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In Vitro Medicinal Properties of Novel Compounds from *Croton steenkampianus*

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Promoter: Prof JJM Meyer

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I declare that the thesis/dissertation, which I hereby submit for the degree PHD Plant Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:

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SUMMARY

In Vitro Medicinal Properties of Novel Compounds from *Croton steenkampianus*

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The effect of infectious diseases on the population in the developing countries is of utmost concern. Malaria, tuberculosis (TB) and human immunodeficiency virus (HIV) are the three major infectious disease threats. They account for approximately half of the mortality caused by infectious diseases, which is almost half of the mortality in the developing countries. With no vaccine likely in the foreseeable future, drugs remain the best means of controlling infectious diseases. In the industrialized nations at the present time, some 50% of all prescribed drugs are derived or synthesized from natural products (animals, marine species, plants and micro-organisms). It has been estimated that plants are the most important source of medicine for more than 80% of the world's population. As previous work on the leaves of *Croton steenkampianus* gave promising results and revealed that it still contained bioactive compounds that could be isolated, it was chosen for further work.

The bioassay guided fractionation of the ethanol crude extract using silica and Sephadex column chromatography resulted in the isolation of six compounds: three flavoniods (quercetin, tamarixetin and eriodictyol), one new indane (**1**) (2,6-dimethyl-1-oxo-4 indanecarboxylic acid) and two new diterpenes (steenkrotin A (**2**) and steenkrotin B (**3**)) with novel skeletons. The structure of the compounds was determined using NMR, IR, UV, MS and X-ray crystallography.

Ethanol crude extract, quercetin, steenkrotin A, steenkrotin B and the indane were tested against four strains of *Plasmodium falciparum* (D6, D10, Dd2 and W2). Quercetin showed good antiplasmodial activity against the D10 and Dd2 strains. The antiplasmodial activity of steenkrotin A and crude extract were moderate. The antimalarial activity of steenkrotin A in particular is promising, as it showed more activity against resistant strains. The indane, and steekrotin B were not active against the strains of *P. falciparum* used (IC_{50} > 10 µg/m). The IC_{50} of the compounds improved when they were combined with chloroquine. However, the IC_{50} of chloroquine was still the lowest. The compounds showed moderate bioactivity against *Bacillus cereus* and *Escherichia coli*. The three new compounds (**1**, **2** and **3**) tested against *Mycobacterium* (H37Rv) were not active (IC_{50} > 10 µg/ml). The indane (**1)** showed anti-HIV activity at 50 µg/ml against reverse transcriptase. The antioxidant activity of the compounds tested ranged from weak to excellent (>280.00 µg/ml for compound **1** and **2** to 0.05 µg/ml for quercetin).

The cytotoxicity of the compounds and extract were determined against Vero cells lines. Their IC_{50} values ranged from 34.0 to 305.9 µg/ml, which is higher and better than that of chloroquine. The IC_{50} values obtained are: chloroquine (25.0), quercetin (33.6), steenkrotin A (35.0), ethanol extract (45.0), tamarixetin (53.8), indane (248.2) and steenkrotin B (305.9).

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LIST OF ABBREVIATIONS

^{13}C-NMR: Carbon nuclear magnetic resonance

1H-NMR: Proton nuclear magnetic resonance

AIDS: Acquired immune deficiency syndrome

APAD: 3-Acetylpyrimidine adenine dinucleotide

COSY: Correlated spectroscopy

DEPT: Distortionless enhancement by polarization transfer

DHFR: Dihydrofolate reductase

DHODase: Dihydroorotate dehydrogenase

DHPS: Dihydropteroate synthase

DMSO: Dimethylsufoxide

DPP: Dimethylallyl pyrophosphate

EDTA: Ethylenediaminotetra-acetic acid

FPIX: Ferriprotoporphyrin IX

HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

HIV: Human immunodeficiency virus

HMBC: Heteronuclear multiple bond correlation

HMQC: Heteronuclear multiple quantum correlation

HSQC: Heteronuclear singlequantum coherence

IPP: Isopentenyl pyrophosphate

IR: Infrared

LD_{50}: 50% Lethal dose

MS: Mass spectroscopy

MTCT: Mother-to-child transmission

MTT: 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide

NBT: Nitroblue tetrazolium

NMR: Nuclear magnetic resonance

NOESY: Nuclear overhauser effect spectroscopy

NSP: National strategic plan

PBS: Phosphate buffer saline

PEP: Post-exposure prophylaxis

PF: Potentiating factor

SP: Sulphadoxine-pyrimethamine

STD: Sexual transmitted disease

STI: Sexual transmitted infection

TLC: Thin layer chromatography

TMS: Tetramethylsilane

TRIS: N-tris (hydroxymethyl) aminomethane

UNAIDS: Joint United Nations programme on HIV/AIDS

UNGASS: United Nations general assembly session on HIV/AIDS

UNICEF: United Nations children's fund

USAID: United States agency for international development

UV: Ultraviolet

WHO: World health organization

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Medicinal plants

Throughout the ages, humans have relied on nature for their basic needs, for the production of food, shelter, clothing, transportation, fertilizers, flavours and fragrances, and medicines (Cragg and Newman, 2005). Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and probably thousands of years of use. The first records, written on clay tablets in cuneiform, are from Mesopotamia and date from about 2 600 BC (Heinrich *et al*., 2004). Among the substances that were used are oils of *Cedrus* species (cedar) and *Cupressus sempervirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh) and *Papaver somniferum* (poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation. In ancient Egypt, bishop's weed (*Ammi majus*) was reported to be used to treat vitiligo, a skin condition characterized by a loss of pigmentation (Staniszewska, *et al.,* 2003; Beissert and Schwarz, 2002). More recently, a drug (β-methoxypsoralen) has been produced from this plant to treat psoriasis and other skin disorders, as well as T-cell lymphoma (Beissert and Schwarz, 2002).

The interest in nature as a source of potential chemotherapeutic agents continues. Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world today. Higher plants contribute no less than 25% of the total (Farnsworth *et al*., 1985; Cragg and Newman, 2005). In the last 40 years, many potent drugs have been derived from flowering plants; including for example *Dioscorea* species (diosgenin), from which all anovulatory contraceptive agents have been derived; reserpine and other anti-hypertensive and tranquilizing alkaloids from *Rauwolfia* species; pilocarpine to treat glaucoma and 'dry mouth', derived from a group of South American trees

(*Pilocarpus* spp.) in the Citrus family; two powerful anti-cancer agents from the Rosy Periwinkle (*Catharanthus roseus*); laxative agents from *Cassia* sp. and a cardiotonic agent to treat heart failure from *Digitalis* species (Newman *et al.,* 2000).

Approximately half (125 000) of the world's flowering plant species are found in the tropical forests. Tropical rain forests continue to support a vast reservoir of potential drug species. They continue to provide natural product chemists with invaluable compounds as starting points for the development of new drugs. The potential for finding more compounds is enormous as to date only about 1% of tropical species have been studied for their pharmaceutical potential (Cragg and Newman, 2005). This proportion is even lower for species confined to the tropical rain forests. To date about 50 drugs have come from tropical plants. The probable undiscovered pharmaceuticals for modern medicine has often been cited as one of the most important reasons for protecting tropical forests. Therefore the high annual extinction rate is a matter for concern.

Although discovered through serendipitous laboratory observation, three of the major sources of anti-cancer drugs on the market or completing clinical trials are derived from North American plants used medicinally by native Americans: the papaw (*Asimina* spp); the western yew tree (*Taxus brevifolia*), effective against ovarian cancer and the mayapple (*Podophyllum peltatum*) used to combat leukaemia, lymphoma lung and testicular cancer (Gurib-Fakim, 2006).

1.2 Traditional medicine

Plants have been utilized as medicines for thousands of years (Samuelsson, 2004). These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Balick and Cox, 1997; Samuelsson, 2004). The specific plants to be used and the methods of application for particular ailments were passed down through oral tradition. Eventually information regarding medicinal plants was recorded in herbal phamacopoeias (Balunas, 2005).

Modern allopathic medicine has its roots in ancient medicine, and it is likely that many important new remedies will be discovered and commercialized in the future, as it has been till now, by following the leads provided by traditional knowledge and experiences. While European traditions are particularly well known and have had a strong influence on modern western pharmacognosy, almost all societies have well-established herbal traditions, some of which have hardly been studied at all. The study of these traditions will not only provide an insight into how the field has developed but it is also a fascinating example of our ability to develop a diversity of cultural practices.

In some countries, the use of medicinal plants is often associated with witchcraft and superstition, because people do not have the scientific insight to explain and predict the curative action of plants. One example of such an irrational concept is the Doctrine of Signatures, elements of which are found in many of the healing cultures of the world (Boehme, 1982). It is based on the assumption that the appearance of plants may give clues to their medicinal properties—it is interpreted as God's signature on the plant. Red juice and sap, for example, is associated with blood and menstrual ailments; yellow flowers with bile and jaundice; the human shape of certain roots with the female form of fertility and so on. Sometimes this concept however, worked: *Chelidonium majus*, contains yellow flowers and a yellow alkaloid containing latex, and has been used successfully to treat jaundice (Gurib-Fakim, 2006).

1.2.1 African traditional medicine

African traditional medicine is ancient and perhaps the most diverse of all medicinal systems. Africa is considered to be the cradle of humankind, with a rich biological and cultural diversity and marked regional differences in healing practices. Unfortunately, even today the systems of medicines are poorly recorded. The documentation of medicinal uses of African plants is becoming increasingly urgent because of the rapid loss of the natural habitats of these plants due to human activities. The African continent is reported to have one of the highest rates of deforestation in the world. This loss is all the greater because the continent has a high rate of endemism, with Madagascar topping the list at 82% (Green and Sussman, 1990).

African traditional medicine in its varied forms is holistic, involving both the body and the mind. The healer typically diagnoses and treats the psychological basis of an illness before prescribing medicines to treat the symptoms. Well known African medicinal plants include *Acacia senegal* (gum arabic), *Agathosma betulina* (buchu), *Aloe ferox* (Cape aloes), *Aloe vera* (north African origin), *Artemisia afra* (African wormwood), *Aspalanthus linearis* (rooibos tea), *Boswellia sacra* (frankincense), *Catha edulis* (khat), *Commiphora myrrha* (myrrh), *Harpagophytum procumbens* (devil's claw), *Hibiscus sabdariffa* (hibiscus, roselle), *Hypoxis hemerocallidea* (African potato), *Prunus africana* (African cherry). Madagascar has contributed *Catharanthus roseus* (rosy periwinkle) and has the potential of contributing more in view of the diversity of the flora and fauna (Newman *et al., 2000;* Neuwinger, 2000).

1.2.2 American traditional medicine (North, Central and South)

1.2.2.1 North America

In the USA, just like in many other cultures, the indigenous healer or Shaman treated illnesses by addressing both the physical and spiritual dimension of diseases. These Shamanistic ceremonies involve chanting, dancing and other rituals aimed at expelling evil forces so that the patient or the community as a whole can be healed (Fabricant and Farnsworth, 2001). Early settlers learnt from native practices and they eventually adopted many of the herbal remedies, which later formed the basis of the early United States Pharmacopoeia. Among the well known medicinal plants of the United States are Echinacea (*Echinacea purpurea*) and Goldenseal (*Hydrastis canadensis*). During most of the 20th century, herbs or botanicals have been regarded with skepticism and the practice of herbal medicine went into decline. Plants were viewed mainly as a potential source of pure chemical compounds for the development of medicine. In recent years, herbs and botanicals have become very popular in the USA and Canada but they are still considered as nutritional supplements rather than medicines in their own rights (Pieroni *et al.,* 2000; Heinrich *et al.,* 2004; Gurib-Fakim, 2006).

1.2.2.2 Central and South America

Just like Africa, Central and South American countries have rich and diverse healing cultures, which are poorly known and have not been properly recorded. They will no doubt be a source of new herbal remedies in the years to come. South and Central America have made enormous contributions to agriculture and a large number of food crops such as maize, potatoes, tomatoes, pumpkins, cassava, peanuts and sweet potato originate from there. Traditional American Indian medicinal herbs are used extensively but the influence of Spanish, European, East Asian and African medical systems is obvious. Notable examples of medicinal plants are: *Cinchona pubescens* (peruvian bark), *Erythroxylum coca* (coca), *Ilex paraguariensis* (maté), *Myroxylon balsamum* (tolu balsam), *Paullinia cupana* (guarana), *Peumus boldus* (boldo), *Psidium guajava* (guava), *Spilanthes acmella* (Brazilian cress), *Tabebuia impetiginosa* (lapacho) and *Uncaria tomentosa* (cat's claw) (Fabricant and Farnsworth, 2001; Gurib-Fakim, 2006).

1.2.3 Australian and Southeast Asian medicine

This region has witnessed a resurgence of interest in traditional medicine and many countries now promote research into medicinal plants as a potential source of new remedies. The Aborigines had a complex healing system but much of the traditional knowledge in Australia was lost before it could be systematically recorded. In contrast, many healing practices such as those of Malaysia, Thailand, Vietnam, New Zealand, Borneo, and the Polynesian Islands remain intact and are being recorded and developed. A strong Chinese influence is being observed in most countries. Among the well-known medicinal products originating from this region are *Croton tiglium* (purging croton), *Duboisia hopwoodii* (pituri), *Eucalyptus globulus* (bluegum), *Melaleuca alternifolia* (tea tree), *Myristica fragrans* (nutmeg and Mace), *Piper methysticum* (kava kava), *Strychnos nux-vomica* (strychnine), *Styrax benzoin* (benzoin) and *Syzygium aromaticum* (cloves) (Maher, 1999; Kapoor, 1990; Newman, 2000; Gurib-Fakim, 2006).

1.2.4 Ayurvedic medicine (Indian traditional medicine)

Ayurveda is perhaps, the most ancient of all medicinal traditions. It is probably older than traditional Chinese medicine and is considered to be the origin of systemized medicine. It is actually a practical and holistic set of guidelines to maintain balance and harmony in the system. Dioscorides (who influenced Hippocrates) is thought to have taken many of his ideas from India. Ancient Hindu writings on medicine contain no references to foreign medicines whereas Greek and Middle Eastern texts refer to concepts and drugs of Indian origin (Magner, 1992; Chopra, 2000).

Ayurveda is derived from the Indian words '*Ayar*' (life) and '*veda*' (knowledge or science) and hence means the science of life. Following the system would help ensure a long life, which is considered to be the instrument for achieving righteousness (*dharma*), wealth (*artha*) and happiness (*sukha*).

In India, knowledge and wisdom have been passed on from one generation to the next through songs and poems, which scholars and physicians had to learn and recite by heart. The Veda is an ancient text in four parts (Rig Veda, Sama Veda, Yajur Veda and Atharva Veda), the earliest of which date back to 2 000 years BC. The principles of Ayurvedic medicine and the medicinal uses of plants are contained in thousands of poetic hymns in the Rig Veda. The first school to teach Ayurvedic medicine was at the University of Banaras in 500 BC where the great Samhita (or encyclopedia of medicine) was written. Another great enclyclopedia was written 700 years later, and these two together form the basis of the Ayurveda (Chopra, 2000).

Ayurveda is similar to Galenical medicine in that it is based on body humours (*dosas*) and the inner life force (*prana*) that is believed to maintain digestion and mental activity. The living and the non-living environment, including humans, are considered to be elements: earth (prithvi), water (jada), fire (tejac), air (vaju) and space (akasa). For an understanding of these traditions, the concept of impurity and cleansing is also essential. Illness is the consequence of imbalance between the various elements and it is the goal of the treatment to restore this balance (Magner, 1992).

Famous Ayurvedic medicinal plants include *Azadirachta indica* (neem), *Centella asiatica* (gotu kola), *Cinnamomum camphora* (camphor), *Elettaria cardamomum* (*ela* or cardamomum), *Rauwolfia serpentina* (Indian snake root), *Santalum album* (sandalwood), *Terminalia* species (myrobolan) and *Withania somnifera* (aswargandha) (Kapoor, 1990; Magner, 1992; Padua de, 1999; Gurib-Fakim, 2006).

1.2.5 Chinese traditional medicine

The civilizations of China and India were flourishing when only modestly sophisticated cultures were developing in Europe. Expectedly writings on medicinal plants and the aesthetics of vegetation were numerous. This ancient system of medicine, believed to be more than 5 000 years old, is based on two separate theories about the natural laws that govern good health and longevity, namely *yin* and *yang*, and the five elements (*wu xing*) (Kapoor, 1990; Patwardhan, 2005).

