the complexity

of the disease. Many symptoms and several biochemical pathways are involved. The symptoms rather than cancer are usually treated. The use of plants boosting the immune system, anti-pathogenic agents (anti-microbial, anti-fungal and anti-viral) and plants controlling inflammation is recommended. Some of the plants that are used traditionally in South Africa for cancer are tabulated in Table 1.1 (Steenkamp and Gouws, 2006).

Numerous plants mentioned in Table 1.1 are being used in herbal formulations to treat mainly the symptoms associated with cancer, rather than cancer. By law herbal formulations may only be advertised as treating certain illnesses and not as a cure. *Bidens pilosa* occurs in tropical regions and infusions as well as alcoholic preparations are regarded as medicinal. This plant is traditionally used for the treatment of pain, fever, diabetes, edema (water retention), infections and inflammation. The flavonoid, quercetin isolated from *B. pilosa* has been shown to suppress tumours in mice. Upon testing *B. pilosa* extracts on Ehrlich tumour cells *in vitro* using MTT assay, it was found that only one of the samples showed activity. Pre-clinical studies were done on male isogenic Balcb/c mice housed under controlled conditions. Significant anti-tumour activity was found when Ehrlich ascites carcinoma mice were treated with chloroform extract of *B. pilosa*. Thus it was concluded that *B. pilosa* possessed anti-tumour activity when extracts were prepared with chloroform compared to less active extracts as used in the traditional preparation (Kviecinski *et al.,* 2008).

Table 1.1: Traditionally used plants for the treatment of cancer (Steenkamp and Gouws, 2006)

Scientific name	Family	Plant parts	Traditional use
Bidens pilosa L.	Asteraceae	Leaves and stems	Prostate gland tumours & inflammation
Centella asiatica (L.) Urb.	Araliaceae	Leaves	Skin complaints, rheumatoid arthritis, cancer & fever
Cnicus benedictus L.	Asteraceae	Decoctions	Internal cancers
Dicoma capensis Less.	Asteraceae	Leaves and twigs	Cancer, high blood pressure & fever
Hypoxis hemerocallidea Fisch. and C.A. Mey	Hypoxidaceae	Corms	Bladder disorders & testicular tumours
Sutherlandia frutescens (L.) R.Br.	Fabaceae	Leaves decoctions	Stomach problems, internal cancers, inflammation & viral infections

Centella asiatica also called Gotu Kola has been traditionally used especially in Malaysia and South Africa (Steenkamp and Gouws, 2006) for aiding in wound healing, memory improvement, bronchitis, asthma, uretheristis and cancer (Figure 1.5). Interestingly this plant is commonly used as porridge in Sri Lanka, feeding pre-school children and preventing nutritional deficiencies. This sample has very good antioxidative properties. Phenolic compounds are known to exhibit a wide range of biological activities. These activities include antibacterial, antiviral, anti-inflammatory and anti-allergic. Some

phenolics have been shown to be useful in preventing several diseases such as arteriosclerosis, cancer, diabetes and neurodegenerative diseases (Zainol *et al.,* 2003). Gotu kola is readily available from health shops and *via* the internet.

Figure 1.2: Gotu Kola and capsules (Treklens and CDPcholine, 2010)

Sutherlandia frutescens is endemic to South Africa and is known as cancer bush or "kankerbos'. Extracts of this plant has been used by Koi San and Nama tribes to treat stomach cancers and other ailments such as eye infections and coughs for centuries. Tai and colleagues (2004), Chinkwo (2005) as well as Stander and colleagues (2009) had shown that this plant had significant activity during *in vitro* studies on the viability of cancerous cells. Active compounds in *Sutherlandia* were found to be canavanine, pinitol and gamma-aminobutyric acid (GABA). Canavanine and canaline have anti-tumour properties and are in the process of being developed as anti-cancer drugs. *Suterlandia* also has immune-stimulant properties, thus boosting HIV and other immune-suppressed patients. South African doctors and health care workers also found anti-cancer effects when HIV positive patients took the herbal preparation. The South African Medical Research Council conducted a pilot clinical trial during 2002 to confirm the reports of doctors (Tai *et al.,* 2004). Herbal preparations of this plant are readily available, especially in South Africa (Figure 1.6).

Figure 1.3: Cancer bush and capsules (Bidorbuy and Medicoherbs, 2010)

Hypoxis hemerocallidea, is commonly known in South Africa as African potato or yellow star flower. Infusions of the tubers are used in African traditional medicines for the treatment of emetics, dizziness, burns, wounds, anxiety, depression, insanity, diabetes mellitus, hypertension and cancer. Herbal formulations are marketed for the treatment of prostate cancer, urinary tract infections as well as boosting immune system (Figure 1.7). Anti-inflammatory and anti-diabetic activities of aqueous extracts have been demonstrated in mice and rats. Active compounds found in this plant include norligan glycoside, hypoxoside which is hydrolysed in the gastrointestinal tract to rooperol. Rooperol has anti-inflammatory and anti-mutagenic activity. Phytosterols in *Hypoxis* are used for the treatment of testicular tumours and benign prostatic hyperplasia (Katerere and Eloff, 2008). In several studies it was evident that rooperol had anti-cancer activity. Experiments were conducted on MCF-10A (non-invasive breast epithelial cell line) and transformed invasive MCF-10A neoT cells (activated c-Ha-ras oncogene). Hypoxide concentrations up to 25 µM was tolerated by MCF-10A cells, while concentrations higher than 3.12 µM hypoxide showed lower cell counts and definite DNA fragmentations (180kDa base pairs) after 24 hour incubation. Products usually contain only *Hypoxis* or combinations with *Aloe* or *Sutherlandia* extracts (Drewes *et al*., 2008).

Figure 1.4: African potato aerial parts (left), tubers (middle) and capsules (right) (Drewes *et al.,* 2008 and Medicoherbs, 2010)

One newly marketed product available on the internet is causing huge concern. This product is called FAITH (manufactured by Miracle Mineral Solutions) and claims to cure AIDS, hepatitis A, B and C, malaria, herpes and most cancers. An internet advertisement lists the following plant extracts within the marketed concoction: *Centella asiatica, Cnicus benedictus, Dicoma capensis, Hypoxis hemerocallidea, Sutherlandia frutescens, Bidens pilosa, Catharanthus roseus, Myrcene, Camptotheca, Motherwort, Leonotis leonurus* and *Elytropappus Rhinocerotis.* Some of these plants have been discussed and are known for immunomodulatory and anti-cancer activity *via* traditional knowledge and some scientific investigations (Pienaar, 2010).

The combination of these plant extracts used together, to my knowledge have not been scientifically evaluated, although this "miracle" formulation is being sold in pharmacies! Most interestingly the FAITH treatment consists of an activator containing sodium chloride and the immune fortifier (containing the plant extracts) (Figure 1.8). A news paper article recently published reported that users of this product are instructed to mix the activator with citric acid such as found in citrus fruits (Appendix A). Chlorine dioxide is produced in this mixture, which is used as bleach in industries to clean textiles and clean industry water. Several side effects have been observed which includes nausea, vomiting and severe dehydration. The Food and Drug Administration (FDA) of the United States of America has warned all users of the product to immediately stop using the product. The FDA has also warned users that no research has been done to prove the effectiveness of the product (Pienaar, 2010).

Figure 1.5: The miracle medicine, FAITH (Cancer Cure, 2010)

2.5 Natural compounds derived from plants used in chemotherapy

Some of the most popular chemotherapeutic agents are derived from plants. These agents are known as phytochemicals. Phytochemicals that are known to inhibit tumours includes flavonoids and alkaloids. A summary of some of the popular naturally derived products that are currently used in chemotherapy is listed in Table 1.2. The mechanism of action is also indicated. More than 100 drugs are now being used to eradicate cancer. Drug resistant cancers are common and some drugs need to be used synergistically to have an impact on the growth and spread of cancer (Neustadt, 2006).

Cell cycle specific agents generally disrupt the cell cycle during the synthesis of DNA or during mitosis. For these types of agents to be effective, tumour cells need to be growing. These agents depend on the proliferation of cells to exercise their actions. Thus S-phase drugs inhibit DNA synthesis, while M-phase drugs inhibit mitosis or affect the mitotic spindle. These two types of agents are used against fast proliferating cancer cells, which enter the S-phase and M-phase more often than other types of cancer cells. An example of fast proliferating cells is leukaemia. Cell-cycle non-specific agents interrupt the cancer cells regardless of the cell cycle phase. These agents damage DNA even when cells are not proliferating rapidly. Unfortunately these drugs don't discriminate between cancerous and non-cancerous cells, but luckily they still have a greater impact on rapid proliferating cells. The agents are incorporated into the chemotherapy for slow growing tumours such as solid tumours (Hoffman, 2003).

Section 2.2 and 2.3 and 2.5 confirm the need in modern society to scientifically evaluate traditionally used plants. Although herbal preparations can still be poisonous, side effects may be less severe when medicine is being used under the supervision of traditional medicine practitioners.

Table 1.2: Compounds derived from plants used in chemotherapy (Neustadt, 2006)

Mode of action	Compounds	Natural source
Microtubule interfering	Vinblastine	*Cantharanthus roseus*
	Vincristine	
	Vinorelbine	*Colchicum autumnale*
	Colchicine	
	Paclitaxel	*Taxus brevifolia*
	Docetaxel	
Topoisomerase poisons	Topotecan	*Camptotheca acuminate*
	Irinotecan	
Topoisomerase II poisons	Podophyllotoxin	*Podophyllum species*
DNA synthesis inhibitors	Polyphenol Resveratrol	*Vitis vinifera* *Arachis hypogaea* *Morus spp*
Protein Synthesis inhibitors	Ligans and lectins	*Viscum album*

3. Problem statement:

Globally 22.5 million people are living with cancer. The approximate number of people each year dying of cancer is six million. Cancer annually kills more people than Acquired Immune Deficiency Syndrome (AIDS), malaria and tuberculosis (TB) combined. According to Harrington (2005) one in four people will be diagnosed with cancer some or other time during their lives. Twenty-five percent of individuals that are diagnosed with cancer develop malignant tumours.

Figure 1.9 indicates the occurrence of cancer for each continent. It is clear that North America has the highest prevalence of cancer. The reason for this sky rocketing data may be due to the fact of poor diets, lack of exercise and bad habits, such as smoking and drinking excessively and as well as stress (WHO, 2008).

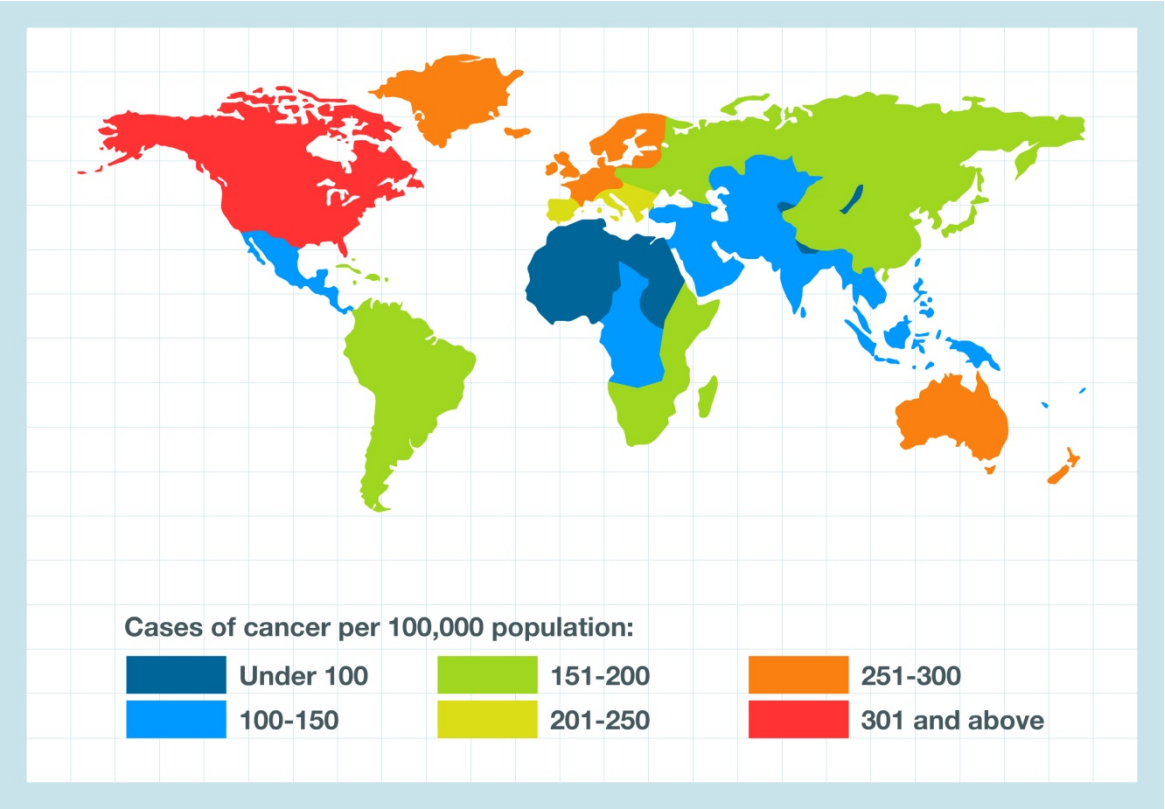


Figure1. 6: Map indicating the cases of cancer worldwide annually (Positive Health, 2008)

Other continents where high prevalence of cancer cases is found include Australia, Greenland and a small part of Europe. Africa, South America and the rest of Europe have much lower incidences of cancer. The cases of cancer each year are most probably underestimated because of the failure to detect and correctly diagnose cancer in third world countries which do not have the finances for proper medical staff and machinery (Underwood, 2005).

When countries were classified into income categories, the World Health Organisation (WHO) found that during 2007, the top ten causes of death in low income countries were mostly due to lower respiratory tract diseases and heart diseases. Another type of disease collectively called diarrhea disease, usually caused by contaminated drinking water and food accounted for the third most prevalent cause of death. Communicable diseases, such as human immunodeficiency virus (HIV) and tuberculosis (TB) infections were common causes of death in low income countries. Cancer was not part of the top ten diseases causing mortality in this income group during 2007. In middle income countries, people were most likely to die of stroke cerebro-vascular disease, heart disease and chronic obstructive pulmonary disease. Interestingly more people were likely to die in road accidents than from stomach cancer. The risk of lung cancer was however still common. Lung cancer, breast cancer and stomach cancer were part of the top ten causes of deaths in high income countries. Thus the world map and statistics given by the WHO correlate. The WHO states that 40% of cancer cases can be prevented by following a healthy lifestyle. It can be postulated that people in high income

countries have bad habits such as over indulging in fast foods, which contains large amounts of saturated fat, smoking, drinking and lack of sufficient exercise, due to long working hours in office buildings. This is not the case with people living in low income countries. In these countries physical work and less bad habits due to the lack of money and healthy eating contributes to less occurrences of diseases. Cancer can also be caused by contaminated injections in health care settings, unsafe sex and indoor smoke from fires (WHO, 2008).

In South Africa it is estimated that one in every six men and one in every seven women will be diagnosed with cancer. Cancer is not restricted to race, age or gender. Thus everyone is at risk. Cancers that are generally widespread in South African women include breast cancer, cervical cancer, colon-rectal cancer, lung cancer and oesophageal cancer. Cancers generally widespread across South African men include prostate cancer, lung cancer, oesophageal cancer and colo-rectal cancer (Health24, 2007). The World Health Organization (2008) predicts that by 2030 the mortality rate of South Africans due to cancer will rise from 5.3% (2005) to 5.9%. The estimates for cancer related mortality for South African men are given as was found during 2005 (Figure 1.10). The most fatal cancers included that of the trachea, bronchus, lungs and oesophagus. These cancers are associated with polluted air as well as smoking.

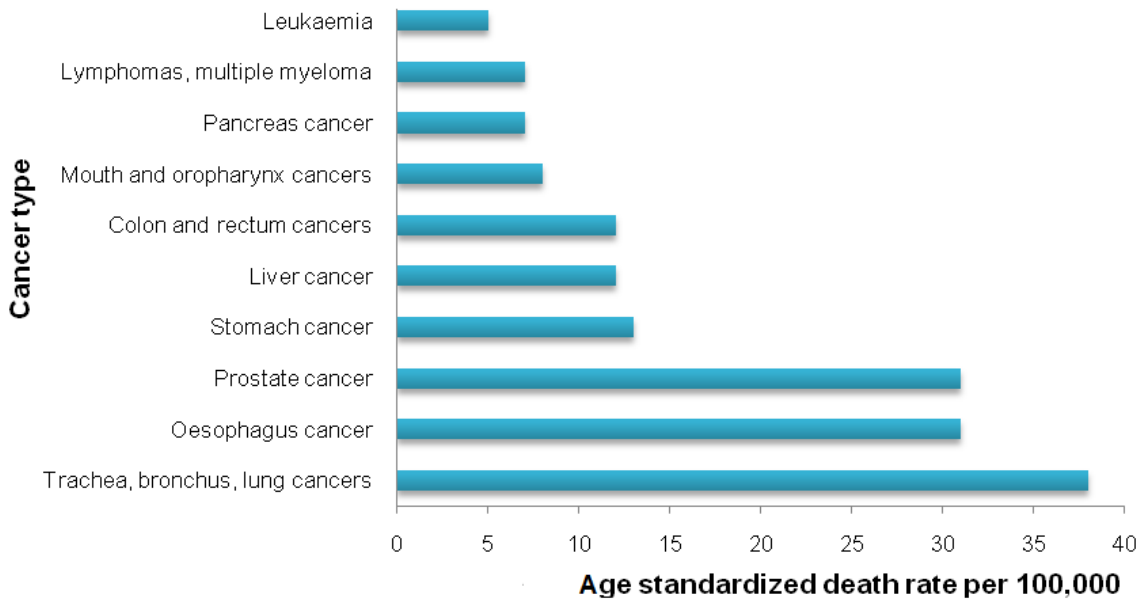


Figure 1.7: Cancer mortality in South African males during 2005 (WHO, 2008)

The highest mortality rates among females were cancers of the cervix, uterus and breast. It can be postulated that these cancers are sometimes caused by unsafe sex (cervical and uterus) or by genetically disadvantaged (breast) individuals (Figure 1.11). During 2007, it was announced that a vaccine has been developed to prevent cervical cancer, thus giving young girls immunity against

human papilloma virus, the causative agent of cervical cancer. The problem with vaccination is that young girls might be more promiscuous and behave sexually inappropriate. The vaccination is also still quite expensive, thus still not realistic in preventing cervical cancer in developing countries such as found in Africa. The vaccination might prevent high incidences of new cervical cancer patients, but will not have an impact on patients living presently with this disease.

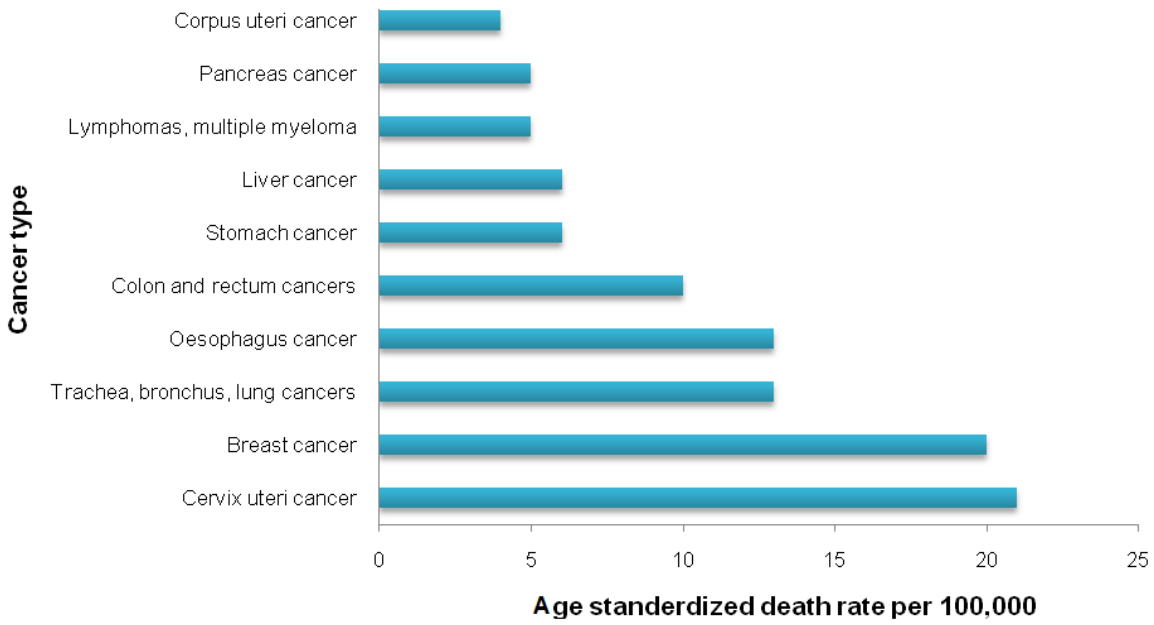


Figure 1.8: Cancer mortality in South African females during 2005 (WHO, 2008)

Statistics are also available for the common cancers that children suffer from. Twenty-four percent of children that are diagnosed with cancer suffer from leukemia. Twenty-one percent of them are diagnosed with brain cancer, while sixteen percent of them suffer from lymphomas. Cancer of the kidneys as well as cancer of the symphatic system (neuroblastoma) is less prevalent (Health24, 2007).

4. Synopsis of research

During the initial stages of this investigation during 2008, nine plants found in South Africa were preliminarily screened to determine their cytotoxicity (Honours' project, 2008). The ethanol extracts were tested on brine shrimps to determine the general toxicity *in vivo,* after which all extracts were tested on cancerous HeLa cells and the most active extracts tested on "non-cancerous" Vero cells. The selectivity index was calculated for the most active extracts, after which one extract was chosen for DNA fragmentation analysis.

The aims of the previous study were to determine the general toxicity of the selected plants, the cytotoxicity of the extracts on cancerous cells and non-cancerous cells and to determine the

mechanism of action for the most effective plant. Nine southern African plants were selected based on phytochemical constituents for the present study. These plants were evaluated for toxicity using brine shrimp assays and cancer cell lines. Ethanol extracts of leaves of eight plants were prepared except in one case (*Crotalaria agatiflora* subspp *agatiflora*) where leaves and flowers were respectively extracted with ethanol. The pre-screening of the samples was done using brine shrimp bioassays. Out of the ten samples, eight extracts were found to be relatively non-toxic (Lethal concentration, $LC_{50} > 1$ mg/ml) and two extracts showed moderate toxicity (Table 1.3). All extracts were tested for anti-cancer activity on cervical epithelial carcinoma cells (HeLa cells). The fifty percent inhibitory concentration was determined using *in vitro* Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) cytotoxicity assays. Five extracts namely, *Ghomphocarpus fruticosus* subspp*. fruticosus, Crotalaria agatiflora* subspp. *agatiflora* (leaves), *Euphorbia damarana, Hedera helix* and *Leucosidea sericea* exhibited IC_{50} values ≤ 80 µg/mL and were further tested on non-cancerous cells, African green monkey kidney cells (Vero cells). *Crotalaria agatiflora* flower extract was not further investigated due to the unsustainable nature of the resource.

Table 1.3: Lethal Concentration for 50% of the brine shrimp population (LC_{50}) of the extracts

Sample	LD_{50} (µg/mL)
Crotalaria agatiflora flowers	1195 ± 0.001
Crotalaria agatiflora leaves	625 ± 0.219
Euphorbia damarana	1040 ± 0.042
Ghomphocarpus fruticosus	1030 ± 0.028
Hedera helix	925 ± 0.049
Leucosidea sericea	1500 ± 0.042
Podocarpus falcatus	175 ± 0.001
Podocarpus henkelii	2255 ± 0.178
Podocarpus latifolius	1490 ± 0.071
Scadoxus puniceus	1265 ± 0.001
Podophyllotoxin	**3.419 ± 1.98**

Extracts that had higher IC_{50} values than 80 µg/mL were not further tested on Vero cells. The remaining five samples were tested on Vero cells and the selectivity index calculated. Leaves of *Crotalaria agatiflora* showed the weakest activity of the five extracts tested on these cells. The IC_{50} of *C. agatiflra* leaves was found to be 352.4 µg/ml. Selective indexes were calculated for the five samples. Due to the nature of this study, samples were not only elected on the degree of toxicity, but also the selectivity of the sample on cancerous cells. *Euphorbia damarana* and *Leucosidea sericea* had the same and lowest selectivity indices. Although both these samples were classified as very

active, these samples failed to be selective, compared to the other samples and the positive control. *Ghomphocarus fruticosus* had moderately higher selective toxicity and was much more active against the cell lines (Table 1.4). The Council of Science and Industrial Research (CSIR) published an article in 2008 which included toxicities of *G. fruticosus* on several cell lines. It was decided not to carry on with a plant whose full potential had been already discovered by one of South Africa's leading research institutes (Fouche *et al.,* 2008). *Hedera helix*, an exotic ornamental plant in South Africa, had the second best selectivity index. The SI value was still not comparable to either doxorubicin or *C. agatiflora* and was thus not further investigated. Although *C. agatiflora* did not have the best activity on the cancerous cells, it still showed the least toxicity on Vero cells and had an SI value comparable to doxorubicin. *Crotalaria agatiflora* was also selected for further investigation due to the lack of pharmacological and phytochemical data. The observed results also indicated that this plant had the potential to be used as an alternative chemotherapy drug which theoretically would have fewer side effects than current chemotherapeutics.

Table 1.4: Summary of cytotoxicities of plant extracts on cell lines and selectivity index

Sample	HeLa cells IC_{50} (µg/mL)	Vero cells IC_{50} (µg/mL)	Selectivity Index
Crotalaria agatiflora flowers	50 ± 2.05	ND	ND
Crotalaria agatiflora leaves	72.6 ± 3.56	352.4 ± 4.85	4.85
Euphorbia damarana	19.86 ± 0.44	15.07 ± 0.75	0.76
Ghomphocarpus fruticosus	32.27 ± 7.33	36.01 ± 2.44	1.12
Hedera helix	53.25 ± 1.5	108.80 ± 2.47	2.04
Leucosidea sericea	80.46 ± 1.19	61.30 ± 1.09	0.76
Podocarpus falcatus	98.38 ± 0.2	ND	ND
Podocarpus henkelii	130.1 ± 2.05	ND	ND
Podocarpus latifolius	157.6 ± 1.4	ND	ND
Scadoxus puniceus	>400	ND	ND
Doxorubicin	**0.617 ± 0.23**	**2.90 ± 0.12**	**4.70**

Crotalaria agatiflora leaf extract which showed selectively less toxicity on non-cancerous cells and good anti-cancer activity was evaluated for its mechanism of action using DNA fragmentation analysis. Results showed that the leaf extract exhibited moderate signs of apoptosis and necrosis in cultured HeLa cells (Figure 1.12). Further studies in terms of identification of bioactive principles of this plant and mechanism involved were recommended.

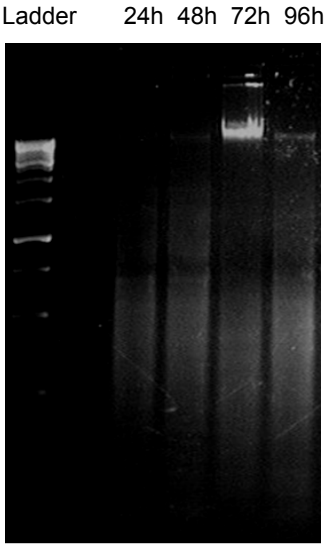


Figure 1.9: DNA fragmentation gel with different treatment times of the IC_{50} value of *C. agatiflora*

5. Crotalaria agatiflora subspp. *agatiflora*

5.1 Taxonomic classification

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Super division	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabaceae* – Pea family
Genus	*Crotalaria* L. – rattlebox
Species	*Crotalaria agatiflora* Schweinf. – canary bird-bush

(United States department of agriculture, 2009)

5.2 Morphology and distribution

This small tree or shrub can grow to a height of approximately three meters. The leaves have long stalks, are compound and consists each of three leaflets on short stalks. A raceme inflorescence is usually terminal on stout stalks and many yellow bird-like flowers occur. The flowers look like birds, the calyx is purplish and the corolla is yellow (Figure 1.13). The pods of this tree are inflated (Van

Wyk *et al.,* 2005). The canary bird shrub is native to East Africa and Northern Africa and is usually found in disturbed areas, for example on the side the roads (Van Wyk and Malan, 1998).

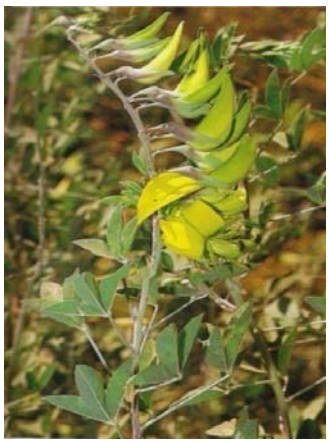


Figure 1.10: The flowers and the leaves of the canary bird shrub (Van Wyk and Malan, 1998)

All the parts of the shrub contain pyrrolizidine alkaloids (Van Wyk *et al.,* 2005), which are used as food and herbal remedies. The traditional healers, neuropaths and herbalists prepare medicine concoctions from plants rich in pyrrolizidine alkaloids. Experiments have shown that pyrrolizidine alkaloids and their N-oxides in some cases are tumour inhibitors. The pyrrolic metabolites that are produced in the liver have an anti-mitotic effect (IPCS-INTOX Database, 1988).

5.3 Traditional use and phytochemistry

Very few literature exploring this plant's medicinal properties were found. Only one applicable article could be found, which describes pharmacological investigations of *C. agatiflora* Schwienf. Sharma *et al*. (1967) found that this plant can be used as a relaxant with hypotensive properties. It was also found that extracts of this plant was able to inhibit spasms in dogs. Traditionally *C. agatiflora* extract is used for the treatment of sexually transmitted diseases (STDs) and ortitis media, specific bacteria causing ear infections (Njoroge *et al.,* 2004 and Njoroge and Bussmann, 2006). Hardly any compounds have been isolated from *C. agatiflora*. To the author's knowledge only two parent compounds have been isolated which are madurensine and anacrotine and their derivatives (Dictionary of Natural Products, 2010).

According to previous researchers' madurensine had been reported in the seeds, twigs and leaves of *Crotalaria agatiflora* subspp*. imperialis* and *C. laburnifolia* subspp. *tenuicarpa*. Anacrotine has been identified in *C. agatiflora* supspp. *imperialis, C. gillettii, C. incana* subspp*. purpurascens and C. laburnifolia* subspp*. tenuicarpa* (Asres *et al.,* 2004). Madurensine had been screened for anti-cancer activity by the National Cancer Institute (NCI). Different yeast stains such as mlh1 rad18, bub3, cln2

rad14, sgs1 mgt1, mec2-1 and rad50 were used to test the compound's anti-cancer activity. The bioassay is based on growth inhibition of yeast strains with defined genetic alterations. Compound treatments which inhibited the growth of the yeast by 70% were considered active. All strains tested negative for anti-cancer activity (PubChem, 2009). Anacrotine had been given orally to rats in which case the pyrrolizidine alkaloid was metabolized to its pyrrole metabolite, which caused liver and lung damage in the animals (Mattocks and Driver, 1987). Due to the lack of pharmacological and phytochemical research on this plant, it was decided to select this plant for further evaluation. The genus is known in homeopathy for their antirheumatic, antiphlogistic and expectorant activities (Ko *et al*., 2004). The plants are also used for cooling and purifying blood in impetigo and psoriasis (Bahar *et al.*, 2006). Alkaloids found in *Crotalaria spp.* are said to have anti-cancer activity, accompanied with high toxicity. Table 1.5 summarizes the traditional uses of *Crotalaria*. The bioactivity as found by various researchers previously has been depicted in Table 1.6. It was found that *Crotalaria spp.* had very weak anti-microbial activity. Thus although cancer patients obtain many opportunistic pathogens during chemotherapy, the anti-microbial activity of *Crotalaria* will not be investigated in this study. *Crotalaria* extracts were found to be active against parasites such as malaria and leishmans, thus showing toxicity for higher organisms (Pillay *et al*., 2008 and Rocha *et al*., 2005).

Table 1.5: Traditional uses of *Crotalaria*

Plant	*Part used*	*Therapeutic application*	*Preparation*	*Country*	*Reference*
Crotalaria ramosissima Roxb.	Flowers	Eczema	Fresh flowers crushed and paste applied externally	India	Ram *et al*., 2004
Crotalaria mesopontica Taub.	Leaves and stems	Antiviral (Anthrax, laryngitis, sores)	-	Rawanda	Vlietinck *et al*., 1995
Crotalaria alata Buch-Ham. Ex. Roxb.	Whole plant	Fever and respiratory tract infections	Decoction	Nepal	Shrestha and Dhillion, 2003
Crotalaria pumila Ortega *Crotalaria fulva* Roxb.	Aerial	Yellow fever and rash	-	United States of America	Adonizio *et al*., 2006
Crotalaria prostrate Rottl. Ex. Willd.	Leaves	Cuts	-	India	Kumar *et al*., 2007
Crotalaria sp. L.	Leaves	Cancer, stomach pain, hepatic pain	Aqueous infusion of fresh material	Ecuador	Tene *et al*., 2007
Crotalaria cf. caudate Welw. Ex. Baker	Leaves	Skin diseases, gonorrhea, threatened miscarriages, insanity	Powdered leaves mixed with oils and facts (topical), Decoctions	Tanzania	Maregesi *et al*., 2007
Crotalaria retusa L.	Leaves	Eye infections, skin diseases, fever, haemoptysis and colic, paste applied to wounds	Infusions Leaf juice Infusions Leaf paste	Tanzania	Maregesi *et al*., 2007
Crotalaria emarginella Vatke.	Whole plant	Scabies, impetigo, fever and colic as well as haemoptysis	-	India and African countries	Bahar *et al*., 2006
Crotalaria assamica Benth.	Whole plant	Anticancer	-	China	Graham *et al.,* 2000
Crotalaria ferruginea	Whole plant	Anticancer	-	China	Graham *et al.,* 2000
Crotalaria sessiliflora	Whole plant	Skin and cervical cancer	-	China	Graham *et al.,* 2000
Crotalaria agatiflora Schwienf.	-	STD	-	Kenya	Njoroge *et al.,* 2004
Crotalaria agatiflora Schwienf.	Leaves	Otitis media (bacterial ear infection)	-	Kenya	Njoroge and Bussmann, 2006

Table 1.6: Research by other institutions

Plant	*Part used*	*Research*	*Result*	*Reference*
Crotalaria burkeana Benth.	Leaves, roots	Malaria (pLDH) Strain = D10	DCM root extract best, IC_{50}=9.5µg/mL	Pillay *et al*., 2008
Crotalaria pallida Ait.	Leaves	Antimicrobial –disc diffusion method (*E. coli* and *S. aureus*)	No activity for methanol extracts at 1mg/mL	Melendez and Capriles, 2006
Crotalaria ramosissima Roxb.	Flowers	Anitmicrobial – disc diffusion method (*Pseudomonas aeruginosa, Staphylococcus aureus, micrococcus luteus, Micrococcus roseus* and *Candida albicans*)	Weak activity	Ram *et al*., 2004
Crotalaria deserticola Taub. Ex. Bak	Leaves Stems Fruits		Weak or no activity	Vlietinck *et al*., 1995
Crotalaria incana L. var. *purpurescens* (Lam) Milne. Redh.	Leaves Stems Fruits Roots	Antimicrobial, antifungal (agar diffusion method) and antiviral (EPTT method) – Bacteria: *S. areus, P. aeruginosa, E. coli.* Yeast: *Candida albicans*. Fungi: *Miorosporum canis, Trichophyton mentagrophytes*. Viruses: Polio, Coxsackie, Semliki forest, herpes and measles.	Weak or no activity	Vlietinck *et al*., 1995
Crotalaria mesopontica Taub. Var. Mesopontica	Leaves Stems		Stem extract: reduction factor of viral titre of 10^3 against polio virus	Vlietinck *et al*., 1995
Crotalaria barbata R. Grah.	Whole plant	Antileishmanial – test organism *L. donovani*	Ethanol extract active	Rocha *et al*., 2005
Crotalaria pallida AIT.	Bark	Anti-inflammatory	MetOH extract active compounds	Ko *et al*., 2004
Crotalaria assamica Benth.	Seeds		MetOH extract active compounds	
Crotalaria emarginella Vatke.	Aerial	Anti-inflammatory, anti-hepatoxic and anti-diabetic	Ethanol extracts and isolated compounds significant anti-inflammatory, and anti-hepatoxic activity.	Bahar *et al.,* 2006

6. Hypotheses

Screening of leaf extracts made from *Crotalaria agatiflora* subspp *agatiflora* using different solvents against cancerous and a non-cancerous cell lines will result in different selectivity indexes, because the extracts' composition will not be the same. Water is very polar and thus will extract most of the polar compounds, while ethanol is less polar as compared to water and will extract both polar and nonpolar compounds (Houghton and Raman, 1998). *Crotalaria agatiflora* will show *in vitro* anti-neoplasmic activity as postulated by Graham *et al*., 2000 and Dictionary of Natural Products for related species belonging to the genus *Crotalaria*. Taxonomists have found that many species within a given genus will have similar groups of chemical constituents (Wink, 2003) for example the anti-neoplasmic alkaloid, monocrotaline, found in several *Crotalaria* spp. (Dictionary of Natural Products, 2010). Thus this specific species under investigation may either also contain traces of monocrotaline or derivatives that will have anti-cancer activity.

Extracts will show potential as chemo-preventative agents, having anti-oxidant characteristics as was found for *C. sessiflora* by Mun'im *et al.,* 2003. Phenolic compounds shows moderate to high anti-oxidant activity. *Crotalaria* is from the family *Fabaceae* which are known chemo-taxonomically for the presence of several types of flavonoids, isofavonoids and terpenes (Wink, 2003).

Isolation of alkaloids (Wink, 2003) from *C. agatiflora* subspp. *agatiflora* will show anticancer activity because alkaloids may be cytotoxic (Wink, 2007; Hoffman, 2003; Levin and York, 1978). Related species, *C. agatiflora* subspp. *erlangeri* contained 1.5% alkaloids in its leaves (Asres *et al*., 2004).

The mechanism of action of *C. agatiflora* leaf extract / compounds will be determined by basic ultrastructure analysis, flow cytometry and Raman spectroscopy. Similar investigations were conducted by Stander and his colleagues (2009) using *Sutherlandia frutescens* water extracts to investigate the ultrastructure of breast cancer cell lines, as well as cell progression and apoptosis. Guo *et al.,* 2009 used Raman spectroscopy to investigate interactions between malignant hepatocytes with doxorubicin, to conclude submolecularly mechanisms of cell death *in vitro*. The bioactive samples will show signs of necrosis / apoptosis or autophagy.

7. Aims and objectives

The objectives of the present study were as follows:

- Determine the cytotoxicity of differently extracted *C. agatiflora* subspp. *agatiflora* by doing *in vitro* cytotoxicity assays on cancerous (U-937, MCF-7, SNO and HeLa cells) and noncancerous (Vero) cell lines conducting XTT colorimetric assays.

- Determine the most effective extract for further investigations by analyzing cytotoxicity results and calculating selective index (SI) values.

- Isolate alkaloids from *C. agatiflora* subspp. *agatiflora*, by various chromatographic techniques.

- Determine the antioxidant potential of the extracts and isolated compounds' by DPPH scavenging capabilities.

- Determine the mechanism of action for the extract *in vitro* by ultrastucture investigation, flow cytometry and Raman spectroscopic investigation.

8. Chapter layout

Chapter 1: Phytotherapy and cancer

Chapter 2: Investigation of cytotoxicity and chemo-preventative activity of crude extracts of *C. agatiflora* subspp. *agatiflora*

Chapter 3: Bioassay-guided fractionation of *C. agatiflora* subspp. *agatiflora*

Chapter 4: Ultra structure and flow cytometric analysis of the ethanolic extract and madurensine's cytotoxicity

Chapter 5: Raman spectroscopic analysis of *Crotalaria* ethanolic extract and madurensine treated leukemic U-937 cells

Chapter 6: Conclusions and future recommendations

Appendix A: News paper report

Appendix B: Dictionary of Natural Products

Appendix C: NMR data

9. References

Adonizio, A.L., Downum, K., Bennet, B.C. and Mathee, K. 2006. Anti-quorum sensing activity of medicinal plants in southern Florida. *Journal of Ethnopharmacology* **105**: 427-435.

Asres, K., Sporer, F. and Wink, M. 2004. Patterns of pyrrolizidine alkaloids in 12 Ethiopian *Crotalaria* species. *Biochemical Systematics and Ecology* **32**: 915-930.

Bahar, A., Al-Howiriny, T.A. and Mossa, J.S. 2006. Crotalic acid and emarigellic acids: Two tripenes from *Crotalaria emarginella* and anti-inflammatory and anti-hepatoxic activity of crotalic acid. *Phytochemistry* **67**: 956-964.

Balunas, M.J. and Kinghorn, A.D. 2005. Drug discovery from medicinal plants. *Life Sciences* **78:** 431-441.

Bertram, J.S. 2001. The molecular biology of cancer. *Molecular Aspects of Medicine* **21**: 167-223.

Bid or buy. 2010. "*Sutherlandia frutescens* seeds." [Online]. Available: http://www.bidorbuy.co.za/item/18634216/20_Sutherlandia_frutescens_Seeds_Kankerbos_Cancerbush_Wildegansie_Indigenous_Medicinal.html [Cited 14 August 2010].

Cancer Association of South Africa. 2010. "Research projects." [Online]. Available: http://www.cansa.org.za/cgi-bin/giga.cgi?cat=867&limit=10&page=0&sort=D&cause [Cited 5 September 2010].

Cancer Cure. 2010. "FAITH Drops". [Online]. Available: www.cancercure.co.za/images/faith_drops [Cited 3 August 2010].

Cancer Initiative of South Africa. 2010. "About CARISA – background on the establishment of CARISA." [Online]. Available: https://secure.carisa.org.za/about.htm [Cited 5 September 2010].

CDPcholine. 2010. "*Centella asiatica*" [Online]. Available: http://www.cdpcholine.net/centellaasiatica.html [Cited 1 August 2010].

Chabner, B.A. and Chabner Thompson E. 2007. "Surgery". The Merck manuals, online medical library. [Online]. Available: http://www.merck.com/mmhe/sec15/ch182/ch182d.html [Cited 25 May 2010].

Chinkwo, K.A. 2005. *Sutherlandia frutescens* extracts can induce apoptosis in cultured carcinoma cells. *Journal of Ethnopharmacology* **98**: 163-170.

Cotton, C.M. 1996. Ethnobotany principles and applications. John Wiley and Sons, Chichester. P 218.

Council for Scientific and Industrial Research. 2010. "Scouring indigenous knowledge and plants for medicinal advancement." [Online]. Available: http://www.csir.co.za/biosciences/staff/vinesh.html [Cited 5 September 2010].

Dictionary of Natural Products. 2010. "Search engine." [Online]. Available: http://www.dnp.chemnetbase.com [Cited 1 March 2010]

Drewes, S.E., Elliot, E., Khan, F., Dhlamini, J.T.B. and Gcumisa, M.S.S. 2008. *Hypoxis hemerocallidea* – Not merely a cure for benign prostate hyperplasia. *Journal of Ethnopharmacology* **119**: 593-598.

Fennell, C.W., Lindsey, K.L., McGaw, L.J., Sparg, S.G., Stafford, G.I., Elgorashi, E.E., Grace, O.M., Van Staden, J. 2004. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. *Journal of Ethnopharmacology* **94:** 205-217.

Fouche, G., Cragg, G.M., Pillay, P., Kolesnikova, N., Maharaj, V.J. and Senabe, J. 2008. In vitro anticancer screening of South African plants. *Journal of Ethnopharmacology* **119**: 455-461.

Genitope Corporation. 2010. "Personalized immunotherapy – Manufacturing." [Online]. Available: http://www.genitope.com/product/manufacturing/ [Cited 26 May 2010].

Graham, J.G., Quinn, M.L., Fabricant, D.S. and Farnsworth, N.R. 2000. Plants used against cancer – an extension of the work of Jonathan Hartwell. *Journal of Ethnopharmacology* **73**: 347-377.

Guo, J., Cai, W., Du, B., Qjan, M. and Sun, Z. 2009. Raman spectroscopic investigation on the interaction of malignant hepatocytes with doxorubicin. *Biophysical Chemistry* **140**: 57-61.

Gurib-Fakin, A. 2007. Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine* **27**: 1-93.

Haematology and oncology associates of Rhode Island. 2010. "Side effects of chemotherapy." [Online]. Available: http://www.cancercenterofri.org/side.html [Cited 26 May 2010].

Hanahan, D. and Weinberg, R.A. 2000. The hallmarks of cancer. *Cell* **100**: 57-70.

Harrington, C.L. 2005. Introduction to Cancer, Cancer Research History and the Significance of Plants in Cancer Research. [Online]. Available: www.surfingmedicine.org/research/Introduction-to-cancer.do [Cited 3 February 2008].

Health24. 2007. Types of cancer prevalent in South Africa. [Online]. Available: http://www.health24.com/medical/Condition_centres/777-792-802-1626,14701.asp [Cited 23 February 2008].

Hoffman, D. 2003. An introduction to Photochemistry in Medical Herbalism, Pp 140-168

Houghton, P.J. and Raman, A. 1998. Laboratory Handbook for the Fractionation of Natural Extracts. Chapman & Hall, London, p1-112.

International Program on Chemical Safety INTOX Program. 1988. Pyrrolizidine alkaloids [Online]. Available: http://www.intox.org [Cited 6 April 2008].

Katerere, D.R. and Eloff, J.N. 2008. Anti-bacterial and anti-oxidant activity of *Hypoxis hemerocallidea* (Hypoxidaceae): Can leaves be substituted for corms as a conservation strategy? *South African Journal of Botany* **74**: 613-616.

King, A. T. and Primrose, J.N. 2003. Cancer treatment: Principles of cancer treatment by surgery. *Surgery* **21**: 284-288.

Ko, H-H., Weng, J-R., Tsao, L-T., Yen, M-H., Wang, J-P. and Lin, C-N. 2004. Anti-inflammatory flavonoids and pterocarpanoids from *Crotalaria pallida* and *C. assamica*. *Bioorganic & Medicinal Chemistry Letters* **14**: 1011-1014.

Kumar, B., Vijayakumar, M., Govindarajan, R. and Pushpangadan, P. 2007. Ethnopharmacological approaches to wound healing – Exploring medicinal plants of India. *Journal of Ethnopharmacology* **114**: 103-113.

Kviecinski, M.R. Felipe, K.B., Schoenfelder, T., De Lemos Wiese, L.P., Rossi, M.H., Goncalez, E., D' arc Felicio, J., Filhno, D.W. and Pedrosa, R. C. 2008. Study of the antitumor potential of *Bidens pilosa* (Asteraceae) used in Brazilian folk medicine. *Journal of Ethnopharmacology* **117**: 69-75.

Levine, D.A. and York, B.M. 1978. The toxicity of plant alkaloids: an ecogeographic perspective. *Biochemical Systematics and Ecology* **6**: 61-76.

Light, M.E., Sparg, S.G., Stafford, G.I. and van Staden, J. 2005. Riding the wave: South Africa's contribution to ethnopharmacological research over the last 25 years. *Journal of Ethnopharmacology* **100:** 127-130.

Maregesi, S.M., Ngassapa, O.D., Pieters, L. and Vlietinck, A.J. 2007. Ethnopharmacological survey of the Bunda district, Tanzania: Plants used to treat infectious diseases. *Journal of Ethnopharmacology* **113**: 457-470.

Mattocks, A.R. and Driver H.E. 1987. Metabolism and toxicity of anacrotine, a pyrrolizidine alkaloid in rats. *Chemico-Biological Interactions* **63**: 91-104.

Medical Research Council. 2010. "Our research." [Online]. Available: http://www.mrc.ac.za/research/ourresearch.htm [Cited 5 September 2010].

Medicoherbs. 2010. "*Sutherlandia frutescens*." [Online]. Available: http://www.medicoherbs.co.za/shop/index.php?main_page=product_info&products_id=58 [Cited 1 Augustus 2010].

Medline Plus. 2010. "Radiation therapy." [Online]. Available: http://www.nlm.nih.gov/medlineplus/ency/imagepages/9805.htm [Cited 26 May 2010].

Melendez, P.A. and Capriles, V.A. 2006. Antibacterial properties of tropical plants from Puerto Rico. *Phytomedicine* **13**: 272-276.

Mun'im, A., Negishi, O. and Ozawa, T. 2003. Antioxidative compounds from *Crotalaria sessiliflora. Biosci. Boitechnol. Biochem* **67**: 410-114.

Neustadt, J. 2006. Molecular mechanisms involved in killing cancer cells. [Online]. Available: http://content.herbalgram.org/wholefoodmarket/herbcoip/review.asp?=44249 [Cited 30 March 2008].

Njoroge, G.N. and Bussmann, R.W. 2006. Traditional management of ear, nose and throat (ENT) diseases in Central Kenya. *Journal of Ethnobiology and Ethnomedicine* **2**: 54-62.

Njoroge, N.G., Bussmann, R.W., Newton, B., Eric, L. and Ngumi, V.W. 2004. Utilization of weed species as sources of traditional medicines in Central Kenya. *Lyonia (unpublished)* 1-16. [Online]. Available: www.lyonia.org

Pienaar, A. 2010. "Wonderdruppels? Smyt weg! Bleikmiddel kan jou siek maak en genees nie verkoue, MIV. In Beeld, 3 August 2010.

Pillay, P., Maharaj, V.J. and Smith, P.J. 2008. Investigating South African plants as a source of new antimalarial drugs. *Journal of Ethnopharmacology* **119**: 438-454.

Postive Health. 2008. "Carcinogenesis." [Online]. Available: http://www.positivehealth/carcinogenesis.com [Cited 1 April 2009].

PubChem. 2009. "BioActivity Analysis." [Online]. Available: http://www.pubchem.ncbi.nlm.nih.gov.innopac.up.az.za/assay [Cited 11 September 2009].

Ram, A.J, Bhakshu, M.D. and Venkata Raju, R.R. 2004. *In vitro* antimicrobial activity of certain medicinal plants from Eastern Ghats, India, used for skin diseases. *Journal of Ethnopharmacology* **90**: 353-357.

Rates, S.M.K. 2001. Plants as source of drugs. *Toxicon* **39**: 603-613.

Rocha, L.G., Almeida, J.R.G.S., Macedo, R.O. and Barbosa-Filho, J.M. 2005. A review of natural products with antileishmanial activity. *Phytomedicine* **12**: 514-535.

Russo, G.L. 2007. Ins and outs of dietary phytochemicals in cancer chemo-prevention. *Biochemical pharmacology* **74**: 533-544.

Shands Cancer Center. 2008. Overview of cancer treatments. [Online]. Available: http://www.ufscc.ufl.edu/Patient/content.aspx?section=cancertreatment&id=597 [Cited 3 March 2009].

Sharma, M.L., Singh, G.B. and Ghatak, B.J. 1967. Pharmacological investigations on *Crotalaria agatiflara* Scwienf. *Indian Journal of Experimental Biology* **5:** 149-150.

Shrestha, P.M. and Dhillion, S.S. 2003. Medicinal plant diversity and use in the highlands of Dolakha district, Nepal. *Journal of Ethnopharmacology* **86**: 81-96.

Stander, A., Marais, S., Stivaktas, V., Voster, C., Albrecht, C., Lottering, M-L. and Joubert, A.M. 2009. *In vitro* effects of *Sutherlandia frutescens* water extracts on cell numbers, morphology, cell cycle progression and cell death in a tumorigenic and a non-tumorigenic epithelial breast cell line. *Journal of Ethnopharmacology* **124**: 45 - 60.

Steenkamp, V., Gouws, M.C. 2006. Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer. *South African Journal of Botany* **72:** 630-633.

Tai, J., Cheung, S., Chan, E. and Hasman, D. 2004. *In vitro* culture studies of *Sutherlandia frutescens* on human tumor cell lines. *Journal of Ethnopharmacology* **93**: 9-19.

Tene, V., Malagon, O., Finzi, P.V., Vidari, G., Armijos, C. and Zaragoza, T. 2007. An ethnobotanical survey of medicinal plants used in Loja and Zamora-Chinchipe, Ecuador. *Journal of Ethnopharmacology* **111**: 63-81.

Treklens. 2010. "Pegaga – *Centella asiatica."* [Online]. Available: http://www.treklens.com/gallery/photo409501.htm [Cited 14 August 2010].

Underwood, J.C.E. 2005. General and Systematic Pathology. Churchill Livingstone, United States of America Pp 223-237

United States Department of Agriculture-Natural Resource Conservation Service. 2009. "Plant Database". [Online]. Available: http://plants.usda.gov/java/ClassificationServlet?source=display&classid=CRAG2 [Cited 8/12/09].

Van Wyk, B. and Malan, S. 1998. Field guide to the Wild Flowers of the Highveld. Struik. Cape Town.

Van Wyk, B-E., Van Heerden, F. and Van Oudtshoorn, B. 2005. Poisonous Plants of South Africa. Briza, Pretoria.

Victor, A. 2010. CANSA research projects. [Online]. Available: http://www.cansa.org.za/cgi-bin/giga.cgi?cat=867&limit=10&page=0&sort=D&cause_id=1056&cmd=cause_dir_news [Cited 5 September 2010].

Vlietinck, A.J., Van Hoof, L., Totte, J., Lasure, A., Van den Berghe, D., Rwangabo, P.C. and Mvukiyumwami, J. 1995. Screening of hundred Rwandese medicinal plants for anti-microbial and antiviral properties. *Journal of Ethnopharmacology* **46**: 31-47.

Wink, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64**: 3-19.

Wink, M. 2007. Molecular modes of action of cytotoxic alkaloids: from DNA intercalation, spindle poisoning, topoisomerase inhibition to apoptosis and multiple drug resistance. *The Alkaloids: Chemistry and Biology* **64**: 1-47.

World Health Organization. 2008. "WHO Global Database: Stop the global epidemic of chronic disease." [Online]. Available: http://www.who.int/infobase/report.aspx?rid=126 [Cited 12 May 2008].

Zainol, M.K., Abd-Hamid, A., Yusof, S. and Muse, R. 2003. Antioxidative activity and total phenolic compounds of leaf, root and petiol of four accessions of *Centella asiatica* (L.) Urban. *Food chemistry* **81**: 575-581.

CHAPTER 2:

INVESTIGATION OF CYTOTOXICITY AND CHEMO-PREVENTATIVE ACTIVITY OF CRUDE EXTRACTS OF *C. AGATIFLORA* SUBSPECIES *AGATIFLORA*

1. Introduction:

Leaf extracts of *Crotalaria agatiflora* were prepared using distilled water and ethanol separately. The extracts were tested on four cancerous and one noncancerous cell lines. The selectivity index of each extract was determined which indicated the most effective extract for further investigations. By conducting cytotoxicity on different cell lines, the most sensitive cell line to the samples was found. Antioxidant potential plays an important role to determine whether plant extracts can play a protective or healing role during initial stages of carcinogenesis. The antioxidant activity of the extracts was investigated to determine the chemo-preventative potential of this plant.

1.1 Cytotoxicity

Cytotoxicity was determined by a colorimetric assay using "Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate" (XTT). This tetrazolium salt is used as a non-radioactive marker for quantification of cell proliferation and viability. The assay is based on the cleavage of yellow tetrazolium salt. This salt forms an intense orange formazan dye in metabolically active cells *via* mitochondrial dehydrogenase (Figure 2.1). This conversion takes place only in viable cells. The formazan dye is soluble in aqueous solutions and is directly quantified using a microtitre plate reader. An increase in the number of living cells results in the increase in the overall activity of mitochondrial succinate dehydrogenase in the sample. This increased activity directly correlates to the amount of orange formazan detected (Roche, 2005).

The following four cancerous cell lines and one noncancerous cell line were selected to determine the cytotoxicity of plant extracts:

1.1.1 HeLa

HeLa cells were harvested originally from an adult black female diagnosed with cervical adenocarcinoma, ATCC number: CCL-2 (ATCC, 2009). These epithelial cells adhere to surfaces (Figure 2.2). This cell line was selected because cancer of cervix, is the leading cause of cancer-based mortality in South African women. Approximately 21 women out of 100,000 women in South Africa will died during 2005 from these cancers (WHO, 2008).

1.1.2 SNO

SNO cells (human oesophageal epithelial cancerous cells) was selected due to the high incidences of oesophageal cancer related mortality in South African males and females. Approximately 44

people out of 200,000 South Africans died during 2005 of oesophageal cancer (Health24, 2007 and WHO, 2008).

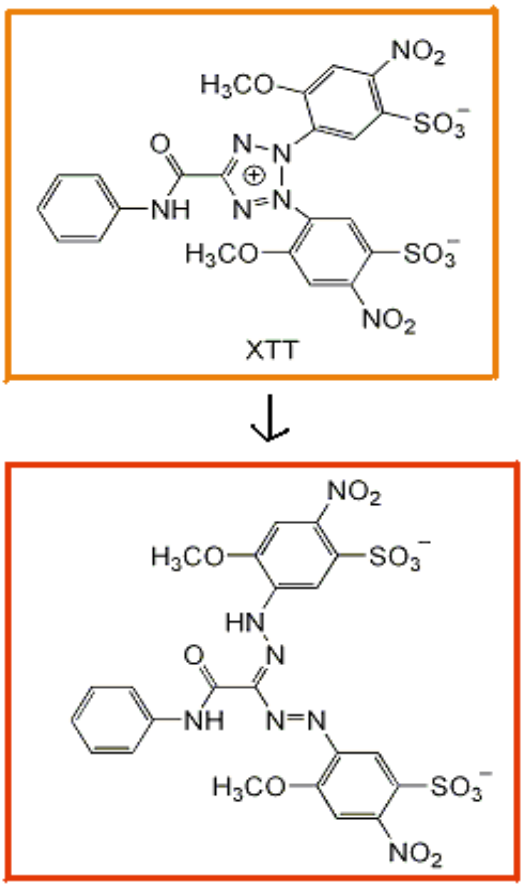


Figure 2.1: Formation of orange formazan dye (Biosynth, 2006)

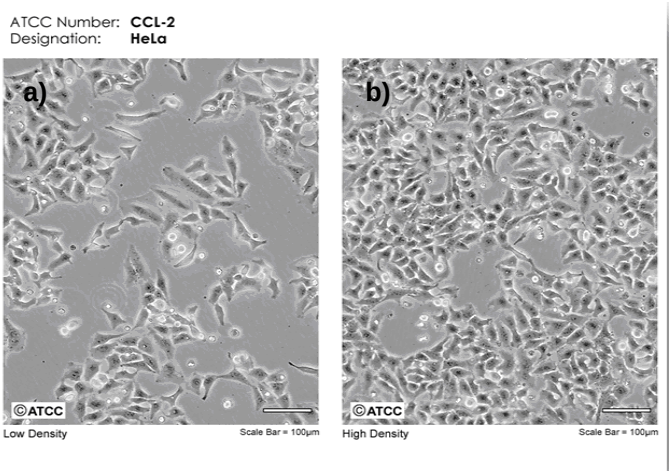


Figure 2.2: HeLa cells at low cell densities (a) and confluent (b) (ATCC, 2009)

1.1.3 MCF-7

MCF-7 cells were originally harvested from an adult Caucasian female suffering from breast (mammary gland) adenocarcinoma, ATCC number: HTB-22 (ATCC, 2009). This adherent cell line was selected because approximately 20 women out of 100,000 women in South Africa are suffering and dying annually of breast cancer (Figure 2.3). This was the second most prevalent cancer during 2005 of which South African women died from (WHO, 2008).

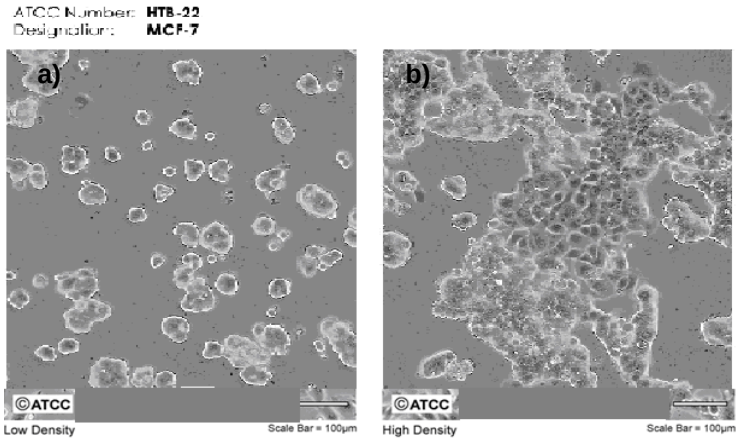


Figure 2.3: MCF-7 cells at low cell densities (a) and confluent (b) (ATCC, 2009)

1.1.4 U-937

U-937 cells were originally harvested from an adult Caucasian male suffering from histiocytic lymphoma, ATCC number: CRL-1593.2. Typical monocyte growth properties and morphology applies, suggesting rapid growth and large multiple nuclei present under homeostatic conditions (Lodish *et al.,* 2005). The cells stay in suspension, unless the cells are differentiated into macrophages (terminal monocytic cells) by phorbol esters, Vitamin D, gamma interferon, tumour necrosis factor or retinoic acid respectively (ATCC, 2009). This cell line was selected because lymphomas, multiple myelomas and leukaemia's contribute to high mortality in South African men and women. These cancers form part of the top ten cancer types diagnosed in both genders and one of the most common secondary cancers diagnosed in patients after treatment of primary cancers (WHO, 2008). It is estimated that 24% of children diagnosed with cancer are suffering from leukaemia, while 16% of diagnosed children suffer from lymphomas (Health24, 2007).

1.1.5 Vero

Vero cells were originally harvested from the kidney of a normal adult African green monkey (*Cercopithecus aethiops* L.), ATCC number: CCL-81. The epithelial cells are adherent (Figure 2.4). This cell line was selected to represent a group of cells with noncancerous characteristics.

This is one of the few cell lines which can be bought at ATCC which was not harvested from a diseased individual (ATCC, 2009). Other noncancerous cells needs to be harvested from volunteers, which is a tedious process, not just to get the primary cell line established, but also to convince the ethical boards that harvesting cells are essential. Thus although these cells are not derived from humans, it was harvested from a closely related lineage.

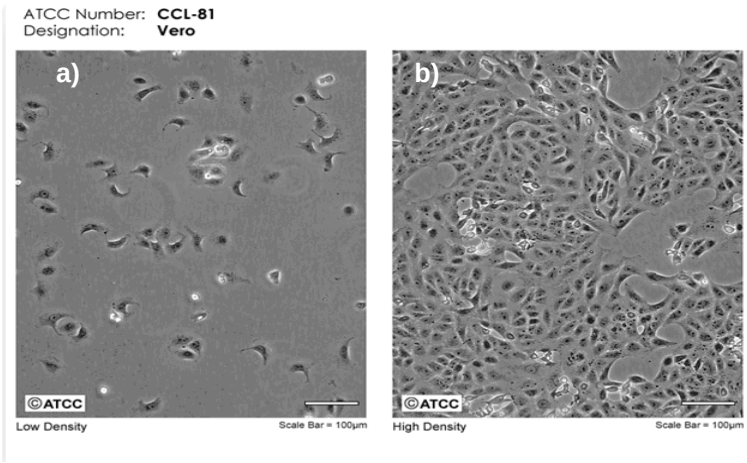


Figure 2.4: Vero cells at low density (a) and high density (b) (ATCC, 2009)

1.1.6 Positive control for cell culture

Actinomycin D is an antineoplastic and antibiotic peptide produced by *Streptomyces* species (Figure 2.5). This antibiotic inhibits cell proliferation by forming stable complexes with DNA. These complexes obstruct the movement of RNA polymerase, which consequently interferes with DNA-dependent RNA synthesis. Not only does RNA synthesis stop, but actinomycin D also causes breaks in DNA. Actinomycin D is thus a potent cytotoxic inducer of apoptosis in tumour cells (Sigma, 2004). It was found that at high concentrations cell divisions are inhibited completely, while at lower, trivial concentrations cell divisions can still take place, up to a certain degree. High levels (>0.5 µg/mL) of actinomycin D is tolerated by cells up to 24 hours of exposure (Reich *et al*., 1962). Not only is this antibiotic being used in cancer research, but also for HIV-replication research. Clinically actinomycin D is used for treating various malignant neoplasms such as Wilm's tumours and sarcomas. Side effects during treatments include bone marrow depression, gastrointestinal toxicity and severe tissue damage (Sigma, 2004).

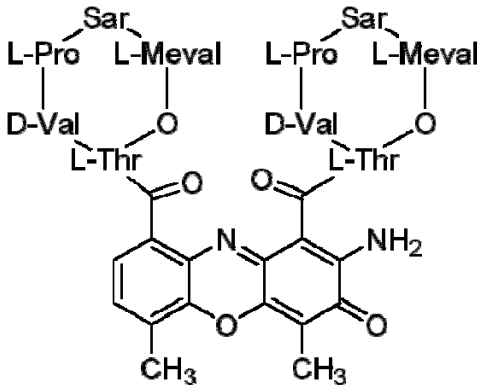


Figure 2.5: Chemical structure of Actinomycin D ($C_{62}H_{86}N_{12}O_{16}$), (Sigma, 2004)

Antioxidants have become very popular as nutritional supplements in Western countries. Antioxidants cannot be produced in most mammals including human, thus antioxidants are an essential part of the diet. In this section the advantages and disadvantages of antioxidants are discussed.

1.2 Antioxidants

Oxidant by-products, superoxide, hydrogen peroxide and hydroxyl radicals are produced during normal metabolism. These same oxidant by-products are the same chemicals produced by radiation. Eating less of the required dietary antioxidants daily, such as vitamin C and E, mimic radiation exposure. Oxidative damage to DNA and other macromolecules appear to have a major role in aging, degenerative diseases and cancer. DNA is oxidized during normal metabolism because antioxidant defences are not always sufficient. When lipids are oxidized, aldehydes are produced, some which are mutagenic. Lymphocytes and other phagocytic cells of the immune system destroy invading bacteria, parasites, viruses and infected cells by destroying them with the mutagenic oxidizing agents, NO, HOCl, and H_2O_2. The burst of oxidants and consequent inflammation from phagocytic cells are a major source of nitrogen oxides and contributes to both cancer and heart disease. These oxidants protect humans from immediate death from infection but they also cause oxidative damage to DNA, chronic cell killing with compensatory cell division and mutation. Antioxidants may explain much of the protective effect of fruit and vegetable intake against stomach and lung cancers caused by inflammation. Antioxidant defences against oxidative damage include ascorbate (vitamin C), tocopherol (vitamin E), carotenoids as β-carotene and lycopene, glutathione, lipoic acid, selenium, zinc and copper-containing proteins (Ames, 1998).

1.2.1 Controversial aspects of antioxidants

The equilibrium of reactive oxygen species (ROS) formation and antioxidative defence level are crucial for cell survival and cell growth. It is very important for the cell to remove ROS effectively to remain viable and to maintain vital functions. Normal cellular function will be altered depending on this balance which will in turn affect the fate of the cell.

Most aerobic bacteria have tolerable antioxidative systems, including SOD and catalase which can eliminate hazardous ROS from aerobic respiration or ambient oxygen derived ROS. The potential damage of ROS upon the bacteria is thus decreased. When an antioxidative enzyme defective mutant is generated the growth of the bacteria in aerobic condition becomes suppressed, although the growth in anaerobic conditions is normal. Facultative bacteria can accommodate this metabolism depending on the given conditions. Similar behaviour can be seen in cancerous cells. Cancer cells, especially those in the centre of the tumour mass, where there is low oxygen pressure, adapt more like anaerobic bacteria, having low levels of mitochondrial oxidative phosphorylation. This has been known as the Warburg-effect. Under such hypoxic conditions, cancer cells produce ATP mainly by glycolysis or even fermentation of amino acids. Recently it has also been reported that acetyl-CoA synthetase played important roles in producing ATP for tumour cells. Generation of ATP in the hypoxic tumour cells is an oxygen independent process. Cancer cells are more frequently deficient in most crucial antioxidative enzymes. These enzymes include catalase, glutathione peroxidase and SOD. The prediction can thus be made that high vulnerability of tumour cells to ROS will be observed. Many conventional anticancer drugs including vinblastine, doxorubicin, camptothecin, cisplatin and inostamycin exhibit anti-tumour activity via ROS generation (Fang *et al.,* 2009).

With regard to ROS and cancer, however, it should be noted that the biological effects of ROS in cancer are multiple and non-linear. High levels of oxidative stress exhibit cytotoxicity, inhibiting cell proliferation and leading to apoptosis or necrosis. Low or intermediate levels of oxidative stress are most effective in DNA damage, causing mutation, inflammation and promoting proliferation of cells and ultimately inducing carcinogenesis *via* initiation progression to cancer development. Convincing evidence indicated that ROS is an endogenous class of carcinogens by triggering the mutation of cells. Moreover, oxidative stress can stimulate the expansion of mutated cell clones by modulating genes related to proliferation and triggering redox-responsive signaling cascades such as epidermal growth factor, tyrosine phosphorylation, protein kinase C and transcription factors that regulate inflammation and apoptosis, including NF-κB, activator protein 1 and NF-E2 related factor (Fang *et al*., 2009). Although cancerous cells can thus benefit from antioxidant treatment, it all depends on when exactly chemoprevention therapy starts. There are two main categories of phytochemicals presenting chemo-preventative agents: cancer-blocking and cancer-suppressing

agents. During the initiation of carcinogenesis, cancer can be prevented by modifying cellular targets by enhancing carcinogen detoxification, changing up take and metabolism of carcinogens, scavenging ROS and other oxidative species and enhancing DNA repair. Cancer-suppressing agents prevent the promotion and progression of cancer by interfering with the cell cycle regulation, signal transduction, transcriptional regulation and apoptosis. Numerous epidemiological investigations suggest a positive correlation between the consumption of a fruit and vegetable rich diet and lowered incidences of stomach, oesophagus, lung, oral cavity and pharynx, endometrium, pancreas and colon cancers. To conclude antioxidant treatment could be more effective during the initiation phase of cancer when a slight increase in ROS can cause genotoxic damage to DNA. Chemo-preventative intervention should be studied in depth and carefully selected for each patient to determine the advantages and disadvantages in each case (Russo, 2007).

In this investigation DPPH (1,1-Diphenyl-2-picryl-hydrazyl), a stable free radical which is reduced to DPPH.H by antioxidants was used for the determination of antioxidant potential of the samples. Antioxidants react with DPPH which reduces the number of DPPH molecules in solution. This reduction is equal to the amount of free hydroxyl groups in solution. Thus the absorption at 517 nm is proportional to the amount of residual DPPH. Purple DPPH is reduced to transparent DPPH.H (Figure 2.6).

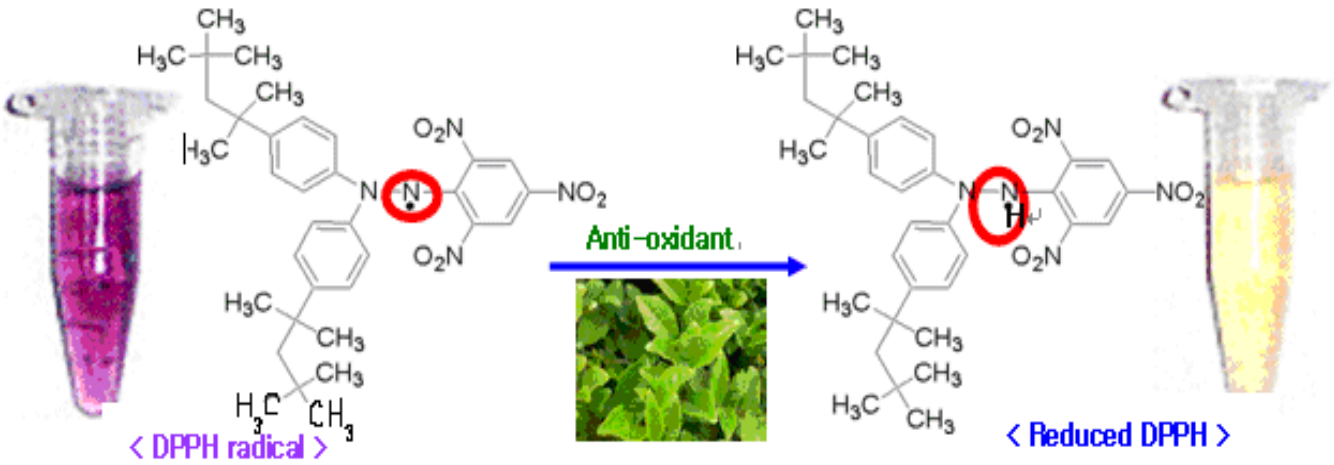


Figure 2.6: Chemical reaction of DPPH (Natural Solutions, 2008)

1.2.2 Positive control for antioxidant assay

Ascorbic acid (vitamin C) is produced by many organisms *via* the hexuronic acid pathway. Production occurs in the liver and kidneys by gulonolactone oxidase. Humans, primates, guinea pigs and some bat species lack this specific enzyme, making it impossible for them to produce ascorbic acid. Ascorbic acid is acquired by eating food rich in Vitamin C, such as fruits and

vegetables. It's a potent water-soluble antioxidant, which have reduction and oxidative properties. Ascorbate and ascorbyl radical are both in one-electron reduction level and thus have the potential to reduce most biologically relevant radicals (Figure 2.7). Oxidants include hydroxyl radicals, superoxide anions, hypochlorous acids and singlet oxygens (Verrax and Calderon, 2008).

Ascorbic acid has several bioactive properties apart from its reduction and oxidation abilities. These activities include donation of electrons to metal cofactors during hydroxylation reactions and iron absorption. It is speculated that ascorbic acid can prevent and cure viral respiratory diseases and that the use of ascorbic acid may be advantageous before and during cardiovascular disease and cancer treatments. No clinical evidence has yet been found for above mentioned benefits for diseases (Verrax and Calderon, 2008).

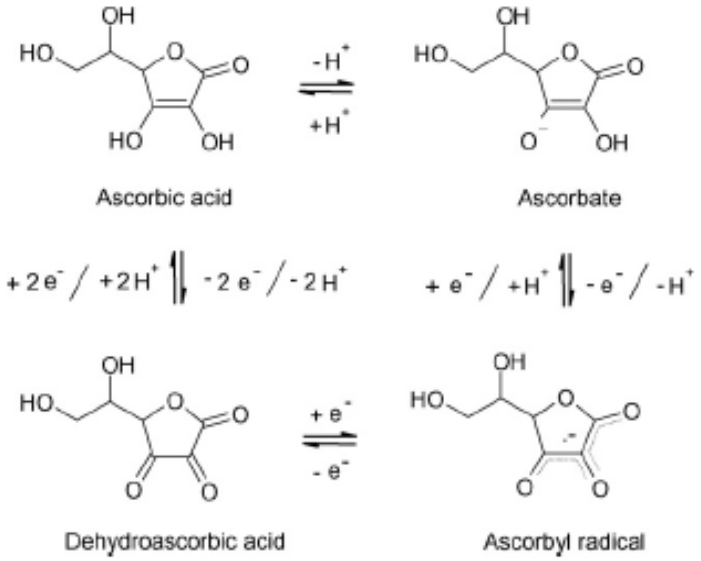


Figure 2.7: Different chemical states of Ascorbic acid (Verrax and Calderon, 2008)

2. Materials and methods

2.1 Materials:

All cell lines, including minimum essential medium Eagle with Earle's salts (MEM), RPMI-1640 medium, trypsin-EDTA, fetal bovine serum (FBS) and antibiotic cocktail (penicillin, streptomycin and fungizone) were supplied by Highveld Biological (Pty) Ltd. (Modderfontein, Johannesburg, RSA). All TPP® sterile plastic ware, cell culture flasks and plates were supplied through Separations (Pty) Ltd. (Randburg, Johannesburg, RSA). All other chemicals were of analytical grade and supplied by Sigma Chemicals Co. (St. Louis, MO, USA).

2.2 Methods:

2.2.1 Plant material

Crotalaria agatiflora subspp. *agatiflora* leaves were collected in Pretoria, South Africa in February 2009. The plant material was identified kindly on the basis of its morphological features with the help of the herbarium staff at the University of Pretoria, and a voucher specimen (PRU 096454) was deposited in the Schweickerdt Herbarium (PRU), Pretoria, South Africa.

2.2.2 Extraction

Leaves were air dried and separated mechanically. Three different leaf extracts were prepared i.e. decoction, infusion and ethanol. The air-dried leaves of *C. agatiflora* subspp. *agatiflora* (22g) were homogenised with 700mL distilled water and extracted for 24h twice. The menstruum was freeze dried to yield a dry brown powder.

For the infusion 20.401g powdered leaves were used. A vacuum rotary evaporator was used for extraction. The water bath was adjusted to 80°C. The ground leaves were added to distilled water (750mL) in a round bottom flask. The leaves were boiled for 15 minutes after which the menstruum was filtered. The menstruum was left to cool, followed by freeze-drying to yield a dry orangey-red powder.

Crotalaria agatiflora subspp. *agatiflora* leaves (1.085 kg) were exhaustively extracted with distilled ethanol (2L) at room temperature. The menstruum was filtered and concentrated under reduced pressure by a vacuum rotary evaporator (Buchi) to yield an ethanol extract (Rahman and Kang, 2009). The data that was collected was then used to determine the yield of extracts. The plant extracts were stored in the cold room (0°C) until use.

2.2.3 Cytotoxicity using XTT kit

Cells were maintained in culture flasks in Minimum Essential Medium, except U-937 cells that were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (56°C, 30min) and antibiotic cocktail (100 U/mL penicillin, 100 µg/mL streptomycin and 250 µg/L fungizone) (Highveld Biological, Jhb, South Africa). Cells were grown and maintained in a humidified atmosphere at 37°C and 5% CO_2 in a Forma Scientific water-jacketed incubator. Subculturing was done every 2-3 days after cells had formed a confluent monolayer. During subculturing, cells that attached to the culture flask were trypsinised (0.25% trypsin containing 0.01% EDTA) for 10 min at 37°C then stopped by the addition of complete medium. Suspension

cells were centrifuged and subdivided into more flasks. Cytotoxicity was measured by the XTT (Sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro) benzene sulfonic acid hydrate) method using the Cell Proliferation Kit II (Roche Diagnostics GmbH) as described by the method of Zheng *et al.* (2001). The cells (100 µl) were seeded (concentration $1x10^5$ cells/mL) into wells of a microtitre plate and incubated for 24h to allow the cells to attach to the bottom of the plate. Dilution series were made of the extracts (stock solution 20 mg/mL, 1.563-400 µg/ml) and added to the microtitre plate and incubated for 72h. The positive drug control (Actinomycin D dissolved in ddH_2O, concentrations 0.002–0.5 µg/mL) was included. Vehicle (DMSO) treated cells were also included in the assay at a single concentration of 2%. This was the highest percentage DMSO found in the dissolved samples, namely at a sample concentration of 400 µg/mL. To test samples at such high concentrations, we found that 2% DMSO was the lowest percentage one could use to dissolve the samples in. The XTT reagent (50 µl) was added to a final concentration of 0.3 mg/mL and incubated for 2-3 hours. After incubation the absorbance of the colour was spectrophotometrically quantified using a BIO-TEK Power-Wave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa), which measured the optical density at 490 nm with a reference wavelength of 690 nm. The assays were carried out in triplicate. The inhibitory concentration of 50% of the cell population (IC_{50} values) were defined as the concentration of the extracts/compounds at which absorbance was reduced by 50%. The results were statistically analyzed with the GraphPad Prism 4 software. The values of DMSO treated cells were used as 100% viable parameter. The analysis was done by selecting the sigmoidal dose response (variable slope) curve fit as well as the 95% confidence interval option. The analysis was limited to values between 0 and 100. The selectivity indexes (SI) of the extracts were defined as the ratio of cytotoxicity on normal cells to cancerous cells (Mena-Rejon *et al.,* 2008).