The legendary emperor Shen Nung discussed medicinal herbs in his works—which were probably written 2 500 years B.P. (Before Present) and not the traditional date of 3 500 B.P. The Traditional Chinese medicine was systematized and written between 100 and 200 BC (Before Christ). The most complete reference to Chinese herbal prescription is the Modern Day Encyclopedia of Chinese *materia medica* published in 1977. It lists nearly 6 000 medicines out of which 4 800 are of plant origin (Magner, 1992).

Treatment is based on symptoms and on a pattern of imbalances, often detected by taking the pulse or observing the patient's tongue. Warming or hot herbs, such as ginger and cinnamon, are used to treat ailments associated with cold symptoms such as cold hands, abdominal pains and indigestion (Kapoor, 1990; Padua de, 1999).

In common with Western and African traditional medicines, Chinese herbs are usually given in fixed mixtures or formulas of up to 20 herbs, carefully prepared according to traditional recipes. There are hundreds such recipes being used alongside with Western medicines. As in other healing cultures,

traditional recipes are used preferentially against chronic illnesses while acute or serious illnesses are cured by Western medicines.

The spread of traditional Chinese medicine to most continents has undoubtedly contributed to the current popularity of herbal medicines throughout the world. Examples of famous Chinese medicinal plants are *Angelica polymorpha* var. *sinensis* (dang gui), *Artemisia annua* (qing hao), *Ephedra sinica* (ma huang), *Paeonia lactiflora* (bai shao yao), *Panax ginseng* (ren shen) and *Rheum palmatum* (da huang) (Magner, 1992; Padua de, 1999; Gurib-Fakim, 2006).

1.2.6 European medicine

In the ancient Western world, the Greeks contributed significantly to the rational development of the use of herbal drugs. However, the European healing system is said to have originated with Hippocrates (460–377 BC) and Aristotle (384–322 BC), whose own ideas were rooted in ancient beliefs from India and Egypt. The philosopher and natural scientist, Theophratus (~300 BC), in his *History of Plants*, dealt with the medicinal qualities of herbs, and noted the ability to change their characteristics through cultivation. Dioscorides, a Greek physician (100 AD), during his travels with Roman armies, recorded the collection, storage and the use of medicinal herbs and Galen (130–200 AD) who practiced and taught pharmacy and medicine in Rome, published no less than 30 books on these subjects, and is well known for his complex prescriptions and formulas used in compounding drugs, sometimes containing dozens of ingredients (“galenicals”) (Weiher *et al.,* 1999).

Greek and Roman medicine was based on the belief that the world is composed of four elements—earth, wind, fire and water. Each of these has its corresponding humours, linked to the four vital fluids in the body. The four humours—blood, phlegm, black bile and yellow bile, influence both health and temperament (respectively sanguine, phlegmatic, melancholic and choleric). In order to restore balance, drastic measures such as blood letting (reducing excess blood) and purging (to remove excess black bile) was used. The four

humours were also associated with cold, heat, dampness and dryness and each of these had a corresponding range of cold, hot, damp or dry herbs that were supposedly able to restore imbalances. European tradition also had many regional influences that influenced local folk practices and traditions (Weiher *et al.,* 1999).

One of the most powerful influences was the famous book *De Materia Medica*, written by the Greek physician Dioscorides in the first century AD. It is generally accepted to be the first European herbal and was the standard reference in Europe for more than 1 000 years, providing the base for most of the later herbals. As early as AD 800, medicinal plants were cultivated according to a standardized layout in monasteries in Central Europe. One of the famous healers of this era was Hildegard of Bingen (1098–1179). In later years a Swiss alchemist known as Paracelsus (1493–1541) emphasized the importance of the correct dosage for medical treatments (Gurib-Fakim, 2006). Herbal medicine was part of everyday life in many countries in Europe and to this day has remained a popular method of treating ailments but is often considered to be supportive rather than curative. To date in several European countries, the use of herbal tea is still very popular. In addition to these, 'natural products' taken in their crude form (unprocessed) as teas or decoctions, more sophisticated phytomedicines (standardized and formulated extracts of plants, often subject to rigorous testing in humans) remain a popular alternative to medicinal products derived from pure synthetic chemicals (Vicker and Zollman, 1999).

A large number of traditional herbal remedies in Europe have become widely known as a result of commercialization and a number of active compounds have been isolated from medicinal plants and are used today as single chemical entities (Pieroni, 2000).

1.2.7 Classical Arabic and North African traditional medicine

The oldest written information in the Arabic traditions comes from the Sumerians and Akkadians of Mesopotamia, thus originating from the same areas as the archeological records of Shanidar IV (Heinrich *et al*., 2004). The earliest documented record, which presumably relates to medicinal plants, dates from 60 000 before the common era (BCE) found in the grave of the Neanderthal man from Shanidar IV, an archeological site in Iraq. Pollen of several species of plants, presumably used as medicines, was discovered among which are: *Centaurea solstitialis* (Asteraceae), *Ephedra altissima* (Ephedraceae), *Althea* sp. (Malvaceae) amongst others. Although this may not be a finding with direct bearing on the culture of Shanidar, these species or closely related ones from the same genus, are still important today in the phytotherapy of Iraq and also known from other cultural traditions. These species may well have been typical for the Neanderthal people and may also be part of a tradition for which Shaidar IV represents the first available record (Cragg and Newman, 2005).

The Middle East is known as the cradle of civilisation and many plants cultivated nowadays were domesticated in this region. The Babylonians, Assyrians and Sumerians recorded herbal remedies in cuneiform writing on numerous clay tablets. Of special interest is the Code of Hammurabi (ca. 1 700 BC), a comprehensive set of civil laws carved in stone and commissioned by the King of Babylon and which lists several medicinal herbs (Spiegel and Springer, 1997).

Similar documents have survived several millennia in Egypt. The Egyptians documented their knowledge (including medical and pharmaceutical) in wall paintings of tombs dating from the Old Kingdom and on papyrus which is made from *Cyperus aquaticus*. The most important of these writings is the Ebers Papyrus, which originates from around 1 500 BC and is reported to contain ancient medicinal knowledge from before 3 000 BC (Oubré *et al,* 1997). This famous 20 m papyrus scroll reputedly found in a tomb is inscribed in Egyptian hieroglyphics and named after Prof. Ebers Georges at Thebes in 1872. It was deposited at the University of Leipzig 1873 and two years later G.

Ebers published a facsimile edition (Ghalioungui, 1987). The Ebers Papyrus is a medical handbook covering all sorts of illnesses and includes empirical as well as symbolic forms of treatment. The diagnostic precision documented in this text is impressive. During the Dark and Middle Ages (5–12th Centuries, AD), the monasteries in countries such as England, Ireland, and Germany were responsible for preserving the remains of Western knowledge. But it was the Arabs who were responsible for the preservation of much of the Greco-Roman expertise, and for expanding it to include the use of their own resources, together with the Chinese and Indian herbs, till then unknown to the Greco-Roman world. The Arabs were the first to establish privately owned drug stores in the 8th century. The Persian pharmacist, physician, philosopher and poet, Avicenna, contributed much to the sciences of pharmacy and medicine throughout works such as *Canon medicinae*, regarded as the "final codification of all Greco-Roman medicine". *Canon medicinae* included elements of other cultures healing system and forms the basis for a distinct Islamic healing system known today as *Unani-Tibb* (Sheehan and Hussain, 2002).

Among the famous medicinal plants of the Middle East and Egypt are: *Allium cepa* (onion), *Astracantha gummifera* (tragacanth), *Carthamus tinctorius* (safflower), *Carum carvi* (caraway), *Ferula assafoetida* (asofoetida), *Lawsonia inermis* (henna), *Papaver somniferum* (opium poppy), *Peganum harmala* (syrian rue), *Prunus dulcis* (almond), *Punica granatum* (pomegranate), *Rosa* x *damascena* (damask rose), *Ricinus communis* (castor oil plant), *Salvadora persica* (toothbrush tree), *Senna alexandrina* (senna), *Sesamum indicum* (sesame), *Trachyspermum ammi* (ajowan), *Trigonella foenum-graecum* (fenugreek) and *Vitis vinifera* (grape) (Padua de, 1999; Neuwinger, 2000; Gurib-Fakim, 2006). A list of some botanical drugs used in traditional medicine, which have led to useful modern drugs are shown in Table 1.1. However, it should be noted that concern has been raised on the toxicity of Kava pyrones and that their anxiolytics have been positively reviewed (Schulze *et al.*, 2003).

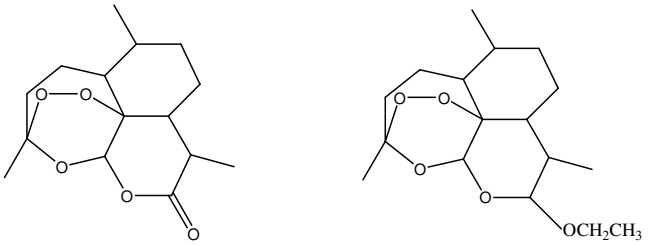
Table 1.1: Botanical drugs used in traditional medicine which led to useful modern drugs (Gurib-Fakim 2006).

Botanical names	English names	Indigenous use	Origin	Uses in biomedicine	Biologically active compounds
Adhatoda vasica	–	Antispasmodic, antiseptic, insecticide, fish poison	India, Sri Lanka	Antispasmodic, oxytocic, cough suppressant	Vasicin (lead molecule for Bromhexin and Ambroxol)
Catharanthus roseus	Periwinkle	Diabetes, fever	Madagascar	Cancer chemotherapy	Vincristine, Vinblastine
Condrodendron tomentosum	–	Arrow poison	Brazil, Peru	Muscular relaxation	D-Tubocurarine
Gingko biloba	Gingko	Asthma, anthelmintic (fruit)	Eastern China	Dementia, cerebral deficiencies	Ginkgolides
Harpagophytum procumbens	Devil's claw	Fever, inflammatory conditions	Southern Africa	Pain, rheumatism	Harpagoside, Caffeic acid
Piper methysticum	Kava	Ritual stimulant, tonic	Polynesia	Anxiolytic, mild stimulant	Kava pyrones
Podophyllum peltatum	May apple	Laxative, skin infections	North America	Cancer chemotherapy, warts	Podophyllotoxin and lignans
Prunus africana	African plum	Laxative, 'Old man's disease'	Tropical Africa	Prostate hyperplasia	Sitosterol

1.3 Drug discovery from medicinal plants

Drug discovery from medicinal plants has evolved to include numerous fields of inquiry and various methods of analysis. The process typically begins with a botanist, ethnobotanist, ethnopharmacologist, or plant ecologist who collects and identifies the plant(s) of interest. Collection may involve species with known biological activity for which active compound(s) have not been isolated (i.e traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program. It is necessary to respect the intellectual property rights of a given country where plant(s) of interest are collected (Baker *et al*., 1995). Phytochemists (natural product chemists) prepare extracts from the plant material, subject these extracts to biological screening in pharmacologically relevant assays, and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation. Molecular biology has become essential to medicinal plant drug discovery through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets. Pharmacognosy encapsulates all of these fields into a distinct interdisciplinary science.

Numerous methods used to acquire compounds for drug discovery include: isolation from plants and other natural sources; synthetic chemistry; combinatorial chemistry, and molecular modeling (Ley and Baxendale, 2002; Geysen *et al*., 2003; Lombardino and Lowe, 2004). Despite the recent interest in molecular modelling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and funding organizations, the natural products, and particularly that of medicinal plants, remain an important source of new drugs, drug leads, and chemical entities (Newman *et al*., 2000; Newman *et al*., 2003; Butler, 2004). In both 2001 and 2002, approximately one quarter of the best-selling drugs worldwide were natural products or were derived from natural products (Butler, 2004). An example is Arteether (Fig. 1.1), a potent antimalaria drug. It is derived from artemisinin, a sesquiterpene lactone isolated from *Artemisia annua* (Asteraceae), a plant used in traditional Chinese medicine (TCM) (van Agtmael *et al*., 1999; Graul, 2001).



Artemisinin Arteether

Figure 1.1: The structure of artemisinin and arteether.

Despite evident successes of drug discovery from medicinal plants, future endeavors face many challenges. Pharmacognosists, phytochemists, and other natural product scientists will need to continuously improve the quality and quantity of compounds that enter the drug development phase to keep pace with other drug discovery efforts (Butler, 2004). The process of drug discovery has been estimated to take an average of 10 years upwards (Reichert, 2003) and cost more than 800 million US dollars (Dickson and Gagnon, 2004). Much of this time and money is spent on the numerous leads that are discarded during the drug discovery process. It has been estimated that only one in 5000 lead compounds will successfully advance through clinical trials and be approved for use. Lead identification is only the first step in a lengthy drug development process (Fig. 1.2). There is also lead optimization (involving medicinal and combinatorial chemistry), development (including toxicology, pharmacology, pharmacokinetics, ADME [absorption, distribution, metabolism, and excretion], and drug delivery), and clinical trials which all take a considerable length of time.

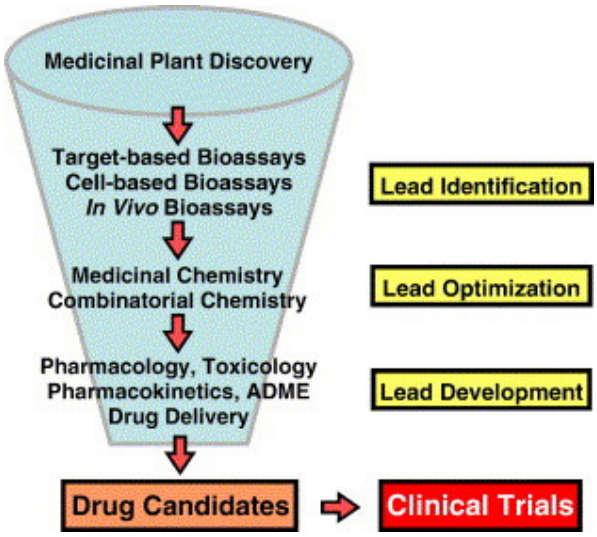


Figure 1.2: Schematic representation of a typical medicinal plant drug discovery process and development (Balunas and Kinghorn, 2005).

Drug discovery from medicinal plants has traditionally been lengthier and more complicated than other drug discovery methods. Therefore, many pharmaceutical companies have eliminated or scaled down their natural product research (Butler, 2004; Koehn and Carter, 2005).

Recently, there has been a rekindling of interest in 'rediscovering natural products'. As stated by one authority "We would not have the top-selling drug class today, the statins; the whole field of angiotensin antagonists and angiotensin-converting enzyme inhibitors; the whole area of immunosuppressives, nor most of the anticancer and antibacterial drugs. Imagine all of these drugs not being available to physicians or patients today". It is clear that nature has played and will continue to play, a vital role in the drug discovery process (Cragg and Newmann, 2005).

1.4 Synthesis and role of plant secondary metabolites

In plants, as a result of metabolic processes, many different kinds and types of organic compounds or metabolites are produced. These metabolites are grouped into primary and secondary metabolites. The primary metabolites like chlorophyll, amino acids, nucleotides, simple carbohydrates or membrane lipids, play recognised roles in photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation. The secondary metabolites also differ from primary metabolites in having a restricted distribution in the plant kingdom. That is, particular secondary metabolites are often found in only one plant species or a taxonomically related group of species, whereas the basic primary metabolites are found throughout the plant kingdom (Taiz and Zeiger, 2006). During the past few decades, experimental and circumstantial evidence has made it clear that many secondary metabolites do indeed have functions that are vital for the fitness of a plant producing them. The main roles are:

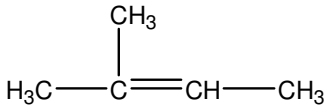
- Defence against herbivores (insects, vertebrates)
- Defence against fungi and bacteria
- Defence against viruses
- Defence against other plants competing for light, water and nutrients
- Signal compounds to attract pollinating and seed dispersing animals
- Signals for communication between plants and symbiotic micro-organisms (e.g. N-fixing Rhizobia or mycorrhizal fungi)
- Protection against UV-light or other physical stress (Wink, 1999)

They have also provided an invaluable resource that has been used to find new drug molecules (Gurib-Fakim, 2006).

Plant secondary metabolites can be grouped into three chemically distinct classes: terpenes, phenolics and nitrogen containing compounds. Figure 1.3 shows, in simplified form, the pathways involved in the biosynthesis of secondary metabolites and their interconnection with primary metabolites.