2.2.4 Determination of antioxidant activity DPPH radical scavenging

The method of du Toit *et al*. (2001) was followed with some modifications for the determination of antioxidant activity. Briefly the positive control, vitamin C was prepared as a 2mg/mL stock solution by adding distilled ethanol to vitamin C. The samples were prepared as stock solutions of 10mg/mL. For each sample, a dilution series was prepared in a 96-well microtitre plate by adding distilled water as a dilution medium. The concentrations tested for the plant extracts ranged between 3.906 - 500 µg/mL and the concentration of Vitamin C between 0.781 - 100 µg/mL. All the samples were prepared in triplicate. After the dilution series was complete, 90 µl DPPH (0.04 mg/mL) was added to all of the wells, except for the extract control in which the DPPH was substituted with distilled water. The plates were covered with aluminium foil and left to develop at room temperature for 30 minutes. The radical scavenger capability of the samples were determined by using an BIO-TEK Power-Wave XS multi-well plate reader (A.D.P., Weltevreden Park, South Africa) to measure the decolouration of DPPH at 515nm, using KC Junior software.

The IC_{50} values for each sample were determined by using GraphPad Prism 4 software in conjunction with Windows Excel 2000.

3. Results and discussion

3.1 Plant extracts

Large quantities of plant material were needed to obtain a substantial amount of plant extracts. The indigestible cellulose contributes towards the weight of the plant. The weights of the plant extracts and dry material were used to calculate the percentage yield obtained (Table 2.1). The formula that was used was as follows:

% Yield = (Dry extract weight / powdered plant weight) x 100

The percentage yields for the different extraction methods were similar. The yields varied between 13% and 23%. The differences in the percentage yields can be due to the amount of compounds which are able to dissolve in the different solvents. The water extracts yielded different coloured extracts. The decoction had a dark brown colour, while the infusion had a bright orange colour. The colour of the extracts could be explained partially by the different amounts of compounds extracted. The yields of these two extraction processes were similar. The infusion had a higher percentage yield, which could be due to heat applied during the extraction process.

Table 2.1: Percentage yield of plant extracts

Type of extract	Powdered weight (g)	Dry extract (g)	% Yield
Ethanol	1085	147.038	13.552
Decoction	22	4.389	19.95
Infusion	20.401	4.746	23.264

3.2 Cytotoxicity

In this preliminary experiment the anti-cancer activity of the three extracts were determined by testing on various cancerous cell lines and one non-cancerous cell line. Developed 96-well plates for cytotoxicity are illustrated in Figure 2.8. The positive control, actinomycin D, was not included in any of the graphs, because of its potent toxicity, but the toxicity was discussed for all of the cell lines. During the analysis it was found that actinomycin D had variable activity on the different cell lines, but the toxicity overall was as expected for such a potent control.

Figure 2.8: XTT developed cytotoxicity plates

3.2.1 U-937 cytotoxicity

As illustrated in Figure 2.9, the water and ethanol extracts inhibited the cell's proliferation in a dose-dependent manner. At all eight concentrations cell survival rates followed the sequence of infusion > decoction > ethanol. The ethanol extract had higher cytotoxicity against U-937 cells as compared to the two water extracts. The results revealed that the water extracts were inactive and had similar toxicities at the highest concentration tested, being relatively non-toxic to the U-937 cells. The Infusion and Decoction had IC_{50} values higher than 400 µg/mL. In contrast to those two extracts, the ethanolic extract was the most active and thus cytotoxic, having an IC_{50} value of 73.9 ± 1.06 µg/mL. Actinomycin D had an IC_{50} value of 0.002 ± 0.0001 µg/mL.

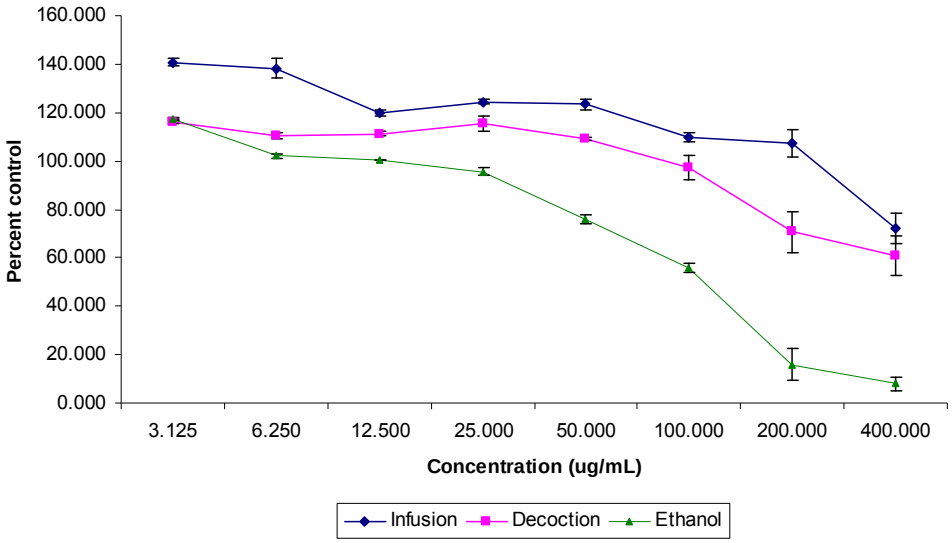


Figure 2.9: Dose-response curves of water and ethanol extracts on cancerous U-937 cells

3.2.2 HeLa cytotoxicity

Illustrated in Figure 2.10 is the dose response of HeLa cells on exposure to the different extracts. Both water extracts did not show dose-dependent responses. It was evident that both these extracts had consistent affects on the cells at different concentrations treated. Between 3.125 µg/mL and 25 µg/mL cell survival rates followed the sequence of decoction > ethanol > infusion. At 12.5 µg/mL there was a sudden decrease in cell viability for the decoction treatment. Ethanol and infusion treatments seemed to be equally active at 50 µg/mL. At approximately 150 µg/mL the decoction and infusion extracts had the same effects on cell survival rates. At 400 µg/mL the sequence of cell survival rates was as follows: Infusion > Decoction > Ethanol. Only 25% of the cells were found to be viable on exposure to the ethanolic extract at 400 µg/mL. Both the water extracts were inactive. The infusion, as well as the decoction water extracts had IC_{50} values higher than 400 µg/mL. In contrast to those extracts, the ethanol extract had a dose-dependent response. It was also clear from the graph that the ethanolic extract had the best activity and thus the lowest IC_{50} value of all the samples. The IC_{50} value was 153.9 ± 0.75 µg/mL. Actinomycin D had an IC_{50} value of 0.075 ± 0.006 µg/mL.

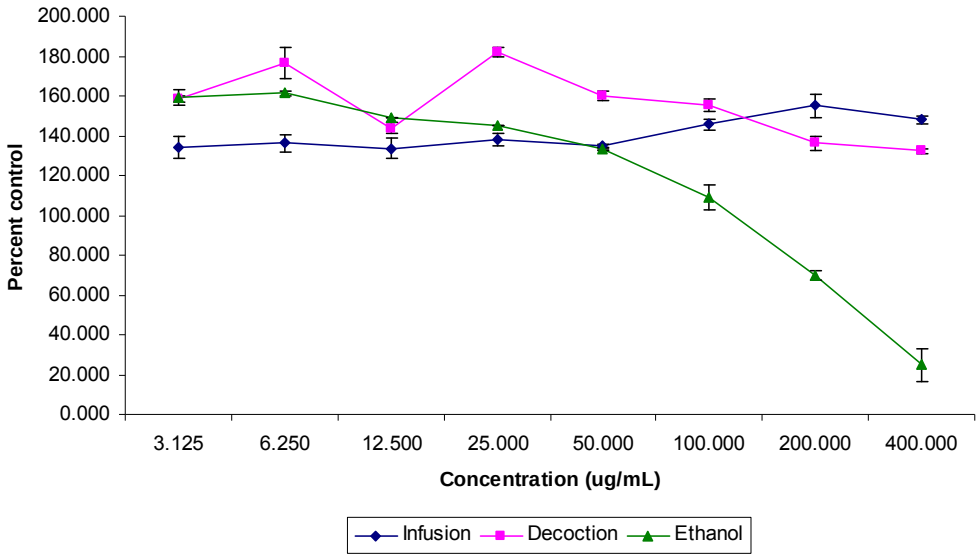


Figure 2.10: Dose-response curves of water and ethanol extracts on cancerous HeLa cells

3.2.3 MCF-7 cytotoxicity

Figure 2.11 illustrates the inhibition of proliferating MCF-7 cells during incubation with extracts. Both water extracts did not follow dose-dependent responses. The ethanolic extract had a dose-dependent affect on the cells' viability. Treatments with the infusion and decoction extracts showed very similar trends at all the concentrations tested. Between the concentration range of 3.125 µg/mL and 100 µg/mL, the sequence of cell survival rate was as follows: Infusion >

Decoction > Ethanol. At a concentration just higher than 100 µg/mL, the sequence changed to Decoction > Infusion > Ethanol up to the highest concentration tested. The cell survival rate at 400 µg/mL was found to be similar for the Decoction and Infusion treatments. The ethanolic extract caused cell survival to decrease sharply between 100 µg/mL and 400 µg/mL, reaching 9.8%. Both the water extracts were found to be inactive. The Infusion and Decoction had IC_{50} values higher than 400 µg/mL. The ethanolic extract exhibited an IC_{50} value of 233.1 ± 6.2 µg/mL. Thus making the ethanolic extract almost twice as active compared to the water extracts. Actinomycin D had an IC_{50} value of 0.15 ± 0.001 µg/mL.

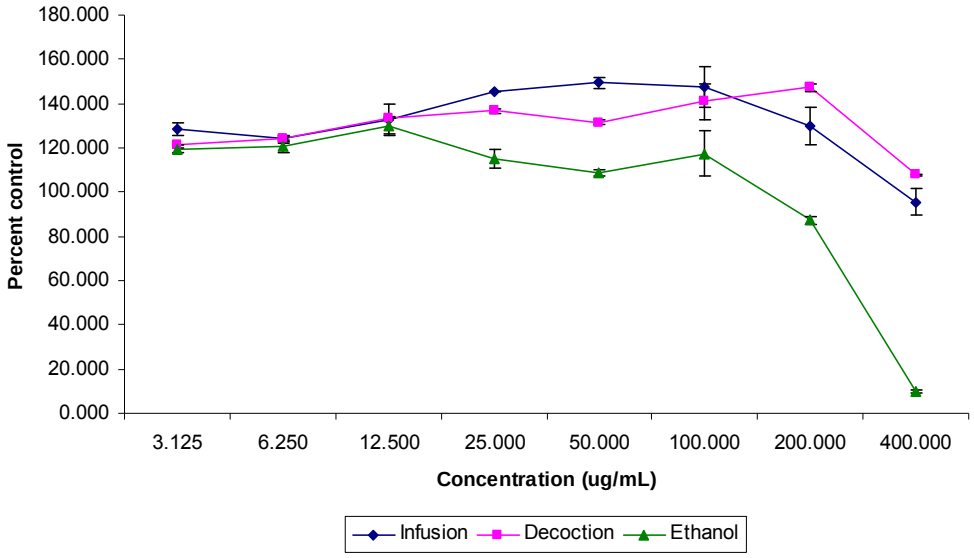


Figure 2.11: Dose-response curves of water and ethanol extract treatments on cancerous MCF-7 cells

3.2.4 SNO cytotoxicity

Cytotoxicity on SNO cells showed similar trends as the cytotoxicity exhibited by the extracts on HeLa cells (Figure 2.12). The cell survival rate was as follows between 3.125 µg/mL and 12.5 µg/mL: Ethanol > Infusion > Decoction. At 25 µg/mL the survival rate was: Infusion > Ethanol > Decoction. Between 50 µg/mL and 400 µg/mL, the survival rate was as follows: Decoction > Infusion > Ethanol. Only 6.9% of the cells could survive during the highest concentration of the ethanolic extract treatment. Both the water extract were not active up to the highest concentration tested. The decoction and infusion had IC_{50} values higher than 400 µg/mL. On the contrary the ethanolic extract had an IC_{50} value of 113.2 ± 2.43 µg/mL, suggesting that the ethanolic extract was once again the most active as compared to the water extracts. Actinomycin D had an IC_{50} value of 0.072 ± 0.004 µg/mL.

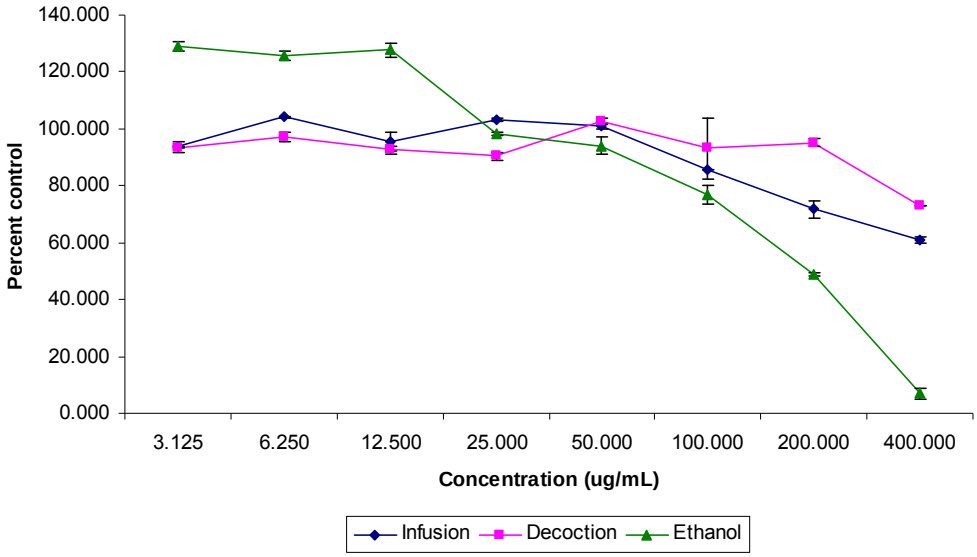


Figure 2.12: Dose-response curves of water and ethanol extract treatments on cancerous SNO cells

3.2.5 Vero cytotoxicity

As illustrated in Figure 2.13 the toxicity on the noncancerous Vero cells were found to be very low. None of the extracts tested showed good dose-dependent responses. The water extracts had similar trends at all of the concentrations tested. The ethanolic extract showed less toxicity at low concentrations, similar toxicity as the water extracts at 200 µg/mL and higher toxicity at 400 µg/mL compared to the water extracts. The cell survival rate during 400 µg/mL ethanolic extract treatment was higher than 80%. Both water extracts had IC_{50} values > 400 µg/mL. More specific values could not be determined during this study. An IC_{50} value of 352.4 ± 5.9 µg/mL was calculated for the ethanolic extract treatment. Thus these values were an indication that all of the extracts were relatively not active, and thus relatively non-toxic *in vitro*. Actinomycin D had an IC_{50} value of 0.080 ± 0.003 µg/mL.

3.2.6 Selectivity Index

The selectivity index (SI) of extracts is defined as the ratio of cytotoxicity on normal healthy cells to cancerous cells. In general it is considered that the biological efficacy is not due to cytotoxicity when the SI value is ≥ 10 (Mena-Rejon *et al*, 2008). In Table 2.2 the SI values are summarized according to the cell lines tested. Results correlated with the respective graphs. The Infusion and Decoction extracts didn't show any preference to any of the cell lines. The ethanolic extract had the best selectivity values as compared to all of the samples tested. Actinomycin D showed high SI values for U-937 cells suggesting the pure compound's toxicity was not due to cytotoxicity, but rather due to another type of mechanism, such as the induction of apoptosis by forming stable complexes with DNA and interfering with DNA-dependent RNA synthesis (Section 1.1.6).

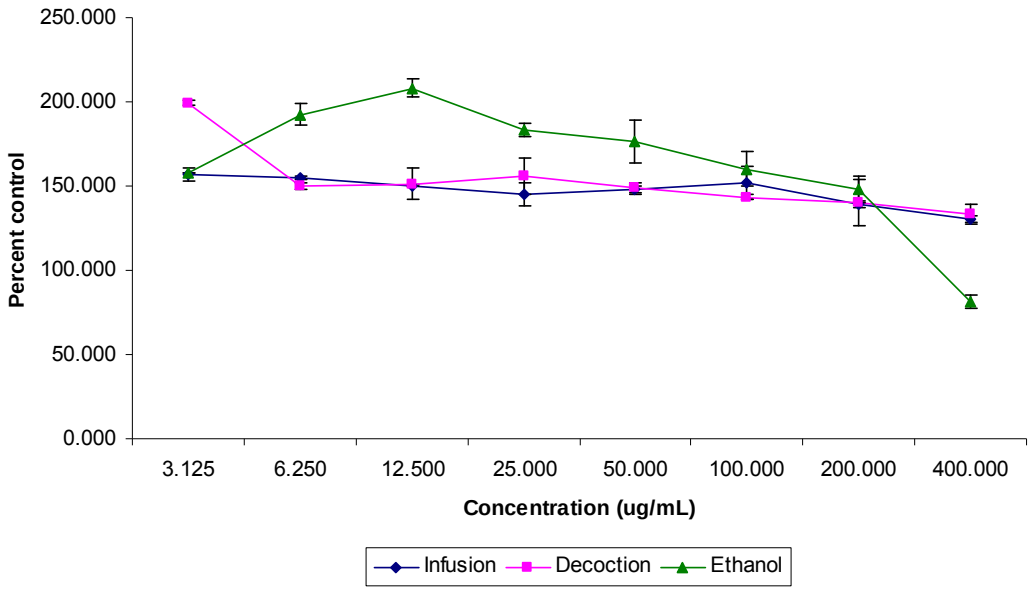


Figure 2.13: Dose-response curves of water and ethanol extract treatments on non-cancerous Vero cells

XTT salt is part of tetrazolium salt family which are metabolically reduced to intensely coloured end products. The bio-reduction of the salts is still poorly understood despite the long historical use of them in viability assays. According to Berridge *et al.* (1996) it was found that XTT in the absence of cell extract was reduced by NADH and NADPH. It was also evident that NADH-dependent reduction of XTT was inhibited by mitochondria. On the other hand succinate-dependent reduction of XTT was dependent on mitochondria. Interestingly it was found that when a colour reaction took place within a few minutes after the tetrazolium salt was added and then the reduction was caused mainly by reduced pyridine nucleotides. If the reduction took more than an hour it was likely to have been either NAD(P)H dependent reduction or succinate-dependent reduction of the salt. It was also found that superoxide was involved in reduction of XTT by NAD(P)H and NADH. Antioxidants can be determined by 3 – (4, 5 –dimethylthiazole-2-yl) – 2, 5 – diphenyltetrazolium bromide (MTT) assay (Muraina *et al.,* 2009). This assay is related to the same mechanism as XTT. This caused concern for the integrity of the results obtained. Blank plates containing the plant extract, complete medium and XTT, limited confusion and thus the antioxidative activity of the extracts was taken into account in the final analysis.

The IC_{50} value of the extract on U-937 cells was the lowest, implicating that the lowest concentration of extract will be necessary to stop proliferation of 50% of the cell population. By using the IC_{50} values it was postulated that the survival rate of the cells was MCF-7 > HeLa > SNO > U-937. The Vero cells were perceived as normal healthy cells, although these cells have been transformed to immortalize them. This was done to ensure prolonged culturing of these cells in laboratories worldwide. Vero cells were used instead of volunteer blood cells due to the controversial and ethical issues surrounding the harvesting and testing of human samples at such

early stages of research. Testing the samples on Vero cells also made comparisons easier, because it is such a popular cell line to work with.

Table 2.2: Selectivity index of extracts on several cell lines

	Selectivity Index			
Samples	**U-937**	**HeLa**	**MCF-7**	**SNO**
Infusion	~ 1	~ 1	~ 1	~ 1
Decoction	~ 1	~ 1	~ 1	~ 1
Ethanol	4.77	2.30	1.45	3.11
Actinomycin D	**40**	**1.07**	**0.67**	**1.11**

Overall the water extracts of *C. agatiflora* preformed poorly during the determination of cytotoxicity. On most of the cell lines these extracts gave similar IC_{50} values, being in most cases higher than 400 µg/mL. This inability of water extracts to kill cancerous cells at low concentrations may be due to the type of compounds extracted during the extraction process. Water is a polar molecule which in theory will then be able to be used when polar compounds are being extracted, such as sugars, amino acids and glycosides (Houghton, 2008). The difference between the infusion and decoction is that during the brewing of the infusion process, heat was applied to the infusion procedure. Heat causes the plant material to heat up which could have accommodated the release of more compounds within the material. On the other hand cold water was used to prepare the decoction extract, thus increasing the time needed to extract the similar amounts of compounds. At the end it was determined that using water as extraction solvent for *Crotalaria* leaves had very little anti-cancer activity. These findings are in contrast with the traditional uses of *Crotalaria spp.* in Ecuador for the use of fresh leaves that are infused and used to treat cancers (Tene *et al*., 2007).

In China a variety of *Crotalaria spp.* are used for treating cancers. Unfortunately little information for preparation of extracts for treatments had been documented. The dictionary of natural products reports on the isolation of compounds from *Crotalaria spp.* (Appendix B). These compounds include alkaloids, non-protein amino acids, flavonoids, flavones, amino acids, pyrrolizidine alkaloids, stilbene polymers, monoterpenoids, isolavones, oligosaccharides, edible fats and oils and cardenolide steroids. Ethanol is a very good extractant, thus it can be postulated that alkaloids and pyrrolizidine alkaloids may have caused the cytotoxicity of the tested cells. *Crotalaria* is known to have high concentrations of alkaloids (Graham *et al*., 2000). Alkaloids are responsible for the fitness of the organisms which produce them. These secondary compounds are used to deter, repel and inhibit a wide variety of predators and pathogens. In many cases one alkaloid may have more than one biological function. For the organism (plants, microorganisms, marine animals) to

protect themselves they need to have secondary compounds that interfere with important cellular and molecular targets. On a cellular level alkaloids may interact with several structures of the cytoskeleton by binding to microtubules and actin filaments. Alkaloids, on a molecular level can interact with DNA, RNA and associated enzymes and processes. More specifically alkaloids could be alkalyting or intercalate into DNA, may interfere with DNA topoisomerase I or II and inhibit telomerase. Other mechanism of cytotoxicity includes the induction of apoptosis through activation of the extrinsic or intrinsic pathways (Wink, 2007).

Looking at the SI values of the samples, one can hypothesize that the ethanolic extract of *C. agatiflora* was the best candidate for further investigations. Although Mena-Rejon and his colleagues (2008) suggested that when the SI value is lower than 10, the sample is cytotoxic, it didn't mean that the mechanism of action would be less interesting. The ethanolic extract had the highest SI value against U-937 cells, as compared to the other extracts and against the other cell lines. Actinomycin D had very high SI values for U-937 and SNO cells. Unfortunately the results of the extracts couldn't be compared to Actinomycin D, because Actinomycin D is a pure compound, while the extracts were a unique unknown combination of compounds. In some cases it has been found that pure compounds have much better activity compared to extracts due to the one specific mechanism of action. On the contrary it is also known that some compounds work synergistically or additive, thus being more active in combination with each other than the activity of the two or more compounds alone (Wink, 2007). It could thus be postulated that either the plants being used in other parts of the world has very different chemical profiles or that those traditionally used plants have anti-cancer applications in the long term. If the second scenario is true than *Crotalaria spp.* have the potential of treating cancer with less side effects than current conventional chemotherapies.

Experiments done on HepG2 (larynx) cells using MTS tetrazolium (Owen's reagent) by Riss and Maravec (2004), it was found that cells treated with tamoxifen citrate showed similar dose and time dependent toxicity trends. Tamoxifen is a naturally derived compound isolated from the Pacific Yew (*Taxus brevifolia*). The IC_{50} values were lower when the treatment exposure time was increased. Riss and Maravec (2004) found that the density of cells in the parent stock solution as well as the concentration cells plated had an impact on the toxicity observed. It was found that cells were more responsive to tamoxifen when the cells were harvested from lower density grown parent stocks and when the cells were seeded in lowered concentrations. During the present study's cytotoxicity assays, cells were grown to very high confluency before they were used in the assays. During Hons. studies (2008), due to time restrictions, cells were grown to sub-optimal confluence before they were used. This could explain the increase in IC_{50} values for samples tested against HeLa cells during 2008 and 2009.

Comparing the anti-cancer activity of the leaf extract of *C. agatiflora* with the other traditionally used plants in South Africa, similarities in toxicities were found. Steenkamp and Gouws (2006) tested six traditionally used plant extracts on four cell lines. The samples were *Sutherlandia frutescens, Bidens pilosa, Hypoxis hemerocalledea, Centella asiatica, Cnicus benedictus* and *Dicoma capensis* extracted with methanol. The four cell lines were DU-145 (prostate carcinoma), MDA-MB-231 (breast cancer cells), MCF-7 (breast cancer cells) and MCF-12A (noncancerous breast cells). The samples were tested at only one concentration i.e. 50 µg/mL. *Crotalaria* samples were also tested at 50 µg/mL and thus its general anti-cancer activity be compared to those traditionally used plants. In Table 2.3 growth percentages are given for cancerous and non-cancerous cell lines at 50 µg/mL. The average percentage growth of the samples were calculated (three cell lines for traditionally used plants and four cell lines for *Crotalaria*), except for noncancerous cells in which case only one cell line's data was available for each as described above. Thus at 50 µg/mL it was found that *Crotalaria agatiflora* induced similar growth percentages as other traditionally used plants in South Africa. The noncancerous cells treated with ethanolic extract of *Crotalaria* was the least affected by treatment compared to other samples.

Crotalaria's cytotoxicity was quite similar to that of *Sutherlandia* (Table 9). *Sutherlandia* is well known for having anti-cancer activity and stimulating the immune systems' effectiveness. Numerous experiments had been conducted by researchers on *Sutherlandia frutescens* (Tai *et al.,* 2004, Chinkwo, 2005 and Stander *et al.,* 2009). Tai and colleagues (2004) conducted cytotoxicity of purchased plant extract tablets from PhytoNova. The tablets were extracted with 70% ethanol. The cell lines that were tested included MCF-7, MDA-MB-468, HL60 (human promyelocytes), RAW 264.7 (murine monocytic cells) and Jurkat (human leukemic cells). Concentrations tested for cytotoxicity was between 113.6 µg/mL – 1.36 mg/mL and the method was trypan blue exclusion assay, which is rapid and inexpensive, but not the most accurate and sensitive assay available. They found that MCF-7 was the most sensitive cell line to the extract and Jurkat cells were the least sensitive cell line. Interestingly, the opposite is true for the cell lines treated with *Crotalaria* ethanolic extract (SI values, Table 2.3). It was estimated that the IC_{50} value for *Sutherlandia* treated MCF-7 cells was approximately 505.4 µg/mL and for Jurkat cells 909.1 µg/mL. Comparing these values to values obtained for *Crotalaria* treated cells; it seemed as if *Crotalaria* was a more effective therapeutic candidate compared to *Sutherlandia*. Results for the ethanol extracted plant samples for the two different species might not be in agreement with each other due to the fact that the two plants have very different chemical profiles and that the molecular mechanism of action might be different in the two species. It had been found that *Sutherlandia* was able to induce apoptosis at high concentrations, thus making it of interest to determine what the mechanism of action will be for the more cytotoxic *Crotalaria* spp.

Table 2.3: Average percentage growth in vitro for traditionally used plants for the treatment of cancer at 50 µg/mL (Steenkamp and Gouws, 2006)

Sample	Average percentage growth	
	Cancerous cells	Noncancerous cells
Sutherlandia frutescens	100 ± 26.5	125
Bidens pilosa	90 ± 5	105
Hypoxis hemerocalledea	96.7 ± 30.5	130
Centella asiatica	109.3 ± 11	115
Cnicus benedictus	90 ± 10	90
Dicoma capensis	62.3 ± 32.5	35
Crotalaria agatiflora subspp agatiflora	**101.3 ± 21.7**	**170**

The two plant species were in agreement based on the fact that ethanol extracts had shown to be much more active that water extracts, especially referring to infusions. This agreement was established by the findings of Chinkwo (2005) and Stander *et al.* (2009) who focussed on the cytotoxicity and anti-cancer activity of *Sutherlandia* water extracts. Cells were treated in both scenarios with extracts at a concentration range of 1.5 – 10 mg/mL. Cell lines treated included CHO (Chinese hamster ovary) cells and Caski (cervical carcinoma) cells (Chinkwo, 2005), MCF-7 cells and MCF12A (noncancerous breast) cells (Stander *et al*., 2009). Cell viability was determined through ApoPercentage dye assay (Chinkwo, 2005) and crystal violet staining (Stander *et al*., 2009). It was determined that the IC_{50} value was about 3.5mg/mL (Chinkwo, 2005) while Stander *et al.* (2009) roughly calculated the IC_{50} value to be between 5 – 10 mg/mL. In both these studies the IC_{50} values were much higher than those obtained from water extracts of *Crotalaria* (Section 3.2). The reason why the results differ between concentrations tested by Chinkwo (2005) and Stander *et al.* (2009) might be due to the fact that different subspecies were used (Chinkwo didn't mention full classification of species) and Stander *et al.* (2009) autoclaved the samples collected before extraction, thus heat unstable compounds might have been lost during that process, giving higher approximate IC_{50} values.

Similar studies were done on *B. pilosa* and *C. asiatica*, which are also used traditionally to treat cancer. The average percentage growth as stipulated in Table 2.3 for *C. asiatica* was 109.3% when different cancerous cell lines were tested at 50 µg/mL. This observation is very similar to the one observed for *C. agatiflora* in the present study, although *C. agatiflora* inhibited the average percentage growth more effectively. It was also evident that *C. agatiflora* was less toxic to noncancerous cell lines by promoting cell proliferation by 170%. When tryphan blue exclusion assay was performed on Ehrlich ascites tumour cells (EAC) and Dalton's lymphoma ascites tumour cells (DLA), it was found that the IC_{50} values were 62 µg/mL and 75 µg/mL respectively after 7

days of treatment with methanol crude (whole plant) extract of *C. asiatica* (Babu *et al.,* 1995). It can be confirmed that cytotoxicity of *C. asiatica* compares well with the cytotoxicity of the ethanol extract of *C. agatiflora* (IC_{50} = 73.9 µg/mL) on similar types of DLA and U-937 cells. The only difference is time of incubation. Longer times of incubation could either have had a positive or negative effect on the results. The positive effect is that due to extra time cells are much longer exposed to the toxin and thus will be more susceptible to die (especially without medium replacement, which are not mentioned), while on the other hand prolonged exposure might give indications of recuperation of the cells, thus after the standard 72 h incubation the IC_{50} value could have been lower. Noncancerous cells (NHL cells – normal human lymphocytes) tested by Babu *et al.,* (1995) and Vero cells tested in the present study both showed to be relatively non-toxic.

Bidens pilosa overall inhibited cell growth the most effectively at 50 µg/mL on different cell lines, although the toxicity was not very selective to cancerous cells (Table 2.3). Kviecinski *et al.* (2008) investigated the anti-tumour activity of *B. pilosa*, by extracting the different chemical constituents with different solvents. Solvents that were used was hydroalcoholic solvent mixture, chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol (MetOH). The cytotoxicity was determined using MTT. Cells were only exposed to the sample for 24h (not the standard 72h) and were plated at much lower cell concentrations than what was used in the present study. Lower concentrations of cells will increase the toxicity of the sample due to more available molecules to interact with the cells (Riss and Maravec, 2004). Cells tested used for that experiment was also EAC cells as mentioned in the previous paragraph. None the less when comparing the results with the present study, it was found that *B. pilosa* was non-toxic to the cells when the extract was prepared with alcohol. The only active extract was the $CHCl_3$ extract, which had an IC_{50} value of 97.0 ± 7.2 µg/mL. Comparing the data with *C. agatiflora* ethanolic extract results it was evident that *C. agatiflora* (range of IC_{50} values between 73.9 – 233 µg/mL for the different cell lines) is much more active compared to methanol extract of *B. pilosa* (IC_{50} > 1000 µg/mL).

3.3 Radical Scavenging Potential

The antioxidant assay (microtitre plate for the ethanolic extract) and the DPPH reduction on exposure to the different concentrations of extracts (infusion, decoction and ethanol) of *C. agatiflora* are illustrated in Figure 2.14 and 2.15.

Figure 2.14: DPPH free radical scavenging activity of ethanol extract including the positive control (Vitamin C)

All three samples (infusion, decoction and ethanolic) demonstrated dose-dependent responses. Both water extracts showed nearly identical capacity of DPPH reduction. At 500 µg/mL and 250 µg/mL all the extracts showed similar activity. The ethanolic extract showed higher activity between 125 µg/mL and 15.625 µg/mL as compared to the water extracts. At the two lowest concentrations tested, all three extracts showed similar activity. The IC_{50} values found were as follows: Ethanolic extract 18.89 ± 0.305 µg/mL, Decoction 27.31 ± 1.59 µg/mL and Infusion 29.63 ± 1.59 µg/mL. The ethanolic extract was thus the most active and effective sample for free radical scavenging. Vitamin C, the positive control had an IC_{50} of 1.368 ± 0.001 µg/mL.

Previous studies done by Fernandes *et al.* (2004) demonstrated that *S. frutescens* had very good anti-oxidant activity. Unfortunately the results obtained in that study was not comparable to the current study due to the nature of the tests conducted. Briefly they found that the hot water extract (infusion) had potent antioxidative activity by scavenging neutrophil derived oxidants effectively at concentrations as low as 10 µg/mL for superoxide and 2.5 µg/mL for hydrogen peroxide / horseradish peroxide mediated chemi-luminescence. The comparisons between these assays and more commonly used assays have not yet been established. Thus it will be interesting to see comparative studies on the different assays and what effect *C. agatiflora* will have on enzymatic assays usings neutrophils.

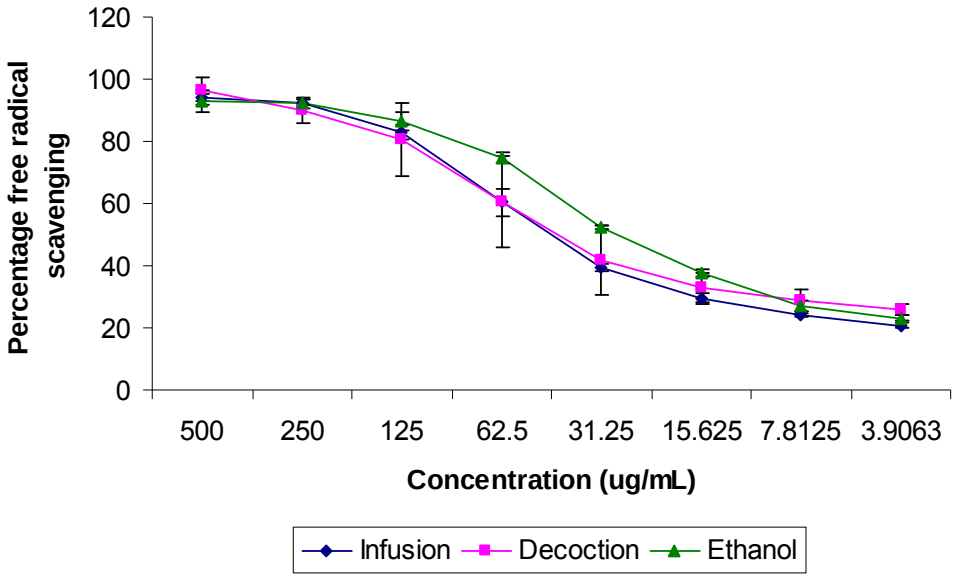


Figure 2.15: Free radical scavenging capability of Ethanol, Infusion and Decoction extracts using DPPH assay

Samples of *C. asiatica* were extracted with 100% MeOH for 24h and antioxidant activity was measured using two assays namely Ferric thiocyamate (FCT) method and the Thiobarbituric acid (TBA). No IC_{50} values were calculated, thus making comparisons very difficult. Zainol *et al.* (2003) determined that there was a positive correlation between the amount of phenolics and the antioxidant activity (at least for *C. asiatica*). They found that the leaves extract contained a high amount of phenolic compounds (8.13 -11.7 g / 100 g plant material). It was also found that leaves extracts had similar activity compared to α-tocopherol which exhibited an IC_{50} value of 8.97 µg/mL, thus implicating that the activity of *C. asiatica* might be in an approximate range of 8.97 µg/mL (Chiang *et al.,* 2004). This results implicated that *C. agatiflora* in the present study was less active than *C. asiatica*.

In previous studies conducted on *B. pilosa* it was found that the ethanol extract had an IC_{50} value of 80.93 µg/mL using the DPPH assay (Chiang *et al.,* 2004). The free radical scavenging properties of this plant is not as significant as compared to the positive controls, ascorbic acid (6.34 µg/mL) and α-tocopherol (8.97 µg/mL). Many other crude extracts had been tested previously for their antioxidant activity, as reported briefly by Drewes *et al.* (2008). It was found that *H. hemerocallidea* extract; another traditionally used plant of South Africa had an IC_{50} value of 75 µg/mL when it was determined by TBA assay. It had been reported that olive leaf oil has an IC_{50} value of more than 30 µg/mL, while green tea has an IC_{50} value of 16 µg/mL. Comparing all of the above mentioned results with *C. agatiflora*, it is clear that *C. agatiflora* had better antioxidant activity than *B. pilosa* and *H. hemerocallidea*. On the other hand the water extracts of *C. agatiflora* had similar antioxidant potential as olive leaf extracts, while the ethanolic extract had similar antioxidant activity as compared to green tea.

Phenolic compounds, such as flavonoids and derivatives thereof have very good antioxidant activity due to the fact that these compounds have hydrogens to donate to the oxidative agent (Verrax and Calderon, 2008). It has been found that hydroxyl groups may dissociate in physiological conditions to form phenolate ions. These ions can form ionic bonds with positively charged amino acids, such as the residues of lysine, arginine and histidine (Wink, 2007). When ionic bonds are formed with amino acids the secondary as well as tertiary structure of proteins *in sito* (in side of the cells) may be jeopardized, causing essential enzymes or proteins to lose their functions. These findings together with the controversial concepts surrounding antioxidants had given enough reason to believe that antioxidants should rather be used as chemo-preventative agents, instead of forming part of chemotherapy, due to the fact that it might be antagonistic to oxidative chemotherapy drugs. Most chemotherapy drugs are inducing the production of reactive oxygen species within the human body, thus forming an important part of the mechanism of action of many of these drugs such as doxorubicin. Thus the question should be asked whether plant extracts could have the ability to be very cytotoxic and at the same time have protective properties such as good antioxidant potential. Considering the present study it seems as if this is impossible which can also be theoretically confirmed. When compounds are toxic they are more than likely to cause to some extent damage to all cells (the degree might differ due to selectivity) and thus highly unlikely that the plant extract will simultaneously contain compounds to protect cells and reduce reactive oxidative species induced by the same sample.

4. Conclusion:

During this investigation it was found that the ethanol extract of *C. agatiflora* was evidently more active with regards to both cytotoxicity and antioxidant activity as compared to water extracts of *C. agatiflora*. The ethanolic extract seemed to have potential for chemo-preventative effect and to a lesser extent anti-cancer effect. The cytotoxicity of the ethanol extract may be due to a variety of mechanisms, thus arousing the curiosity of the precise mechanism of action of the ethanolic extract. Cytotoxicity of *C. agatiflora* was comparable and in some cases better than traditionally used plants in South Africa used for treating cancer.

5. References:

Ames, B.N. 1998. Micronutrients prevent cancer and delay aging. *Toxicology Letters* **102:** 5-18.

American Tissue Culture Collection. 2009. “Cell lines and hybridomas.” [Online]. Available: http://www.atcc.org/CulturesandProducts/CellBiology/CellLinesandHybridomas/tabid/169/Default.aspx [Cited 10 June 2009].

Babu, T.D., Kuttan, G. and Padikkala, J. 1995. Cytotoxic and anti-tumour properties of certain taxa of *Umbelliferae* with special reference to *Centella asiatica* (L.) Urban. *Journal of Ethnopharmacology* **48**: 53-57.

Berridge, M.V., Tan, A.S., McCoy, K.D. and Wang, R. 1996. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica* **4**: 14 – 19.

Biosynth- Biology and Chemistry. 2006. "Diagnostic reagents: XTT formazan salt." [Online]. Available: http://www.biosynth.com/index.asp?topic_id=97&g=19&m=152&cat_id=1189 [Cited 15 June 2009].

Chiang, Y-M., Chuang, D-Y., Wang, S-Y., Kuo, Y-H., Tsai, P-W. and Shyur, L-F. 2004. Metabolite profiling and chemopreventative bioactivity of plant extracts from *Bidens pilosa*. *Journal of Ethnopharmacology* **95**: 409-419.

Chinkwo, K.A. 2005. *Sutherlandia frutescens* extracts can induce apoptosis in cultured carcinoma cells. *Journal of Ethnopharmacology* **98:** 163-170.

Drewes, S.E., Elliot, E., Khan, F., Dhlamini, J.T.B. and Gcumisa, M.S.S. 2008. *Hypoxis hemerocallidea* – Not merely a cure for benign prostate hyperplasia. *Journal of Ethnopharmacology* **119**: 593-598.

Du Toit, R., Volsteedt, Y. and Apostolides, Z. 2001. Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. *Toxicology* **166**: 63-69.

Fang, J, Seki, T. and Maeda, H. 2009. Therapeutic strategies by modulating oxygen stress in cancer and inflammation. *Advanced Drug Delivery Reviews* **61**: 290-302.

Fernandes, A.C., Cromarty, A.D., Albrecht, C. and Jansen van Rensburg, C.E. 2004. The antioxidant potential of *Sutherlandia frutescens*. *Journal of Ethnopharmacology* **95**: 1-5.

Graham, J.G., Quinn, M.L., Fabricant, D.S. and Farnsworth, N.R. 2000. Plants used against cancer – an extension of the work of Jonathan Hartwell. *Journal of Ethnopharmacology* **73**: 347-377.

Health24. 2007. Types of cancer prevalent in South Africa. [Online]. Available: http://www.health24.com/medical/Condition_centres/777-792-802-1626,14701.asp [Cited 23 February 2008].

Houghton, P. 2008. Fractionation of crude extracts: techniques and choices. Workshop – Scientific validation of South African plants with medicinal value workbook.

Kviecinski, M.R. Felipe, K.B., Schoenfelder, T., De Lemos Wiese, L.P., Rossi, M.H., Goncalez, E., D' arc Felicio, J., Filhno, D.W. and Pedrosa, R. C. 2008. Study of the antitumor potential of *Bidens pilosa* (Asteraceae) used in Brazilian folk medicine. *Journal of Ethnopharmacology* **117**: 69-75.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C.A., Krieger, M., Scott, M.P., Zipursky, L. and Darnell, J. 2005. Molecular Cell Biology. 5th ed. New York: Freeman and Co.

Mena-Rejon, G., Caamal-Fuentes, E., Cantillo-Ciau, Z., Cedillo-Rivera, R., Flores-Guido, J. and Moo-Puc, R. 2008. *In vitro* cytotoxic activity of nine plants used in Mayan traditional medicine. *Journal of Ethnopharmacology* doi: 10.1016/j.jep.2008.11.12.

Muraina, I.A., Suleiman, M.M. and Eloff, J.N. 2009. Can MTT be used to quantify the antioxidant activity of plant extracts? *Phytomedicine* **16**: 665-668.

Natural Solutions. 2008. "Antioxidant / Anti-aging." [Online]. Available: http://www.naturalsolution.co.kr/tech21e.html [Cited 8 June 2010].

Rahman, A. and Kang, S.C. 2009. *In vitro* control of food-borne and food spoilage bacteria by essential oil and ethanol extracts of *Lonicera japonica* Thunb. *Food Chemistry* **116:** 670-675.

Reich, E., Franklin, R.M., Shatkin, A.J. and Tatum, E.L. 1962. Action of Actinomycin D on animal cells and viruses. *Biochemistry* **48**: 1238-1245.

Riss, T.L. and Maravec, R.A. 2004. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay and drug development technologies* **2**: 51 – 62.

Roche. 2005. Cell Proliferation Kit II (XTT). CAT number: 11 465 015 001.

Russo, G.L. 2007. Ins and outs of dietary phytochemicals in cancer chemoprevention. *Biochemical pharmacology* **74**: 533 – 544.

Sigma. 2004. "Product Information – Actinomycin D." [Online]. Available: http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=A9415|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC [Cited 12/02/2010].

Stander, A., Marais, S., Stivaktas, V., Voster, C., Albrecht, C., Lottering M-L. and Joubert, A.M. 2009. *In vitro* effects of *Sutherlandia frutescens* water extracts on cell numbers, morphology, cell cycle progression and cell death in a tumorigenic and a non-tumorigenic epithelial breast cell line. *Journal of Ethnopharmacology* **124**: 45-60.

Steenkamp, V. and Gouws, M.C. 2006. Cytotoxicity of six South African medicinal plant extracts used in the treatment of cancer. *South African Journal of Botany* **72**: 630-633.

Tai, J., Cheung, S., Chan, E. and Hasman, D. 2004. In vitro culture studies of *Sutherlandia frutescens* on human tumor cell lines. *Journal of Ethnopharmacology* **93**: 9-19.

Tene, V., Malagon, O., Finzi, P.V., Vidari, G., Armijos, C. and Zaragoza, T. 2007. An ethnobotanical survey of medicinal plants used in Loja and Zamora-Chinchipe, Ecuador. *Journal of Ethnopharmacology* **111**: 63-81.

Verrax, J. and Calderon, P.B. 2008. The controversial place of vitamin C in cancer treatment. *Biochemical pharmacology* **76**: 1644-1652.

World Health Organization. 2008. "WHO Global Database: Stop the global epidemic of chronic disease." [Online]. Available: http://www.who.int/infobase/report.aspx?rid=126 [Cited 12 May 2008].

Wink, M. 2007. Molecular modes of action of cytotoxic alkaloids: from DNA intercalation, spindle poisoning, topoisomerase inhibition to apoptosis and multiple drug resistance. *The Alkaloids: Chemistry and Biology* **64**: 1-47.

Zainol, M.K., Abd-Hamid, A., Yusof, S. and Muse, R. 2003. Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of *Centella asiatica* (L.) Urban. *Food Chemistry* **81**: 575-581.

Zheng, Y.T., Chan, W.L., Chan, P., Huang, H. and Tam, S.C., 2001. Enhancement of the antiherpetic effect of trichosanthin by acyclovir and interferon. *FEBS Letters* **496**: 139–142.

CHAPTER 3: BIOASSAY-GUIDED FRACTIONATION OF *C. AGATIFLORA* SUBSPP. *AGATIFLORA*

1. Introduction

Isolation of compounds from active crude extracts is necessary to determine the identity of compounds responsible for bioactivity. Other compounds present within the crude extract might increase the toxicity of the active compounds, have side effects or even decrease the activity of the active compounds. Standardization of crude extracts within herbal formulations is required before these formulations can be sold commercially. Active compounds' structures can be elucidated and the mechanism of action can be more easily predicted and determined. Chromatography is the most commonly used method to isolate compounds from crude extracts. Based on the evaluation of cytotoxicity of different extracts of *Crotalaria agatiflora*, an ethanolic extract of the plant material was selected for further evaluation. Different chromatographic techniques were used for the identification of bio-active compounds (Figure 3.1). *Crotalaira* species are rich in alkaloids and alkaloids are known to have anti-cancer activity. A number of alkaloidal compounds from plants are anti-cancer agents, such as vincristine and vinblastine etc. The focus of the present study was to isolate alkaloidal compounds from *C. agatiflora*.

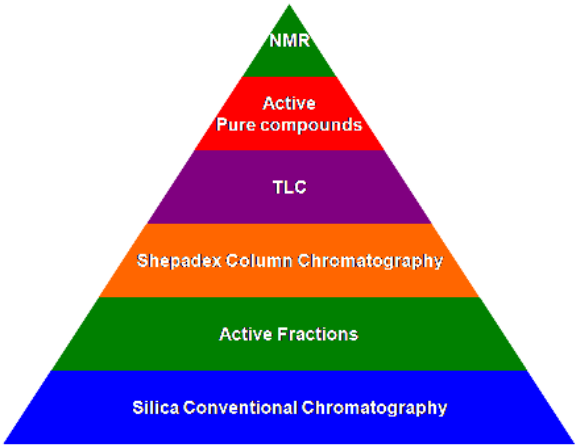


Figure 3.1: Methodology – isolation and identification of pure compounds from extracts

2. Materials and methods

2.1 Materials:

All solvents used were purchased from Merck (Germany) as well as silica TLC plates. Vanillin powder and sephadex were supplied by Sigma-Aldrich (St. Louis, MO, USA). Acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE) from eels (type VI-S lipophilized powder), 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and galathamine were bought at Sigma-Aldrich (St. Louis, MO, USA).

2.2 Methods:

2.2.1 Bioassay-guided isolation of bioactive constituents

A total of 50 g ethanolic extract was subjected to liquid-liquid partition (Figure 3.2). The extract was dissolved in 3.4 L methanol (80%). The filtrate was acidified using 5% HCl. The solution was shaken twice with dichloromethane (DCM) and then the ammonia solution (NH_4OH) was added to the aqueous solution till pH~12.0. The basic alkaline aqueous solution was shaken twice again with DCM. The DCM fractions were collected and concentrated using a rotavapor. Approximately 16.2 g alkaloidal fraction was subjected to silica gel column chromatography (CC, size 10 x 20 cm) using DCM/ MeOH mixtures of increasing polarity (0% - 10%). A total of 40 fractions (250 mL) were collected (Figure 3.3). The fractions were pooled based on their thin layer chromatography (TLC) profile, which led to 8 main fractions. Eight fractions were developed again using TLC; chloroform ($CHCl_3$): MeOH: NH_4OH (15:85:0.1) as an eluent (Figure 3.4). Cytotoxicity assays was carried out against U-937 and non-cancerous Vero cells, as previously described in Chapter 2, Section 2.2.2, with the exception that the fractions were tested only at 50 µg/mL. Leukemic U-937 cells were selected as the primary cancer cells due to the high sensitivity during the crude ethanolic extract treatments compared to the other cell lines. No further work on Fraction 1 (45mg), Fraction 2 (<1mg), Fraction 5 (60mg) and Fraction 6 (68mg) was done due to the small quantities of each fraction. Fraction 8 (461 mg) had the weakest activity on U-937 cells and was thus not further investigated in the present study.

Figure 3.2: Liquid-liquid partition of the ethanolic extract of *C. agatiflora*

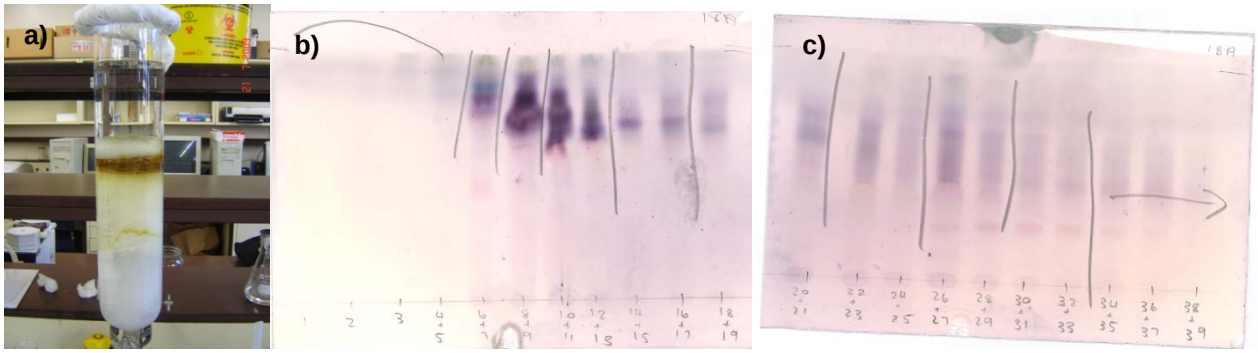


Figure 3.3: Silica column of total alkaloidal fraction (a) and corresponding TLC plates (b and c)

Fraction 3 (215.4 mg) was subjected to sephadex column chromatography (CC, 4 x 15 cm) using EtOH as an eluent. Aliquots of 20 mL were collected and a total of 37 fractions were collected. The collected fractions were spotted on TLC plates and developed using $CHCl_3$: MeOH: NH_4 (95: 5: 0.1) as eluent. After the TLC plates were analyzed under UV, similar fractions were combined, dried and weighed, which resulted in three major subfractions. Subfraction 3.3 contained only three major compound bands on the investigated TLC plate. Subfraction 3.3 was further purified using preparative TLC. Thirty milligram of Subfraction 3.3 was spotted on three TLC plates and developed using $CHCl_3$: MeOH: NH_4OH (95: 5: 0.1) as eluent. Three different bands were observed under UV which was scratched off the aluminium plates using a blade. The silica gel powder was eluted twice with distilled ethyl acetate and three times with distilled MeOH. The compounds were dried and weighed. The structural elucidation of isolated compound (only Band III, 24 mg) was identified by physical (mp. $[\alpha]_D$) and spectroscopic (^1H and ^{13}C NMR) data (Compound I).

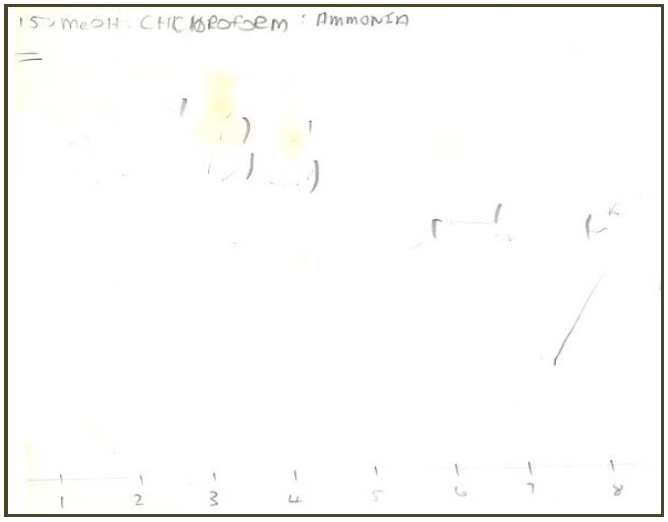


Figure 3.4: Developed plate of 8 main fractions

Fraction 4 when dried yielded white crystalline compound which was washed first with ethyl acetate: hexane (50:50), followed by methanol (100%). The pure crystals obtained were then dissolved in chloroform, spotted on a TLC plate and developed using $CHCl_3$: MeOH: NH_4OH (95: 5: 0.1). The impure crystals before washing and after washing were developed on a TLC plate (Figure 3.5). The precipitated crystals were developed on TLC and showed one clear spot; hence this compound was subjected to NMR analysis (Compound II). A synopsis of the methodology is illustrated in Figure 3.6.

Figure 3.5: Constituents of Fraction 4

Cytotoxicity assays were carried out for the isolated compounds against cancerous U-937 cells and non-cancerous Vero cells. Compounds were tested at eight concentrations ranging from 0.781 - 100 µg/mL. Antioxidant activity was also conducted as previously described in Chapter 2, Section 2.2.4, with the exception that the compounds were tested at concentration ranging between 0.781 - 100 µg/mL.

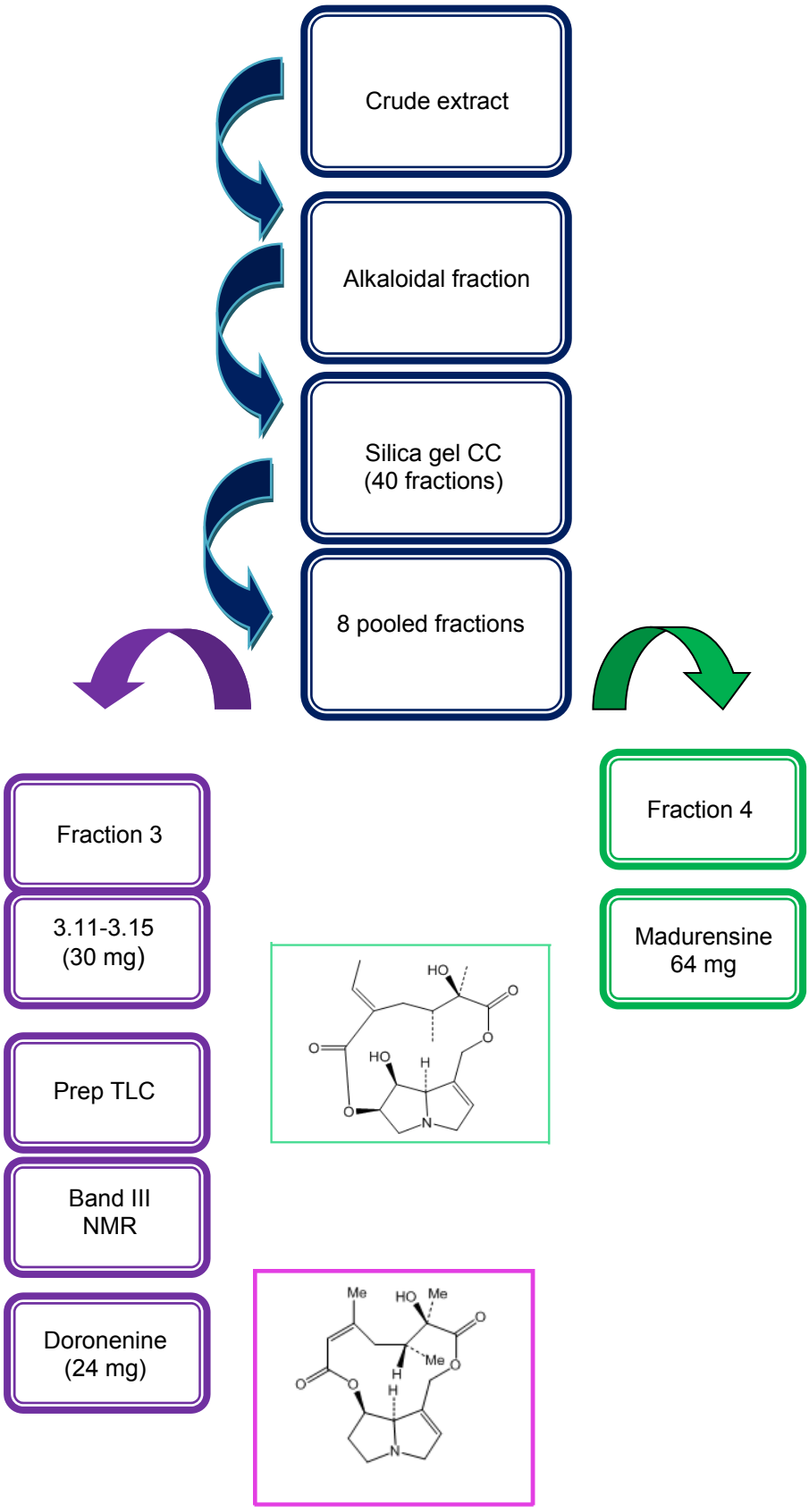


Figure 3.6: Bioassay guided fractionation of *Crotalaria agatiflora*

3. Results and discussion

3.1 Bioassay-guided isolation of bioactive constituents

Two compounds were isolated, both belonging to "pyrrolizidine alkaloids". Compound 1 was isolated from the total alkaloidal fraction using silica column chromatography (Fraction 3). The compound was identified as doronenine (1,2 – Dihydro bulgarsenine), based on NMR data (^{1}H and ^{13}C). The data stipulated in Figure 3.7 and Appendix C is applicable with the given structure and similar with those reported for the same compound in literature (Roder *et al.,* 1980). The ^{1}H NMR of doronenine showed H-18 quartet at δ 0.93 (J = 6.5 Hz). The spectrum also showed three methyl groups at δ 24.6, 11.7 and 28.3 for methyls 17, 18 and 19 respectively. The ^{13}C NMR with DEPT-135 confirmed the structure of compound I as doronenine, which showed 18 carbons of which three were methyls, five CH_2 groups and three CH groups. This is the first report of doronenine being isolated from *C. agatiflora* subspp *agaftiflora*. This compound has been previously isolated from *Senecio doronicum* (Compositae) (Dictionary of Natural Products, 2010).

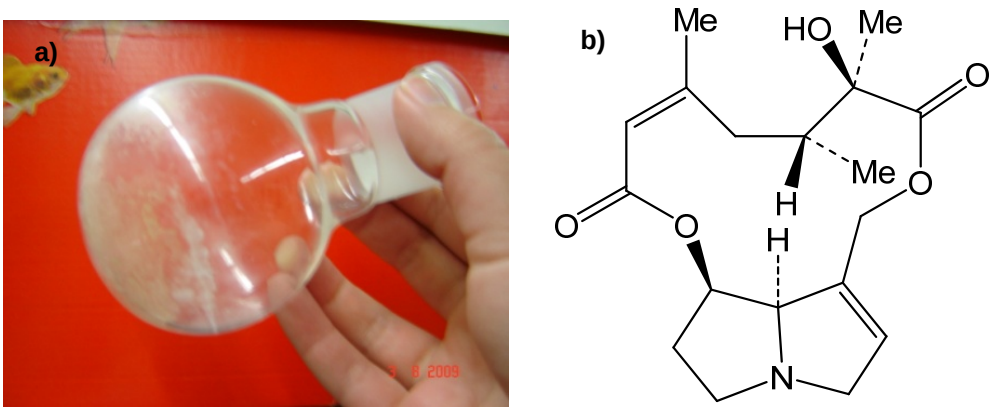


Figure 3.7: Crystalls of pure Doronenine in round bottom flask (a) and chemical structure (b) (Dictionary of Natural Products, 2010)

Compound II was isolated from Fraction 4 and identified as madurensine based on NMR data (^{1}H and ^{13}C) (Figure 3.8 and Appendix C). The ^{1}H NMR of madurensine showed H-18 quartet at δ 7.12 (J = 6.7 Hz), which indicated the *trans* configuration of the carboxyethylidene moiety, two downfield shift protons at 4.98 (t, J = 2.4 Hz) and 4.55 (m) of H-7, H-6, which adjacent to two hydroxyl groups, the proton at 6.17 (br 5) indicative for the olefinic proton at C-2. The spectrum also showed three methyl groups at 0.86 (d, J = 6.7), 1.38 (s) and 1.78 (d, 0.7) for methyls 17, 19 and 16 respectively. The ^{13}C NMR with DEPT-135 confirmed the structure of compound II as madurensine, which showed 18 carbons, three methyls, four CH_2, six CH groups and 5 quaternary carbons. The above mentioned data is similar with those reported for the same compound in literature (Verdoorn and Van Wyk, 1992).

Madurensine had been previously identified in *Crotalaria agatiflora, C. rosenii*, *C. madurensis* and *C. laburnifolia* (Abegaz *et al.,* 1987, Atal and Kapur, 1966 and Flores *et al*., 2009) and was found together with trans-anacrotine to be the only alkaloids in the seeds of *C. capensis* (Verdoorn and Van Wyk, 1992). According to previous researchers' madurensine had been reported in the seeds, twigs and leaves of *Crotalaria agatiflora subspp. imperialis* (Asres *et al.,* 2004).

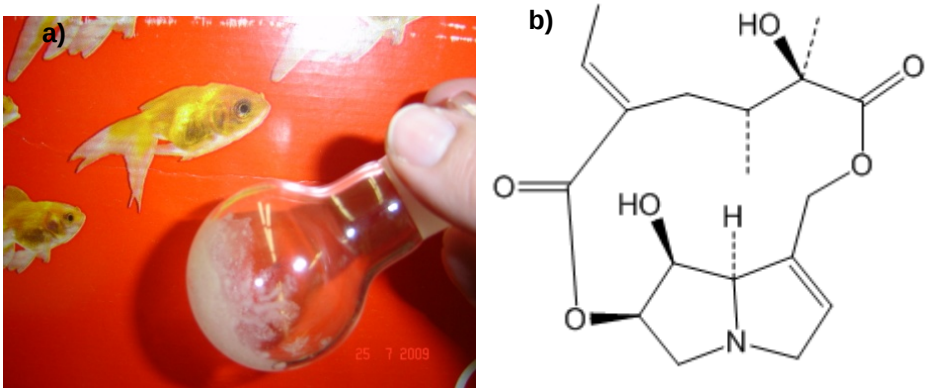


Figure 3.8: Crystalls of Pure madurensine in round bottom flask (a) and chemical structure (b) (Dictionary of Natural Products, 2010)

Some general chemical characteristics of the two isolated compounds are summarized in Table 3.1.

Table 3.1: Chemical characteristics of isolated compounds (Dictionary of Natural Products, 2010)

Characteristic	Madurensine	Doronenine
Molecular formula	$C_{18}H_{25}NO_6$	$C_{18}H_{25}NO_5$
Molecular weight	351.399 g/mole	335.399 g/mole
Physical description	Needles (EtOH)	Crystalline
Type of compound	Pyrrolizidine alkaloid	Pyrrolizidine alkaloid

3.2 Cytotoxicity of different fractions and isolated compounds

All eight fractions obtained from silica gel column chromatography were tested at only one concentration, namely 50 µg/mL. The fractions were all moderately toxic to relatively non-toxic against U-937 cells, except for Fraction 1 (Figure 3.9). Fraction 1 inhibited the growth of the cells by 71.8%, thus only leaving 28.2% viable. Unfortunately Fraction 1 was not enough for further work with regards to isolation of compounds.

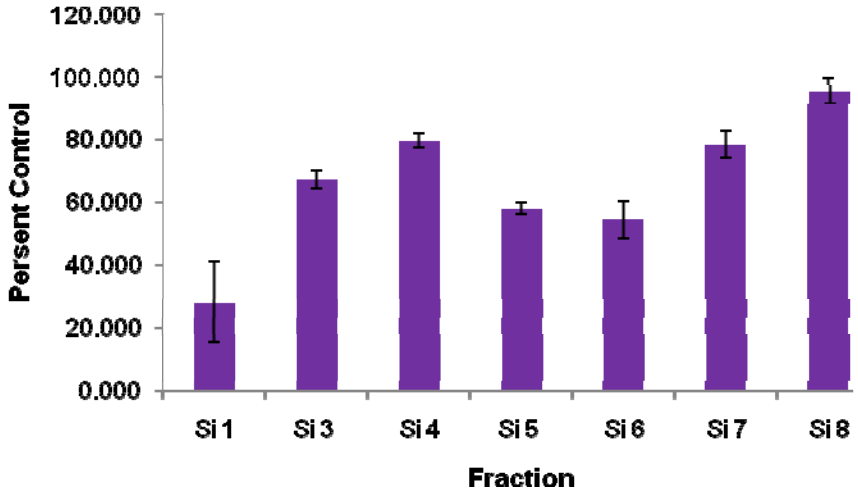


Figure3.9: Cytotoxicity of silica fractions at 50 µg/mL on U-937 cells

All silica fractions were relatively non-toxic on Vero cells (Figure 3.10). The viability of the cells ranged between 100% and 92.0%. Thus none of the fractions showed significant toxicity on noncancerous cells.

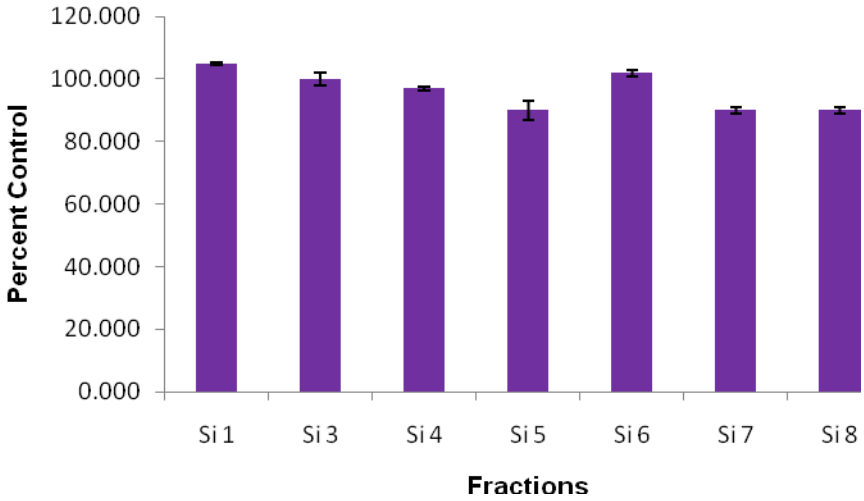


Figure 3.10: Cytotoxicity of silica fractions at 50 µg/mL on Vero cells

Two isolated compounds were tested against U-937 and Vero cells. Madurensine had an IC_{50} value of 136.5 ± 17.92 µg/mL, while doronenine had an IC_{50} value of 87.65 ± 2.715 µg/mL against U-937 cells (Figure 3.11). Doronenine was 1.6 times more active than madurensine. Actinomycin D had an IC_{50} value of 0.002 ± 0.00005 µg/mL. Madurensine had been screened for anti-cancer activity by the National Cancer Institute (NCI). Different yeast stains such as mlh1 rad18, bub3, cln2 rad14, sgs1 mgt1, mec2-1 and rad50 were used to test the compound's anti-cancer activity. The bioassay was based on growth inhibition of yeast strains with defined genetic alterations.

Compound treatments which inhibited the growth of the yeast by 70% were considered active. All strains tested negative for anti-cancer activity (PubChem, 2009). To my knowledge no data was available for any biological activity of doronenine.

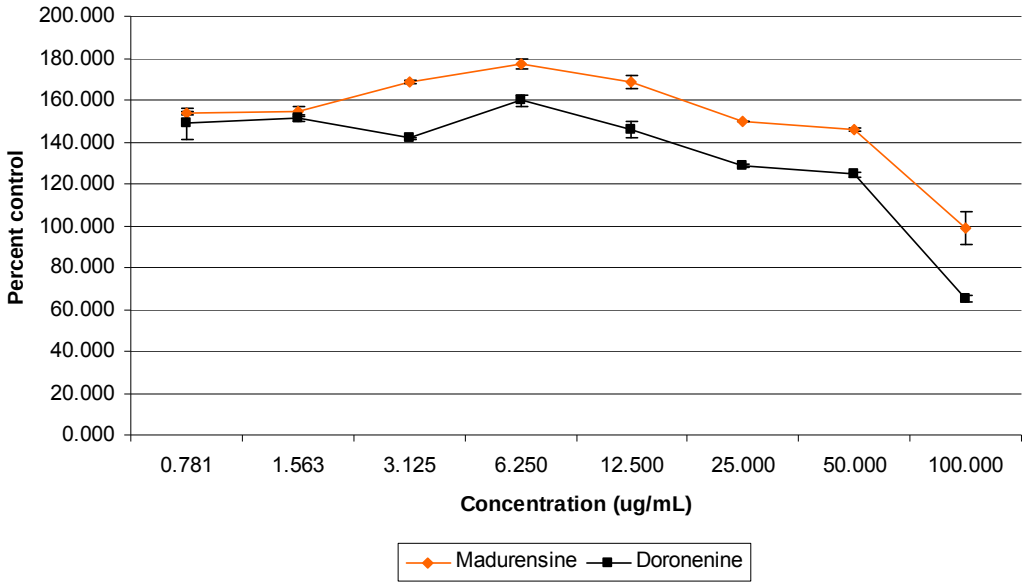


Figure 3.11: Dose-response curves of madurensine and doronenine on U-937 cells

Vero cells were less susceptible to the influence of doronenine and madurensine as compared to that of the compounds on U-937 cells (Figure 3.12). Madurensine and doronenine exhibited an estimated IC_{50} value of 21183 ± 3.334 µg/mL and 2806 ± 1.708 µg/mL respectively. The estimated values were determined using GraphPad Prism 4, which extrapolated the values to predict the IC_{50} values. Due to the predicted values not being determined experimentally, it is safe to say both IC_{50} values are higher than the highest concentration tested, thus being higher than 400 µg/mL. Actinomycin D had an IC_{50} value of 0.080 ± 0.029 µg/mL. Madurensine had a selectivity index (SI) value of 155.2 while doronenine had an SI value of 32. Although doronenine was more active than madurensine on U-937 cells, it was less selectively cytotoxic. Based on the fact that the compound was less selectively cytotoxic and due to unavailability of doronenine, it was decided that madurensine was more applicable for further investigations (mechanistic activity).

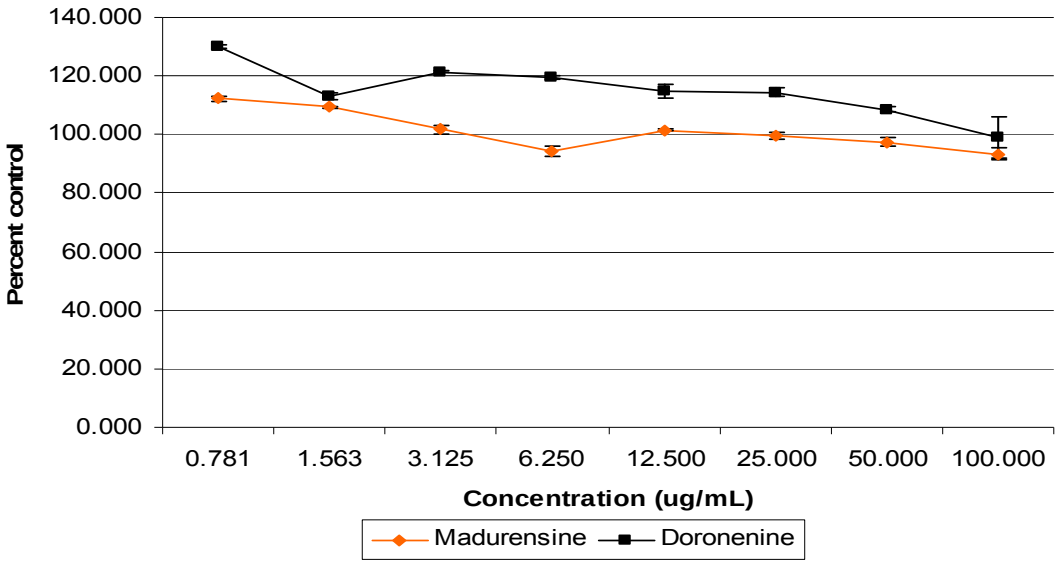


Figure 3.12: Dose-response curves of madurensine and doronenine on Vero cells

Both compounds showed weak DPPH scavenging potential at the highest concentration tested. Both these compounds' IC_{50} values were higher than 100 µg/mL.

Plant alkaloids comprise 15.6% of known natural products and contribute to almost 50% of the plant derived natural products of pharmaceutical and biological significance. Interestingly, 35.9% of alkaloids tested in 20 bioassays or more are pharmaceutically significant (Cordell *et al.,* 2001). The total percentage alkaloids in dried *C. agatiflora* material were determined as follows: seeds 1.88%, twigs 0.78% and leaves 1.50%. Leaves thus have high estimated quantities of alkaloids which can be investigated. Alkaloids don't only have side effects on humans (hepatotixicity, mutagenicity, carcinogenicity and tetratogenicity) (Asres *et al.*, 2004), but can also be used as acetylcholinesterase inhibitors and as stimulants (caffeine being used as a stimulant).

It was also found that Senecionine (N-oxide) a macrocyclic pyrrolizidine alkaloid isolated from the seed of *C. anagyroides* had been identified as an antineoplasmic agent. Interestingly, senecionine had the same molecular formula as compared to madurensine but both compounds had different chemical structures (Dictionary of Natural Products, 2010). On the other hand various plants in *Leguminosae* and *Crotalariaceae* are hepatotoxic due to 1,2-dehydropyrrolizidine alkaloids (Cordell *et al.*, 2001). Doronenine, also known as 1,2-dihyrobulgarsenine (Dictionary of Natural Products, 2010) did not show toxicity against Vero cells in the present study. According to researchers doronenine could still be activated in the liver (*in vivo*) to pyrrole derivates which may be very toxic, especially to the liver and lungs (Prakash *et al.,* 1999). The above mentioned statement however is in contrast with the finding of Bahar and colleagues (2006) who found that *C. emarginella* and

some of its constituents were anti-hepatoxic. However monocrotaline, also a macrocyclic alkaloid which is found in some species of *Crotalaria* is toxic to liver. The cytotoxicity observed in the present study by ethanol extract of *C. agatiflora* could be due to those kinds of alkaloids, such as monocrotaline present in the extract. The other reason could be due to the isolated alkaloids not being metabolically converted by liver enzymes when they were tested.

4. Conclusion

Both isolated compounds madurensine and doronenine, showed moderate cytotoxicity on leukemic cells and exhibited high SI values. Madurensine and doronenine had insignificant antioxidant activity as well as no apparent anti-Alzheimer activity. This is the first report of doronenine being isolated from *C. agatiflora*. Thus these compounds must be further investigated to determine the mode of action.

5. References

Abegaz, B., Atnafu, G., Duddeck, G. and Snatzke, G. 1987. Macrocyclic pyrrolizidine alkaloids of *Crotalaria rosenii*. *Tetrahedron* **43**: 3263-3268.

Asres, K., Sporer, F. and Wink, M. 2004. Patterns of pyrrolizidine alkaloids in 12 Ethiopian *Crotalaria* species. *Biochemical Systematics and Ecology* **32**: 915-930.

Atal, C,K. and Kapur, K.K. 1966. A new pyrrolizidine aminoalcohol in alkaloids of *Crotalaria species*. *Tetrahedron Letters* **6**: 537-544.

Bahar, A., Al-Howiriny, T.A. and Mossa, J.S. 2006. Crotalic acid and emarigellic acids: Two tripenes from *Crotalaria emarginella* and anti-inflammatory and anti-hepatoxic activity of crotalic acid. *Phytochemistry* **67**: 956-964.

Cordell, G.A., Quin-Beattie, M.L. and Fransworth, N.R. 2001. The potential of alkaloids in drug discovery. *Phytotherapy* **15**: 183-205.

Dictionary of Natural Products. 2010. "Search engine." [Online]. Available: http://www.dnp.chemnetbase.com [Cited 16 September 2010]

Eldeen, I.M.S., Elgorashi, E.E. and Van Staden, J. 2005. Antibacterial, anti-inflammatory, anti-cholinesterase and mutagenic effects of extracts obtained from some trees used in South African traditional medicine. *Journal of Ethnopharmacology* **102**: 457-464.

Flores, A.S., de Azevedo Tozzi, A.M.G and Trigo, J.R. 2009. Pyrrolizidine alkaloid profiles in *Crotalaria* species from Brazil: Chemotaxonomic significance. *Biochemical Systematics and Ecology* **37**: 459–469.

Prakash, A.S, Pereira, T.N., Reilly, P.E.B. and Seawright, A.A. 1999. Pyrrolizidine alkaloids in human diet. *Mutation Research* **443**: 53-67.

PubChem. 2009. "BioActivity Analysis." [Online]. Available: http://www.pubchem.ncbi.nlm.nih.gov.innopac.up.az.za/assay [Cited 11 September 2009].

Roder, E., Wiedenfeld, H. and Frisse, M. 1980. Pyrrolizdine alkaloide aus *Senecio doronicum*. *Phytochemistry* **19**: 1275-1277.