1.4.1 Terpenes

Terpenes, terpenoids or isoprenoids are dimmers, trimers or polymers of isoprene units, which are usually jointed in a head to tail fashion. In plants the



Isoprene unit

activated form of the isoprene unit (isopentenyl pyrophosphate) which is the building-block of each type of terpenoid is synthesised either by the mevalonic acid pathway (e.g. sesquiterpenoids) or the methylerythritolphosphate pathway (e.g. mono- and diterpenoids) (Taiz and Zeiger, 2006) (Fig 1.3). Isoprene units usually condense to form linear chain or ring compounds commonly containing carbon atom numbers of 10 (the monoterpenoids), 15 (the sesquiterpenoids, 20 (the diterpenoids), or 30 (the triterpenoids). Terpenoids with 25 carbons are rarely found.

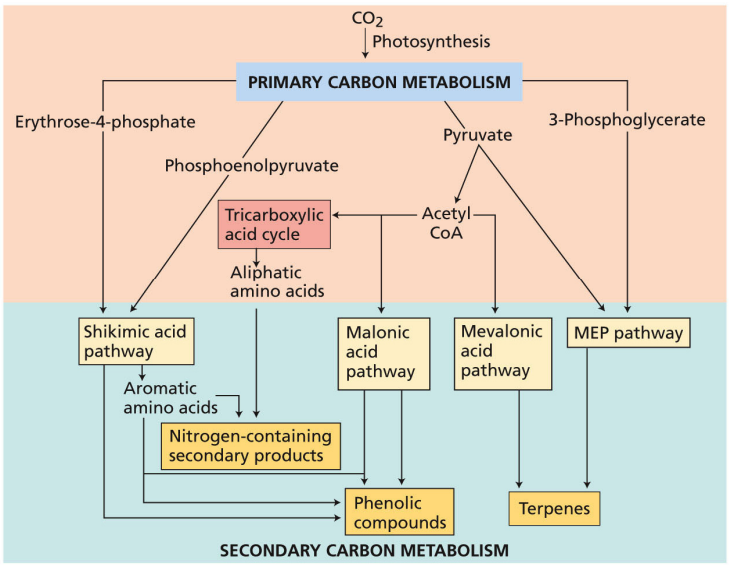
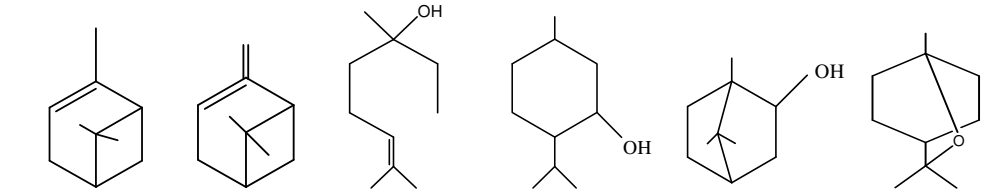


Figure 1.3: Main pathways leading to secondary metabolites (Taiz and Zeiger, 2006).

1.4.1.1 Monoterpenes

Monoterpenes are commonly found in essential oils. Iridoids and pyrethrins are included in this group. Examples of monoterpenes (Fig. 1.4) commonly found in essential oils are shown below (Taiz and Zeiger, 2006):



α- Pinene β-Pinene Linalool Menthol Borneol 1,8-cineol

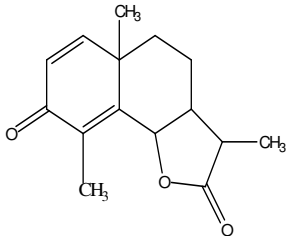
Figure 1.4: Monoterpenes commonly found in essential oils.

They are widely used as insecticides and their pharmacological properties range from analgesic to anti-inflammatory.

1.4.1.2 Sesquiterpenes

Sesquiterpenes are also constituents of essential oils of many plants, e.g. bisabolol, humulene and caryophyllene. Sesquiterpene lactones are well known as bitter principles and occur in families like the Asteraceae.

These compounds possess a broad range of activities due to the α-methylene-γ-lactone moiety and epoxides. Their pharmacological activities are antibacterial, antifungal, anthelmintic, antimalarial and molluscicidal. An example is santonin, which is used as anthelmintic and antimalarial (Gurib-Fakim, 2006).



α- Santonin

1.4.1.3 Diterpenes

Diterpenes are present in animals and plants and have some therapeutic applications, for example, the famous taxol and its derivatives are anticancer drugs. Other examples are forskolin, which has antihypertensive activity; zoapatanol is an abortifacient while stevoside is a sweetening agent (Gurib-Fakim, 2006).

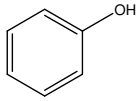
1.4.1.4 Triterpenes

Triterpenes are C_{30} compounds arising from the cyclization of squalene. They are comprised of a variety of structurally diverse compounds, which include steroids. Tetracyclic terpenes and steroids have similar structures but have different biosynthetic pathways (Taiz and Zeiger, 2006).

Steroids contain a ring system of three six-membered and one five-membered ring. Because of the profound biological activities encountered, many natural steroids together with a considerable number of synthetic and semi-synthetic steroidal compounds, are employed in medicine (e.g. steroidal saponins, cardioactive glycosides, corticosteroid hormones and mammalian sex hormones). The pharmaceutical applications of triterpenes and steroids are considerable (Gurib-Fakim, 2006).

1.4.2 Phenolic compounds

All phenolic compounds have an aromatic ring that contains various attached substituent groups such as hydroxyl, and methoxy (-O-CH_3) groups, and often other non-aromatic ring structures.



They range from simple structures with one aromatic ring to complex polymers such as tannins and lignins. Phenolics differ from lipids in being more soluble in water and less soluble in non-polar organic solvents. Some phenolics, however, are rather soluble in ether, especially when the pH is low enough to prevent ionization of any carboxyl and hydroxyl group present.

These properties greatly aid separation of phenolics from one another and from other compounds (Taiz and Zeiger, 2006). Other classes of phenolic compounds include coumarines, quinones and flavonoids.

Phenolic compounds are synthesised via the Shikimic acid or acetate pathway (Fig 1.5) and subsequent reactions. They have a wide range of pharmaceutical activities such as anti-inflammatory, analgesic, antitumour, anti-HIV, anti-infective (antidiarrhoeal, antifungal), antihepatotoxic, antilipolytic, antioxidant, vasodilatory, immunostimulant and antiulcerogenic. In plants they serve as effective defence against herbivores (Wink, 1999 and Gurib-Fakim, 2006).

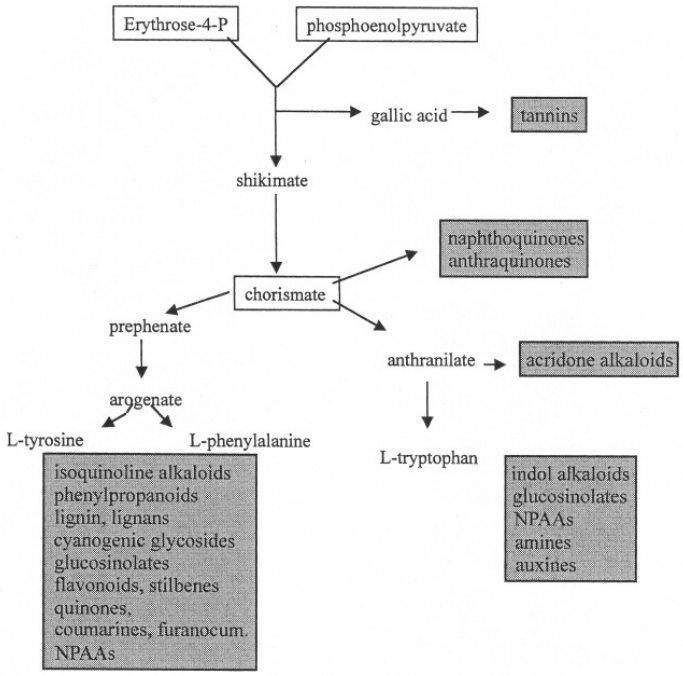


Figure 1.5: The pathways of secondary metabolites derived from precursors in the shikimate pathway (Wink, 1999).

1.4.2.1 Flavonoids

Flavonoids are 15-carbon compounds generally distributed throughout the plant kingdom (Fig. 1.6). More than 2000 have been identified from plants

(Taiz and Zeiger, 2006). They are responsible for the colour of flowers, fruits and sometimes leaves. Some may contribute to the colour by acting as a co-pigment. The name 'flavonoid' refers to the Latin word '*flavus*' meaning yellow.

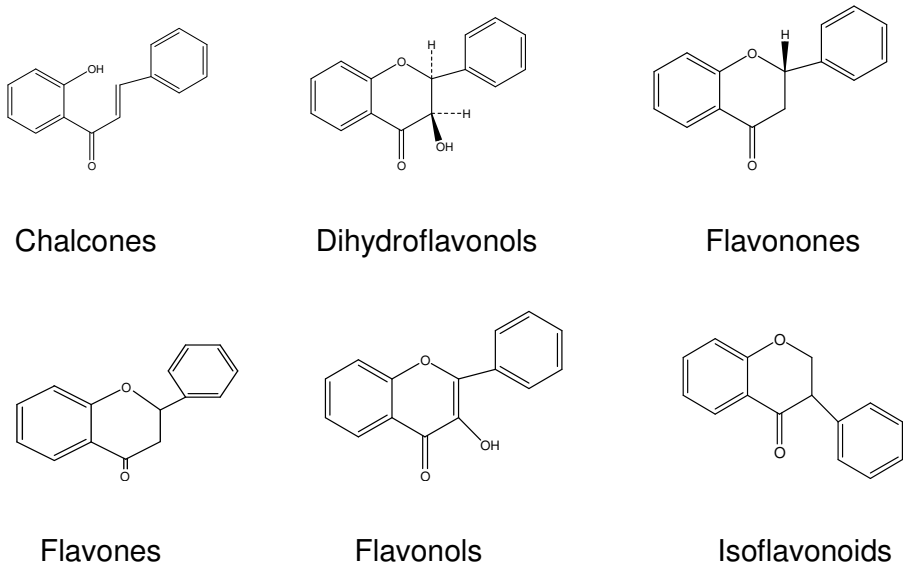


Figure 1.6: Basic structures of some flavonoids.

Flavonoids protect the plant from UV-damaging effects and play a role in pollination by attracting animals with their colours (Gurib-Fakim, 2006). The basic structure of flavonoids is 2-phenyl chromane or an Ar–C3–Ar skeleton. Biosynthetically they are derived from a combination of the Shikimic acid and the acetate pathways. Small differences in basic substitution patterns give rise to several sub-groups. In the plant, flavonoids can either occur as aglycones or as O- or C-glycosides (Gurib-Fakim, 2006). Recently, flavonoids have attracted interest due to the discovery of their pharmacological activities.

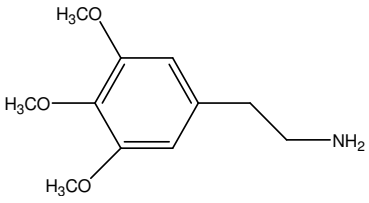
1.4.3 Nitrogen containing compounds

A large variety of plant secondary metabolites have nitrogen in their structures. Included in this category are such well-known antiherbivore compounds such as alkaloids and cyanogenic glycosides, which are of considerable interest because of their toxicity to humans and their medicinal

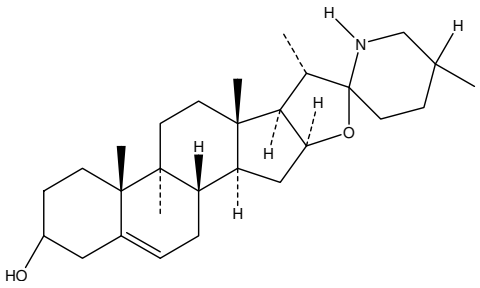
properties. Most nitrogenous secondary metabolites are biosynthesised from common amino acids (Taiz and Zeiger, 2006).

1.4.3.1 Alkaloids

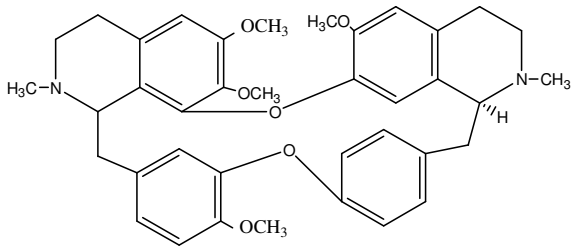
The term 'alkaloid' has been defined as a cyclic organic compound containing nitrogen in a negative oxidation state, which has limited distribution in living organisms (Taiz and Zeiger, 2006). Based on their structures, alkaloids are divided into several subgroups: non-heterocyclic alkaloids and heterocyclic alkaloids, which are again divided into 12 major groups according to their basic ring structure. Mescaline is an example of a non-heterocyclic or pseudo-alkaloid, tetrandrine is an example of a bisbenzylisoquinoline alkaloid while solasodine is a triterpene alkaloid (GuribFakim, 2006) (Fig. 1.7).



Mescaline



Solasodine



Tetrandrine

Figure 1.7: Structures of some alkaloids.

Free alkaloids are soluble in organic solvents and react with acids to form water-soluble salts. There are exceptions like berberine, which is a quartenary ammonium alkaloid. Most alkaloids are solids except for nicotine, which is a liquid.

Alkaloids, usually have a marked physiological action on humans or animals, and are sometimes believed to be waste products and a nitrogen source. They are thought to play an important role in plant protection, germination and plant growth stimulation.

Alkaloids are pharmaceutically significant, e.g. morphine as a narcotic analgesic, codeine in the treatment of coughs and pain, colchicines in the treatment of gout, quinine as an antimalarial, quinidine as an antiarrythmic and L-hyoscyamine (in the form of its racemic mixture known as atropine) as antispasmodic and for pupil dilation (Gurib-Fakim, 2006).

1.4.3.2 Cyanogenic glycosides

Perhaps the most obvious defence-related secondary metabolites are the cyanogenic glucosides (Bennett and Wallsgrove, 1994). They are not in themselves toxic but are readily broken down to give off volatile poisons when the plant is crushed. Cyanogenic glycosides release the well-known respiratory poisonous gas, hydrogen cyanide (Taiz and Zeiger, 2006).

1.5 Infectious diseases

Despite the tremendous progress in medicine, infectious diseases caused by bacteria, fungi, viruses and parasites continue to pose a threatening challenge to public health (Cos *et al.,* 2006). The burden of these diseases is felt the most in developing countries due to poverty, unavailability of medicines and the emergence of widespread resistance of pathogens to the available drugs (Okeke *et al.,* 2005). The World Health Organisation in 2002 has also reported that infectious and parasitic diseases account for 26.2% of the global cause of death, the vast majority of which occurred in the developing countries (WHO, 2003).

Every year, more than half of the deaths associated with infectious diseases continue to be attributed to three illnesses: HIV/AIDS, tuberculosis and malaria. These diseases are present in epidemic proportion, profoundly affecting and serving as major obstacles to the economic growth and development in many of the poorest countries in the world (Mandell *et al.,* 2005). Urgent solutions are required if the poorest regions in the world is to develop.

1.5.1 Malaria

Malaria is a protozoal disease caused by parasitic protozoa of the genus *Plasmodium*. It is transmitted to humans by the female *Anopheles* mosquito. There are over three hundred species of *Anopheles* mosquito, however, only about sixty are able to transmit the malaria parasite. Malaria commonly affects the populations of tropical and subtropical areas world wide, as well as increasing number of travellers to and from these areas. The following four species of *Plasmodium* cause the disease in its various forms: *P. falciparum, P. vivax, P. ovale* and *P. malariae*. *P. falciparum* is the most widespread and dangerous of the four as it can lead to the fatal cerebral malaria, which often results in death (Hyde, 2002). Today some 500 million people in Africa, India, South East Asia and South America are exposed to endemic malaria and it is estimated to cause 2.5 million deaths annually, one million of which are children. Although malaria is found in over 100 countries (Fig. 1.8 and 1.9), the major burden of the disease is carried by the nations of Africa, where over 90% of all falciparum malaria deaths are recorded, and where the high levels of morbidity and transmission place considerable strain on public health services and economic infrastructure (Hyde, 2002). In the absence of effective vaccines, management of the disease has depended largely upon chemotherapy and chemoprophylaxis. Of the various antimalaria drugs available, the aminoquinoline, chloroquine was for several decades the agent of choice, as it was safe, effective and cheap. Parasite resistance to this drug was first observed in Thailand in 1957 and then on the border of Colombia and Venezuela in 1959. By the late 1970s it had spread to East Africa and by the mid-1980s had become a major problem in several areas in Africa (Wernsdorfer and Payne, 1991).

Although the increasing prevalence of drug resistant *P. falciparum* has hindered the ability to control/treat the disease, it has at the same time intensified attempts to develop novel antimalaria drugs and agents to prolong

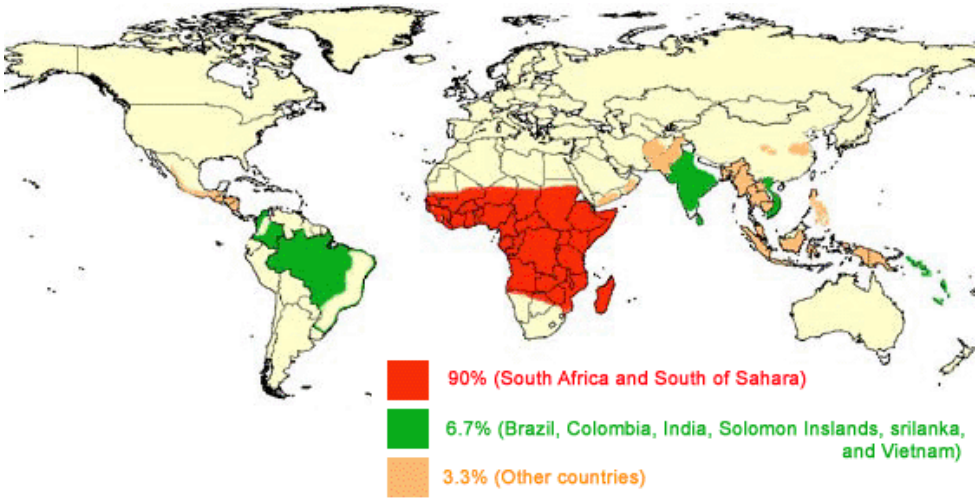


Figure 1.8: Global malaria distribution (WHO global atlas, 2005).

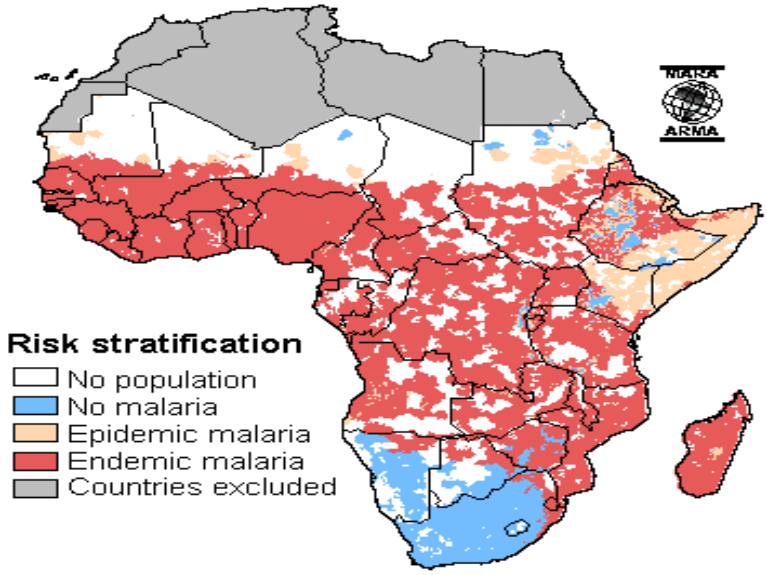


Figure 1.9: Distribution of malaria in Africa (WHO global atlas, 2005).

the clinical usefulness of the few currently available drugs (Singh and Puri, 2000). An increasing number of countries have been compelled to adopt a different class of drug, the antifolates, as the first line of alternatives to

chloroquine. The most widely used combination of this type consist of pyrimethamine (PYR) and sulfadoxine (SDX), known as fansider or SP, which is cheap and, until recently, was effective against the chloroquine-resistant parasites found in Africa. However, resistance to this formulation, long established in parts of south-east Asia and South America (Wernsdorfer, 1994), now threatens to leave Africa with no affordable treatment. Further combinations of antifolates with newer drugs such as the artemisinin derivatives, or the development of alternative combinations, may be the only way to limit the pace of the parasitic resistance to chemotherapy. For example, the antifolate prodrug, proguanil, has now been formulated together with a new type inhibitor, atovaquone, to yield malarone, recently licensed for clinical use (Hyde, 2002).

Developing countries, were malaria is epidemic, still depend on traditional medicine for the treatment of the disease. However, little scientific data are available to assess the efficacy of these herbal remedies. On the other hand, it is accepted that the recognition and validation of traditional medicinal practices could lead to new plant derived drugs, e.g. artemisinin from *Artemisia annua*, a Chinese traditional medicine plant (Ridley, 2002). Therefore it is important that medicinal plants which have a folklore reputation for antimalarial properties are investigated, in order to establish their efficacy and to determine their potential as a source of new antimalarial drugs (Tran *et.al.,* 2003). South Africa is an ideal place to search for a new drug because of its remarkable biodiversity and rich cultural traditions of plant uses.

1.5.2 Human immunodeficiency virus (HIV)

The Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organisation (WHO), reported the number of people living with HIV at the end of 2006 to be 39.5 million globally. Of the people infected worldwide, 64% reside in sub-Saharan Africa, 77% of which are women. The severity of the epidemic has been associated with poverty, low status of women and other socio-economic factors (Nicoll and Gill, 1999; NSP, 2000).

A number of documents have described the seriousness of HIV/AIDS in the Southern Africa region with particular emphasis on Southern Africa being the most affected (UNAIDS, 2000). The prevalent rate for South Africa is estimated to be 12.5%, which is one of the highest national prevalent rates in the world (James *et al.,* 2006). Women are more hit by the epidemic of HIV/AIDS. Of the 5.54 million people living with HIV in South Africa in 2005, 18.8% are adults aged 15-49 years of which women account for approximately 55%. The infection is more pronounced in the age group 20-24 years and 25-29 where the HIV prevalence rates are 23.9% for women to 6% for men and 33.3% for women and 12.2% for men respectively (NSP, 2007). HIV was around 3% among children aged 2-14 year and nearly 4% for people in their sixties (Dinkelman *et al.,* 2007).

The United Nations General Assembly Session on HIV/AIDS (UNGASS) has identified young people of the age group 15-24 years as the target group for reducing new cases of HIV infection and set a global target of reducing incidence of HIV in this group by 20% by 2015. Young people represent the main focus for altering the course of this epidemic. UNAIDS data on the experience of several countries including South Africa, confirm that positive behavioural change is more likely in this group than in older ages (NSP, 2007).

The increase in pregnancy and HIV infection in young school leaving people has been linked with unemployment. The inability to secure a job plus pressure from family members to make financial contributions for the maintenance of their homes, predispose them to sex work as a way to make ends meet (Dinkelman *et al.,* 2007).

Children under the age of 18 comprise 40% of the population of South Africa. In 2004, it was reported that 13% of them have lost either mother or father, half of which was due to AIDS. Children from deeply impoverished households were worst affected by the impact of AIDS (UNAIDS, 2004). Children are exposed to HIV through sexual abuse, blood transfusion and

mother to child transmission (MTCT) which occurs mostly during birth and/or breastfeeding (Nicoll and Gill, 1999).

The immediate determinant of the spread of HIV relates to behaviours such as unprotected sexual intercourse, multiple sexual partnerships, and some biological factors such as sexually transmitted infections, the fundamental drivers of this epidemic in South Africa are deep rooted in the problem of poverty, underdevelopment, and low status of women, including gender-based violence in society (Dinkelman *et al.*, 2007).

Many countries in Africa and Asia have taken urgent steps to curb the epidemic with varying degrees of success. In South Africa, despite the effort invested, the HIV infection rate has increased significantly over the last 5 years (NSP, 2000). The country has the largest number of people enrolled on antiretroviral therapy in the world. There are still many more people in need of this and other related interventions to reduce the morbidity and mortality of HIV/AIDS. In particular, more eligible adults than children have accessed these services. There is need to develop more innovative strategies to improve access for children in schools (primary and secondary) (NSP, 2007).

At the moment there is no cure for HIV. Single drug therapy is no longer effective due the resistance developed by the virus. Combinational therapy is now the method of choice in treatments (Spencer, 2005). The adverse side effects of the available drugs or combination of drugs and non-compliance of patients on treatment did not help the situation (Spencer, 2005). There is a urgent need to develop drugs with less side effects for the treatment and a cure for the disease. The only effective way to combat HIV infection at the moment is in prevention-advocacy and the practice of abstinence, and safe sexual practice (Nicoll and Gill, 1999).

1.5.3 Tuberculosis

The bacterium (*Mycobacterium tuberculosis)* causing tuberculosis first emerged as a major disease threat more than 15 000 years ago. Today about 2 billion people are infected. However, not all these individuals will become ill or develop active tuberculosis (Mandell *et al.,* 2005). HIV infection is the

strongest risk factor for progression to active disease: 46% of people in the developing world with HIV are co-infected with tuberculosis (Nicoll and Gill, 1999; Anthony and Fauci, 2005). About 4 million people have active tuberculosis at any time resulting in about 2 million deaths each year. Most of the deaths occur in the developing countries predominantly in Africa and Asia (Anthony and Fauci, 2005). The fatalities of this disease are worsened by the development of resistance to the available antituberculosis drugs (Mandell *et al.,* 2005).

1.6 Antioxidant activity

Free radical decomposition results in a large number of human diseases, such as heart disease, cataracts, cognitive dysfunction, aging and cancer (Brahmachari and Gorai, 2006). These damages or diseases are caused by free radicals called reactive oxygen species (ROS). Examples of ROS include superoxide anions, singlet oxygens, hydroxyl radicals, lipid peroxyl radicals and peroxynitrite radicals (Erkoç *et al.,* 2003).

The human body has evolved with antioxidant systems to protect it against free radicals. These systems include some antioxidants produced in the body (endogenous), obtained from the diet (exogenous) and repair antioxidant (proteases, lipase, transferases, and DNA repair enzymes). The ones produced in the body are enzymatic defences, such as Se-glutathione peroxidase, catalase, and superoxide dismutase, which metabolize superoxide, hydrogen peroxide and lipid peroxides, thus preventing most of the formation of the toxic hydroxyl radicals. Exogenous antioxidants consist of non-enzymatic defenses, such as glutathione, histidine-peptides, the iron-binding proteins transferring and ferritin, dihydrolipoic acid etc (Erkoç *et al.,* 2003; Brahmachari and Gorai, 2006). Owing to the incomplete efficiency of our endogenous defence systems and the existence of some physiopathological situations (cigarette smoking, air pollutants, UV radiation, high polyunsaturated fatty acid diet, inflammation, ischemia/reperfusion, etc) in which ROS are produce in excess and at the wrong time and place, dietary antioxidants are needed for diminishing the cumulative effects of oxidative

damage over the life span. The antioxidants derived from diet are vitamins C, E and A, and carotenoids. Other antioxidants of value to health derived from plants include phenols, phenolic acids, flavonoids, tannins and lignans (Pietta, 2000).

Antioxidant activity of a drug candidate adds to its medicinal value. In this chapter the antioxidant activity of the compounds isolated has been investigated using both qualitative and quantitative assays.

1.7 *Croton steenkampianus*

Several species of the genus *Croton* (Euphorbiaceae) showed excellent results when crude extracts were tested for antiplasmodial activity previously. Of the species tested, the leaves of *C. steenkampianus* Gestner, had the best activity and was therefore selected for isolation of the active principles in this study (Prozesky, 2004). Before 2004, little or nothing was known regarding the chemical composition and medicinal use of *C. steenkampianus* other than that known for the family in general. However, flavonoids and terpenes that showed promising antiplasmodial activities had been isolated from its leaves (Prozesky, 2004). Therefore it was decided to attempt the isolation of more active principles from this species.

Generally, species in the family Euphorbiaceae have a variety of uses and commercial products include rubber *(Hevea*), tung oil (*Aleurites*), castor oil (*Ricinus*), and cassava (*Manhot*) and many are used as ornamentals (Leistner, 2000). Medicinally, despite reports that many species are poisonous, they are used for ailments such as malaria, hepatic and kidney disorders, obesity, hypertension, fever, dysentery, convulsions, snakebite, chest pains, gastrointestinal disturbances, sterility, eye and respiratory complaints (Pooley, 1993; Ngadjui *et al.,* 2002; Suarez *et al.,* 2006). Chemically, the genus contains very diverse compounds including alkaloids, flavonoids and triterpenes. Many structurally diverse diterpenes have also been isolated from the genus (Prozesky, 2004).

C. steenkampianus is a shrub to small tree (1.5-4 m) (Fig. 1.10), found on the margins of sand forests and thickets in the eastern parts of South Africa and

further north into Africa. The main stem is much branched from the base, with smoothish grey bark. The leaves are large, heart shaped, grey to olive-green above, white beneath, with a pointed tip (Pooley, 1993; Prozesky, 2004).

Figure 1.10 *Croton steenkampianus* leaves.

1.8 Objectives

The objectives of this study were:

- Isolation of biologically active compound(s) from *C. steenkampianus*
- *In vitro* testing of pure compound(s) for antibacterial activity, antiplasmodial activity, anti-HIV activity and antioxidant activity
- *In vitro* testing of the antiplasmodial activity of isolated compound(s) with and without chloroquine
- Cytotoxicity testing of isolated compound(s)

1.9 Scope of the thesis

The isolation and identification of three flavonoids, two new diterpenes (with a newly described skeleton) and one new indane is described in chapter two. The isolation was performed using bioassay-guided fractionation (antibacterial activity). The bio-activities (antiplasmodial, antibacterial and antioxidant, anti-HIV and cytotoxicity of the isolated compounds are described in chapters 3, 4,

5 and 6 respectively. Chapter 7 consists of a general discussion and conclusion.

1.10 Hypothesis

The determination of biological activities and isolation of compounds from *C. steenkampianus* was performed for the first time by Prozesky (2004). This species not being well studied plus the interesting results reported previously led to the conclusion that it contains more active principles and was reselected for further studies. The hypothesis of this study is therefore that *C. steenkampianus* contains compounds with valuable bioactivity.

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CHAPTER 2

BIOASSAY GUIDED FRACTIONATION OF THE CRUDE EXTRACT FROM *CROTON STEENKAMPIANUS*

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2.1 Introduction

Plants from the Euphorbiaceae family are well known for their medicinal properties and are used to treat many diseases around the world (Suarez *et al.,* 2003). This family has about 300 genera and 5 000 species of trees, shrubs and herbs. The genus *Croton* has about 750 species of trees, shrubs and herbs distributed in tropical and subtropical regions and is rich in constituents with biological activity (Suarez *et al.,* 2003). This genus is being used to treat malaria, hepatic and kidney disorders, obesity, hypertension, wounds, inflammation, tumors, diabetes, diarrhoeas, rheumatism, gastric ulcers and pain. A number of biologically active compounds (terpenes, flavonoids and alkaloids) have been isolated from it (Silva *et al.,* 2005; Suarez *et al.,* 2003; Suarez *et al.,* 2006; Ngadjui *et al.,* 2002).

Before 2004, *C. steenkampianus* had no history of medicinal usage or any record of the isolation of an active compound. However, it has since been reported that the crude extracts from the leaves showed antiplasmodial activity and contained several interesting compounds such as a diterpene with a new exoskeleton, one triterpene and two flavonoids (Prozesky 2004). Antibacterial activity was used to guide the isolation of compounds from fractions because it is simpler and quicker to perform. This method is supported by the fact that most compounds showing antibacterial activity also show antiplasmodial activity (Boonphong *et al*., 2007; Zdzislawa, 2007).

2.2 Materials and Methods

2.2.1 Collection of plant materials

Leaves of *C. steenkampianus* Gerstner were collected in April 2003, at Thembe Elephant Park in northern KwaZulu-Natal, South Africa, and a voucher specimen (registry number 92520) is preserved at the HGWJ Schweickerdt Herbarium (PRU) at the University of Pretoria.

2.2.2 Methods

2.2.2.1 Preparation of the crude extract

The leaves were allowed to dry for two weeks at room temperature. The dried leaves (2 kg) were then crushed to a powder and extracted with ethanol at room temperature for 3 days. The resulting mixture was then filtered under vacuum and the residue generated was extracted further with ethanol. The combined filtrate was evaporated under reduced pressure at 37°C to yield an 80 g total extract.