Verdoorn, G.H. and Van Wyk, B-E. 1992. Pyrrolizidine alkaloids from seeds of *Crotalaria capensis*. *Phytochemistry* **31**: 369-371.

CHAPTER 4:

ULTRA STRUCTURE AND FLOW CYTOMETRIC ANALYSIS OF THE ETHANOLIC EXTRACT AND MADURENSINE'S CYTOTOXICITY

1. Introduction

Vero and U-937 cells were investigated with light microscopy to determine morphological changes in the cells on exposure to the ethanol extract of the leaves of *C. agatiflora* subspp *agatiflora*. This investigation was conducted to determine whether the cytotoxicity of the ethanolic extract is selective to leukemic U-937 cells. The mechanism of cell death in U-937 cells and Vero cells was postulated determined using light microscopy. Flow cytometry was further used for quantitative analysis and for the evaluation of cell death (apoptosis / necrosis) after exposure to the samples.

1.1 Light microscopy

Different microscopes are used to investigate samples depending on the detail to be investigated. After cytotoxicity was determined, the cancerous cells need to be investigated on a subcellular level. The light microscope has 500 times more resolution than the human eye (Solomon *et al*., 2005). Light microscopy can be used to identify and quantify cell death (Tinari *et al*., 2008). In this investigation cell death will not be quantified due to the biased nature of microscopy and will be quantified by flow cytometry. The type of cell death can be predicted. Cell death is well characterized in higher eukaryotes which includes apoptosis, autophagy, necrosis and oncosis (Menna-Barreto *et al.,* 2009). In the present study, the different types of cell death namely apoptosis, autophagy and necrosis were analysed on Vero and U-937 cells in the precence of tested samples.

Apoptosis is a regulated process of cell suicide involved in several diseases. Programmed cell death is critically important in homeostasis of multicellular organisms, playing a fundamental role in morphogenesis, physiology and host defence against pathogens. Apoptotic features in mammalian cells include proteolytic cleavage by caspases, cell shrinkage, DNA inter-nucleosomal fragmentation, phosphatidylserine exposure, blebbing of the plasma membrane, formation of apoptotic bodies and loss of mitochondrial membrane potential with cytochrome c release to the cytosol (Figure 4.1 and Table 4.1). Autophagic cell death involves the autophagosomal-lysosomal system. Autophagosomes are double-membrane vesicles responsible for the engulfment of cytoplasmic components, during turnover of organelles. After autophagosome-lysosome fusion, an autophago-lysosome is formed in which cellular residues are digested. The morphological identification of this type of cell death involves autophagosome formation, with the appearance of membranes surrounding organelles and cytosolic structures. The early degradation of organelles is accompanied by cytoskeleton maintenance until later stages, without inflammation. Necrosis is described as non-apoptotic cell death or chaotic cell death, displaying aspects of programmed cellular suicide. The interplay between apoptosis and necrosis is altered in response to different

stimuli, being observed under conditions of chemical or genetic inhibition of apoptosis. Originally necrosis was decribed as accidental death of cells and tissues (Figure 4.1). The hallmarks of this type of death involve dramatic alterations in mitochondria which include mitochondrial depolarization, ATP depletion, generation of reactive oxygen species (ROS), loss of calcium homeostasis, cytoplasmic vacularization and finally breakdown of the plasma membrane (Menna-Barreto *et al.,* 2009).

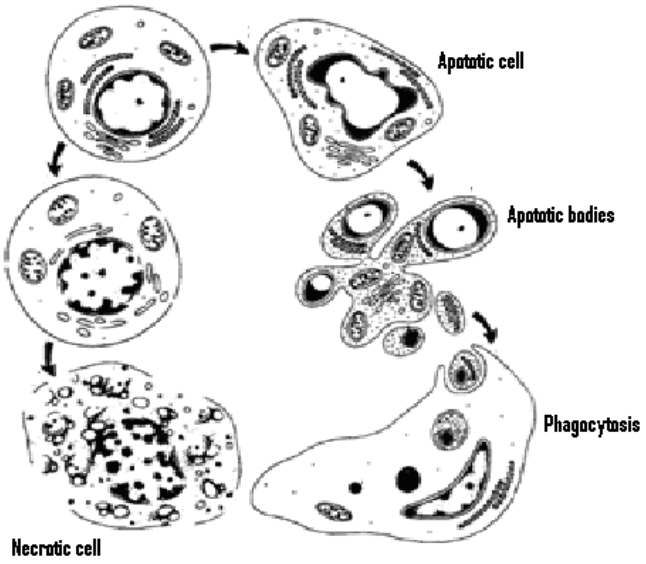


Figure 4.1: Major characteristics of apoptosis and non-apoptotic cell death (Van Cruchten and Van den Broeck, 2002)

Table 4.1: Morphological and biochemical characteristics of cell death (Tinari *et al.,* 2008)

Cell death	Cell compartment			
	Nucleus	**Plasma membrane**	**Cytoplasm**	**Characteristics**
Apoptosis	Chromatin condensation Nuclear fragmentation DNA laddering	Blebbing Smoothing	Apoptotic body formation	Caspase dependent
Autography	**Partial chromatin condentation** **No DNA laddering**	**Blebbing**	**Increased number of autophagic vacuoles**	**Caspase independent** **Increased lysosomal activity**
Necrosis	Clumping DNA degradation	Swelling Ruptures	Vacuoles increase Organelle degeneration Mitochondrial swelling	-

Flow cytometric analysis was conducted to quantify cell death of U-937 cells after treatment with the ethanolic extract of the leaves of *C. agatiflora* and the isolated compound madurensine for the two most common types of cell deaths' progressive stages namely apoptosis and necrosis.

1.2 Flow cytometry

Flow cytometry measures both biochemical and physical characteristics of individual cells. Flow cytometry can be used for both cell analysis and separation of labelled cells without changing the viability of the sample. Biological particles are sorted usually by multiple parameters, but usually by size and fluorescence. Flow cytometry has various applications ranging between immunological, functional, biochemical and molecular investigations of biological particles (Boeck, 2001). One of the biochemical hall marks of apoptosis is the rearrangement of phosphatidylserine (PS). This plasma membrane phospholipid is generally found on the inner leaflet of the lipid bilayer. During apoptosis the cell membrane consituents are redistributed and PS becomes exposed on the outer leaflet of cell membranes (Trahtemberg *et al.,* 2007). Different fluorochomes are used to detect cell death using flow cytometry.

2 Materials and method

2.1 Materials:

Bouin's fixative, haematoxylin, eosin and xylene were of analytical grade and supplied by Sigma Chemicals Co. (St. Louis, MO, USA). BD Biosciences' Annexin-V-FITC apoptosis kit was provided by BDBiosciences (Johannesburg, South Africa (Pty) Ltd).

2.2 Methods:

2.2.1 Cell morphology – light microscopy (haematoxylin and eosin staining)

Haematoxylin and eosin staining were conducted to provide a qualitative analysis of the morphological characteristics of U-937 and Vero cells after exposure to the ethanolic extract of the leaves of *C. agatiflora.* Leukemic U-937 cells were exposed to 73.9 µg/mL (IC_{50}) and 147.8 µg/mL ($2IC_{50}$). Vero cells were exposed to the ethanolic extract at 73.9 µg/mL (IC_{50}) and 147.8 µg/mL ($2IC_{50}$) and additionally to 352.4 µg/mL (IC_{50}) and 704.8 µg/mL ($2IC_{50}$). The latter concentrations were chosen since dose-dependent anti-proliferative effects were observed at these concentrations. Madurensine was not analysed using light microscopy due to the small quantity of the compound. Madurensine was retained for more crucial analysis.

U-937 cells are suspension cells and therefore it was necessary to manipulate the cells to adhere to the coverslips. U-937 cells were washed three times with phosphate buffer to remove all traces of fetal bovine serum (FBS) and then resuspended in complete medium lacking FBS. This treatment allowed U-937 cells to adhere to the coverslip. Exponentially growing U-937 and Vero cells were seeded at one million and 250,000 cells per well respectively on sterilized coverslips. After 24h incubation (37°C, 5% CO_2) to allow the cells to adhere, U-937 cells were exposed to 73.9 µg/mL (IC_{50}) and 147.8 µg/mL ($2IC_{50}$) of ethanolic extract of the leaves of *C. agatiflora* including vehicle-treated control (0.74%), actinomycin D (0.002 µg/mL), cells propagated in growth medium and incubated for 72h at 37°C. After 24h incubation (37°C, 5% CO_2), Vero cells were exposed to 73.9 µg/mL (IC_{50} of U-937 cells) and 147.8 µg/mL ($2IC_{50}$ of U-937 cells), 352.4 µg/mL (IC_{50}) and 704.8 µg/mL ($2IC_{50}$) of ethanolic extract of the leaves of *C. agatiflora* including vehicle-treated control (3.5%), actinomycin D (0.080 µg/mL), cells propagated in growth medium and incubated for 72h at 37°C. Cells were consequently fixed in Bouin's fixative for 60min and stained by standard haematoxylin and eosin staining procedures (Figure 4.2), (Stander *et al*., 2009). The cells were investigated using Nikon Stereo Light microscope equipped 1.4 Apo oil lense (Microscopy Unit, University of Pretoria). The magnification was x 1000.

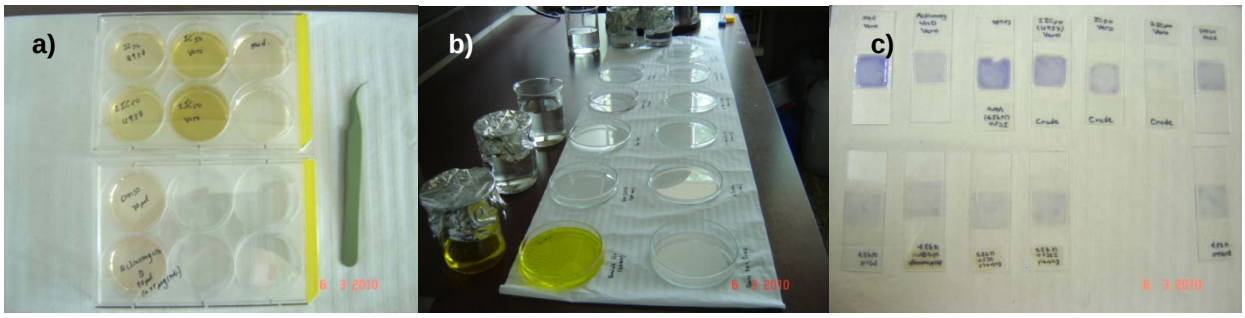


Figure 4.2: Treatment of cells (a), preparation for staining cells (b) and prepared microscope slides (c)

2.2.2 Flow cytometry – apoptosis detection assay

Exponentially growing U-937 cells were seeded at 0.5 x 10^6 cells per 25 cm^2 flask. Cells were exposed to 73.9 µg/mL (IC_{50}) and 147.8 µg/mL ($2IC_{50}$) of the ethanol extract of the leaves of *C. agatiflora* and exposed to 136.5 µg/mL isolated compound, madurensine respectively and incubated for 72h and 96h respectively, before the cells were stained. The analysis included vehicle-treated control (0.74%) and actinomycin D (0.2 µg/mL) treated cells. One million cells were double-stained with annexin – V (FITC) and propodium iodide, according to the manufacturer's instructions (BD Biosciences, Johannesburg, South Africa (Pty) Ltd.). Annexin-V and propidium iodide fluorescence were measured with a fluorescence-activated cell sorting (ARIA) System flow cytometer (BD Biosciences, Johannesburg, South Africa (Pty) Ltd.) equipped with an air-cooled argon laser excited at 488nm (Figure 4.3), (Stander *et al.,* 2009). Data from at least 10,000 cells

were analyzed with BD FACS Diva Software Version 6.1 (BD Biosciences, South Africa (Pty) Ltd (Kang *et al.*, 2009).

Figure 4.3: BD Bioscience's FACSAria flow cymeter (BD Biosciences, 2010)

Actinomycin D was tested at 100 times higher concentration compared to the IC_{50} value because during trial runs, little cell death was observed. It was known that cells can survive at concentrations of 0.1 µg/mL for 24h before dying (Sigma, 2004), so to make sure of definite positive results, the concentration was increased. Stander *et al.* (2009) used the same concentration as a positive apoptosis inducer during their studies, stating nowhere in the publication that that concentration was the calculated IC_{50} value. Thus that concentration was merely used at a standard concentration to prove that induced apoptosis could be detected. For all of the samples, dot plots were used for illustrating the results. The dot plots were divided into four quadrants. These quadrants were allocated to the positive and negative staining of annexin-V and propidium iodide. In Table 4.2 the staining sequence is summarized.

Table 4.2: Allocations of stained and unstained cells (Tinari *et al.*, 2008)

State of cells	*Annexin-V (FITC)*	*Propidium iodide (PE)*
Viable / unstained	Negative	Negative
Early apoptosis	Positive	Negative
Late apoptosis	Positive	Positive
Necrosis	Negative	Positive

3. Results and discussion

3.1 Cell morphology – light microscopy (haematoxylin and eosin staining)

A qualitative investigation on the influence of 73.9 µg/mL (IC_{50}) and 147.8 µg/mL ($2IC_{50}$) of the ethanol extract of the leaves of *C. agatiflora* was determined by analysing cell morphology in cancerous U-937 cells after 72h of exposure and compared to the vehicle-treated controls (0.74% DMSO). Non-cancerous Vero cells morphology were also analysed on exposure to 73.9 µg/mL (IC_{50} of U-937 cells) and 147.8 µg/mL ($2IC_{50}$ of U-937 cells) and additionally to 352.4 µg/mL (IC_{50}) and 704.8 µg/mL ($2IC_{50}$) of the ethanolic extract of the leaves of *C. agatiflora* after 72h of exposure and compared to vehicle-treated control (3.5% DMSO).

Heamatoxylin and eosin staining (Figure 4.4) provides information regarding the cytoplasm (pink) and nucleus (dark blue / purple). This staining method was useful in cell death detection in monolayered cells. Thus by looking at treated and untreated cells under the microscope, ultra structural changes may give clues on what type of cell death took place and which of the cell components were affected dramatically (Sigma, 2010).

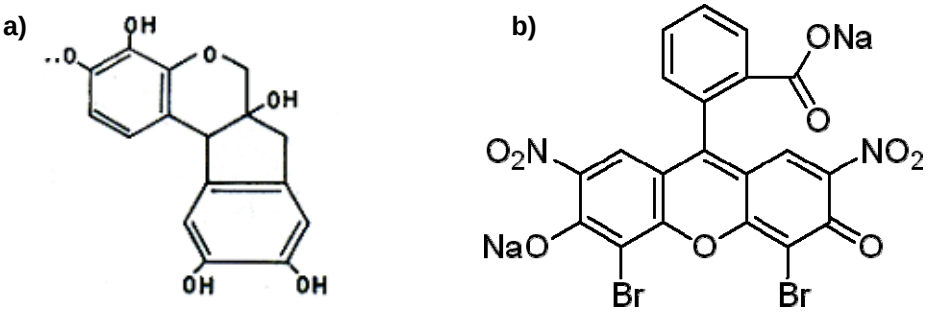


Figure 4.4: Chemical structures of haematoxylin (a) and eosin (b) (Sigma, 2010)

3.1.1 U-937 Cells

Large multiple nuclei were observed in the present study (Figure 4.5a). The cells had intact cell membranes and large amounts of cytoplasm. Vehicle control cells were viable and still able to grow (Figure 4.5b). Actinomycin D (0.002 µg/mL) showed severe signs of cell death (Figure 4.5c). The density of cells decreased as compared to the untreated cells. The density reduction gave an indication that cells detatched during incubation time. Remaining cells contained little cytoplasm, indicating either that the cell membranes ruptured or that apoptotic bodies formed leaving less cytoplasm in the treated cells. Nuclear material of treated cells, chromatin condensation and fragments were visible. Necrosis was also observed in the positive control treated cells to some extent (Figure 4.5c). *Crotalaria agatiflora*-treated U-937 cells revealed an increase in

morphological features of cell death in a dose-dependent manner, which included decresed cell density, hypercondensed chromatin, apoptotic bodies and shrunken cells (Figure 4.5d and 4.5e). Those features are characteristic of apoptosis and autophagy.

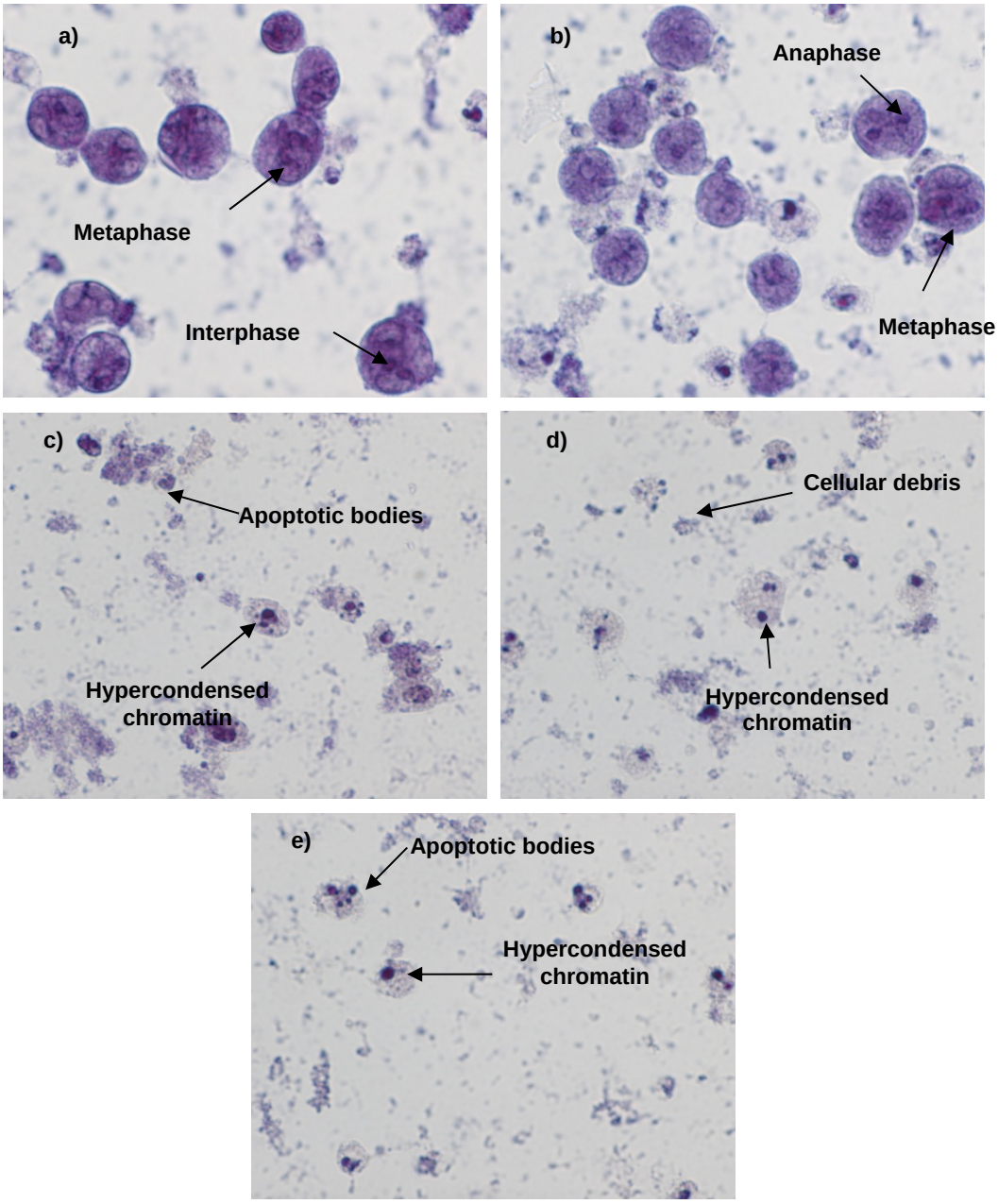


Figure 4.5: Haematoxylin and eosin staining of U-937 medium only control cells (a), vehicle-treated control (b), 0.002 µg/mL actinomycin D (c), 73.9 µg/mL *C. agatiflora*-treated cells (d) and 147.8 µg/mL *C. agatiflora*-treated cells (e) after 72h of exposure (1000x magnification).

3.1.2 Vero cells

Vehicle control cells (3.5%) were viable and still able to grow (Figure 4.6b) as compared to untreated Vero cells (Figure 4.6a). Actinomycin D (0.08 µg/mL) showed severe signs of cell death (Figure 4.6c). The non-cancerous Vero cells revealed less prominent features of cell death when the cells were treated with 73.9 µg/mL and 147.8 µg/mL (IC_{50} and twice the of IC_{50} of U-937 cells) the ethanol leaves extract of *C. agatiflora* (Figure 4.6d and 4.6e). Cells treated with 352.4 µg/mL and 704.8 µg/mL of the ethanol leaves extract showed dose-dependent features of cell death. The density of cells decreased as compared to the untreated cells. Those features included reduction in cell size and hypercondensed chromatin (Figure 4.6f and 4.6g).

When cancerous U-937 cells and non-cancerous Vero cells morphological changes were compared, we found that with 73.9 µg/mL treatment the U-937 cells were much more susceptible and sensitive to the treatments compared to the same concentration on Vero cells. In agreement with the findings observed by Chinkwo (2005), who explored cervical carcinoma (Caski) and Chinese hamster ovary (CHO) cells treated with *Sutherlandia frutescens,* the cells in the present study at the respective IC_{50} values had condensed nuclei and decreased amount of cytoplasm. Conclusions are in agreement with the conclusions made by Stander *et al.* (2009) who observed similar selectivity between cancerous breast adenocarcinoma (MCF-7) and non-cancerous epithelial mammary gland (MCF-12A) cells treated with aqueous extracts of *S. frutecens.*

The affects of treatment were much more severe in U-937 cells and thus the mechanism of action will be determined in the cancerous cells before investigating Vero cells in future studies. It should be mentioned that the results found with light microscopy was insufficient in determining the type of cell death, due to the fact that apoptosis and autophagy looks very similar in light microscopy investigations. Apoptosis and necrosis were quantified, because the techniques were established already in our labs and due to the rapid and simple analysis of samples.

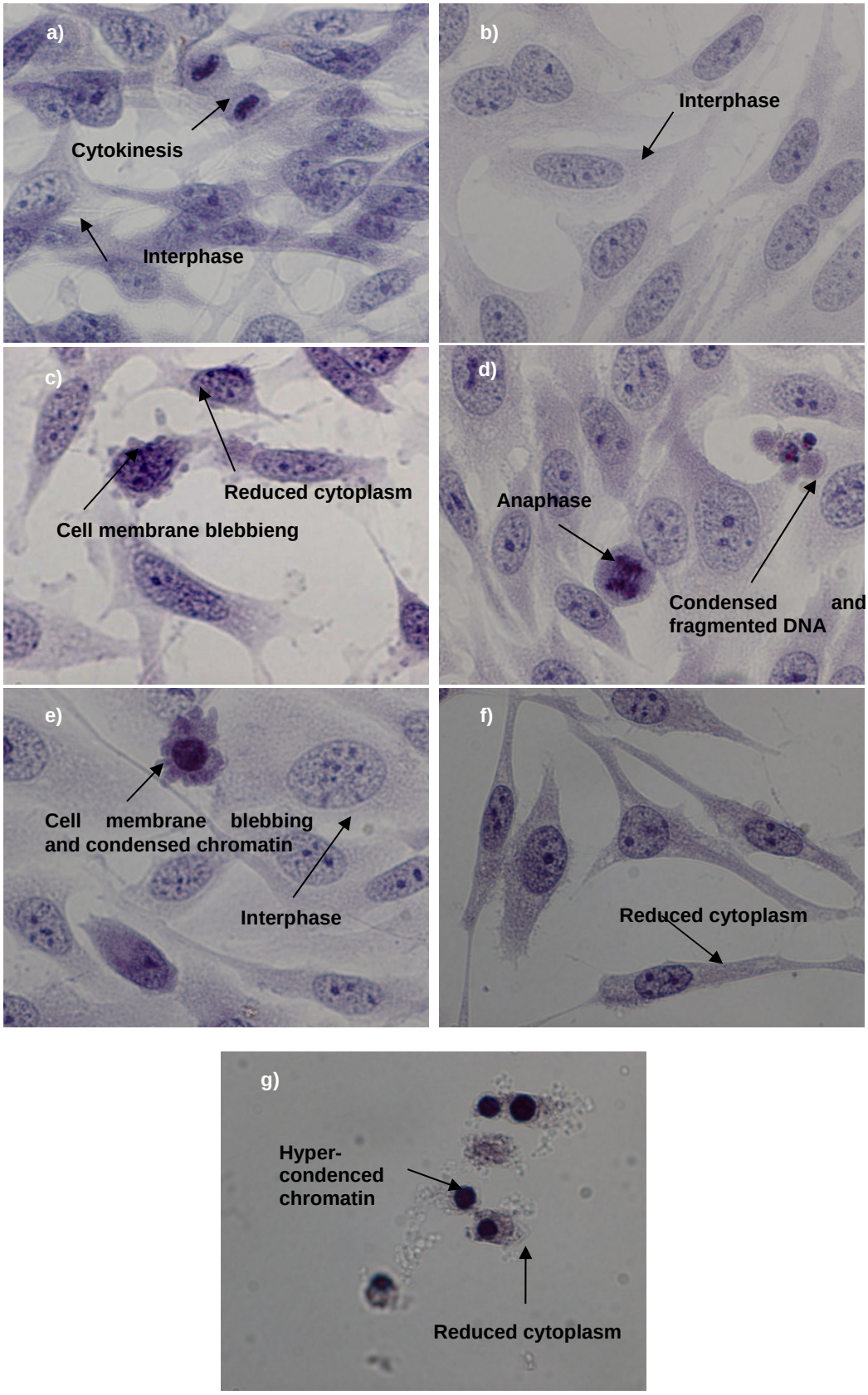


Figure 4.6: Haematoxylin and eosin staining of U-937 medium only control cells (a), vehicle-treated control (b), 0.08 µg/mL actinomycin D (c), 73.9 µg/mL *C. agatiflora*-treated cells (d) and 147.8 µg/mL *C. agatiflora*-treated cells (e), 352.4 µg/mL *C. agatiflora*-treated cells (f) and 708.4 µg/mL *C. agatiflora*-treated cells (g) after 72h of exposure (1000x magnification).

3.2 Flow cytometric investigation to determine percentage apoptosis

Double staining of untreated and treated cells with Anexin-V and propidium iodide (PI) was used to quantify cell death that took place (Figure 4.7). Annexin-V has stong anti-coagulation activity. This protein has a high calcium ion dependent affinity for aminophospholipids, having thus high affinity for phosphatidylserine. Propidium iodide is a cationic dye, excluded by intact cell membranes (Figure 4.8). Necrotic cell membranes are extensively damaged and PI thus enters cells to intercalate within DNA (Tinari *et al.,* 2008).

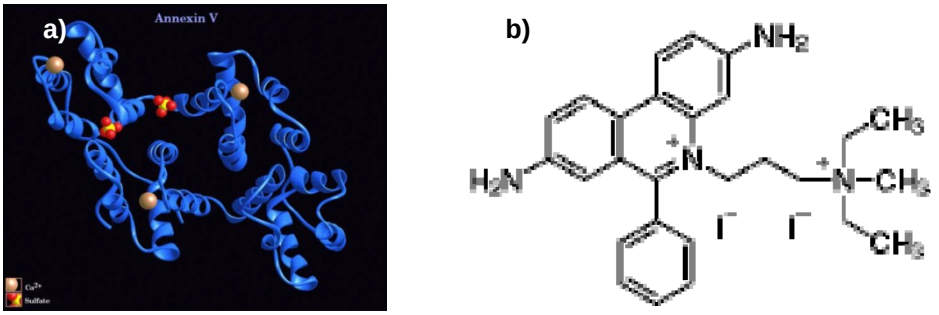


Figure 4.7: Protein structure of Annexin-V (a) and chemical structure of propidium iodide (b) (Imgenex, 2010; Sigma, 2010)

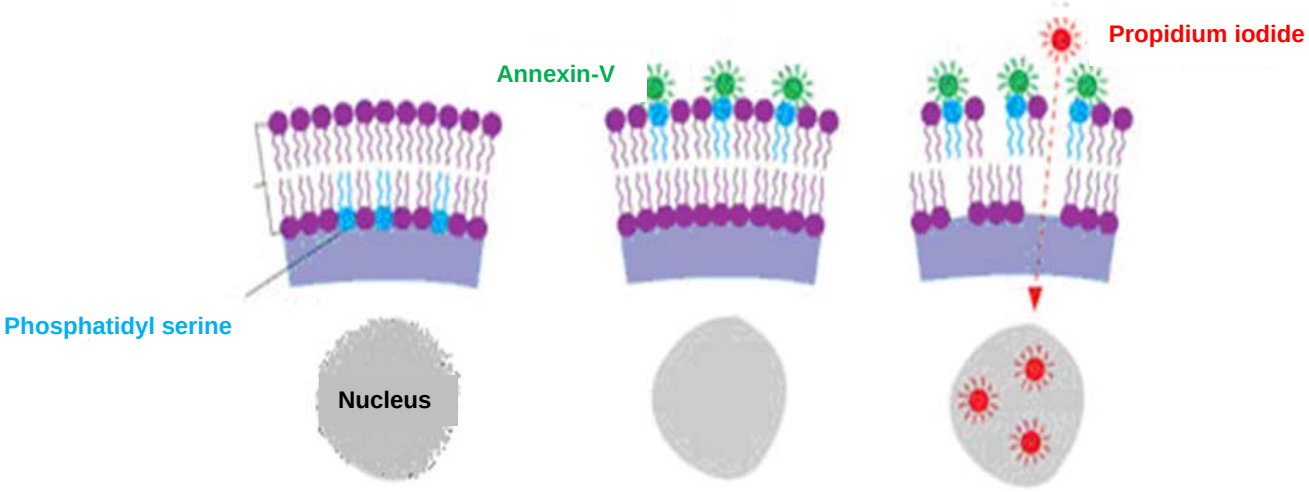


Figure 4.8: Binding and distribution of Annexin-V and Propidium iodide in stages of cell death (Imgenex, 2010)

3.2.1 Apoptosis detection analysis after 72h incubation

To demonstrate the mechanism of cell death, the effect of the ethanol leaves extract of *C. agatiflora* was tested at 73.9 µg/mL (IC_{50}) and 147.8 µg/mL ($2IC_{50}$) and madurensine at 136.5 µg/mL (IC_{50}) to determine the percentage binding of Annexin-V and PI.

After 72 hours, untreated cells were 98.8% unstained with Annexin-V and PI and thus viable, with only minute percentages of cells in stages of cell death (Figure 4.9a and Table 4.3). Similar to the findings observed by Stander *et al.* (2009) who explored MCF-7 cells during flow cytometric analysis. Viability obtained during the analysis of untreated MCF-7 cells was 91.4%. The increased viability in the U-937 cells could be due to the fact that MCF-7 cells were trypsinized to detach the cells from the flask surfaces. During trypsinaztion cells can be damaged due to the nature of the enzyme trypsin. Vehicle control, DMSO (0.74%) treated cells were analysed to determine the amount of damage and induced cell death caused by the agent during treatment. It was found that the viability (97.3%) decreased slightly after 72h incubation in the present study. The decrease in viability was small but confirmed that DMSO had negative effects on cell cultures (Figure 4.9b and Table 4.3). These results were in disagreement with the findings of Stander *et al*. (2009) who found that MCF-7 cells' viability increased during the treatment with DMSO, however the percentage DMSO used during treatments were not mentioned. Actinomycin D induces apoptosis and thus was a good treatment to detect apoptosis. Cells were treated at a very high concentration of 0.2 µg/mL and showed severe cell death characteristics. Only 3.9% treated cells were still viable (unstained by Annexin-V and PI) in the present study (Figure 4.9c, Table 4.3). This was in agreement with Stander *et al*. (2009), which found that 5.8% cell were viable after 0.2 µg/mL actinomycin D treatment. Interestingly the stages of apoptotic cell death were opposite in the two different cell lines. MCF-7 cells were mostly classified in late apoptosis (76.1%) while U-936 cells were classified in the majority as being in early apoptosis (77.1%). Thus it can be speculated that U-937 cells might be less sensitive for the induction of apoptosis after 72h, compared to adherent cells. Low percentage of necrosis was induced in U-937 cells compared to MCF-7 cells.

Cells treated with different concentrations of *C. agatiflora* leaves' extract showed dose-dependent responses. Viability of the samples decreased as the concentration of the samples increased (Figure 4.9d and 4.9e). Considering that IC_{50} and double IC_{50} concentrations were used, the results were supposed to have reflected the results of XTT assay (Chapter 2). Although the viability decreased and cell death increased during treatments, 50% of the cells should have died during IC_{50} treatments. The same scenario was seen when U-937 cells were treated with madurensine. These results it was evident that cells' viability was not affected by the treatments and that little cell death *via* apoptosis and necrosis took place (Figure 4.9f). These conflicting

results might have been due to the fact that different targets were tested. The XTT salt was metabolically reduced by viable cells by dehydrogenase enzymes, thus giving an indication of enzyme activity, while Annexin-V binds to rearranged phosphatidylserine phospholipids externalized on the double layered cell membrane during apoptosis. Propidium iodide is a cationic dye which intercalates into DNA only when the cell membrane is damaged, usually during late stages of apoptosis or necrosis. Thus the two assays had different endpoint targets, enzymatic activity (characteristic of viable cells) *versus* the membrane integrity (very specific characteristic of cell death). Flow cytometry, using Annexin-v and PI is a very sensitive assay used and focuses entirely on the membrane integrity, while the XTT assay is less specific and focuses on the enzymatic abilities of treated cells. Thus Annexin-V together with propidium iodide quantifies cell death while the XTT salt quantifies the viability and thus proliferation of cells.

For interest sake the incubation time was increased to 96h to determine the affect of longer incubation times on the cells. Ninety-six hours was selected because longer incubation time might have had severe negative effects on the viability of untreated cells due to the decrease of nutrients in the medium and the increase of pH. Cell death must be caused by treatment and not primary negligence. Longer incubation time, beyond 96 hours was also seen as inappropriate due to the uniformity of experiments thus far (72 hours).

The difference in activity observed by the isolated molecule in comparison to the extract may be due to the incorrect molecules being isolated, thus leaving the active compounds unidentified in the ethanolic extract. Another possibility could have been that the compounds in the crude extract worked in synergy, giving the crude extract higher toxicity and the ability to induce cell death. Madurensine is clearly one of the less active (toxic) compounds found in *C. agatiflora*.

3.2.2 Apoptosis detection analysis after 96h incubation

Viability decreased slightly as seen in untreated cells after 96h of incubation (Figure 4.10a and Table 4.4). The decrease in viability could be due to the increased density of cells, thus fewer nutrients for cells to survive and slightly more acidic medium contributed by the respiration of cells. Comparing the viability the cells were still more viable than MCF-7 cells investigated by Stander *et al.* (2009) after 72h incubation. Similar trends were seen in 96h compared to 72h. Actinomycin D showed higher percentages early apoptotic cells compared to 72h (Figure 4.10c, Table 4.3 and 4.4). Small changes in percentage cell death were observed in 96h compared to 72h treatments. Cells treated with *C. agatiflora* samples had similar responses as in the case of 72h exposure (Figure 4.10d and 4.10e). On the other hand madurensine samples had a small increase in the percentage viable cells (Figure 4.10f). The percentage change was so small that the increase

could have been seen as coincidence instead of recuperation of cells. Further studies should be done to explore this phenomenon.

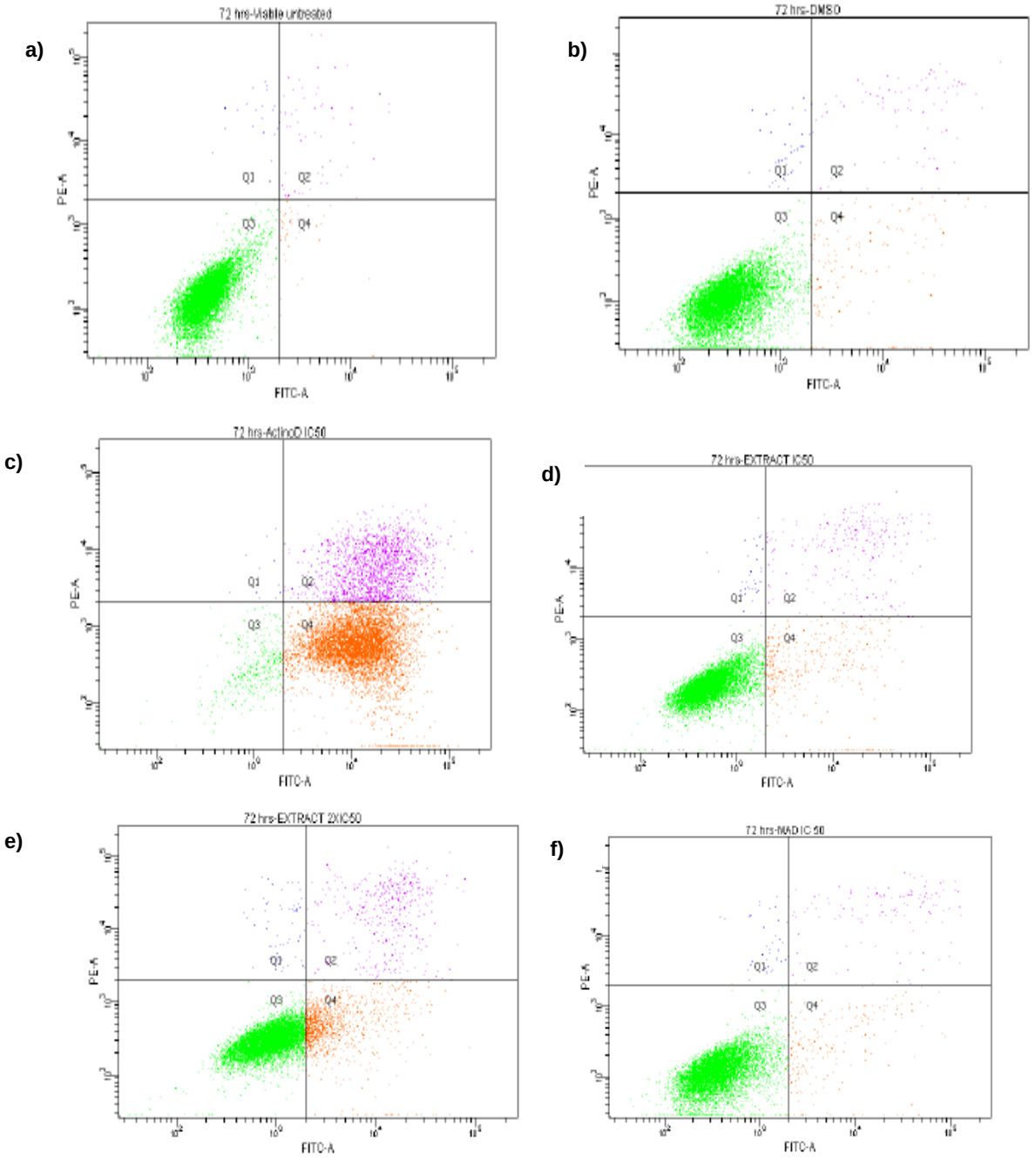


Figure 4.9: Annexin-V (FITC) versus Propidium iodide (PE) dot plots of: a) untreated U-937 cells, b) DMSO treated cells, c) actinomycin D treated cells, d) 73.9 µg/mL *C. agatiflora* treated cells, e) 147.8 µg/mL *C. agatiflora* treated cells and f) madurensine treated cells after 72h exposure

Table 4.3: Pecentage phosphatidylserine externalization and membrane permeability of samples exposed for 72h

	Viable	Early apoptosis	Late apoptosis	Necrosis
Viable (Untreated)	98.8	0.5	0.5	0.2
DMSO	97.3	1.5	0.8	0.4
Actino D	**3.9**	**77.1**	**18.8**	**0.1**
Extract IC50	92.9	4.2	2.5	0.4
Extract 2 IC50	81.8	13.5	4.1	0.6
Madurensine	96.0	2.2	1.3	0.4

Comparing light microscopy results with flow cytometric results it could be predicted that although signs of cell death were seen with light microscopy it was confirmed with flow cytometry to be found in the minority of the cells. Light microscopy can be misleading and thus the reason why much more assays should be performed before any conclusions can be made. The cytotoxicity using XTT assay gave an indication that the viability of the cells was reduced by 50% during treatments. Light microscopy indicated that the cells looked less viable, while flow cytometry confirmed that the cells were viable with intact plasma membranes.