2.2.2.2 Bacterial culturing and antibacterial testing

Bacillus cereus and *Escherichia coli* were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. These bacteria were maintained on nutrient agar slant and were recovered for testing by culturing them in a nutrient broth. A sterile loop was used to transfer some bacterial colonies to the nutrient broth (50 ml) in conical flasks under sterile conditions (in a laminar flow cabinet). The opening of the flask was then plugged with cotton wool, covered with aluminium foil, placed on a shaker and incubated for 24 hours at 37°C. After 24 hours, the bacterial culture was centrifuged at 3 000 rpm for 20 minutes. The supernatant was discarded and the sedimented bacteria resuspended in fresh nutrient broth (Lund and Lyon, 1975).

The isolation of the possible antiplasmodial active fraction and compound was guided by antibacterial testing using direct bioautography on TLC plates. This is because the bioassay is simpler and quicker to perform than an antiplasmodial bioassay and most compounds showing antibacterial activity also show antiplasmodial activity (Boonphong, 2007; Zdzislawa, 2007). The extract or fraction (~5 µl) was applied to silica gel 60 plates (Merck), developed in 3-10% methanol in chloroform and observed under ultraviolet light (254 and 366 nm). The developed TLC plate was then left to dry overnight (for the solvent to evaporate completely). It was then sprayed with bacterial suspension until it appeared translucent and incubated at 25°C for 24 hours in humid conditions. The plates were then sprayed with an aqueous solution of 2.0 mg/ml p-iodonitrotetrazolium violet (Sigma) and reincubated at

25°C for 3 hours. Any inhibition of bacterial growth could be clearly seen as white spots on a red background (Begue and Kline, 1972; Eloff, 1998).

2.2.2.3 Isolation and identification of compounds

The total ethanol extract (80 g) was applied (Fig. 2.1) to a flash silica gel column (10 x 50 cm). The column was developed with a solvent gradient of hexane : ethyl acetate, in order of increasing polarity (100:0 to 0:100) and the fractions were collected. Similar fractions were pooled together according to the TLC profile to yield 4 fractions, which was then tested for antibacterial activity. Fractions 2 and 4 showed antibacterial activity.

Fraction 2 (10 g) was applied to a flash silica gel column (3.0 x 50 cm) and eluted with solvent gradient of hexane : ethyl acetate mixture (95:5 to 90:10). Similar fractions obtained were pooled together resulting in six main fractions (2.1-2.6) while 2.4 to 2.6 showed antibacterial activity.

Fraction 2.5 (2.4 g) was applied to a flash silica gel column (2.5 x 30 cm), and eluted with a solvent gradient of hexane : ethyl acetate in a 95:5 to 90:10. Sub-fraction 2.5.13 that showed antibacterial activity was purified further with a Sephadex LH-20 column, using methanol as eluent to yield the pure compound **1** (100 mg).

Fraction 2.6 (2.4 g) was further purified on a silica column. A solvent mixture of hexane : ethyl acetate was applied to the column in a 1:19 to 1:1 ratio. Sub-fraction 2.6.4 was further purified with a Sephadex LH-20 column with methanol as eluent to yield compound **1** (48 mg) and eriodictyol (5 mg).

Fraction 2.4, upon standing overnight, gave a crystalline compound which was washed with hexane : ethyl acetate (1:1) to give compound **2** (150 mg). The remaining part of fraction 2.4 was further purified with a flash silica gel column (2.5 x 30 cm) which was developed with a solvent system of hexane : ethyl acetate in ratio 7:3 (100 ml fractions collected), to yield more of the pure compound **2** (100 mg).

Fraction 4 (5 g) was applied to a flash silica gel column (3.0 x 50 cm) and developed with a solvent gradient of hexane : ethyl acetate in a 95:5 to 90:10. Sub-fraction 4.2 that showed antibacterial was further purified with a Sephadex LH-20 column, which was developed with solvent mixture methanol : chloroform mixture (1:9) to yield tamarixetin (5 mg) and quercetin (10 mg) respectively. Fraction 4.2.1 showed antibacterial activity and was purified further on a silica gel column (2.5 x 30 cm) and eluted with a solvent mixture of methanol : chloroform (1:19). The resulting sub-fraction 4.2.1.4 which still indicated antibacterial activity was then purified further with a Sephadex LH-20 column and eluted with water : ethanol mixture (1:19) to yield the pure compound **3** (50 mg).

2.2.2.4 Structure elucidation

The isolated compounds were identified by their proton-nuclear magnetic resonance (^{1}H-NMR), carbon-nuclear magnetic (^{13}C-NMR), two dimensional NMR, mass spectroscopy (MS), infra red (IR), UV spectra and X-ray crystallography (Fig. 2.5-2.8) (section 2.3). The nuclear magnetic resonance (NMR) data were obtained at 300 MHz on a Bruker ARX 300 NMR spectrometer using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal standard.

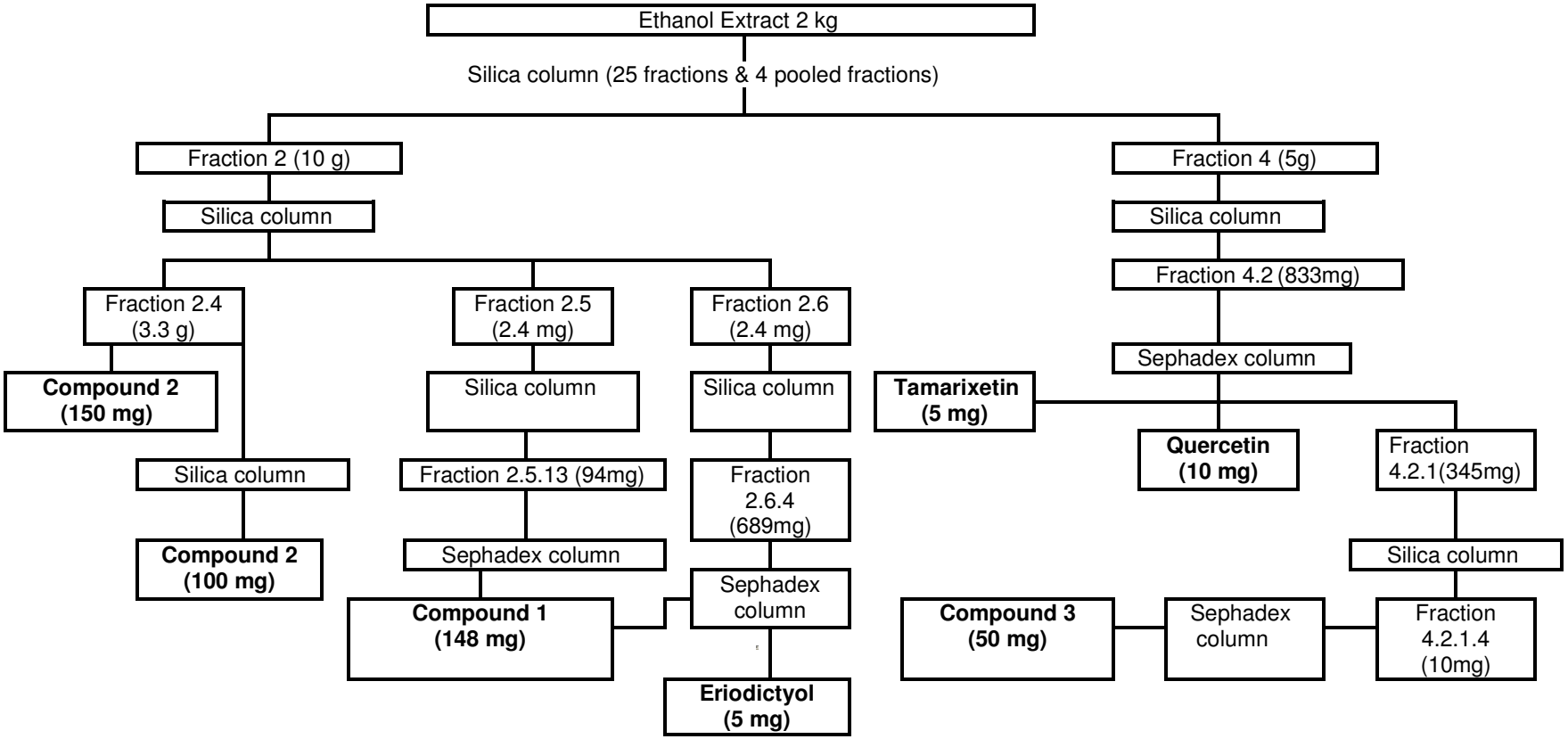
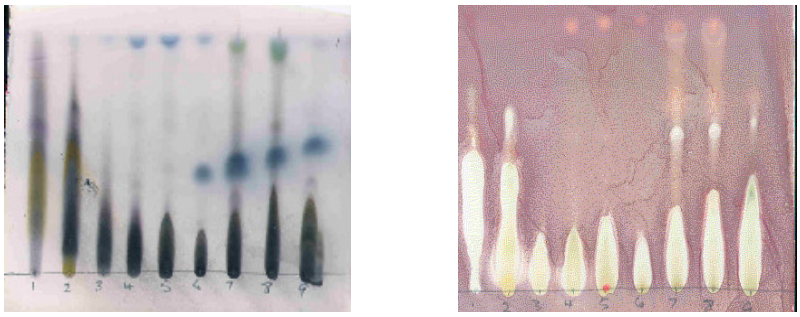


Figure 2.1: Schematic representation of the bioassay guided isolation of active compounds from *C. steenkampianus.* Only fractions with antibacterial activity are shown.

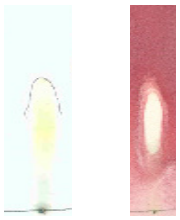
2.3 Results and Discussion

Fractions with antibacterial activity have been reported to show antiplasmodial activity (Prozesky, 2004; Boonphong *et al.,* 2007; Zdzislawa, 2007). Therefore, antibacterial-guided fractionation was used to monitor and direct the isolation of compounds with possible antiplasmodial activity from the crude ethanol extracts of the leaves of *C. steenkampianus*. Pooled fractions from the columns were then collected and tested against *Bacillus cereus*. A typical result of this method is shown in Fig. 2.2 and 2.3. This method has led to the isolation of six compounds: two diterpenes with new skeletons, three flavonoids and one indane (Fig. 2.4). Compounds showed moderate to good antibacterial activity. The antibacterial activity observed in fractions (Fig. 2.2b) is more pronounced than those of pure compounds (Fig. 2.3). This could be due to other compounds not isolated having synergistic effects in the fractions.



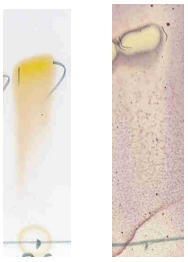
(a) **(b)**

Figure 2.2: Typical results obtained from the pooled fractions from the silica column tested for antibacterial activity. Both TLC plates were developed with chloroform : methanol (9:1). The white zones on TLC plate (b) indicate antibacterial activity.



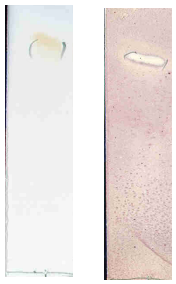
(1a) (1b)

Purified fraction of the indane.



(2a) (2b)

Purified fraction of quercetin.



(3a) (3b)

Purified fraction of tamarixetin.

Figure 2.3: TLC plates showing antibacterial activity of pure compounds. The TLC for the purified fractions were developed in solvent mixture: hexane : ethyl acetate (1:1) for indane, chloroform : methanol (9:1) for quercetin and tamarixetin. a) sprayed with vanillin reagent. b) the white zone indicates antibacterial activity.

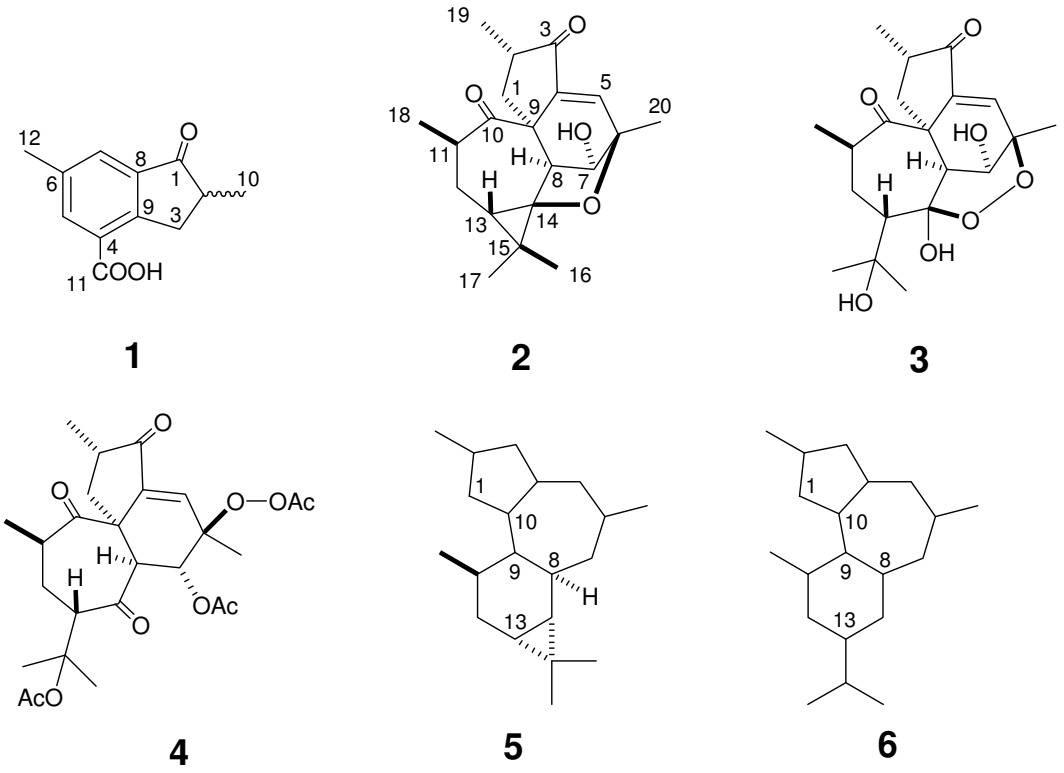


Figure 2.4: Structures of isolated compounds.

2,6-Dimethyl-1-oxo-4-indanecarboxylic acid (**1**) was isolated as a amorphous white solid, it showed in UV (MeOH) spectra absorptions at λ_{max} 215, 248, 310 nm, the IR (KBr) showed absorption peaks at $_{max}$ 3400-2300, 2924, 1709, 1580, 1459, 1423, 1250, 1182, 1133, 927, 836, 693 cm^{-1}. Low-resolution mass spectrometry and combustion analysis indicated a molecular formula $C_{12}H_{12}O_3$ for **1** and its NMR spectroscopic data (Table 2.1) were in agreement with a structure of 2,6-dimethyl-1-oxo-4-indanecarboxylic acid. In particular, the HMBC (Fig. 2.13) correlations observed for **1** (C-1 with H-2, H_2-3, H-7, and Me-10; C-12 with H-5 and H-7; C-9 with H-2, H_2-3, H-5 and H-7; C-11 with H-5, and C-12 with H-5 and H-7) established a 1-indanone skeleton and a 2,6-dimethyl-4-carboxyl substitution pattern for this new substance. Since **1** was devoid of optical

rotation between 589 and 365 nm, it was evident that the isolated compound is a racemic mixture of the two enantiomers at the C-2 asymmetric center, which can be easily racemized through the enolic form of the 1-indanone.

Table 2.1: NMR spectroscopic data [400 (^{1}H) and 100 (^{13}C) MHz, $CDCl_3$] for compound **1**.[a]

Position	δ_C, mult.	δ_H (*J* in Hz)	HMBC[b]
1	209.1, qC	-	-
2	42.0, CH	2.72, ddq (7.9, 7.0, 3.7)	1, 3, 8, 9, 10
3	36.1, CH_2	3.77, dd (18.6, 7.9)	1, 2, 4, 8, 9, 10
		3.02, dd (18.6, 3.7)	1, 2, 4, 8, 9, 10
4	127.1, qC	-	-
5	138.0, CH	8.18, d (2.0)	4, 6, 7, 9, 11, 12
6	138.1, qC	-	-
7	129.4, CH	7.79, d (2.0)	1, 5, 6, 8, 9, 12
8	137.9, qC	-	-
9	153.1, qC	-	-
10	16.2, CH_3	1.31, d (7.0)	1, 2, 3
11	171.3, qC	8.60, br[c]	[d]
12	20.9, CH_3	2.45, s	5, 6, 7

[a]These assignments were also in agreement with the COSY and HSQC spectra. [b]HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon. [c]Carboxylic proton. [d]Not observed (Fig. 2.9-2.14).

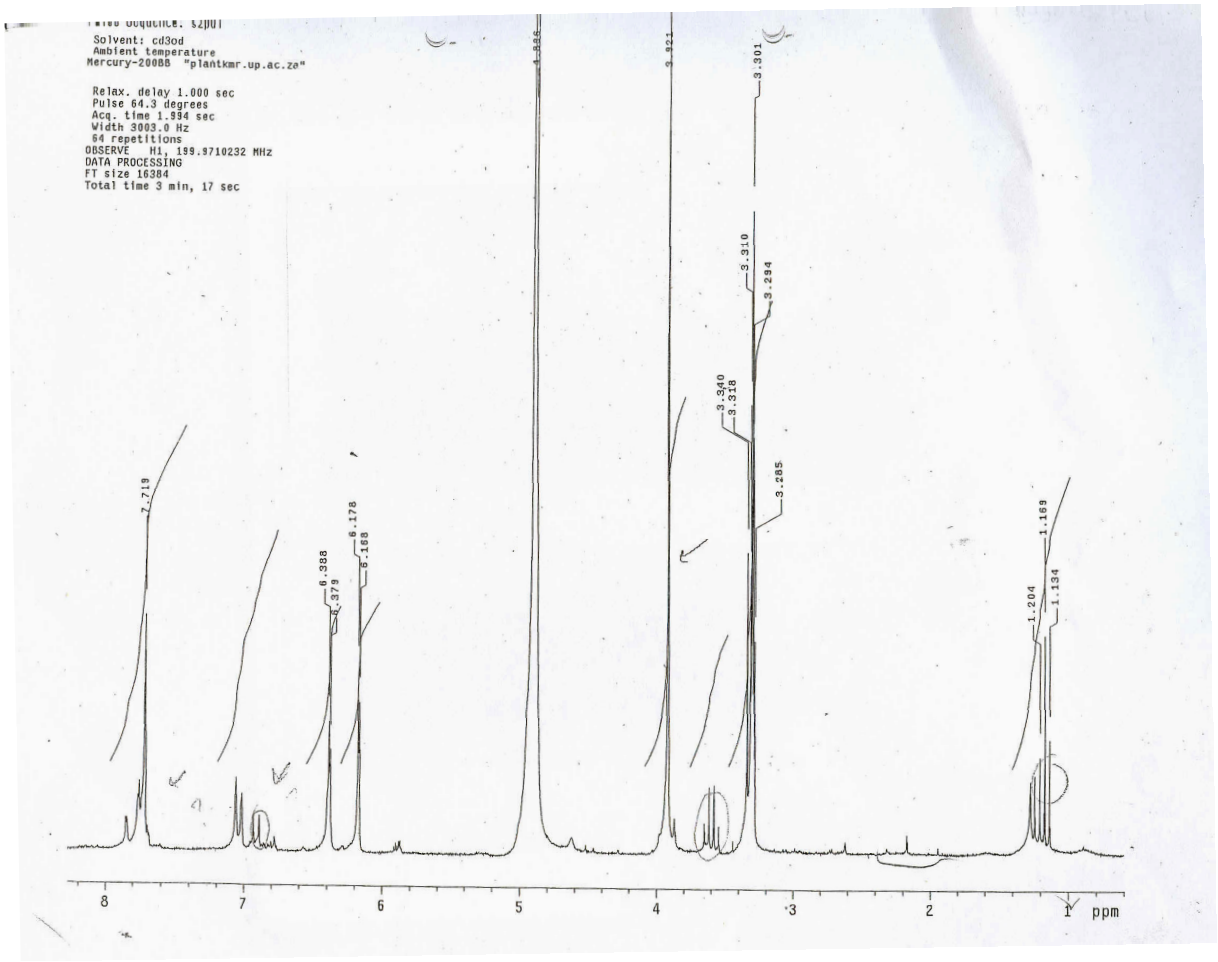


Figure 2.5: ^{1}H-NMR spectrum of tamarixetin.

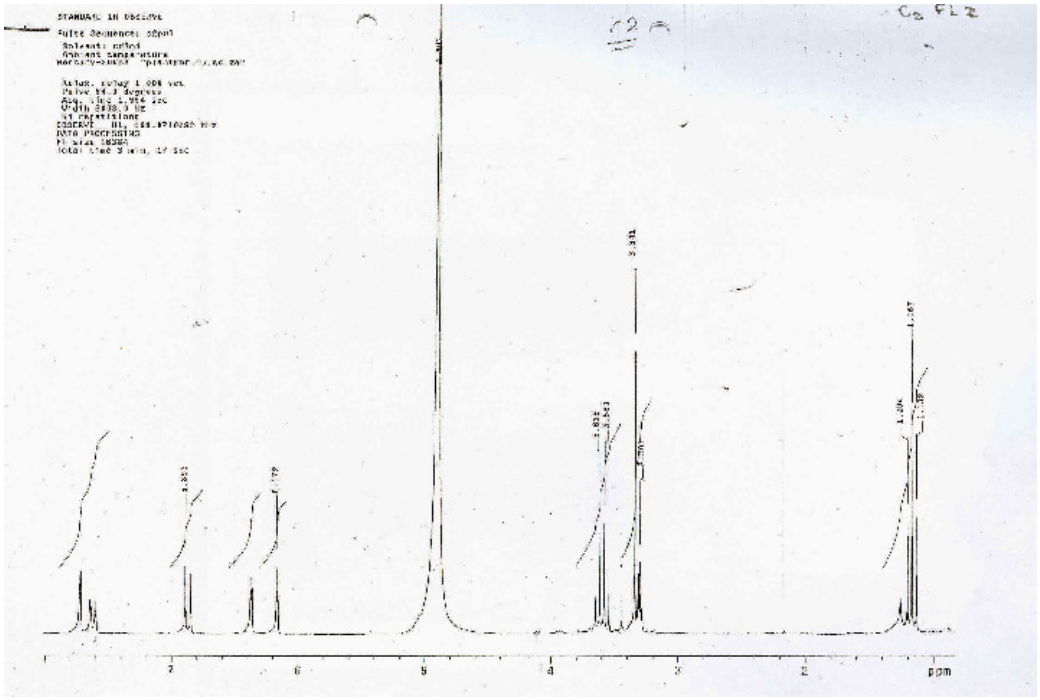


Figure 2.6: ^{1}H-NMR spectrum of quercetin.

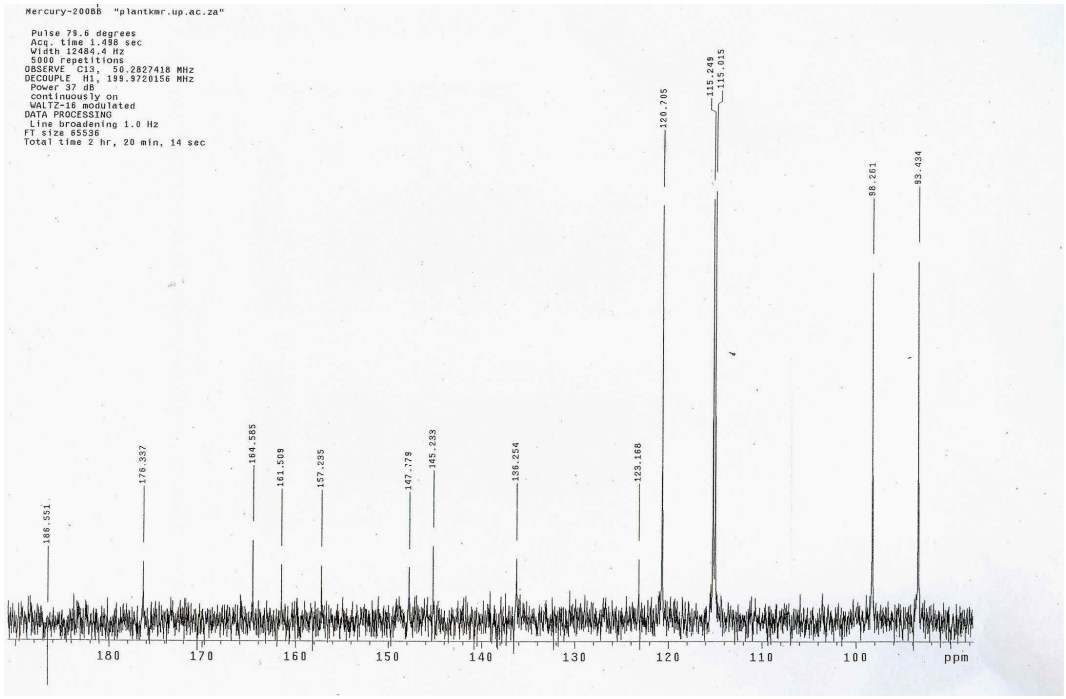


Figure 2.7: ^{13}C-NMR spectrum of quercetin.

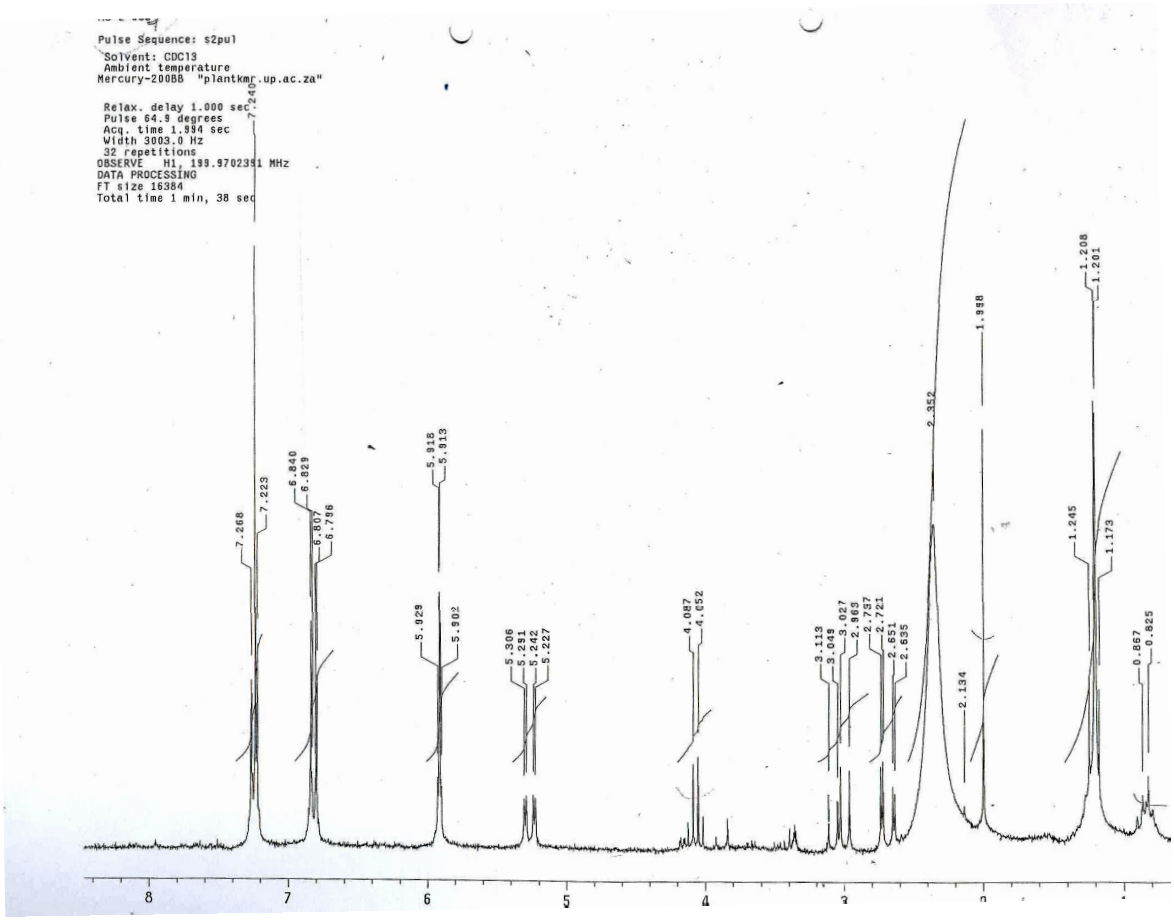


Figure 2.8: ^{1}H-NMR spectrum of eriodictyol.

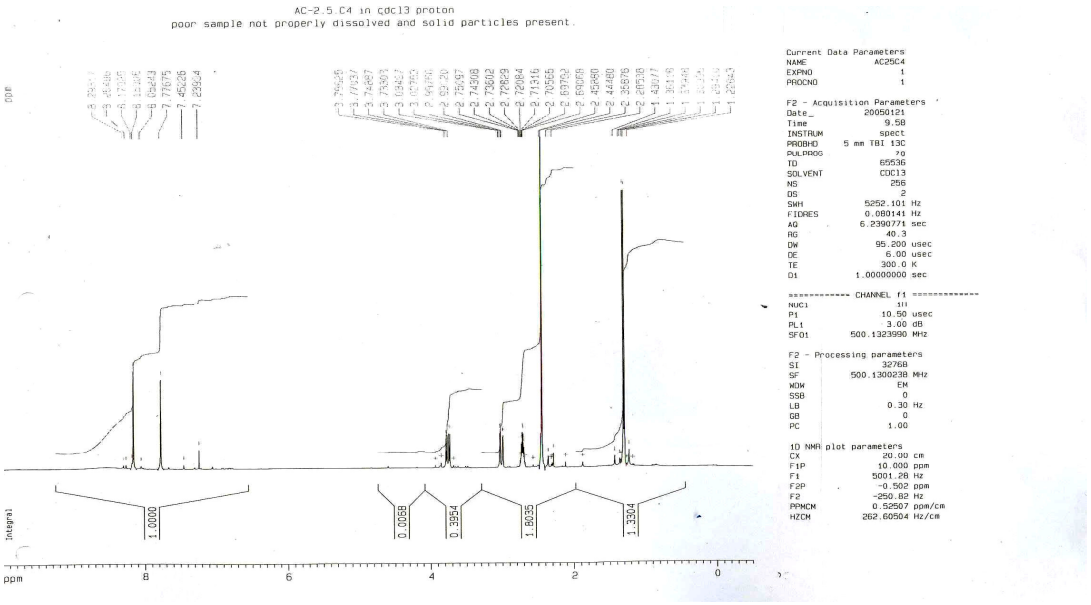


Figure 2.9: ^{1}H-NMR spectrum of indane.

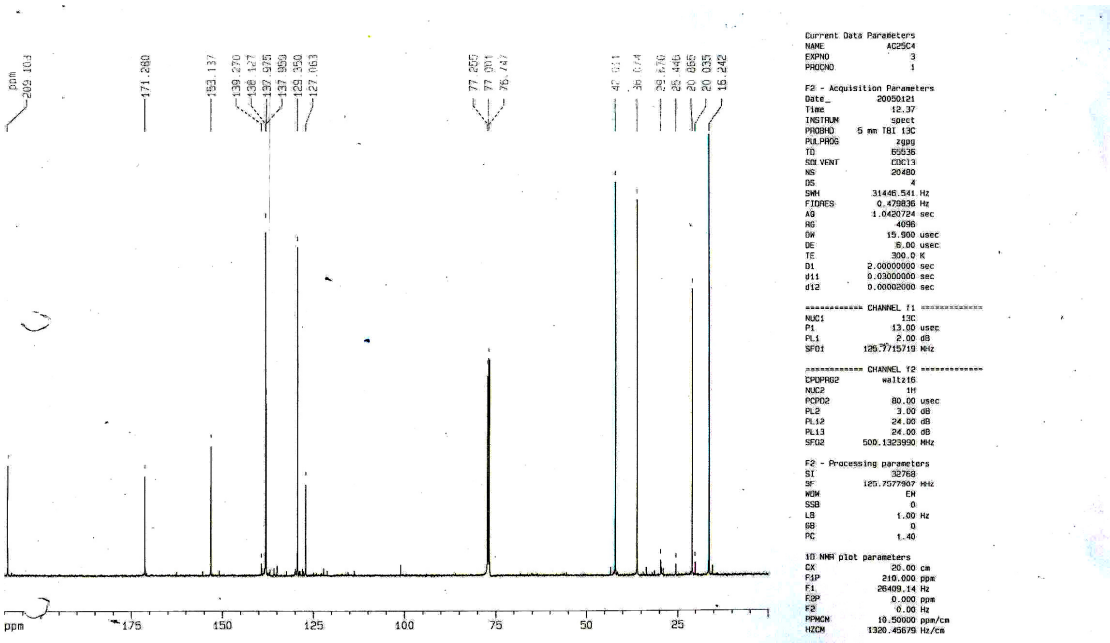


Figure 2.10: ^{13}C-NMR spectrum of indane.