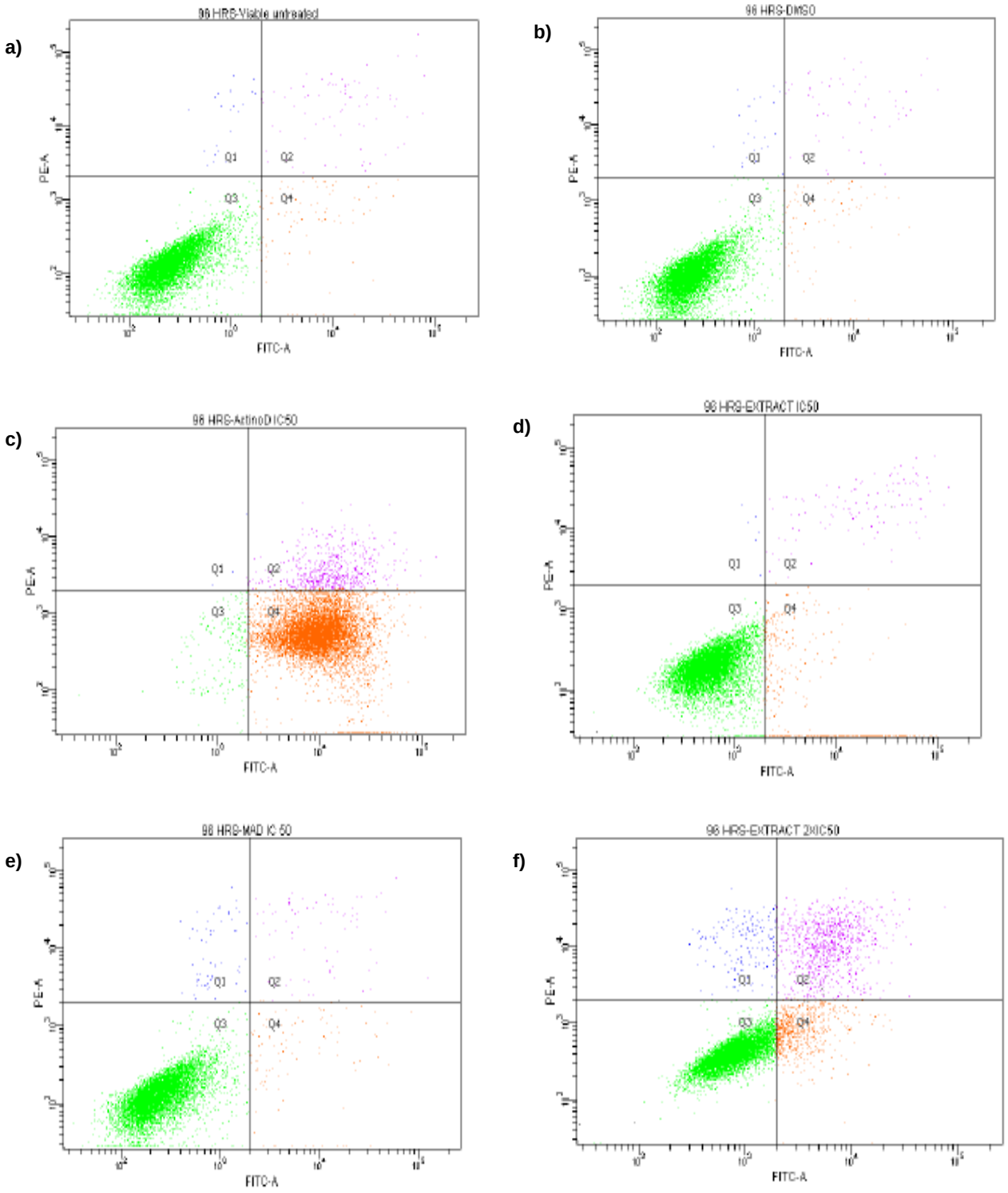


Figure 4.10: Annexin-V (FITC) versus Propidium iodide (PE) dot plots of: a) untreated U-937 cells, b) DMSO treated cells, c) actinomycin D treated cells, d) 73.9 µg/mL *C. agatiflora* treated cells, e) 147.8 µg/mL *C. agatiflora* treated cells and f) madurensine treated cells after 96h exposure

Table 4.4: Pecentage phosphatidylserine externalization and membrane permeability of samples exposed for 96h

	Viable	Early apoptosis	Late apoptosis	Necrosis
Viable (Untreated)	98.1	0.9	0.8	0.2
DMSO	98.4	0.8	0.6	0.3
Actino D	**2.0**	**91.9**	**6.1**	**0.0**
Extract IC50	94.3	4.4	1.2	0.1
Extract 2 IC50	81.1	7.7	9.5	1.7
Madurensine	97.6	1.1	0.7	0.6

For cytotoxicity more types of mechanisms must be tested due to the fact that tetrazolium salt is taken up by cells unspecifically, thus viable, damaged and dead cells are able to take up the salt. The XTT is then converted by enzymes in the mitochondria, more specifically mitochondrial dehydrogenase, to form soluble formazan orange salt. Emphasis must be put on damaged cells. Cells might not have had compromised cell membranes, but damaged mitochondria. Thus the complete XTT reaction will be unable to take place in damaged mitochondria, thus giving low IC_{50} values. Preliminary assays using XTT was thus only an indication of the fitness of mitochondrial metabolism and is not sensitive enough to compare to other very sensitive assays such as flow cytometry (especially in cases where apoptosis is not induced).

4. Conclusion

Light microscopy alone was insufficient in determining cell death and needs to be confirmed with other more sensitive assays. Flow cytometry using Annexin-V and propidium iodide is a very sensitive and specific method used to quantify cell death, excluding autophagy. It was determined that apoptosis and necrosis made a very small contribution to cell death occurring in treated cells. Autophgy must be investigated to determine the degree of autophagy present in treated cells.

5. References

BD Biosciences. 2010. "Product Information." [Online]. Available: http://www.bdbiosciences.com/FACS [Cited 13 June 2010].

Boeck, G. 2001. Current status of flow cytometry in cell and molecular biology. *International review of cytology* **204**: 239-298.

Chinkwo, K.A. 2005. Sutherlandia frutescens extracts can induce apoptosis in cultured carcinoma cells. *Journal of Ethnopharmacology* **98:** 163-170.

Imgenex. 2010. Annexin-V apoptosis kits. [Online]. Available: www.imgenex.com/apop_kits_list.php?id=76 [Cited 14 July 2010].

Kang, K.B., Zhu, C., Yong, S.K., Gao, Q. and Wong, M.C. 2009. Enhanced sensitivity of celecoxib in human glioblastoma cells: Induction of DNA damage leading to p53-dependent G_1 cell cycle arrest and autophagy. *Molecular Cancer* **8**: 66 – 82.

Menna-Barreto, R.F.S., Salomao, K., Dantas, A.P., Sant-Rita, R.M., Soares, M.J., Barbosa, H.S. and de Castro, S.L. 2009. Different cell death pathways induced by drugs in *Trypanosoma cruzi*: An ultrastructural study. *Micron* **40**: 157-168.

Sigma. 2004. "Product Information – Actinomycin D." [Online]. Available: http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=A9415|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC [Cited 12/07/2010].

Sigma. 2010. "Product search". [Online]. Available: http://www.sigmaaldrich.com/catalog/ProductDetail/ [Cited 5 September 2010].

Solomon, E.P., Berg, L.R. and Martin, D.W. 2005. Biology. 7th ed. Brooks/Cole. United States of America.

Stander, A., Marais, S., Stivaktas, V., Voster, C., Albrecht, C., Lottering, M-L. and Joubert, A.M. 2009. *In vitro* effects of *Sutherlandia frutescens* water extracts on cell numbers, morphology, cell cycle progression and cell death in a tumorigenic and a non-tumorigenic epithelial breast cell line. *Journal of Ethnopharmacology* **124**: 45 - 60.

Tinari, A., Giammarioli, A.M., Manganelli, V., Ciarlo,L. and Malorni. W. 2008. Analyzing morphological and ultrastructural features in cell death. *Methods in Enzymology* **442**: 1-26.

Trahtemberg, U., Atallah, M., Krispin, A., Verbovetski, I and Mevorach, D. 2007. Calcium, leukocyte cell death and the use of annexin V: fatal encounters. *Apoptosis* **12**: 1769-1780.

Van Cruchten, S. and Van den Broeck., W. 2002. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. *Anatomy, Histiology and Embryology* ***31***: 214-223.

CHAPTER 5:

RAMAN SPECTROSCOPIC ANALYSIS OF *CROTALARIA* ETHANOLIC EXTRACT AND MADURENSINE TREATED LEUKEMIC U-937 CELLS

1. Introduction

In the previous chapters it was established that the ethanolic extract of the leaves of *Crotalaria agatiflora*, as well as madurensine are moderately toxicity on leukemic U-937 cells. It was evident that the ethanolic extract had good antioxidant activity, while madurensine was not toxic up to a concentration of 100 µg/mL. Light microscopic investigations indicated that symptoms of cell death were induced during treatments, but flow cytometry analysis of treated cells, using annexin-V and propidium iodide, showed that apoptosis and necrosis were insignificantly induced. Due to inconclusive results obtained regarding the mechanism of action of the ethanolic extract and madurensine it was decided to make use of Raman spectroscopy in an attempt to understand the mechanism of action on a molecular level.

Raman spectroscopy has been used in industry and engineering since its development as a laboratory technique during the 1970s. Raman spectroscopy characterizes and identifies materials according to their vibrational spectra, which is unique for each material. A laser source which interacts with a variety of vibrational and other modes leads to light scattering of an elastic and inelastic nature. The energy spectrum of inelastic scattered light is directly related to vibrational, rotational and other low energy modes specific to the biochemical composition of the substance investigated. These energy modes serve as very specific molecular fingerprints and can be rapidly translated into unique spectral signatures using minute amounts of sample (Brown *et al.,* 2009). Biomedical sciences, including biochemical and biophysical intracellular investigations of diseases are increasingly making use of Raman spectroscopy and recently the focus has been on cancer research. The fundamental understanding of intracellular events, such as mitosis, cell differentiation, cell death, phagocytosis and interactions of drugs within living cells are important considerations when investigating cancer. Conventional assays are widely used in cancer research, notwithstanding the fact that these experiments have long preparation times, use large amounts of cells which need to be labelled and fixed and are damaged after the experiment, rendering them useless for further analysis. In contrast, Raman spectroscopy is a rapid and non-destructive technique for studying biological systems and neither labels nor fixation of cells is required, thus measurements can be taken in physiological conditions. Proteins, nucleic acids and lipids can be studied within cells and as Raman spectroscopy is sensitive to chemical and physical changes of biological molecules it can also differentiate between normal and abnormal cells in tissues; for example the detection of cancerous cells. The distribution of chemicals, induced by physical or chemical conditions, can be measured within the cells at sub-cellular levels as recent research focussing on drug interactions with proteins and DNA has illustrated. (Guo *et al.,* 2009).

2. Materials and methods

2.1 Sample preparation

The method published by Guo *et al*. (2009) was used as a guideline during analysis. U-937 cells are suspension cells and therefore it was necessary to manipulate the cells to adhere to the coverslips. U-937 cells were prepared on coverslips as previously discussed (Chapter 4) and treated with the cells were exposed to 73.9 µg/mL (IC_{50}) and 147.8 µg/mL ($2IC_{50}$) of the ethanolic extract of the leaves of *C. agatiflora* and exposed to 136.5 µg/mL of the isolated compound, madurensine, also included was vehicle-treated control (0.74%) cells and actinomycin D (0.002 µg/mL) treated cells. Cells were then propagated in growth medium and incubated for 72h at 37°C. Vero cells (noncancerous African green monkey kidney cells), on exposure to 147.8 µg/mL ethanolic extract of *C. agatiflora* ($2IC_{50}$ of U-937 cells) during microscopic investigations (Chapter 4) did not show any signs of apoptosis/necrosis, hence treatments involving Vero cells were not included for the Raman investigation.

2.2 Raman spectroscopy

Micro-Raman spectroscopy was performed with a T64000 micro-Raman spectrometer from HORIBA Scientific, Jobin Yvon Technology (Villeneuve d'Ascq, France). The Raman spectra were excited with the 514.5 nm line of an Innova 70v argon ion laser from Coherent and the 50x objective of an Olympus microscope was used to focus the laser beam (spot size ~ 10 µm) on the air-dried individual cells and also collected the backscattered Raman signal. An integrated triple spectrometer was used in the double subtractive mode to reject Rayleigh scattering and dispersed the light onto a liquid nitrogen cooled Symphony CCD detector. The spectrometer was calibrated with the silicon phonon mode at 520 cm^{-1}. The laser power at the sample was kept at 3 mW, to ensure that no sample degradation occurred, for the control samples (untreated, DMSO and Actino D), but had to be reduced to 2mW for the treated samples due to an increase in sensitivity to the laser light. These experimental conditions were kept constant for all measurements and three cells per sample were analysed at three different positions in a cell. All the spectra were recorded with a 150 s acquisition time and 10 accumulations.

2.3 Data processing

The Labspec 6.0 software was used to fit and subtract a fifth order polynomial function from all spectra to obtain a zero baseline in order to make comparisons between spectra possible. A 5 degree linear Savitsky-Golay smoothing procedure was applied to all the spectra. The intensity of the spectra was adjusted to make comparisons possible between spectra recorded with a laser power of 3 mW and 2mW respectively.

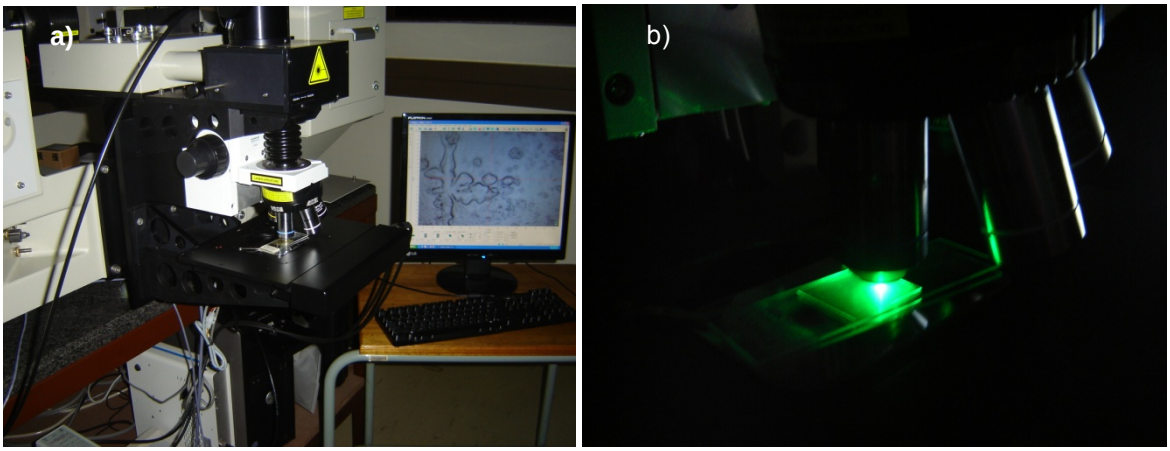


Figure 5.1: Raman spectroscopy equipment (a) and 514nm laser on microscope slide containing the organic sample (b)

3. Results:

3.1 Micro-Raman spectroscopic investigation of U-937 cells

Raman spectra did not show any difference when comparing spectra of different cells for each treatment and also spectra taken at different positions within the cells were the same. This is in accordance with the findings of Zinin *et al*. (2010), which suggested that U-937 cells are homogenous. The Raman spectrum of the glass slides was not observed in the spectra, as previously reported by Shachaf *et al.,* 2009.

3.1.1 Overview of cancerous U-937

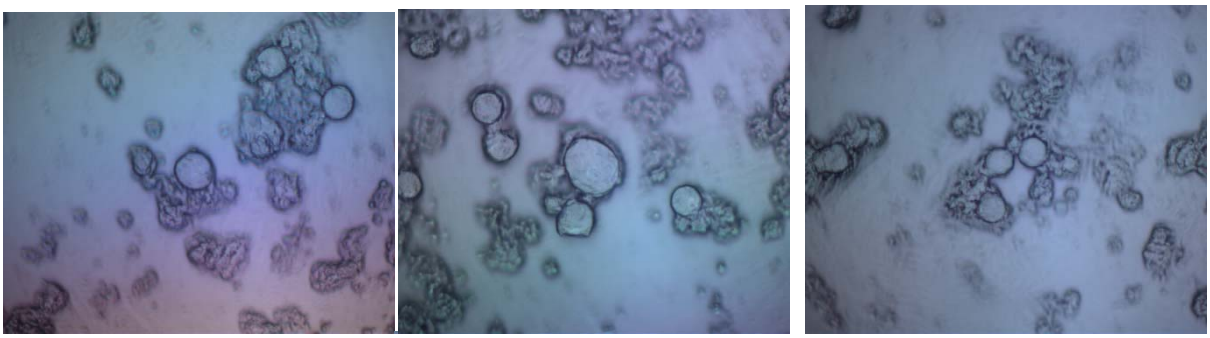


Figure 5.2: Photomicrographs of dried untreated U-937 cells

There was no morphological damage observed and all the cells looked healthy (Figure 5.2). A representative Raman spectrum of U-937 cells is shown in Figure 5.3 and tentative assignments of the bands are listed in Table 5.1. The key biochemical constituents that occur in U-937 cells are proteins / amino acids, nucleic acids (DNA) and lipids. A Raman spectrum of a cell is therefore a

superimposition of the Raman spectra of many different chemical entities and not always easy to analyse as many bands occur at the same positions and overlap. Marker peaks, which are specific to a certain type of molecule, could be assigned for some of the components. The strongest peaks of a specific compound can not always be used as a marker as it may overlap with bands from other molecules and not all the constituents can always be identified through their Raman spectra as all the peaks may be hidden.

Representative peaks for DNA consists of the DNA backbone vibrations at 830 and 1093 cm^{-1} as well as adenine (1490 cm^{-1}) and guanine (1579 cm^{-1}). The Raman spectrum of a protein is characterised by Amide I (~1658 cm^{-1}), Amide II (~1607 cm^{-1}) or Amide III (~1255 cm^{-1}) vibrations of the peptide backbone (Petersen and Nielsen, 2009, Zinin *et al.,* 2010 and Krafft *et al*., 2003). Amide I and Amide III bands are classical markers for protein conformation as the Amide II band is usually weak in a Raman spectrum and can thus in practice not be used as a marker. Spectroscopic markers for lipids can be divided into bands originating from the acyl chain and the lipid head group. Acyl chain markers include a prominent band at 1440 cm^{-1} corresponding to CH_2 bending vibrations, while a peak at 1460 cm^{-1} is representative of CH_3 bending modes. All 'trans' skeletal vibrations are represented around 1133 cm^{-1} and 1064 cm^{-1} by CC stretching bands. Head group markers include the band at 860 cm^{-1} which is assigned to a phosphate group general to most phospholipids (Petersen and Nielsen, 2009). A marker band which is specific to monocytes (U-937 cells are monocytic) is centred on ~1206 cm^{-1} (Zinin *et al.,* 2010).

In untreated leukemic U-937 cells, amino acids that could be identified by their Raman peaks were tryptophan (759 and 1340 cm^{-1}), tyrosine (830, 855, 1172 and 1207 cm^{-1} - ($C\text{-}C_6H_5$) stretching)), phenylalanine (1003 – ring breathing mode and 1207 cm^{-1}), proline and valine as well as other protein backbones (920 cm^{-1}) (Figure 5.3). Two peaks were assigned to the Amide III protein bands (1255 and 1555 cm^{-1}) of which the 1555 cm^{-1} peak refers to the C-N stretching mode. The peak at 1447 cm^{-1} was assigned to CH_2 bending vibrations of long chained amino acids, but the strongest contribution to this peak is from lipids. Bands at 1125 and 1657 cm^{-1} were assigned to C-C stretching and C=C stretching of lipids respectively. Above mentioned peaks gives an indication of the protein and lipid distribution within U-937 cells (Kraft *et al.,* 2003 and Zinin *et al.,* 2010). The band centred at ~783 cm^{-1} was also previously found in bacteria and HeLa cells and assigned to the C-C breathing vibrations of cytosine, thymine and uracil. The weak band at ~1580 cm^{-1} was assigned to nucleic acid ring stretches of guanine and adenine. The band at the peak ~1096 cm^{-1} is associated with O-P-O backbone vibrations of DNA. The peak at 783 cm^{-1} suggested that the DNA is B-form. In B-form DNA, guanine is exposed to the major groove. The N7 and N3 positions of guanine are proton-acceptor sites. Thus it should be kept in mind that guanines in the major grooves are candidates for hydrogen bond formation with zenotoxins, such as doxorubicin. It was found that due to the stereo-hindrance effect, N7 of guanine is more

reactive (Guo *et al.,* 2009). Bands published by Benevides and colleagues (2005) were similar in comparison with mentioned characteristic peaks of B-DNA. Benevides *et al.* (2005) found that B-DNA has a unique backbone conformation marker at 835 ± 7 cm^{-1}. Interestingly, predominant GC

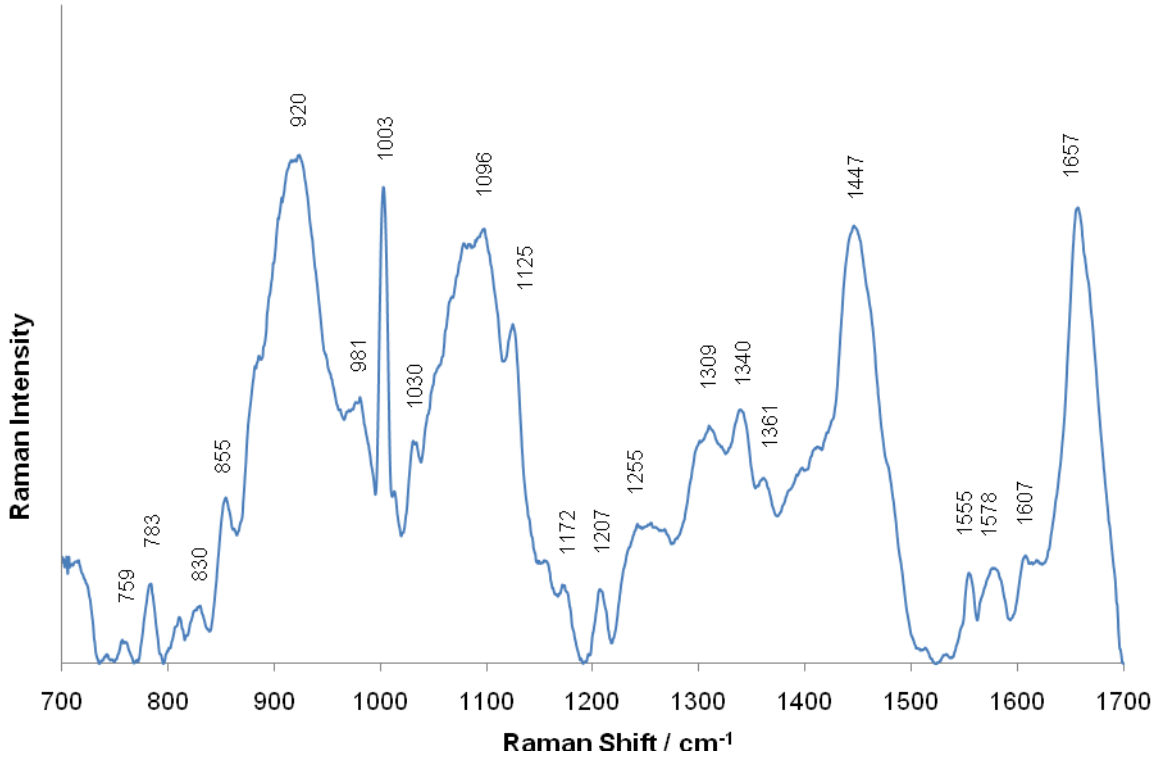


Figure 5.3: Representative Raman spectrum of cancerous U-937 cells

base pairs exhibit the B-marker at ~830 cm^{-1}, while predominantly AT base pairs exhibit the marker near 840 cm^{-1}. According to this untreated cells are in the B-DNA form (Table 5.1, Fig. 5.2). For interest sake a band at ~1602 cm^{-1} is called the Raman spectroscopic signature of life and reflects the metabolism of mitochondria (Zinin *et al.,* 2010). Mitochondria produce energy for cells to transcribe DNA and translate RNA to proteins. Proteins form the main building blocks for enzymes, microtubules, spindles and actin, all essential components of viable cells. Energy as well as protein is required for cells to be able to stay metabolically active and for mitosis to take place. Unfortunately no band could be clearly recognized in the Raman spectrum, due to the proximity of the band representing Amide II protein conformation at 1607 cm^{-1}. Sharp peaks were seen at 981 and 1031 cm^{-1} which were assigned to cell culture media (RPMI medium) by Zinin *et al.,* 2010. The 981 cm^{-1} peak was due to the vibrations of phosphates (PO_4 at 960 cm^{-1}) in solution. All peaks assigned and most prominent were in agreement with the Raman spectrum published for dried U-937 cells by Zinin *et al.,* 2010. Slight changes in the positions of bands may be due to the confocal laser wavelength differences and the resonance.

3.1.2 Raman spectra of vehicle treated (DMSO) cells

Vehicle treated cells appeared to have had minimal damage (Figure 5.4). Raman spectra recorded for untreated and vehicle treated cells looked very similar (Figure 5.5) with only a few bands at 710, 920, 1447 and 1657 cm^{-1} noticeably different. . Affected bands were assigned to proteins and lipids (Table 5.1). A new band appeared at 1420 cm^{-1} which is representative for guanine and adenine base pairs. Thus some changes occurred in the DNA of treated DMSO cells. Two opposing interpretations have been used to discuss intensity changes of peaks during treatments. The first interpretation states that as the intensity of Raman peaks depends linearly on the concentration of molecules in the sample, a lower concentration of a certain molecule will imply that the associated Raman peak will have a lower intensity (Pupples *et al*., 1993). The second interpretation suggests that when molecules denature or lose their form and function some of the Raman peaks' intensities will increase (Zinin *et al.,* 2010). Both these interpretations were considered in our discussions for all treatments.

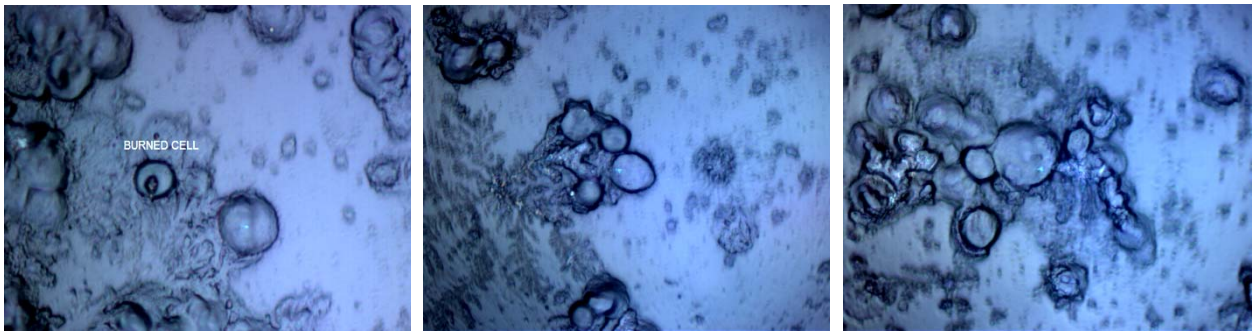


Figure 5.4: Photomicrographs of dried U-937 cells treated with 0.74% DMSO for 72h

Some cells were more sensitive to the laser power in comparison to untreated cells. Considering the peak intensity changed it could be speculated that the slight increase of intensity of peaks positioned around 710 and 1657 cm^{-1} (cholesterol and lipids) could be due to degradation during DMSO treatment. When small amounts of stress are put on viable organisms, one usually finds that as a response, products such as lipids and proteins can be produced in higher concentrations and thus the increase of peak intensities of lipids could be interpreted as an increase in the amount of lipids, but this is unlikely due to the sensitivity of the cell membrane. A decrease in intensity of the peak at 920 cm^{-1} indicated that protein distribution and protein functions might have been also affected by the treatment with DMSO. The peak at ~1003 cm^{-1} associated with phenylalanine slightly increased in intensity, possibly an indication of the denaturing of the amino acid.

Table 5.1: Tentative Raman assignments for untreated U-937 cells

Peak position (cm^{-1})	*Assignments*			
	Proteins	***Nucleic acids***	***Lipids***	***References***
759 wn	Tryptophan			Krafft *et al.,* 2003
783 wn		Cytosine, Thymine, Uracil, O-P-O backbone		Krafft *et al.,* 2003, Guo *et al.,* 2009 & Yao *et al*., 2009
830 mn	Tyrosine			Krafft *et al.,* 2003
855 mn	Tyrosine			Guo *et al.,* 2009
860 wn			Phospholipids	Petersen and Nielsen, 2009
920 sb	α-helix conformation (C-C stretch of proline ring, valine and protein backbones			Zinin *et al.,* 2010 and Yao *et al.,* 2009
981 mn	***Cell culture medium***			Zinin *et al.,* 2010
1003 sn	Phenylalanine (ring breathing mode)			Krafft *et al.,* 2003, Guo *et al.,* 2009 & Yao *et al*., 2009
1031 mn	***Cell culture medium***			Zinin *et al.,* 2010
1096 mb		DNA ($O-P-O^-$ stretch)		Zinin *et al.,* 2010 & Yao *et al*., 2009
1125 mn			C-C stretching	Guo *et al.,* 2009
1172 wn	Tyrosine, Phenylalanine			Guo *et al.,* 2009 & Zinin *et al.,* 2009
1193-1207 mn	Tyrosine, Phenylalanine			Krafft *et al.,* 2003 & Zinin *et al.,* 2009
1255 - 1268 wn	Amide III			Krafft *et al.,* 2003 & Zinin *et al.,* 2009
1309 wn		Guanine		Guo *et al.,* 2009
1340 wn	C-H deformation, Tryptophan	Polynucleotide chain (DNA purine bases, specially Adenine)		Krafft *et al.,* 2003, Zinin *et al.,* 2010, Guo *et al.,* 2009 & Yao *et al*., 2009
1361mn		Thymine and adenine		Zinin et al., 2010
1447 sb	CH_2 stretching		CH_2 stretching	Krafft *et al.,* 2003 & Zinin *et al.,* 2009
1490 wn		dAdenine		Thomas, 1999
1555 mn	Amide III (C-N stretching mode)			Matthaus *et al*., 2008
1573-1578 mn		Guanine, Adenine ring stretch		Krafft *et al.,* 2003 & Thomas, 1999
1607 wn	Amide II			Zinin *et al.,* 2010
1657 sb	Amide I		C =C stretch	Bankapur *et al*., 2010 & Krafft *et al.,* 2003

Abbreviations regarding Raman peaks: s- strong, m-medium, w-weak, n-narrow and b-broad

Thus a decrease in intensity of some of the amino acids peaks implied that the production of amino acids was affected negatively, while the increase of the phenylalanine peak implied that phenylalanine denatured. This result made it clear that even lower percentages of DMSO should be used to keep treated cells unaffected by the vehicle solvent.

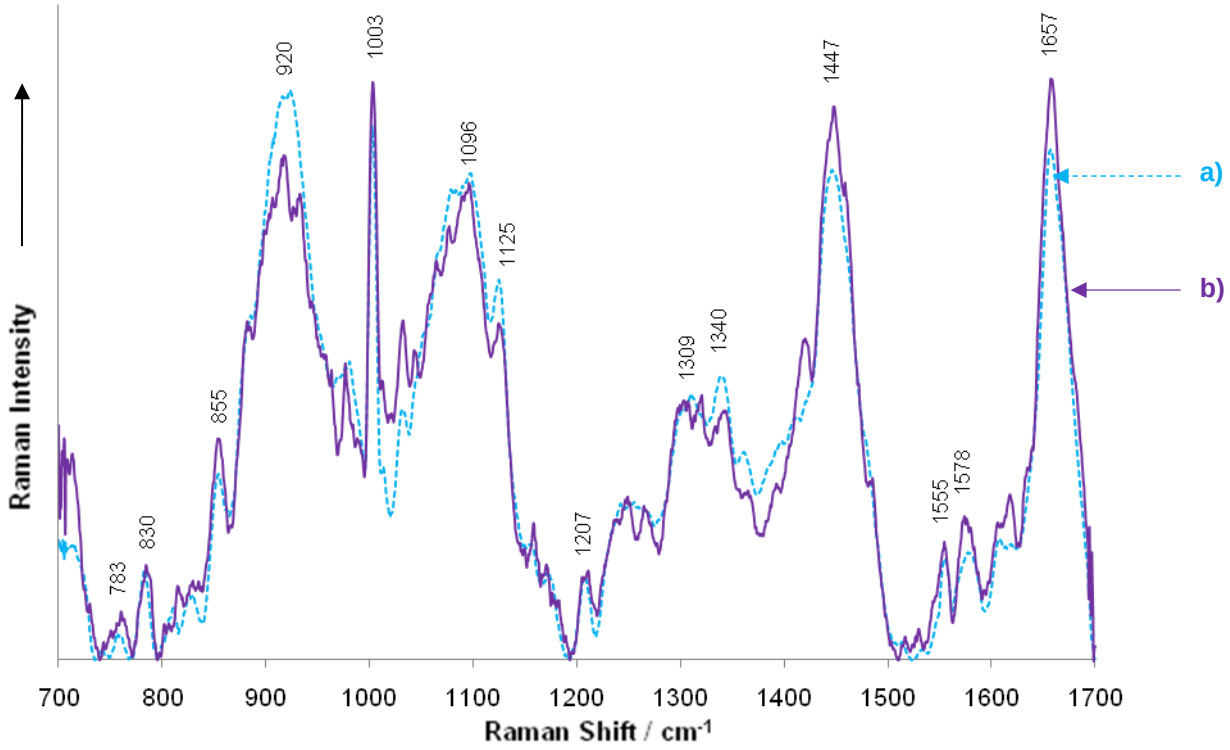


Figure 5.5: Respresentative Raman spectra of untreated (a) and vehicle (DMSO) treated (b) U-937 cells

3.1.3 Impact of Actinomycin D on U-937 cells

Morphologically, treated cells were found to be much smaller compared to untreated cells (Figure 5.6). Cells showed symptoms of apoptosis after 72 hours incubation, which was expected as this compound is known to induce apoptosis (Sigma, 2004, Stander *et al.,* 2009).

Amino acid / protein bands at 759, 830, 855, 920, 1003, 1340, 1447 and 1555 cm^{-1} decreased in intensity, which were interpreted as lower concentrations of molecules within the cells (Figure 5.7). The same trend was observed for lipid representative molecules with peaks at 860, 1125, 1447 and 1657 cm^{-1}. Some bands represent both protein and lipid vibrations (Table 5.1). The decrease in proteins as well as lipids had a direct relationship with changes in DNA, since those molecules are products of RNA translation. Bands assigned to nucleotides and elements of DNA all decreased (1096, 1309, 1340, and 1578 cm^{-1}).

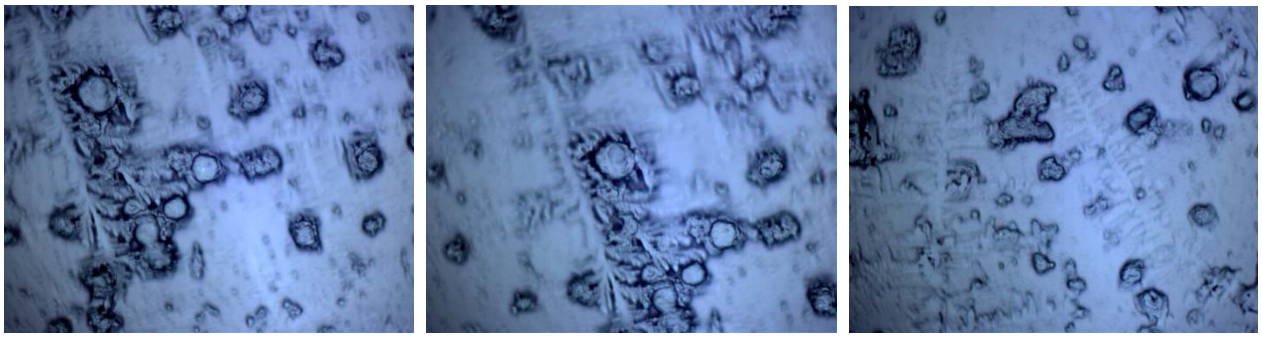


Figure 5.6: Photomicrographs of dried U-937 cells treated with 0.002 µg/mL Actinomycin D for 72h

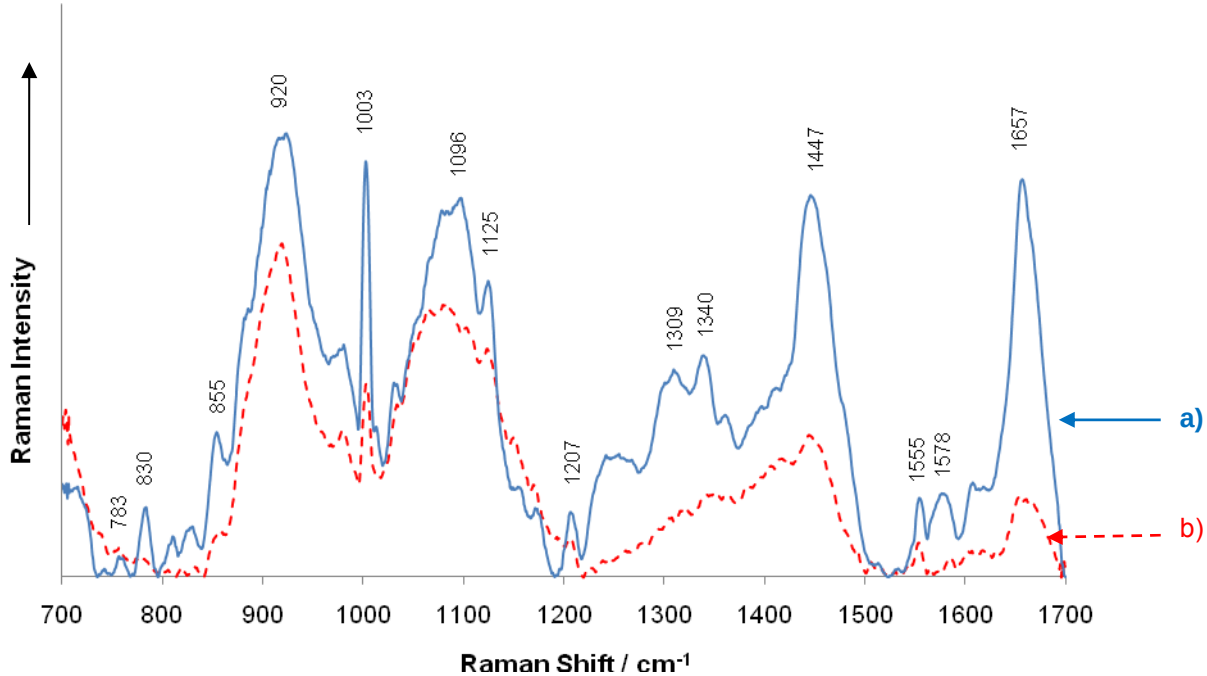


Figure 5.7: Representative spectra of untreated dried U-937 cells (a) and actinomycin D treated dried U-937 cells (b)

The mechanism of action of actinomycin D had been investigated previously with crystallography and it was found that this antibiotic is capable of forming complexes with DNA (Sigma, 2004). More specifically actinomycin D intercalates into DNA. Both amino acids (Threonine) reside in the the minor groove of DNA (see Chapter 2, Section 1.1.6). Hydrogen bonds are formed to N2 and N3 atoms of guanosine (guanine bound to sugar). This compound is sequence specific (GpX) for example d(GAA**GC**TTC) (Neidle, 2002). This specific intercalation causes an obstruction for RNA polymerase movement and consequently DNA-dependent RNA synthesis. This compound is also able to cause breaks in double stranded DNA (Sigma, 2004). The decrease of peak intensity in the Raman spectrum was in agreement with the established fact that actinomycin D prevents amino acid and protein transcription and evidently translation as well as DNA strands breaks. The same result have previously been observed on human gastric cells (SGC-7901 cells) after induction of apoptosis with 5-fluoro uracil where peak intensities (782, 1092, 1320, 1340, 1578 and

1655 cm^{-1}) also decreased. These changes within spectra were used as a second parameter in defining apoptosis after it was confirmed in the first instance with confocal microscopy by staining the DNA of treated cells with Hoechst 33258 (DNA intercalator). It was concluded that a drop in the intensitiy of peaks assigned to nucleic acids, proteins and lipids are associated with apoptosis (Yao *et al.,* 2009). Our Raman results were in agreement with this study and the results from previous flow cytometry studies (Chapter 4).