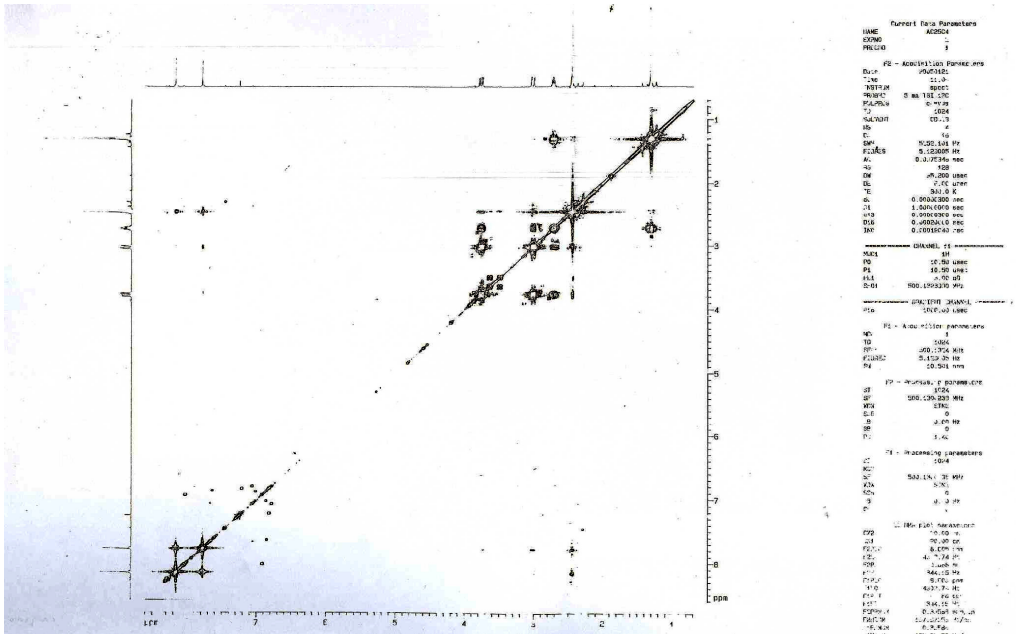


Figure 2.11: COSY spectrum of indane.

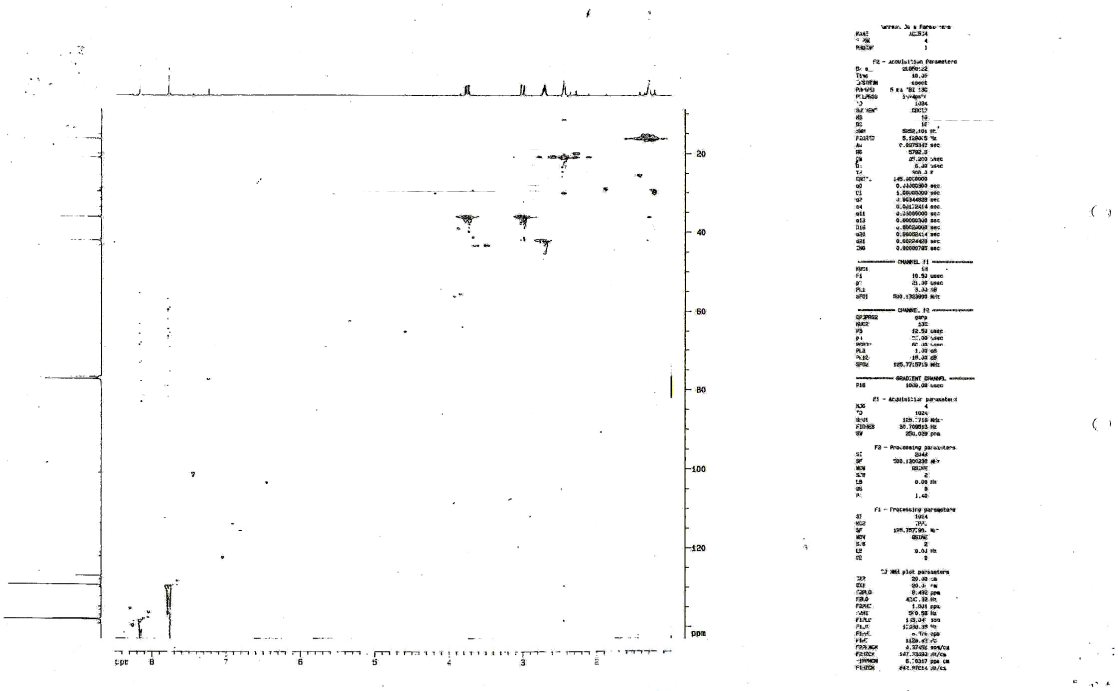


Figure 2.12: HMQC spectrum of indane.

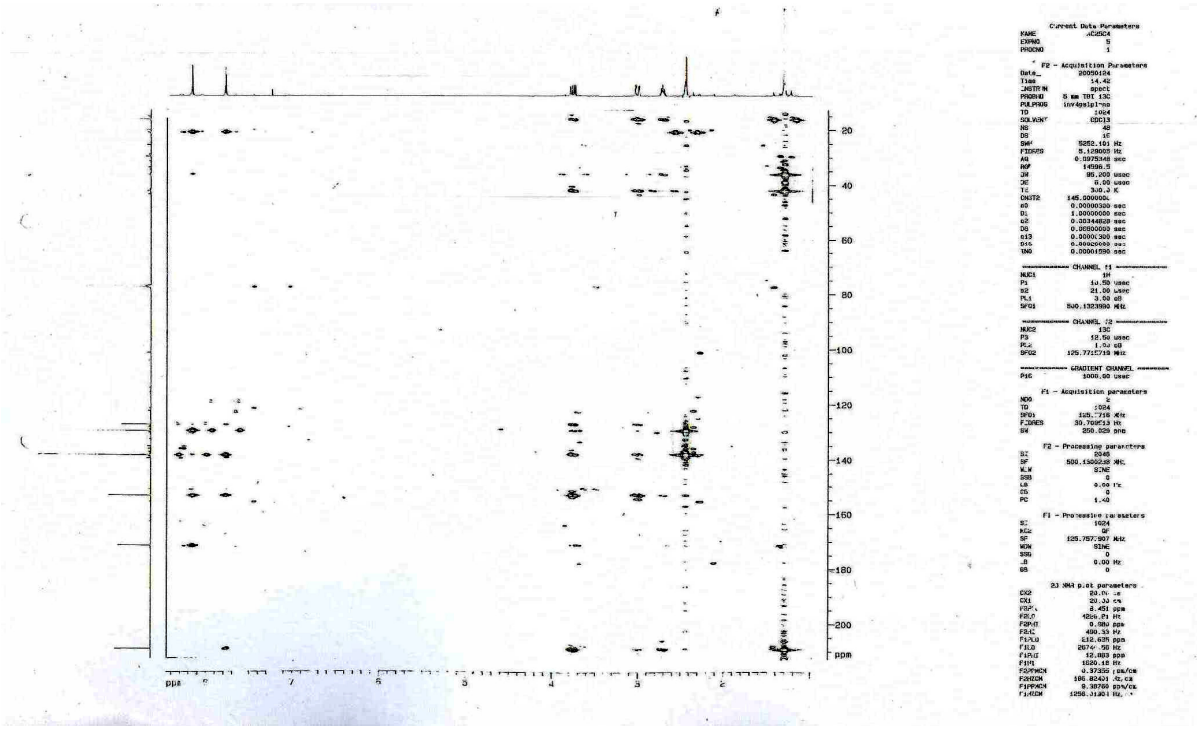


Figure 2.13: HMBC spectrum of indane.

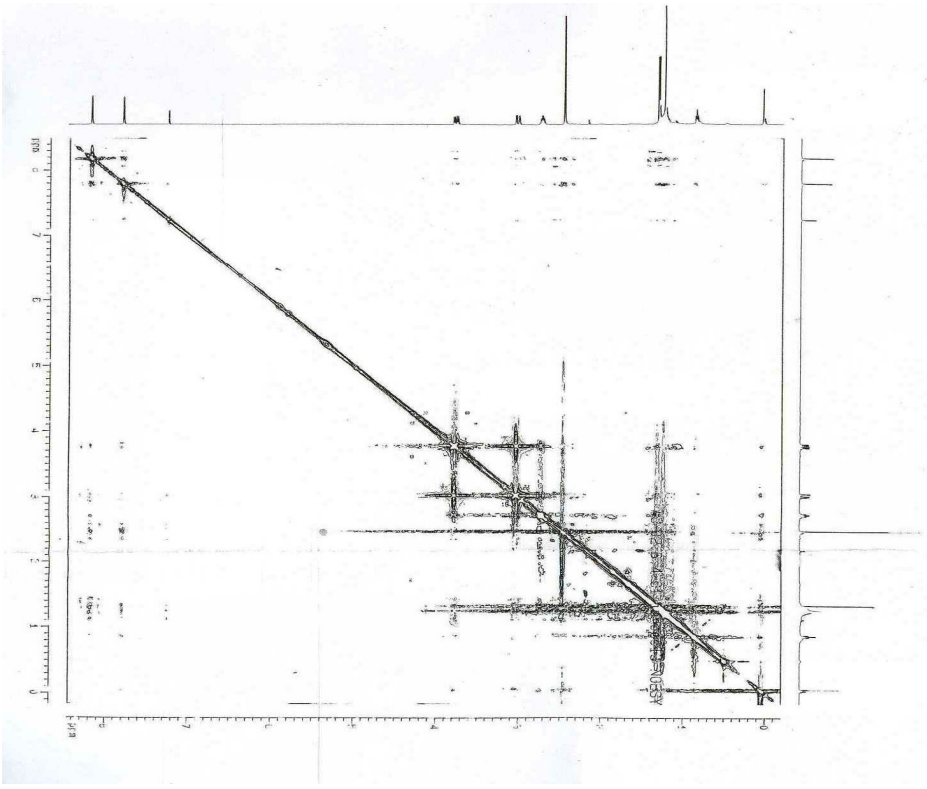


Figure 2.14: NOESY spectrum of indane.

Steenkrotin A (2): colourless needles (EtOAc-petroleum ether), mp 219-221 ºC; $[\alpha]_D^{20}$ +188.6 (*c* 0.324, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 227 (3.96) 250 shoulder (3.68) nm; IR (KBr) ν_{max} 3359, 3050, 3020, 2971, 2940, 1702, 1649, 1454, 1375, 1286, 1231, 1112, 1055, 1019, 905, 743, 650 cm^{-1}; ^1H and ^{13}C NMR: see Table 2.2; EIMS *m/z* 330 $[M]^+$ (3)(Fig. 2.21), 315 (0.5), 312 (2), 297 (0.5), 262 (60), 244 (27), 227 (14), 187 (18), 177 (62), 161 (30), 160 (26), 145 (16), 121 (29), 97 (35), 91 (14), 83 (13), 77 (14), 69 (100), 55 (29); *anal.* C 72.43%, H 8.25%, calcd for $C_{20}H_{26}O_4$, C 72.70%, H 7.93%.

The ^1H and ^{13}C NMR data (Fig. 2.16 and 2.17, Table 2.2) of steenkrotin A indicated the presence of five methyl groups 1.42s (δ_c 21.7), 1.01s (δ_c 20.6), 1.06s (δ_c 16.9), 1.03, d (*J*=3.1 δ_c 16.4) and 1.05d (*J*=3.1 δ_c 14.8), two ketonic group at δ_c 205.1s, 212.7s, one olefinic proton at δ_H 6.55s (δ_c 134.3), two methylene group at δ_H 1.96dd, 2.19dd (δ_c 35.9t), 1.66ddd, 1.84dd (δ_c 29.6), protons at 4.21 d (*J*=3.7, δ_c 79.3) in addition to 0.68dd (*J*=2.3, 10.0, δ_c 25.4) characteristic of a cyclopropyl group (Table 2.2).

The HMBC cross peaks showed correlation of the methyl group at 1.05 with carbons at δ_c 205.1 and 35.9 and the ethylene protons at δ_H 1.96, 2.19 showed correlations with δ_c 62.5, 205.1, 212.7, and 143.7, and correlations between δ_H 6.55 and δ_C 205.1, 62.5; furthermore, the band at 1424 cm^{-1} in IR indicated the presence of αβ-unsaturated ketone. These data clearly confirm the existence of the partial structure (Fig. 2.15).

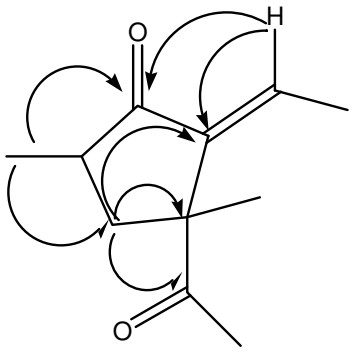


Figure 2.15: HMBC correlation of partial structure.

The other structural parts were confirmed from the other HMBC correlations as follows: the methyl group at 1.03 showed correlations with carbons at δ_c 62.5, 212.7, 29.6 in addition to the correlations δ_H 0.68/ δ_c 74.7, 39.8, 20.6, 21.7; δ_H 6.55/ δ_c 134.9, 75.1, 63.3, 21.7. All the foregoing information in addition to the other data of HMBC, DEPT 135, COSY 45, and HMQC (Fig. 2.18 and 2.20) confirm the plane structure of **2**.

The NOESY correlations of the compound (Fig. 2.19) showed correlations between methyl-20, H-8, H-6, H-5, H-11, H-3 (δ_H 2.19) with H-12 (δ_H 1.66); H-13/H-12, and finally between H-3 (δ_H 1.96)/H-2. These data indicate that the groups; methyl-20, H-8, H-6, H-5, H-11, H-3 (δ_H 2.19) and H-12 (δ_H 1.66) are on one side and the groups H-13, H-12, H-3 (δ_H 1.96) and H-2 are on the other side.

Finally the structure of steenkrotin A (**2**, $C_{20}H_{26}O_4$) was confirmed from X-ray diffraction studies. Figure 2.22 is a perspective view of the molecule of **2** showing its relative configuration. Moreover, the 1D and 2D NMR spectroscopic data of **2** (Tables 2.2 and 2.3) were in complete agreement with the proposed structure.