3.1.4 Impact of increasing concentrations of ethanol extract

Extreme morphological differences were observed between vehicle treated cells (Figure 5.4) and the cells after treatment with the ethanolic extract (Figure 5.8 and 5.9). Cells treated with the extract had shrunken quite significantly during treatments, indicating cell death (Uzunbajakava *et al*., 2003). Convinced signs of cell death were observed and membrane blebbing was clear on most of the photos as well as apoptotic bodies. The cells were overall very sensitive to the laser and so the laser power was decreased to 2 mW to prevent the cells from burning. Although the morphological signs corresponded to apoptosis, flow cytometry measurements established that apoptosis only took place in 10 – 20% of the cells analyzed (Chapter 4). Autophagy displays similar morphological changes corresponding to apoptosis, which include partial chromatin condensation and plasma membrane blebbing (De Bruin and Medema, 2008). Thus the only other mechanism of action of the treatment can be autophagy.

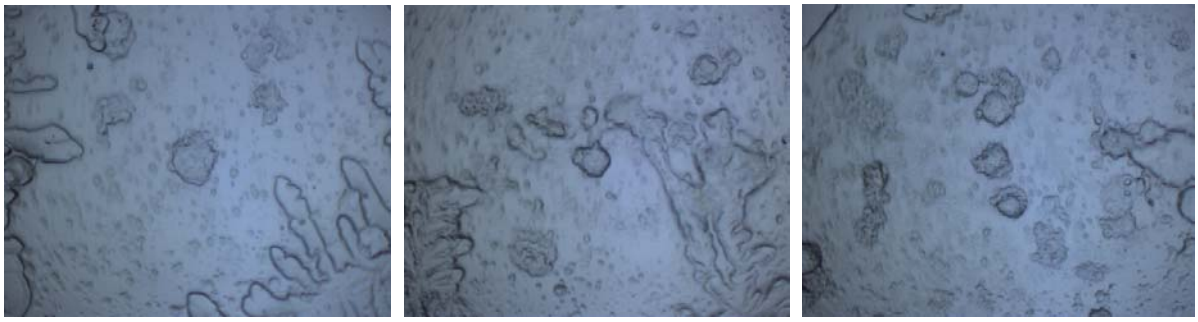


Figure 5.8: Photomicrographs of dried U-937 cells treated at IC_{50} of *C. agatiflora* for 72h

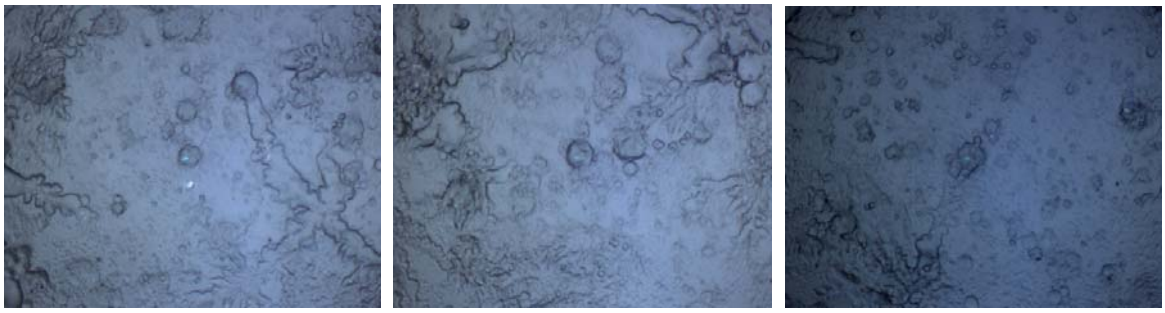


Figure 5.9: Photomicrographs of dried U-937 cells treated at $2IC_{50}$ of *C. agatiflora* for 72h

No publications could be found describing induced autophagic cells investigated with micro-Raman spectroscopy. Our results showed firstly that the treatments had variable affects on the Raman spectra in comparison to DMSO treated cells (Figure 5.10). A decrease in intensity of peaks at 855, 920, 1003, 1657 cm^{-1}, were observed, indicating that some of the major protein and lipid distribution and concentrations diminished while other protein and lipid peaks increased in intensity, namely peaks at 706, 1125, 1193, 1340, 1447, 1555 and 1691 cm^{-1}. Interestingly, 706 and 1671 cm^{-1} are assigned to cholesterol. Cholesterol is one of three classes of membrane lipids and is very abundant in eukaryotic cell membranes (Lodish *et al.,* 2005). Three hypotheses were considered for the increase in the cholestrol peaks. Firstly cholesterol production might be up regulated due to stress signals within the cells during treatment. Secondly, cholesterol lost its function and structure, similar to the denaturing of proteins which lead to increased peak intensities. But the most logical explanation would be that during autophagy, autophogosomes are formed, enclosing whole defective organelles and parts of the cytoplasm. Those organelles and cytosol components are degraded to primary building blocks to supply nutrients to the cells. As cholesterol forms a substantial part of all membranes (plasma membrane, organelle membranes and endoplasmic reticulum) a huge increase in the cholesterol peak intensity can be observed upon degradation of membranes and organelles. A peak at ~ 1692 cm^{-1} is assigned to the upper region of amide I band (Swain et al., 2010 and Matthaus et al., 2008). Further increased peak intensities could be attributed to either degradation of lipids and proteins, or such in the case of cholesterol, specific aspects of the amino acids and lipid structures may be highlighted and thus show increased intensities due to degradation inside the autophagosomes.

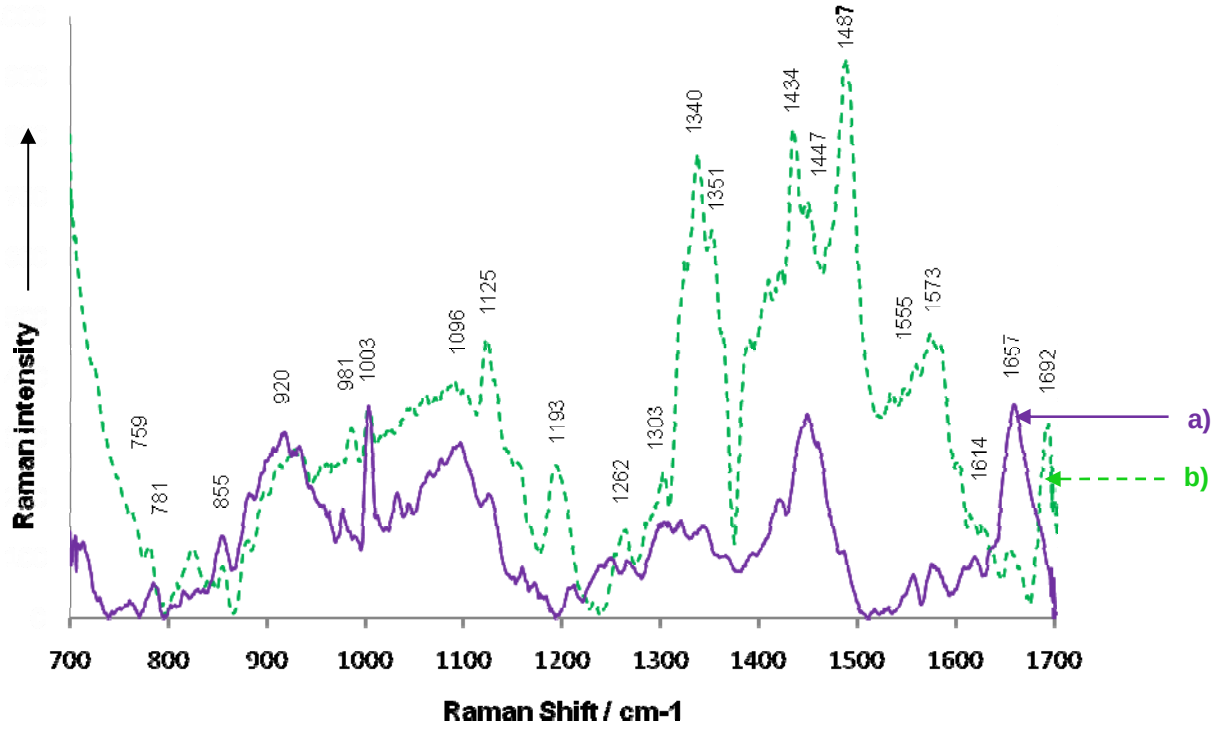


Figure 5.10: Representative Raman spectra of DMSO treated (a) and extract IC_{50} (b)

Briefly, when the focus was shifted to the affect of the extract on DNA, severe changes were seen in concentrations of different nucleic acids. Peaks 729, 1340, 1487 and 1573 cm^{-1} represents purine bases. Peaks 729 and 1378 cm^{-1} represent pyrimidine bases (Krafft *et al.,* 2003 and Taleb *et al.,* 2006). All of these peaks increased in intensity which might be due to the partial condensation of chromatin (De Bruin and Medema, 2008) during autophagy. Another reason for increased peak intensities might be due to compounds binding (especially intercalation of alkaloids) to DNA (Wink, 2003). The phosphate backbone of DNA also changed during sample treatment as evidenced by the peak at 1096 cm^{-1}.

Upon treatment with twice the IC_{50} concentration it seemed as if the concentrations of molecules followed a dose-dependent response, as was also found with the cytotoxicity assay in Chapter 2. Only one peak, centred around 1262 cm^{-1}, assigned to the Amide III band of the protein backbone, relatively increased in comparison to the spectrum of the IC_{50} treated sample (Figure 5.11).

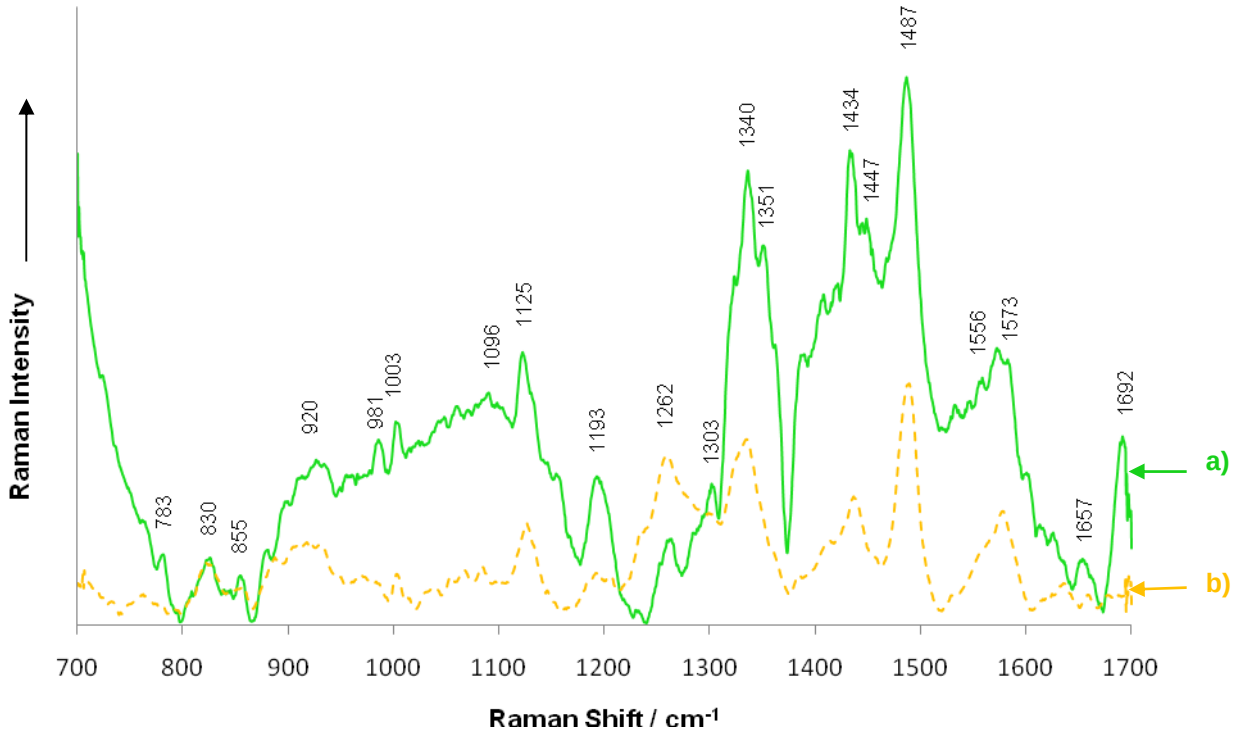


Figure 5.11: Representative Raman spectra of *C. agatiflora* IC_{50} (a) and $2IC_{50}$ (b) treated U-937 cells

This is an indication that during treatment the Amide III bonds were disrupted causing a change in relative peak intensity. The rest of the peaks indicated protein and lipid distribution which all decreased in intensity, suggesting lower concentrations of these molecules in the treated cells. The large decrease in concentrations of molecule might be due to the fact that higher concentrations ethanolic extract caused an enhanced stress response by the cells, leading to more

autophagosomes and thus quicker degradation of molecules to be exocytosed. When those materials are excreted by the cells, lowered concentrations would be left inside the cells. Lower cholesterol content was also indicative of the onset of degradation and removal of defective organelles such as mitochondria.

The ethanolic treated cells were not compared to actinomycin D due to the fact that the ethanolic extract consists of an unknown mixture of compounds, while actinomycin D was a pure compound with known mechanism of action. Thus one can easily misinterpret results when comparing pure compound's results with extract results.

3.1.5 Impact of the isolated compound, madurensine on U-937 cells

Madurensine treated cells showed less prominent signs of plasma membrane disruption, although the cells were shrunken and showed some blebbing of the cell membranes (Figure 5.12). Treated cells, as in the case of extract treated cells were sensitive to 2mW laser power and thus the laser was adjusted to 2mW.

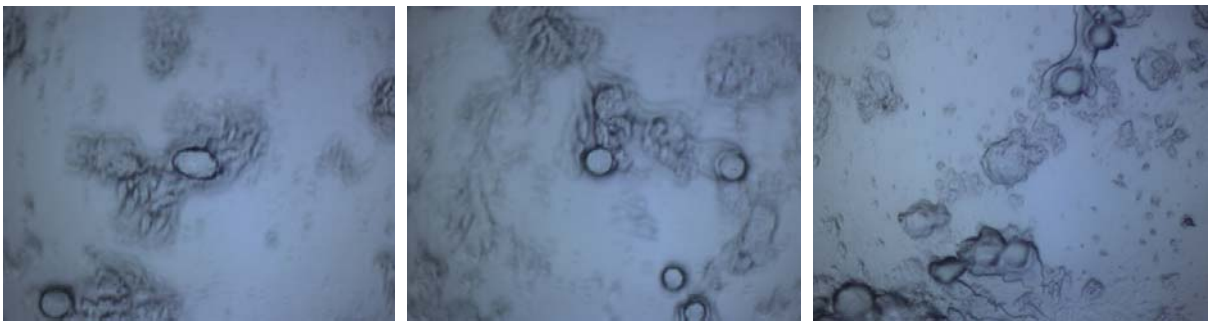


Figure 5.12: Photomicrographs of dried U-937 cells treated 72h with madurensine at IC_{50} value

All the Raman peaks decreased significantly in comparison to the Raman spectrum of DMSO treated cells (Figure 5.13), which is indicative of lower concentrations of proteins and lipids. The peak at 835 cm^{-1}. assigned to the phosphate backbone of DNA and tyrosine had disappeared and another peak was observed at ~808 cm^{-1} which is the marker band for A-DNA (807 ± 3 cm^{-1}) (Benevides *et al*., 2005). A-form DNA is known in Gram positive bacteria during sporolation. In eukaryotic cells, genomic DNA (B-DNA) can become A-DNA when the humidity inside of the cell is lowered (60 -70% humidity) and could also be formed during transcription and replication with DNA-RNA hybrids. The last mentioned scenario is not highly likely due to the hypothesis that the cells are under chemical stress and autophagic. This theory is more plausible due to the low concentrations of proteins and lipids (Neidle, 2002).

These interesting results motivated us to compare the results of the positive control actinomycin D with that of madurensine as both are pure single compounds and the differences might confirm any observed changes in peak intensities for apoptotic and predicted autophagic cells. In Figure 5.14, it was clear that madurensine treated cells were less metabolically active, thus had lower concentrations of proteins and lipids as compared with actinomycin D treated cells. Thus at the end of the analysis it seemed as if proposed autophagy could had caused the "metabolic fingerprints" of treated cells to change dramatically, even though the cells were likely to recover completely when treatment was stopped.

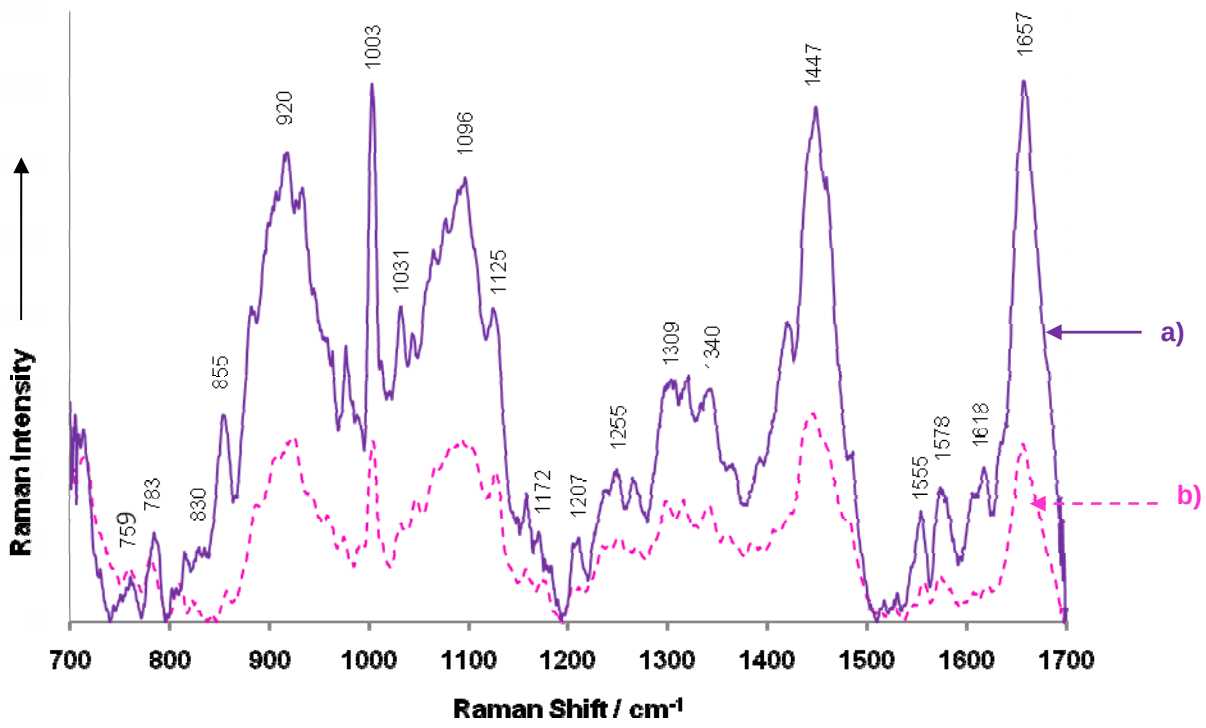


Figure 5.13: Representative Raman spectra of DMSO treated (a) and madurensine (b) treated cells

It seemed as if alkaloids which are theoretically known to be very toxic were not the most active compounds within *Crotalaria*, although the active fractions could not have been determined in this study, we have proven that without metabolic activation alkaloids in *Crotalaria agatiflora* subspp *agatiflora* is not severely cytotoxic in noncancerous cells. Mechanism of action that were previously documented includes enlarged cells (megalocytosis) with chronic poisonings of animals and even in *in vitro* cultured bovine pulmonary artery endothelial cells exposed to monocrotaline pyrrole, inhibition of mitosis and the interactions of pyrrolizidine alkaloids with cell components such as DNA and proteins. During DNA damage apoptotic pathways may be activated (Prakash *et al.,* 1999).

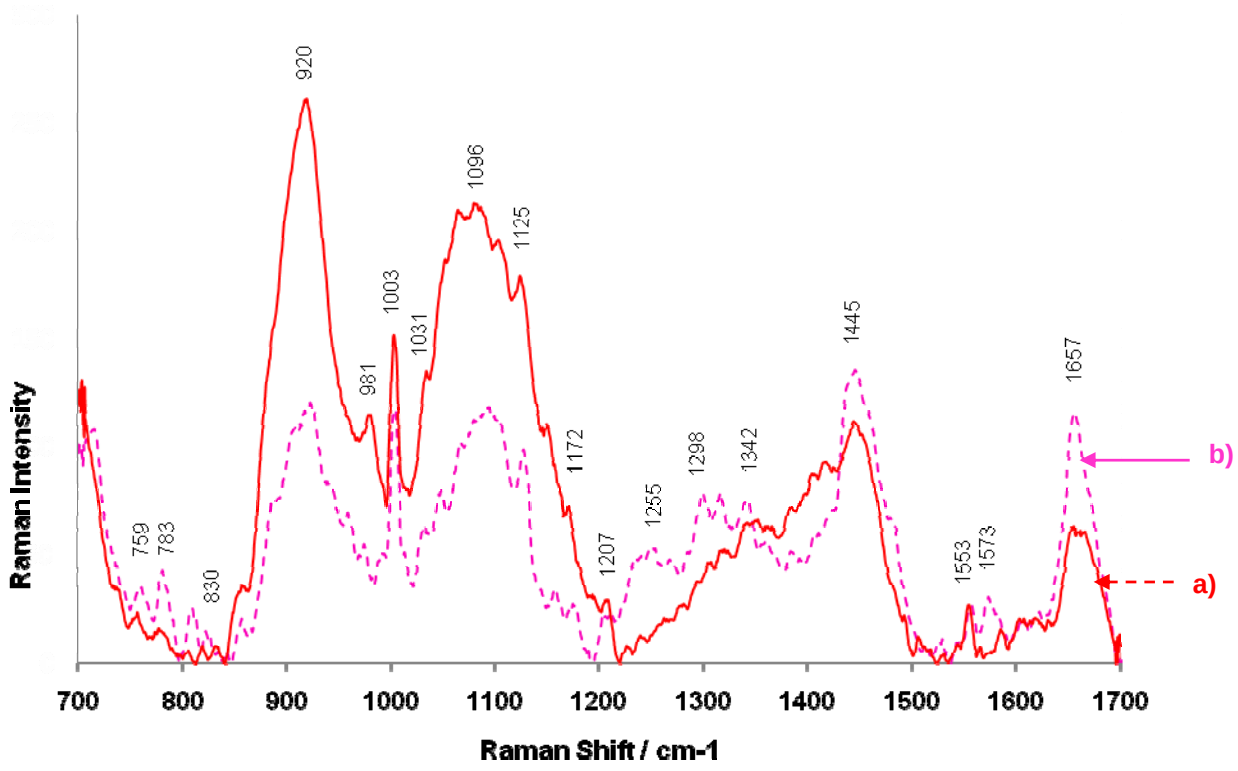


Figure 5.14: Representative Raman spectra of actinomycin D (a) and madurensine (b) treated cells

Only two similar investigations could be found, one of which was a Brazilian MSc dissertation (in Portuguese) and an article published by Saha and Yakovlev (2009). Saha and Yakovlev (2009) investigated the affects of *Nerium oleander* on prostate cancer cells (PANC1) and found that treatment with high concentrations affected protein and lipid composition dramatically. They also found that Raman spectroscopy was a powerful interogation instument to determine the variation of chemical composition in cells. Both findings were confirmed in the present study. Autophagy and apoptosis had very similar spectra and thus could not be distuingished by just micro-Raman spectroscopy.

4. **Conclusion:**

Increasing concentrations of *C. agatiflora* had a severe affect on the composition of the biochemical material of the treated cells. Autophagy was hypothezided due to consequential results seen thus far. Decreased cell size were due to the decreased concentration of proteins and lipids in the cells. During the investigation it was established that micro-Raman spectroscopy can be used for initial screening of samples to determine the effects of different treatments on cancerous cell lines. Apoptosis and autophagy cannot be distinguished by only using micro-

Raman spectroscopy. Raman spectra gave indications on what aspects of molecular cell biology should be futher investigated to conclude autophagy as the mechanism of action.

5. References:

Benevides, J.M, Overman, S.A. and Thomas, G.J. 2005. Raman, polarized Raman and ultraviolet resonance Raman spectroscopy of nucleic acids and their complexes. *Journal of Raman Spectroscopy* **36**: 279 – 299.

Brown, K.L., Palyvoda, O.Y., Thakur, J.S., Nehlsen-Cannarella, S.L., Fagoaga, O.R., Gruber, S.A. and Aumer, G.W. 2009. Raman spectroscopic differentiation of activated versus non-activated T lymphocytes: An in vitro study of an acute allograft rejection model. *Journal of Immunological Methods* **340**: 48-54.

De Bruin, E.C. and Medema, J.P. 2008. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treatment Reviews* **34**: 737-749.

Guo, J., Cai, W., Du, B., Qjan, M. and Sun, Z. 2009. Raman spectroscopic investigation on the interaction of malignant hepatocytes with doxorubicin. *Biophysical Chemistry* **140**: 57-61.

Krafft, C., Knetschke, T., Siegner, A., Funk, R.H.W. and Salzer, R. 2003. Mapping of single cells by near infrared Raman microspectroscopy. *Vibrational spectroscopy* **32**: 75 – 83.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C.A., Krieger, M., Scott, M.P., Zipursky, L. and Darnell, J. 2005. Molecular Cell Biology. 5th ed. New York: Freeman and Co.

Matthaus, C., Bird, B., Miljkovic, M., Chernenko, T., Romeo, M. and Diem, M. 2008. Infrared and Raman microscopy in cell biology, Chapter 10 in *Methods in cell biology* **89**: 275-308.

Neidle. S. 2002. Nucleic structure and recognition. Oxford: Oxford University Press. Pp 150-180.

Petersen, N.R. and Nielsen, C.H. 2009. "Raman spectroscopy as a tool for investigating lipid-protein interactions" [Online]. Available: http://spectroscopyonline.findanalytichem.com/spectroscopy/article/articleDetail.jsp?id=637198 [Cited 19 January 2011].

Prakash, A.S., Pereira, T.N., Reilly,P.E.B. and Seawright, A.A. 1999. Pyrrolizidine alkaloids in human diet. *Mutation Research* **443**: 53-67.

Pupples, G.J., Garritsen, H.S.P., Kummer, J.A. and Greve, J. 1993. Carotenoids located in human lymphocyte subpopulations and natural killer cells by Raman microspectroscopy. Cytometry 14: 251 – 256.

Saha, A. and Yakovlev, V.V. 2009. Towards a rational drug design: Raman micro-spectroscopy analysis of prostate cancer cells treated with an aqueous extract of Nerium Oleander. Journal of Raman Spectroscopy 40: 1459-1460.

Shachaf, C.M., Elchuri, S.V., Koh, A.L., Zhu, J., Nguyen, L.N., Mitchell, D.J., Zhang, J., Swartz, K.B., Sun, L., Chan, S., Sinclair, R. and Nolan, G.P. 2009. A novel method for detection of phosphorylation in single cells by surface enhanced Raman scattering (SERS) using composite organic-inorganic nanoparticles (COINs).*PLoS ONE* **4:** 1-12, doi:10.1371/journal.pone.0005206.

Sigma. 2004. "Product Information – Actinomycin D." [Online]. Available: http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=A9415|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC [Cited 12/08/2010].

Stander, A., Marais, S., Stivaktas, V., Voster, C., Albrecht, C., Lottering, M-L. and Joubert, A.M. 2009. *In vitro* effects of *Sutherlandia frutescens* water extracts on cell numbers, morphology, cell cycle progression and cell death in a tumorigenic and a non-tumorigenic epithelial breast cell line. *Journal of Ethnopharmacology* **124**: 45 - 60.

Swain, R.B., Kemp, S.J., Goldstraw, P., Tetley, T.D. and Stevens, M.M. 2010. Assesment of cell line models of primary human cells by Raman spectral phenotyping. *Biophysical Journal* **98**: 1703-1711.

Taleb, A., Diamond, J., McGarvey, J.J., Beattie, J.R., Toland, C. and Hamilton, P.W. 2006. Raman microscopy for the chemometric analysis of tumor cells. *Journal of physical chemistry* B **110**: 19625 – 19631.

Thomas, G.J. 1999. Raman spectroscopy of protein and nucleic acid assemblies. *Annual Review of biophysical and biomolecules structures* **28**:1-27.

Tolkovsky, A.M., Xue, L., Fletcher, G.C. and Borutaite, V. 2002. Mitochondrial disappearance from cells: a clue to the role of autophagy in programmed cell death and disease? *Biochimie* **84**: 233-240.

Uzunbajakava, N., Lenferink, A., Kraan, Y., Volokhina, E., Vrensen, G., Greve, J. and Otto, C. 2003. Nonresonant confocal Raman imaging of DNA and protein distribution in apoptotic cells. *Biophysical Journal* **84:** 3968-3981.

Wink, M. 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64**: 3-19.

Yao, H., Tao, Z., Ai, M., Peng, L., Wang, G., He, B. and Li, Y. 2009. Raman spectroscopic analysis of apoptosis of single human gastric cancer cells. *Vibrational spectroscopy* **50:** 193-197.

Zinin, P.V., Misra, A., Kamemoto, L., Yu, Q., Hu, N. and Sharma, S.K. 2010. Visible, near-infrared and ultraviolet laser-excited Raman spectroscopy of the monocyte/marcrophages (U-937) cells. *Journal of Raman spectroscopy* **41**: 268-274.

CHAPTER 6:

CONCLUSIONS AND FUTURE RECOMMENDATIONS

Flow cytometry and Raman spectroscopy were used to determine the mechanism of action, but instead of confirming the best known type of cell death, apoptosis, we were surprisingly relieved to have stumbled across a much more interesting plant than was expected. Autophagy should still be confirmed with more conventional assays, but for this investigation enough evidence were collected to suspect autophagy.

Combining microscopy, flow cytometry and Raman spectroscopy the mechanism of action was predicted. After the analysis of flow cytometry, it was evident that apoptosis occurred minimally in treated samples. With Raman spectroscopy it was found that the cells were severely affected biochemically by the treatments, which lead to variable conditions noticed on the molecular level. The mitochondrial results observed using Raman spectroscopy suggested that faint correlations could be made with XTT assay results. The reason for that statement is due to the fact that one of the main focus end points of XTT is the conversion of salt to formazan by mitochondrial enzymes. Thus XTT gave an idea on the fitness of mitochondria, which in this investigation could not be confidently correlated with micro-Raman spectroscopy. Results suggested that autophagy may be the mechanism of action for the observed results. It could not be determined during the investigation whether the cancerous cells would in prolonged conditions survive or die during treatments.

Recommendations for improving future investigations would be to not only focus on the XTT assay as a predictor for cell viability, but also other assays such as crystal violet and neutral red, which combined would give a more holistic perspective of cell viability. Other microscopy techniques should be done in combination with light microscopy to be able to see more specific traits of cell morphology and cellular components. Cell cycle analysis could be included, together with acridine orange stained cells to quantify these assays with flow cytometry. Other experiments of interest would be western blotting, immunocytochemistry, and investigations to determine exactly which genes are induced or suppressed during autophagy (Kang *et al.,* 2009). To add value to micro-Raman spectroscopic investigation, the Raman spectra could be refined with surface enhanced techniques as well as infrared spectroscopy analysis, which is complementary to Raman spectroscopy. The chemo-preventative anti-oxidant characteristic of *C. agatiflora* should be further investigated, because together with autophagy this plant extract will be a very good candidate to use as a cancer preventative supplement. Autophagy is thought of being involved in physiological processes including the response to starvation, cell growth control, anti-aging mechanism and innate immunity. Deregulation of autophagy has been proposed to play a role in certain diseases including cancer, cardiomyophathy, muscular diseases and neurodegenerative disorders (Levine and Klionsky, 2004). Thus by inducing autophagy, cancer could be prevented by removing mutated proteins, defective DNA and organelles. But before the supplement is manufactured, the

necessary experiments should be done, such as the long-term effect of the pyrrolizidine alkaloids and whether the absence of pyrrolizidine alkaloids would change the chemo-preventative activity of *C. agatiflora*. Last but not least *C. agatiflora's* ability to induce autophagy must be investigated as an anti-aging treatment. Autophagy are induced by a restricted calorie intake (similar to starvation), (Hosokawa *et al.,* 2006) and together with *C. agatiflora* supplementation, autophagy could be maximized to reap the most of the anti-aging benefits. When its determined that *C. agatiflora* changes the form of DNA temporarily to A-form DNA, the extract could be used in sunscreens, because A-DNA is UV-protected (Neidle, 2002). The change in form should be temporary due to the fact that permanent change could be fatal.

Out of all the obtained data, it can be concluded that *Crotalaria agatiflora* subspp *agatiflora* has the potential to be used as a chemopreventative agent due to low toxiticity on healthy cells, induction of autophagy and good antioxidant potential. The ethanolic extract and madurensine can be used in lotions, creams and even sunscreens to convert B-DNA to A-DNA temporarily which is protected from ultra-violet rays.

Review of conclusions:

- The ethanol extract of *C. agatiflora* was evidently more active with regard to both cytotoxicity and antioxidant activity as compared to water extracts of *C. agatiflora*.
- Cytotoxicity of *C. agatiflora* was comparable and in some cases better than traditionally used plants in South Africa to treat cancer.
- Apoptosis and necrosis were found to be insignificantly involved in treated samples' cell death.
- Both isolated compounds madurensine and doronenine, showed moderate cytotoxicity on leukemic cells and exhibited high SI values.
- This is the first report of doronenine being isolated from *C. agatiflora*.
- Increasing concentrations of *C. agatiflora* had a severe affect on the composition of the biochemical material of the treated cells as analysed with micro-Raman spectroscopy - autophagy was hypothezided.
- Ethanol extract and madurensine have potential for herbal supplementation, ALTHOUGH *in vivo* investigations should first be conducted

Future recommendations:

- Determination that autophagy is predominately activated during ethanolic treatments of cancerous cells with *C. agatiflora*, by means of more specific microscopy which includes confocal and electron microscopy and flow cytometry.

- Western blotting can be utalized to investigate the decrease in protein concentrations during treatment of the cells.
- Micro-array analysis can aid in the understanding of autophagic genes which are expressed during and after treatments.
- *In vivo* animal studies will help determine the degree of toxicity of the pyrrolizidine alkaloids present in the samples. The probability of herbal supplementation using the extract of *Crotalaria agatiflora* can be further explored.

1. References:

Hosokawa, N., Hara, Y. and Mizushima, N. 2006. Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size. *FEBS letters* **580**: 2623-2629.

Kang, K.B., Zhu, C., Yong, S.K., Gao, Q. And Wong, M.C. 2009. Enhanced sensitivity of celecoxib in human glioblastoma cells: Induction of DNA damage leading to p53-dependent G1 cell cycle arrest and autophagy. *Molecular Cancer* **8**: 66-81.

Levine, B. and Klionsky, D.J. 2004. Development by self-digestion: Molecular mechanisms and biological functions of autophagy. *Developmental Cell* **6**: 463-477.

Neidle. S. 2002. Nucleic structure and recognition. Oxford: Oxford University Press. Pp 150-180.

Presentations and future publications

Fanie de Meillon Post-graduate Symposium (November 2010) - Department of Plant Science, UP

Title: Selective cytotoxicity of *Crotalaria agatiflora* subspp. *agatiflora* Schweinf. and mechanistic studies using micro-Raman spectroscopy

Authors: K. le Roux, N. Lall, A.A. Hussein and L. Prinsloo

National Conference:

37th annual conference of the South African Association of Botanists (SAAB) - (January 2011) - Rhodes University

Title: Selective cytotoxicity of *Crotalaria agatiflora* subspp. *agatiflora* Schweinf. and mechanistic studies using micro-Raman spectroscopy

Authors: K. le Roux, N. Lall, A.A. Hussein and L. Prinsloo

Proposed article:

In vitro chemopreventative activity of *Crotalaria agatiflora* subspecies *agatiflora* Schweinf

Preparing for Journal of Phytomedicine (Impact factor 2.1)

Authors: K. le Roux, N. Lall and A.A. Hussein

Raman spectroscopy investigation of leukemic U-937 cells treated with *Crotalaria agatiflora* subspecies *agatiflora* Schweinf

Preparing for Journal of Raman Spectroscopy (Impact factor 3.2)

Authors: K. le Roux, N. Lall and L. Prinsloo

Acknowledgements

I would like to thank our Lord God, for planting passion in my heart for conducting cancer research.

Prof. Lall, for her encouraging meetings and advice.

Prof. Hussein, for his guidance during isolation of bio-active compounds.

Dr. Linda Prinsloo from the Department of Physics, University of Pretoria, for the opportunity to use the Raman spectroscopy and for sharing my enthuasiasm on new organic analyses in South Africa.

Dr. Esam Elgorashi from the Department of Paraclinical Sciences, University of Pretoria, Onderstepoort for his guidance in conducting acetylcholinesterase assay.

Prof. Joubert and Andre Stander from the Department of Physiology, University of Pretoria, for their valuable advice and help during microscopy and flow cytometric experiments.

Wayne Barnes from the Department of Biochemistry, University of Pretoria, for helping me conduct flow cytometry analysis.

Allan Hall and the Microscopy Unit from the University of Pretoria, for helping me with usage of the microscopy equipment.

Garland More for ordering and receiving all of the reagents needed for my project.

Brenda Mapunya for her guidance during the antioxidant assay.

Rene Swart for always caring and trying to help where she can.

Jacques Kleynhans for enhancing the colours of the world map in Chapter 1.

University of Pretoria and NRF for their generous funding for the durantion of my project.

Parents, siblings and friends for always having my best interest in mind and supporting me during good and bad times.

APPENDIX A:

NEWSPAPER REPORT

Appendix A

APPENDIX B:

DICTIONARY OF NATURAL PRODUCTS

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Crotalaria retusa Alkaloid			Isol. from *Crotalaria retusa*	Alkaloids of unknown or partially unknown structure	
Alpinumisoflavone; 3,4-Dihydro	$C_{20}H_{18}O_5$		Isol. from *Crotalaria madurensis* and *Erythrina variegata*	Isoflavones; three O substituents Cyclised *C*-isopentenylated flavonoids	
2-Amino-5-hydroxyhexanoic acid	$C_6H_{13}NO_3$		Isol. from Crotalaria juncea, component of llamycins	Non-protein α-aminoacids Non-protein α-aminoacids	
2-Amino-3-hydroxypentanoic acid; (2ξ,3ξ)-form, N-Me			Isol. from seeds of *Crotalaria juncea* (Leguminosae)	Non-protein α-aminoacids Non-protein α-aminoacids	
2-Amino-3-(oxalylamino)propanoic acid; (S)-form	$C_5H_8N_2O_5$		Isol. from *Crotalaria* spp., *Lathyrus sativus* (chickling pea) and *Panax notoginseng* (sanchi)	Non-protein α-aminoacids Tea Non-protein α-aminoacids Amino acids	
Anacrotine	$C_{18}H_{25}NO_6$		Alkaloid from *Crotalaria laburnifolia* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Anacrotine; O6-Ac	$C_{20}H_{27}NO_7$		Alkaloid from *Crotalaria agatiflora* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Anacrotine; (E)-Isomer, O6-Ac	$C_{20}H_{27}NO_7$		Alkaloid from *Crotalaria agatiflora* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Anacrotine; (E)-Isomer, O6-angeloyl, N-oxide	$C_{23}H_{31}NO_8$		Alkaloid from *Crotalaria agatiflora* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Anacrotine; (E)-Isomer	$C_{18}H_{25}NO_6$		Minor alkaloid from seeds of *Crotalaria capensis* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Apigenin 4′,7-diglycosides; 4′,7-Di-O-β-D-glucopyranoside	$C_{27}H_{30}O_{15}$		Constit. of *Salvia uliginosa*, pollen of *Taxus baccata* and seeds of *Crotalaria juncea*	Flavones; three O substituents	
Assamicadine	$C_{16}H_{23}NO_5$		Minor alkaloid from seeds of *Crotalaria assamica* (Leguminosae)	Simple pyrrolizidine alkaloids	
Axillarine	$C_{18}H_{27}NO_7$		Alkaloid from the seed of *Crotalaria axillaris*. Major alkaloid from *Crotalaria scassellatii* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Axillarine; 1′-Deoxy	$C_{18}H_{27}NO_6$		From *Crotalaria axillaris* and *Crotalaria scassellatii* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Axillarine; 12-Deoxy	$C_{18}H_{27}NO_6$		Minor alkaloid from seeds of *Crotalaria scassellatii* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crotalaria goreensis Base C	$C_8H_{13}NO$		Alkaloid from *Crotalaria goreensis* whole plant (Leguminosae)	Simple pyrrolizidine alkaloids	
N,N′-Bis(4-aminobutyl)-1,4-butanediamine, 9CI	$C_{12}H_{30}N_4$		Prod. by a *Rhizobium* sp. Also found in *Vicia sativa*, *Vicia villosa* and *Crotalaria spectabilis*	Polyamine alkaloids PA44 (homospermidines) Other 3-residue polyamine alkaloids	
1,2-Bis(3,4-dimethoxyphenyl)-3,4-bis(3,5-dimethoxyphenyl)cyclobutane; (1α,2α,3β,4β)-form	$C_{36}H_{40}O_8$		Constit. of *Crotalaria madurensis*	Stilbene polymers	
1,2-Bis(3,4-dimethoxyphenyl)-3,4-bis(3,5-dimethoxyphenyl)cyclobutane; (1α,2β,3α,4β)-form	$C_{36}H_{40}O_8$		Constit. of *Crotalaria madurensis*	Stilbene polymers	
1,3-Bis(3,4-dimethoxyphenyl)-2,4-bis(3,5-dimethoxyphenyl)cyclobutane; (1α,2α,3β,4β)-form	$C_{36}H_{40}O_8$		Constit. of *Crotalaria madurensis*	Stilbene polymers	
Croaegyptine	$C_{17}H_{25}NO_6$		Alkaloid from *Crotalaria aegyptiaca* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Croalbidine	$C_{18}H_{29}NO_7$		Alkaloid from *Crotalaria albida* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crobarbatine	$C_{15}H_{21}NO_5$		Alkaloid from *Crotalaria barbata* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crocandine	$C_{16}H_{25}NO_5$		Alkaloid from the seeds of *Crotalaria candicans* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crocandine; Stereoisomer	$C_{16}H_{25}NO_5$		Alkaloid from seeds of *Crotalaria candicans* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Cronaburmine	$C_{17}H_{25}NO_5$		Alkaloid from *Crotalaria nana* seeds (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Cropodine	$C_{16}H_{25}NO_6$		Alkaloid from the pericarps of *Crotalaria candicans* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crosemperine	$C_{19}H_{29}NO_6$		Alkaloid from *Crotalaria semperflorens* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crotafoline	$C_{18}H_{25}NO_6$		Alkaloid from *Crotalaria laburnifolia* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crotafuran B	$C_{19}H_{14}O_5$	Antiinflammatory agent	Constit. of the bark of *Crotalaria pallida*	Simple pterocarpan flavonoids Cyclised *C*-isopentenylated flavonoids	
Crotafuran B; 6a-Hydroxy	$C_{19}H_{14}O_6$		Constit. of the seeds of *Crotalaria assamica*	6*a*-Hydroxypterocarpan flavonoids Cyclised *C*-isopentenylated flavonoids	
Crotalarine	$C_{18}H_{27}NO_6$		Alkaloid from *Crotalaria burhia* and *Crotalaria aegyptiaca* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crotalarine lactone	$C_{18}H_{27}NO_6$		Alkaloid from *Crotalaria aegyptiaca* (Leguminosae)	Simple pyrrolizidine alkaloids	
Crotaleschenine	$C_{16}H_{23}NO_5$		Alkaloid from *Crotalaria leschenaultii* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crotananine	$C_{17}H_{25}NO_5$		Alkaloid from *Crotalaria nana* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Crotarin	$C_{20}H_{16}O_6$		Constit. of *Crotalaria madurensis*	Isoflavones; four O substituents Cyclised *C*-isopentenylated flavonoids	
Crotasteroiridocin	$C_{39}H_{64}O_3$		Constit. of *Crotalaria emarginella*	Iridoid monoterpenoids Stigmastane steroids	
Crotmadine	$C_{20}H_{20}O_4$		Constit. of *Crotalaria madurensis*	Chalcone flavonoids; three O substituents Cyclised *C*-isopentenylated flavonoids	
Crotmarine	$C_{20}H_{20}O_4$		Constit. of *Crotalaria madurensis*	Isoflavans Cyclised *C*-isopentenylated flavonoids	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Deguelin; (-)-form	$C_{23}H_{22}O_6$		Isol. from *Crotalaria burhia*, *Derris elliptica*, *Derris malaccensis*, *Lonchocarpus* spp., *Millettia ferruginea*, *Millettia pachycarpa*, *Mundulea sericea*, *Chadsia grevei*, *Piscidia mollis* and *Tephrosia* spp. (all Leguminosae, Papilionoideae)	Simple rotenoid flavonoids Cyclised *C*-isopentenylated flavonoids	
Derrone; 1″,2″-Dihydro			Isol. from *Crotalaria madurensis*	Isoflavones; three O substituents Cyclised *C*-isopentenylated flavonoids	
β-D-Glucopyranosyl-(1→4)-β-D-mannopyranosyl-(1→4)-D-glucose, 9Cl	$C_{18}H_{32}O_{16}$		Isol. from the glucomannan from the fibres of Sunn hemp (*Crotalaria juncea*)	Trisaccharides Oligosaccharides	
Dicrotaline	$C_{14}H_{19}NO_5$		Alkaloid from *Crotalaria dura* and *Crotalaria globifera* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Dicrotaline; Ac	$C_{16}H_{21}NO_6$		Minor alkaloid from leaves of *Crotalaria lachnosema* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
5,7-Dihydroxy-4′-methoxyflavone, 8Cl; 7-O-[β-D-Apiofuranosyl-(1→6)-β-D-glucopyranoside]	$C_{27}H_{30}O_{14}$		Constit. of *Crotalaria podocarpa* and from the seeds of *Carthamus tinctorius* (safflower)	Flavones; three O substituents Herbs, condiments and spices Edible fats and oils Other oxygen-contg. natural products	
1-(3,4-Dihydroxyphenyl)-2-(3,5-dihydroxyphenyl)ethylene; (E)-form, Tetra-Me ether	$C_{18}H_{20}O_4$		Constit. of *Crotalaria madurensis*	Stilbenes	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Elliptone; (-)-form	$C_{20}H_{16}O_6$		Constit. of roots of *Derris elliptica*. Also isol. from *Derris malaccensis, Crotalaria burhia, Crotalaria medicaginea, Tephrosia falciformis, Tephrosia purpurea, Tephrosia strigosa, Tephrosia rogelii* and *Lonchocarpus* spp. (all Leguminosae, Papilionoideae)	Simple rotenoid flavonoids Cyclised *C*-isopentenylated flavonoids	
1,2-Epoxy-1-hydroxymethylpyrrolizidine	$C_8H_{13}NO_2$		Alkaloid from *Crotalaria trifoliastrum, Crotalaria aridicola, Crotalaria grantiana, Heliotropium ternatum, Heliotropium molle*, *Heliotropium angiospermum* (major alkaloid) and *Heliotropium subulatum* (major alkaloid) (Leguminosae, Boraginaceae)	Simple pyrrolizidine alkaloids	
1,2-Epoxy-1-hydroxymethylpyrrolizidine; Me ether	$C_9H_{15}NO_2$		Alkaloid from *Crotalaria trifoliastrum* and *Crotalaria aridicola* (Leguminosae)	Simple pyrrolizidine alkaloids	
Erystagallin C; 3′-Deoxy, 3′,4′-didehydro	$C_{20}H_{18}O_4$		Constit. of the aerial parts of *Crotalaria barbata*	Simple pterocarpan flavonoids Cyclised *C*-isopentenylated flavonoids	
Erystagallin C; 3′-Deoxy, 6a-hydroxy, 1′,2′,3′,4′-tetradehydro	$C_{20}H_{16}O_5$		Constit. of the bark of *Crotalaria pallida*	6*a*-Hydroxypterocarpan flavonoids Cyclised *C*-isopentenylated flavonoids	
Erystagallin C; 3′-Deoxy, 1′,2′,3′,4′-tetradehydro	$C_{20}H_{16}O_4$	Antiinflammatory agent	Constit. of the bark of *Crotalaria pallida*	Simple pterocarpan flavonoids Cyclised *C*-isopentenylated flavonoids	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Erystagallin C; 4′-Hydroxy, 1′,2′-didehydro	$C_{20}H_{18}O_6$		Constit. of the bark of *Crotalaria pallida*	Simple pterocarpan flavonoids Cyclised *C*-isopentenylated flavonoids	
Fulvine	$C_{16}H_{23}NO_5$		Alkaloid from *Crotalaria fulva, Crotalaria crispata, Crotalaria madurensis* and *Crotalaria paniculata* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones) Antineoplastic agents	Shows antineoplastic props.
Fulvine; 12-Epimer or 13,14-diepimer (1)	$C_{16}H_{23}NO_5$		Alkaloid from *Crotalaria madurensis* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Fulvine; 12-Epimer or 13,14-diepimer (2)	$C_{16}H_{23}NO_5$		Alkaloid from *Crotalaria madurensis* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Fulvine; 13-Epimer	$C_{16}H_{23}NO_5$		Alkaloid from *Crotalaria crispata* and *Crotalaria madurensis* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones) Antineoplastic agents	Shows antineoplastic props.
Fulvine; N-Oxide	$C_{16}H_{23}NO_6$		Alkaloid from *Crotalaria fulva* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Gangetinin; 1,2-Dihydro, O-de-Me	$C_{25}H_{26}O_5$	Antifungal agent	Constit. of the seeds of *Crotalaria mucronata*	Simple pterocarpan flavonoids Cyclised *C*-isopentenylated flavonoids	
Grantaline	$C_{18}H_{25}NO_6$		Alkaloid from *Crotalaria virgulata* subsp. *grantiana* and from the seeds of *Crotalaria globifera* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Grantianine	$C_{18}H_{23}NO_7$		Alkaloid from *Crotalaria virgulata* ssp. *grantiana (Crotalaria grantiana)* and *Crotalaria globifera* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
3-Hydroxycarda-14,16,20(22)-trienolide; (3β,5β)-form	$C_{23}H_{30}O_3$		Constit. of *Crotalaria juncea*	Cardanolide steroids	
3-Hydroxycarda-14,16,20(22)-trienolide; (3β,5β)-form, 3-O-β-D-Xylopyranoside	$C_{28}H_{38}O_7$		Constit. of *Crotalaria juncea*	Cardanolide steroids	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
2-Hydroxy-3,5-dimethylpyrazine, 9CI; OH-form, Me ether	$C_7H_{10}N_2O$		Isol. from *Crotalaria ochroleuca* seeds and prod. by microorganisms, e.g. *Chondromyces crocatus*	Off-flavours and taints Alkaloids Pyrazine and quinoxaline alkaloids	Causes objectionable odour in foods.
7-Hydroxy-1-methylenepyrrolizidine; (7R,7aR)-form	$C_8H_{13}NO$		Minor alkaloid from *Crotalaria goreensis* (Leguminosae)	Simple pyrrolizidine alkaloids	
7-Hydroxy-1-methylenepyrrolizidine; (7R,7aS)-form	$C_8H_{13}NO$		Major alkaloid from *Crotalaria goreensis*, also isol. from *Crotalaria podocarpa, Crotalaria maypurensis* and *Crotalaria aegyptiaca* (Leguminosae)	Simple pyrrolizidine alkaloids	
3-(5-Hydroxymethyl-5-methyl-2-oxo-5H-furan-3-yl)-2-methylpropanoic acid	$C_{10}H_{14}O_5$		Constit. of *Crotalaria verrucosa*	Butanolides	
3-Hydroxy-3-methylpentanedioic acid, 9CI	$C_6H_{10}O_5$	Biosynth. precursor of isoprenoids. Antihyperlipidaemic agent. Found in some cases of malonic aciduria. Shows hypoglycaemic activity	Occurs free in *Crotalaria dura* and *Crotalaria globifera* and bound in Dicrotaline. Also occurs in the moss *Tillandsia usneoides*	Hemiterpenoids Antihyperlipidaemic agents	
3-Hydroxy-12-neohopen-28-oic acid; (3α,17β,18α,21β)-form	$C_{30}H_{48}O_3$		Constit. of *Crotalaria emarginella*	Neohopane triterpenoids	
Isobavachromene; 2′,3′-Dihydro, O4″-Me	$C_{21}H_{22}O_4$		Constit. of *Crotalaria ramosissima*	Dihydrochalcone flavonoids Cyclised *C*-isopentenylated flavonoids	
Isobavachromene; 2′,3′-Dihydro	$C_{20}H_{20}O_4$		Isol. from *Crotalaria ramosissima*	Dihydrochalcone flavonoids Cyclised *C*-isopentenylated flavonoids	
Isobavachromene; 3″-Hydroxy, 2′,3′-dihydro	$C_{20}H_{20}O_5$		Constit. of *Crotalaria ramosissima*	Dihydrochalcone flavonoids Cyclised *C*-isopentenylated flavonoids	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Isovitexin; 2″,6″-Di-Ac	$C_{25}H_{24}O_{12}$		Constit. of *Crotalaria thebaica*	Flavones; three O substituents	
Kaempferol 7-glycosides; 7-O-[β-D-Glucopyranosyl-(1→4)-β-D-xylopyranoside]	$C_{26}H_{28}O_{15}$		Constit. of the seed of *Crotalaria laburnifolia*	Flavonols; four O substituents	
Madurensine, 9CI	$C_{18}H_{25}NO_6$		Alkaloid from *Crotalaria madurensis, Crotalaria agatiflora* and *Crotalaria rosenii* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Madurensine, 9CI; O7-Ac	$C_{20}H_{27}NO_7$		Alkaloid from *Crotalaria agatiflora* and *Crotalaria rosenii* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Madurensine, 9CI; 18-Hydroxy	$C_{18}H_{25}NO_7$		Alkaloid from *Crotalaria agatiflora* and *Crotalaria rosenii* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Madurensine, 9CI; (15Z)-Isomer, O7-Ac	$C_{20}H_{27}NO_7$		Alkaloid from *Crotalaria agatiflora* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
1-Methylenepyrrolizidine; (S)-(?)-form	$C_8H_{13}N$		Alkaloid from *Crotalaria anagyroides, Crotalaria damarensis, Crotalaria rhodesiae, Crotalaria stolzii, Crotalaria verrucosa, Crotalaria lachnophora* and *Crotalaria natalitia* (Leguminosae)	Simple pyrrolizidine alkaloids	
1-Methylenepyrrolizidine; (S)-(?)-form, N-Oxide	$C_8H_{13}NO$		Alkaloid from seeds of *Crotalaria anagyroides* (Leguminosae)	Simple pyrrolizidine alkaloids	
Monocrotalic acid; (R,R,R)-form	$C_8H_{12}O_5$		Formed by *Crotalaria spectabilis* and *Crotalaria retusa*. Necic acid component of pyrrolizidine alkaloids	Butanolides	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Monocrotaline	$C_{16}H_{23}NO_6$	Hepatotoxin. Causative agent of much seneciosis, e.g. accidental poisoning by *S.* by weed residues in bread, and characterised by venoocculosive disease. Shows antineoplastic activity esp. vs. adenocarcinoma. Male insect sterilant. Used in China as an antineoplastic agent	Alkaloid from *Crotalaria retusa, Crotalaria spectabilis, Crotalaria aegyptiaca, Crotalaria burhia* and *Lindelofia spectabilis* (Leguminosae, Boraginaceae)	Endogenous toxins/natural metabolites Anticarcinogenic/antitumorigenic agents Alkaloids Pyrrolizidine alkaloids (macrocyclic lactones) Antineoplastic agents	
Monocrotaline; 12,13-Cyclic acetaldehyde acetal	$C_{18}H_{25}NO_6$		Alkaloid from *Crotalaria grahamiana* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Monocrotaline; O13-(2-Methylbutanoyl)	$C_{21}H_{31}NO_7$		Alkaloid from *Crotalaria grahamiana* seeds (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Monocrotaline; N-Oxide	$C_{16}H_{23}NO_7$		Isol. from *Crotalaria sagittalis* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Munchiwarin	$C_{30}H_{36}O_4$		Constit. of *Crotalaria trifoliastrum*	Chalcone flavonoids; four O substituents Monocarbocyclic aldehydes and ketones	
Nilgirine	$C_{17}H_{23}NO_5$		Alkaloid from *Crotalaria mucronata*, *Crotalaria naragutensis*and *Crotalaria striata* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Nilgirine; Ac	$C_{19}H_{25}NO_6$		From *Crotalaria striata* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
12-Oleanene-3,22-diol; (3β,22β)-form, 3-O-[β-D-Xylopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside]	$C_{47}H_{76}O_{17}$		Isol. from the aerial parts of *Crotalaria albida*	Oleanane triterpenoids	
12-Oleanene-3,22-diol; (3β,22β)-form, 3-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside]	$C_{48}H_{78}O_{17}$	Shows anti-herpes virus and anticomplementary activity	Constit. of *Crotalaria albida, Dalbergia hupeana, Pueraria lobata* and *Sophora japonica*	Oleanane triterpenoids	
12-Oleanene-3,22,24-triol; (3β,22β)-form, 3-O-[α-L-Rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→6)]-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside]	$C_{54}H_{88}O_{23}$		Isol. from the aerial parts of *Crotalaria albida*	Oleanane triterpenoids	
12-Oleanene-3,22,24-triol; (3β,22β)-form, 3-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-β-D-glucuronopyranoside]	$C_{47}H_{76}O_{17}$		Isol. from roots of *Astragalus membranaceus*, *Astragalus danicus*, *Wisteria brachybotrys* and *Crotalaria thebaica*	Oleanane triterpenoids	
3,3′,4′,5,7-Pentahydroxyflavanone; (2R,3R)-form, 3-O-[β-D-Galactopyranosyl-(1→6)-β-D-glucopyranoside]	$C_{27}H_{32}O_{17}$		Constit. of *Crotalaria prostrata*	Dihydroflavonols; five O substituents	
2,2′,4,4′,5-Pentahydroxy-3′-prenylchalcone; (E)-form	$C_{20}H_{20}O_6$		Constit. of the inflorescences of *Crotalaria ramosissima*	Chalcone flavonoids; five O substituents	
Retrorsine	$C_{18}H_{25}NO_6$		Alkaloid from *Senecio* spp, *Crotalaria* spp. and *Erechtites quadridentata* (Compositae, Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Retrorsine; 15E-Isomer	$C_{18}H_{25}NO_6$		Alkaloid from *Crotalaria brevifolia, Crotalaria incana, Crotalaria mucronata* and *Crotalaria usaramoensis* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Retrorsine; Stereoisomer	$C_{18}H_{25}NO_6$		Alkaloid from *Crotalaria mucronata* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Retusamine, 9CI	$C_{19}H_{25}NO_7$		Alkaloid from *Crotalaria retusa, Crotalaria crassipes, Crotalaria mitchellii* and *Crotalaria novae-hollandiae* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Retusine	$C_{16}H_{25}NO_5$		Alkaloid from *Crotalaria retusa* and *Crotalaria spectabilis* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Rotenone, ISO, BSI	$C_{23}H_{22}O_6$	Antineoplastic agent. Contact insecticide and pesticide. Potent mitochondrial poison. Toxic against *Artemia salina* (brine shrimp)	Constit. of the root of *Derris elliptica*. Widely distrib. in the Leguminosae (Papilionoideae) e.g. in many other *Derris* spp. *Lonchocarpus* spp. *Millettia* spp., *Tephrosia* spp., *Amorpha fruticosa, Antheroporum pierrei, Crotalaria burhia, Crotalaria medicaginea, Mundulea pauciflora, Mundulea sericea, Neorautanenia amboensis, Neorautanenia ficifolia, Ormocarpum glabrum, Pachyrrhizus erosus* (yam bean), *Piscidia erythrina, Piscidia mollis, Poiretia tetraphylla* and *Spatholobus roxburghii*. Also in *Verbascum thapsus* (Scrophulariaceae)	Simple rotenoid flavonoids Cyclised *C*-isopentenylated flavonoids Legumes Insecticides Antineoplastic agents	
Senecionine; 15E-Isomer, O-Ac	$C_{20}H_{27}NO_6$		Alkaloid from the seeds of *Crotalaria naragutensis* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Senecionine; 15E-Isomer	$C_{18}H_{25}NO_5$	Strong insect antifeedant agent	Alkaloid from *Senecio* spp., *Crotalaria incana* and other *Crotalaria* spp., *Cacalia hastata* and others (Leguminosae, Compositae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Senecionine; N-Oxide	$C_{18}H_{25}NO_6$	Antineoplastic agent	Alkaloid from seeds of *Crotalaria anagyroides* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones) Antineoplastic agents	
Seneciphylline	$C_{18}H_{23}NO_5$	Cause of grazing toxicity in animals	Alkaloid from numerous *Senecio* and *Crotalaria* spp., also *Erechtites hieracifolia* and *Erechtites quadridentata* (Compositae, Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Seneciphylline; 18-Hydroxy	$C_{18}H_{23}NO_6$		Alkaloid from *Senecio riddellii, Senecio longiflorus, Senecio eremophilus, Crotalaria juncea* etc. (Compositae, Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Senkirkine	$C_{19}H_{27}NO_6$		Alkaloid from *Nardosmia laevigata*, (preferred genus name *Petasites*), *Farfugium japonicum*, *Crotalaria laburnifolia*, *Senecio anonymus*, *Senecio kirkii* and others (Compositae, Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Senkirkine; 18-Hydroxy	$C_{19}H_{27}NO_7$		Alkaloid from *Crotalaria laburnifolia* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Senkirkine; 15E-Isomer, 12-Ac, stereoisomer	$C_{21}H_{29}NO_7$		Alkaloid from *Crotalaria verrucosa* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Senkirkine; 15E-Isomer, stereoisomer	$C_{19}H_{27}NO_6$		Alkaloid from *Crotalaria verrucosa* and *Crotalaria walkeri* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Senkirkine; Stereoisomer	$C_{19}H_{27}NO_6$		Alkaloid from *Crotalaria walkeri* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Sumatrol	$C_{23}H_{22}O_7$		Isol. from *Crotalaria burhia, Derris malaccensis, Millettia auriculata, Piscidia erythrina* and *Tephrosia toxicaria*	Simple rotenoid flavonoids	
Supinidine; (S)-form, Me ether	$C_9H_{15}NO$		Alkaloid from *Crotalaria trifoliastrum* and *Crotalaria medicaginea* (Leguminosae)	Simple pyrrolizidine alkaloids	
Tephrosin	$C_{23}H_{22}O_7$	Ichthyotoxin, tumour promotion inhibitor	Found in *Crotalaria burhia*, *Crotalaria medicaginea*, *Amorpha fruticosa*, *Tephrosia toxicaria*, *Tephrosia vogelii*, *Millettia dura*, *Derris elliptica*, *Derris malaccensis*, *Lonchocarpus* spp., *Mundulea sericea*, *Piscidia mollis* and others	12*a*-Hydroxyrotenoid flavonoids Cyclised *C*-isopentenylated flavonoids	
2,3,5,7a-Tetrahydro-1-hydroxy-1H-pyrrolizine-7-methanol, 9CI; (7R,8R)-form	$C_8H_{13}NO_2$		Necine base from numerous pyrrolizidine alkaloids. Trace constit. in seedlings of *Crotalaria scassellatii* (Leguminosae)	Simple pyrrolizidine alkaloids	
2,3,5,7a-Tetrahydro-1-hydroxy-1H-pyrrolizine-7-methanol, 9CI; (7S,8R)-form, O7-Angeloyl	$C_{13}H_{19}NO_3$		Alkaloid from *Crotalaria officinale*, other *Crotalaria* spp., *Heliotropium eichwaldii, Senecio crispatis, Senecio rivularis* and other spp. (Leguminosae, Boraginaceae)	Simple pyrrolizidine alkaloids	
2,3,5,7a-Tetrahydro-1-hydroxy-1H-pyrrolizine-7-methanol, 9CI; (7R,8R)-form, O9-Me, O7-Ac	$C_{11}H_{17}NO_3$		Alkaloid from *Crotalaria trifoliastrum* (Leguminosae)	Simple pyrrolizidine alkaloids	
2,3,5,7a-Tetrahydro-1-hydroxy-1H-pyrrolizine-7-methanol, 9CI; (7R,8R)-form, O9-Me	$C_9H_{15}NO_2$		Constit. of *Crotalaria* spp. (Leguminosae)	Simple pyrrolizidine alkaloids	
2,3,5,7a-Tetrahydro-1-hydroxy-1H-pyrrolizine-7-methanol, 9CI; (7S,8R)-form, O9-Me	$C_9H_{15}NO_2$		Alkaloid from *Crotalaria trifoliastrum, Crotalaria aridicola* and *Crotalaria medicaginea* (Leguminosae)	Simple pyrrolizidine alkaloids	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
2,3,5,7a-Tetrahydro-1-hydroxy-1H-pyrrolizine-7-methanol, 9CI; (7R,8R)-form, 7-O-(3-Methylbutanoyl), N-oxide	$C_{13}H_{21}NO_4$		Constit. of *Crotalaria scassellatii*	Simple pyrrolizidine alkaloids	
2,3,5,7a-Tetrahydro-1-hydroxy-1H-pyrrolizine-7-methanol, 9CI; (7R,8R)-form, 7-O-(3-Methylbutanoyl)	$C_{13}H_{21}NO_3$		Constit. of *Crotalaria scassellatii*	Simple pyrrolizidine alkaloids	
2,3,5,7a-Tetrahydro-1-hydroxy-1H-pyrrolizine-7-methanol, 9CI; (7R,8R)-form, 7-O-(3-Methyl-2-butenoyl), N-oxide	$C_{13}H_{19}NO_4$		Constit. of *Crotalaria scassellatii*	Simple pyrrolizidine alkaloids	
2′,3,4,5-Tetrahydroxychalcone; (E)-form, 3,4,5-Tri-Me ether	$C_{18}H_{18}O_5$		Constit. of the aerial parts of *Crotalaria prostrata*	Chalcone flavonoids; four O substituents	
3,4′,5,7-Tetrahydroxyflavanone; (2R,3R)-form, 7-O-[α-L-Rhamnopyranosyl-(1→4)-β-D-galactopyranoside]	$C_{27}H_{32}O_{15}$		Constit. of *Crotalaria laburnifolia*	Dihydroflavonols; four O substituents	
3,4′,5,7-Tetrahydroxyflavone; 4′,7-Di-O-α-L-Rhamnopyranoside	$C_{27}H_{30}O_{14}$		Isol. from *Crotalaria verrucosa*	Flavonols; four O substituents	
2′,4′,5,7-Tetrahydroxyisoflavone	$C_{15}H_{10}O_6$		Isol. from *Apios tuberosa*, *Argyrocytisus battandieri*, *Cajanus cajan*, *Crotalaria juncea*, *Dolichos biflorus*, *Hardenbergia violacea*, *Lablab niger*, *Laburnum anagyroides*, *Lupinus albus*, *Moghania macrophylla*, *Neonotonia wightii*, *Phaseolus vulgaris*, *Phaseolus coccineus*, *Spartium junceum*, *Stizolobium deeringianum* and others	Isoflavones; four O substituents Legumes Other fruits Other oxygen-contg. natural products	
2′,4′,5,7-Tetrahydroxyisoflavone; 2′-Me ether	$C_{16}H_{12}O_6$		Constit. of the bark of *Crotalaria pallida*	Isoflavones; four O substituents	
2′,3,4,4′-Tetrahydroxy-3′-prenylchalcone; (E)-form, 3-Me ether	$C_{21}H_{22}O_5$	Antimalarial agent	Constit. of the aerial parts of *Crotalaria orixensis*	Chalcone flavonoids; four O substituents	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
α-Toxicarol	$C_{23}H_{22}O_7$		Obt. from *Derris malaccensis*, *Derris elliptica*, *Crotalaria burhia*, *Tephrosia odorata* and *Tephrosia toxicaria*	Simple rotenoid flavonoids Cyclised *C*-isopentenylated flavonoids	Fish poison closely related in props. to Rotenone
Trichodesmine	$C_{18}H_{27}NO_6$		Alkaloid from *Trichodesma incanum, Heliotropium arguzioides, Crotalaria juncea, Crotalaria tetragona* and *Crotalaria rubiginosa* (Boraginaceae, Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Trichodesmine; 17-Hydroxy	$C_{18}H_{27}NO_7$		Alkaloid from *Crotalaria juncea* and *Crotalaria rubiginosa* (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
Trichodesmine; 19-Hydroxy	$C_{18}H_{27}NO_7$		Alkaloid from *Crotalaria globifera* seeds (Leguminosae)	Pyrrolizidine alkaloids (macrocyclic lactones)	
3,11,14-Trihydroxycarda-5,20(22)-dienolide; (3β,11α,14β)-form, 3-O-[β-D-Glucopyranosyl-(1→6)-β-D-galactopyranoside]	$C_{35}H_{52}O_{15}$		Constit. of *Crotalaria retusa*	Cardanolide steroids	
3,5,7-Trihydroxy-3′,4′-dimethoxyflavone; 3-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranoside]	$C_{29}H_{34}O_{16}$		Constit. of the stem of *Crotalaria verrucosa*	Flavonols; five O substituents	
2′,4,4′-Trihydroxy-3′,5′-diprenylchalcone; (E)-form	$C_{25}H_{28}O_4$		Constit. of *Crotalaria medicagineaand Crotalaria trifoliastrum*	Chalcone flavonoids; three O substituents	
4′,5,7-Trihydroxyflavanone; (S)-form, 4′-O-α-L-Rhamnopyranoside	$C_{21}H_{22}O_9$		Constit. of *Crotalaria striata* and *Impatiens bicolor*	Flavanones; three O substituents	
2,3,21-Trihydroxy-12-neohopen-29-oic acid; (2β,3β,17β,18α,21α,22ξ)-form	$C_{30}H_{48}O_5$		Constit. of *Crotalaria emarginella*	Neohopane triterpenoids	
3,22,24-Trihydroxy-12-oleanen-29-oic acid; (3β,22β)-form, 3-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranoside], 22-O-α-L-rhamnopyranoside, di-Me ester	$C_{56}H_{90}O_{24}$		Constit. of aerial parts of *Crotalaria thebaica*	Oleanane triterpenoids	
12-Ursene-3,15,16,22,28-pentol; (3β,15α,16α,22α)-form	$C_{30}H_{50}O_5$		Constit. of the leaves of *Crotalaria saltiana*	Ursane triterpenoids	

Appendix B: Dictionary of Natural Products - *Crotalaria*

Name	Molecular Formula	Biological Use	Biological Source	Type of Compound Words	Use/Importance
Vitexin; 4′-O-β-D-Galactopyranoside	$C_{27}H_{30}O_{15}$		Isol. from *Crotalaria retusa*	Flavones; three O substituents	
Vitexin; 4′-O-D-Xyloside	$C_{26}H_{28}O_{14}$		Isol. from *Crotalaria verrucosa*	Flavones; three O substituents	

APPENDIX C:

NMR DATA

Appendix C: NMR Data

Table 1: NMR spectra summary of madurensine

^{1}H NMR spectrum of Madurensine

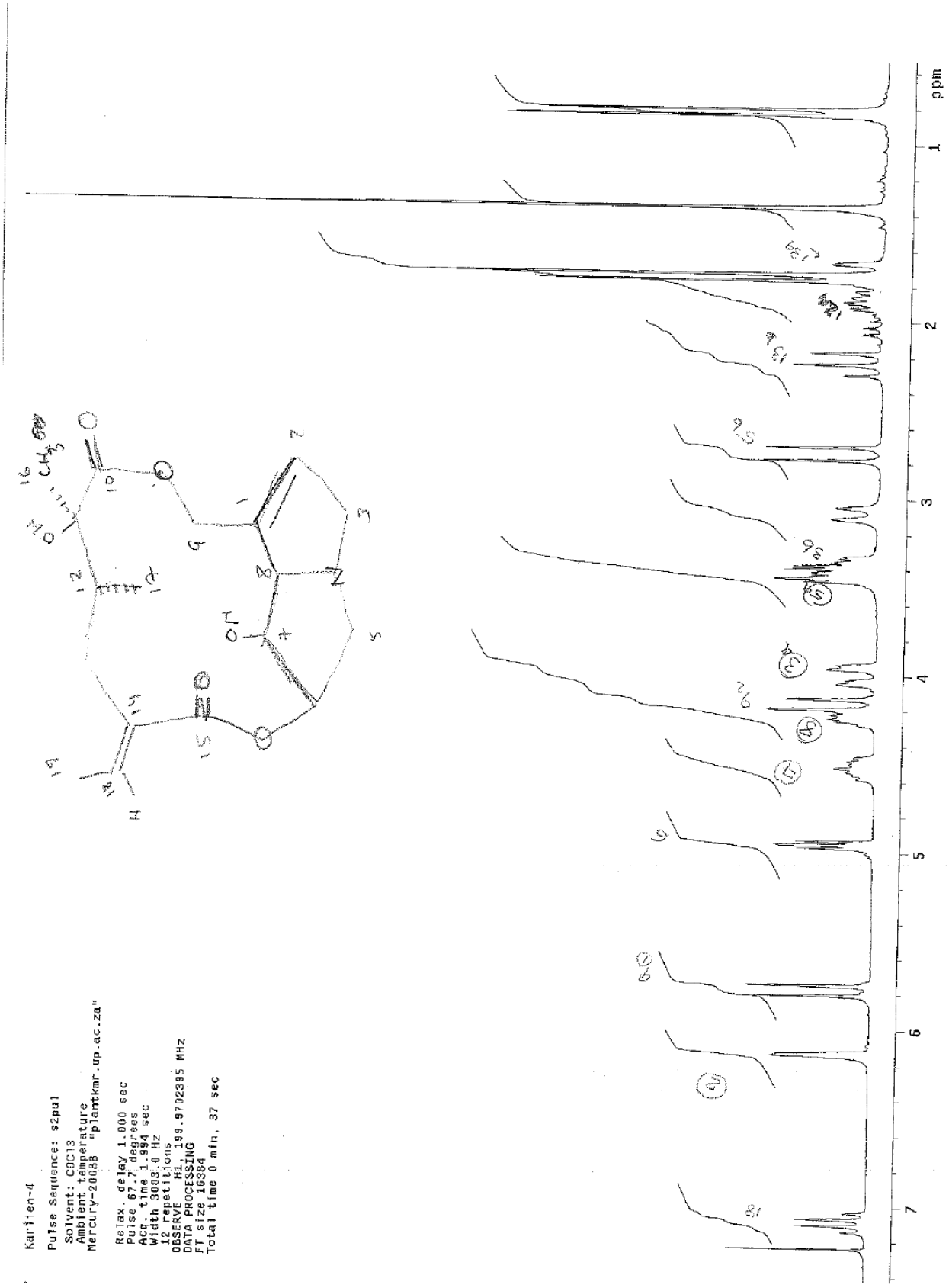
Carbon	δ (ppm)
1	135.4
2	136.1
3	66.3
5	61.3
6	75.0
7	74.5
8	73.6
9	59.9
10	176.9
11	76.2
12	40.3
13	27.4
14	129.6
15	166.9
16	24.4
17	10.6
18	142.5
19	14.9

^{13}C NMR spectrum of Madurensine

Proton	δ (ppm), Proton
C2-H	7.12 q (*J* = 6.7)
C3-H_a	4.02 *dm* (*J* = 14.4)
-H_b	3.42 *dd* (*J* =14.4)
C5-H_a	3.45 *dddd* (*J* = 14.4; 6.2)
-H_b	2.77 *d* (*J* = 14.4)
C6-H_α	4.98 t (*J* = 2.4)
C7-H_α	4.55 *m*
C8-H_α	4.26 *m*
C9-H_1	5.81 *d* (*J* = 10.3)
C9-H_2	4.19 *d* (*J* = 10.3)
C12-H	1.91 *m*
C13-H_a	1.73 *d* (*J* = 11.7)
C13-H_b	2.26 *d* (*J* = 11.7)
C16-Me	1.38 *s*
C17-Me	0.86 *d* (*J* = 6.7)
C18-H	7.12 *q* (*J* = 6.7)
C19-Me	1.28 *dd* (*J* = 6.7; 1.8)

Appendix C: NMR Data

Appendix C: NMR Data



Appendix C: NMR Data

Table 2: NMR spectra summary of doronenine

^{1}H NMR spectrum of Doronenine

Proton of Carbon	δ (ppm)	Proton
1	-	-
2	6.13	1H, *m*
3	3.97; 3.42	2H, *qd*. J = 50 Hz; J = 15 Hz
5	3.23; 2.49	2H, *d, m*
6	2.29; 2.10	2H, *d, m*
7	5.14	1H, *m*
8	4.25	1H, *m*
9	5.56; 4.11	2H, *dd*, J_{AB} = 130 Hz: J_{ab} = 13 Hz
11	3.19	1H, *s*, OH-Gruppe
12	2.19	1H, *m*
13	2.65; 1.96	2H, *d, m*
15	5.65	1H, *m*
17	1.32	3H, *s*
18	0.93	3H, *d*, J = 6.5 Hz
19	1.84	3H, *d*, J = 1.5 Hz

^{13}C NMR spectrum of Doronenine

Carbon	δ (ppm)
1	132.0
2	135.4
3	63.4
5	52.6
6	34.1
7	77.1
8	75.7
9	60.5
10	177.7
11	75.7
12	34.6
13	41.1
14	151.8
15	118.5
16	167.7
17	24.6
18	11.7
19	28.3

Appendix C: NMR Data

Appendix C: NMR Data