Table 2.2: NMR spectroscopic data [400 (^{1}H) and 100 (^{13}C) MHz, $CDCl_3$] for Compounds **2** – 4.[a]

		2			**3**			**4**	
position	δ_C,mult.	δ_H (*J* in Hz)	HMBC[b]	δ_C,mult.	δ_H (*J* in Hz)	HMBC[b]	δ_C,mult.	δ_H (*J* in Hz)	HMBC[b]
1[c]	35.9, CH_2	1.98, dd (12.5, 11.4)	2, 8, 9, 10, 19	39.5, CH_2	2.13, dd (13.1, 12.5)	2, 3, 8, 9, 10, 19	38.8, CH_2	1.72, dd (12.8, 11.3)	2, 8, 9, 10, 19
		2.20, dd (12.5, 8.4)	2, 3, 9, 10, 19		2.63, dd (13.1, 7.2)	2, 3, 4, 9, 10		2.15, dd (12.8, 8.4)	2, 3, 4, 8, 9, 10
2	39.73, CH	2.31, ddq (11.4, 8.4, 7.0)	1, 3, 4, 9, 19	39.2, CH	2.27, ddq (12.5, 7.2, 7.0)	1, 3, 9, 19	38.7, CH	2.52, ddq (11.3, 8.4, 7.0)	1, 3, 19
3	206.5 qC	-	-	206.5, qC	-	-	204.7, qC	-	-
4	143.7, qC	-	-	144.6, qC	-	-	142.7, qC	-	-
5	134.5, CH	6.50, s	3, 6, 7, 9, 20	129.7, CH	6.36, d (0.8)	3, 6, 7, 9, 20	130.4, CH	6.69, d (1.0)	3, 6, 7, 9, 20
6	75.1, qC	-	-	68.6, qC	-	-	74.9, qC	-	-
7	79.2, CH	4.22, d (3.9)	5, 6, 8, 9, 20	75.6, CH	4.30, dd (2.5, 0.8)	5, 6, 8, 9, 20	71.4, CH	5.60, dd (2.3, 1.0)	5, 6, 8, 9, 14, 20
8	40.8, CH	2.69, d (3.9)	1, 4, 6, 7, 9, 10, 13, 15	36.5, CH	3.05, d (2.5)	1, 9, 10, 13, 14	44.6, CH	4.44, dd (2.3, 0.8)	1, 9, 10, 13, 14
9	62.6, qC	-	-	55.6, qC	-	-	52.8, qC	-	-
10	213.1, qC	-	-	212.4, qC	-	-	211.3, qC	-	-
11	39.73, CH	2.95, ddq (12.5, 6.8, 6.4)	10, 12, 18	44.5, CH	3.08, ddq (7.4, 7.1, 3.4)	9, 10, 12, 13, 18	43.1, CH	3.79, ddq (10.7, 2.4, 6.7)	10, 12, 13, 18
12[c]	29.6, CH_2	1.67, ddd (14.3, 6.8, 2.4)	10, 11, 13, 14, 15, 18	29.4, CH_2	1.93, ddd (15.7, 8.9, 3.4)	10, 11, 13, 14, 15, 18	29.3, CH_2	2.22, ddd (16.3, 4.5, 2.4)	10, 11, 13, 14, 15, 18
		1.83, ddd (14.3, 12.5, 10.1)	11, 13, 15, 18		1.79, ddd (15.7, 7.1, 4.9)	10, 11, 13, 14, 15, 18		2.07, ddd (16.3, 6.7, 4.5)	10, 11, 13, 14, 15, 18
13	25.3, CH	0.68, dd (10.1, 2.4)	11, 12, 14, 15, 16, 17	60.3, CH	2.93, dd (8.9, 4.9)	11, 12, 14, 15, 16, 17	61.2, CH	2.66, td (4.5, 0.8)	8, 11, 12, 14, 15, 16, 17
14	74.1, qC	-	-	110.1, qC	-	-	208.2, qC	-	-
15	22.8, qC	-	-	87.6, qC	-	-	87.4, qC	-	-
16	20.7, CH_3	1.03, s	13, 14, 15, 17	22.3, CH_3[d]	1.45, s[d]	13, 15, 17	24.4, CH_3[d]	1.48, s[d]	13, 15, 17
17	16.5, CH_3	1.07, s	13, 14, 15, 16	25.74, CH_3[d]	1.34, s[d]	13, 15, 16	24.7, CH_3[d]	1.44, s[d]	13, 15, 16
18	17.0, CH_3	1.06, d (6.4)	10, 11, 12	18.0, CH_3	1.27, d (7.4)	10, 11, 12	18.3, CH_3	1.20, d (6.7)	10, 11, 12
19	14.9, CH_3	1.04, d (7.0)	1, 2, 3	14.7, CH_3	1.07, d (7.0)	1, 2, 3	15.6, CH_3	1.06, d (7.0)	1, 2, 3
20	21.8, CH_3	1.43, s	5, 6, 7	25.70, CH_3	1.47, s	5, 6, 7	20.9, CH_3	1.52, s	5, 6, 7
6β-OOAc	-	-	-	-	[e]	-	167.0, qC 17.3, CH_3	2.08, s	[f]
7α-OAc	-	-	-	-	[e]	-	169.9, qC 21.1, CH_3	2.05, s	[f]
15-OAc	-	-	-	-	[e]	-	168.5, qC 21.0, CH_3	1.87, s	[f]

[a]All these assignments were in agreement with COSY and HSQC spectra, and with 1D NOESY experiments. [b]HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon. [c]For methylene groups, the first reported δ value belongs to the α-proton and the second δ value is assigned to the β-proton. [d]Interchangeable assignments. [e]Hydroxyl protons at δ 5.89 br s, 3.56 br, and 2.16 s. [f]HMBC correlations for the OAc groups: δ 167.0, 169.9, and 168.5 with the *Me*COO group at δ 2.08, 2.05, and 1.87, respectively, and δ 169.9 also with H-7.

Table 2.3: Significant NOE Data for Compounds **2** – **4**.

Compound	Irradiated proton (s) (δ)	Observed NOEs
2	H-1α (1.98)	H-1β,[a] H-8α,[b] Me-19[b]
	H-5 (6.50)	Me-20[b]
	H-7β (4.22)	H-8α,[b] Me-16 + Me-17,[c] Me-20[b]
	H-8α (2.69)	H-1α,[c] H-1β,[c] H-7β,[b] H-11α,[a], Me-17[b]
	H-11α (2.95)	H-1β,[b] H-8α,[a] H-12α,[b] H-12β,[c] Me-18[a]
	H-13β (0.68)	H-12α,[c] H-12β,[a] Me-16[b]
	Me-20 (1.43)	H-5,[c] H-7β,[b] Me-16[c]
3	H-1β (2.63)	H-1α,[a] H-2β,[a] Me-18[b]
	H-5 (6.36)	Me-20[b]
	H-7β (4.30)	H-8α,[b] Me-20[b]
	H-13β (2.93)	H-12α,[c] H-12β,[b] Me-18[c]
	Me-18 (1.27)	H-1β,[c] H-11α,[c] H-13β,[c] H-12α + H-12β[c]
	Me-19 (1.07)	H-1α,[b] H-1β,[c], H-2β[b]
4	H-1α (1.72)	H-1β,[a] H-2β,[b] Me-19[b]
	H-2β (2.52)	H-1α,[c] H-1β,[b] Me-19[b]
	H-5 (6.69)	Me-20[a]
	H-7β (5.60)	H-8α,[a] Me-20[a]
	H-8α (4.44)	H-5,[c] H-7β,[a] H-11α,[a] H-12α,[b] Me-16 + Me-17,[b] 15-OAc[c]
	H-11α (3.79)	H-8α,[a] H-12α,[b] H-12β,[c] Me-18,[b] 15-OAc[c]
	H-13β (2.66)	H-12α + H-12β,[a] Me-16 + Me-17,[a] Me-18[b]
	Me-18 (1.20)	H-11α,[c] H-13β,[c] H-12α + H-12β[b]
	Me-19 (1.06)	H-1α,[b] H-1β,[c] H-2β,[b] 7α-OAc[c]

[a]Strong NOE enhancement (>4%). [b]Medium NOE enhancement (1.5 - 4%). [c]Weak NOE enhancement (0.5 – 1.5%).

X-Ray Structure Determination of 2. The crystallographic data set was collected at 20 °C on a Siemens P4 diffractometer fitted with a Bruker 1K CCD detector and SMART control software using graphite-monochromated, Mo-Kα radiation by means of a combination of phi and omega scans. Data reduction was performed using SAINT+ and the intensities were corrected for absorption using SADABS. The structures were solved by direct methods using SHELXTS and refined by full-matrix least squares using SHELXTL and SHELXL-97. All hydrogen atoms for the structure of **2** were located experimentally. In the refinement, the hydrogen atoms were refined without any positional constrains. Isotropic displacement parameters for the non-methyl hydrogen atoms were calculated as 1.2 × U_{eq} of the atom to which they were attached, and the corresponding value for methyl hydrogen atoms was 1.5 × U_{eq} of the carbon atom to which they were attached. All non-hydrogen atoms were refined with anisotropic displacement parameters. Drawings of the structure (Fig. 2.22) were produced using Ortep-3 for Windows (Farruggia, 1997), Mercury and POV-Ray for Windows.

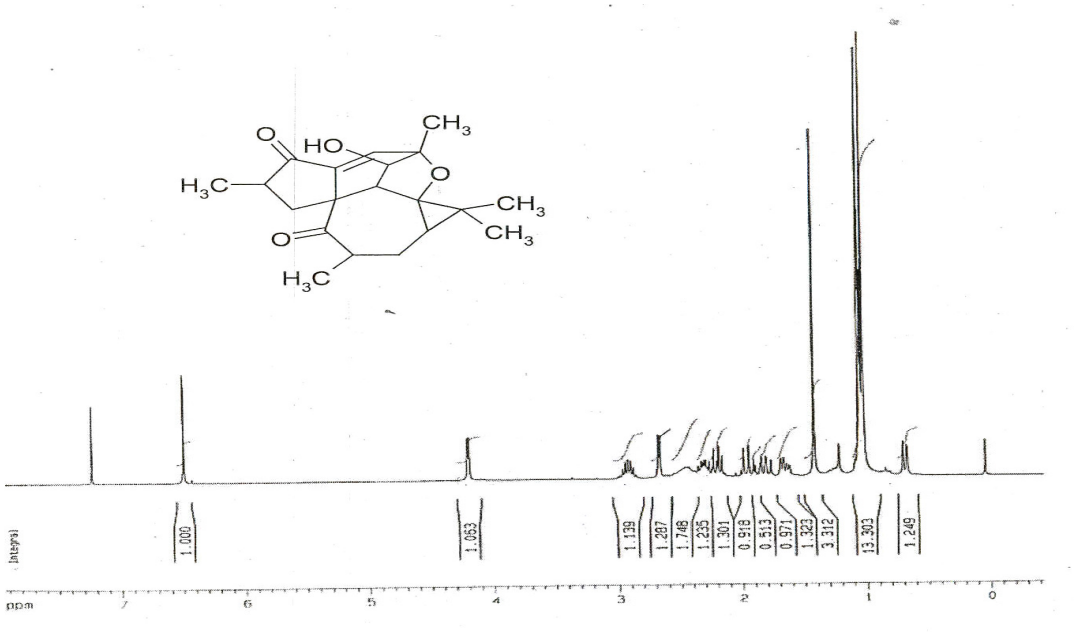


Figure 2.16: [1]H-NMR spectrum of steenkrotin A.

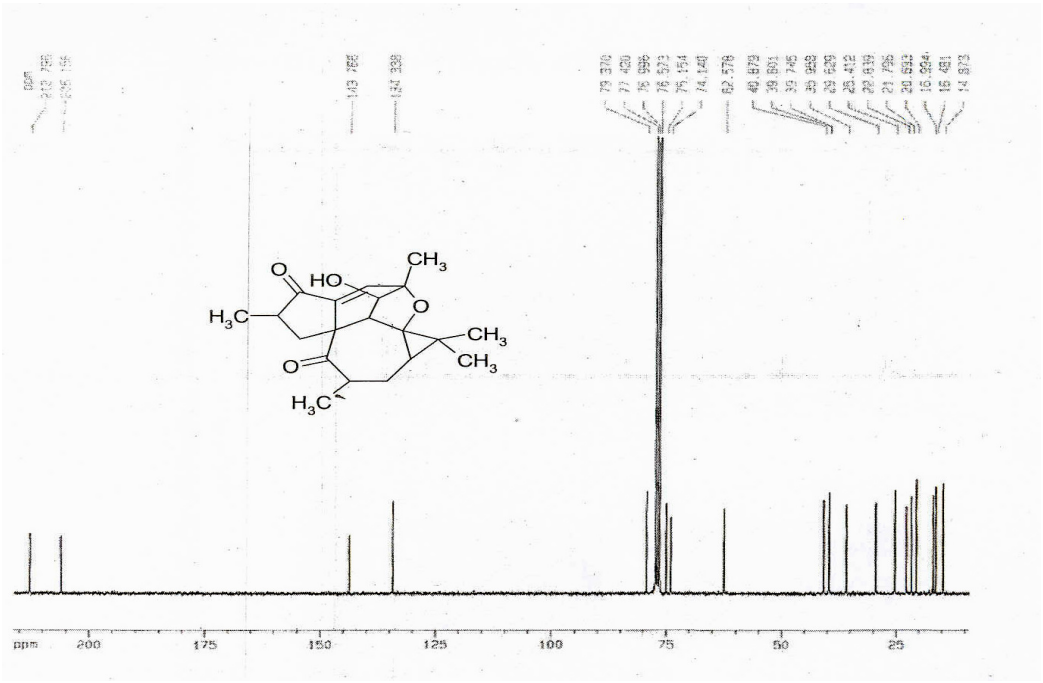


Figure 2.17: ^{13}C-NMR spectrum of steenkrotin A.

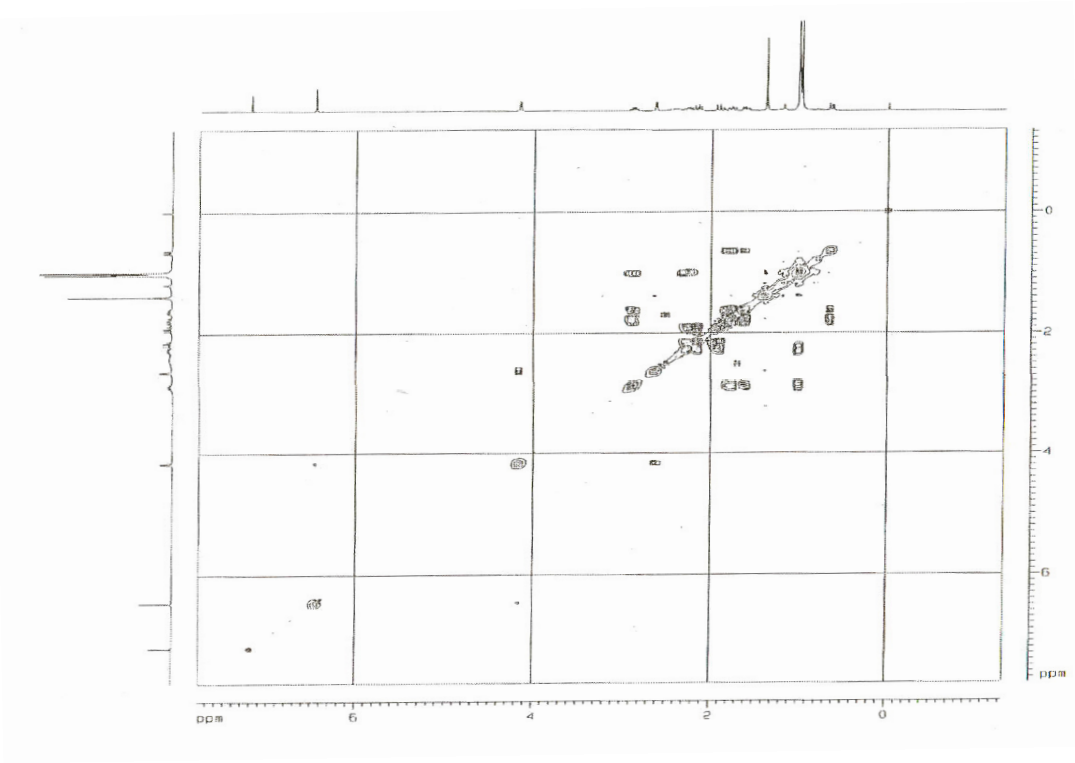


Figure 2.18: COSY spectrum of steenkrotin A.

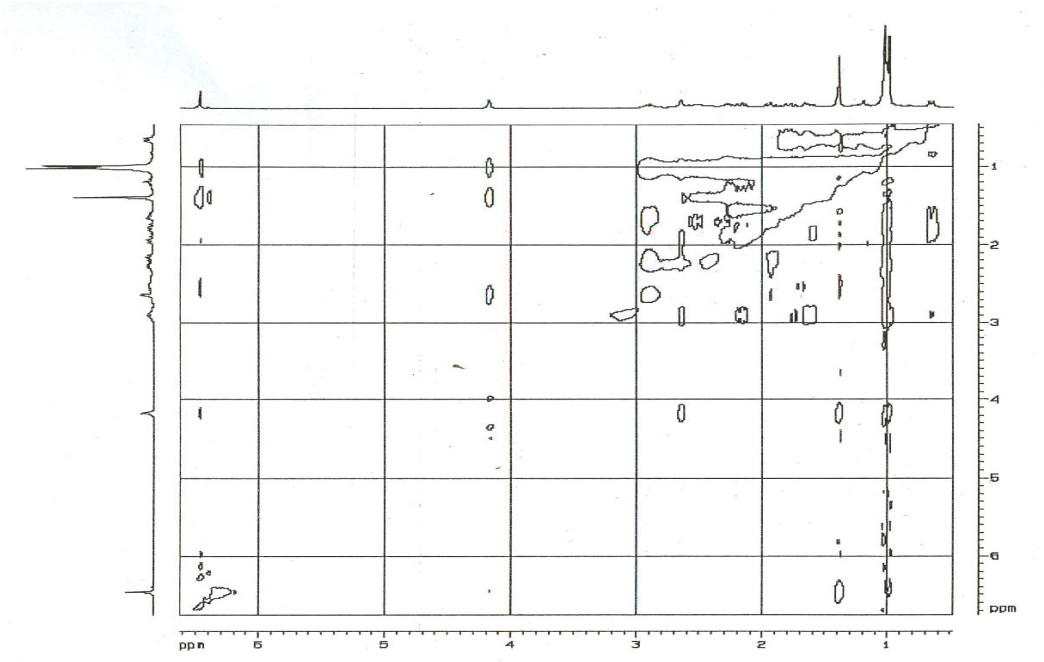


Figure 2.19: NEOSY spectrum of steenkrotin A.

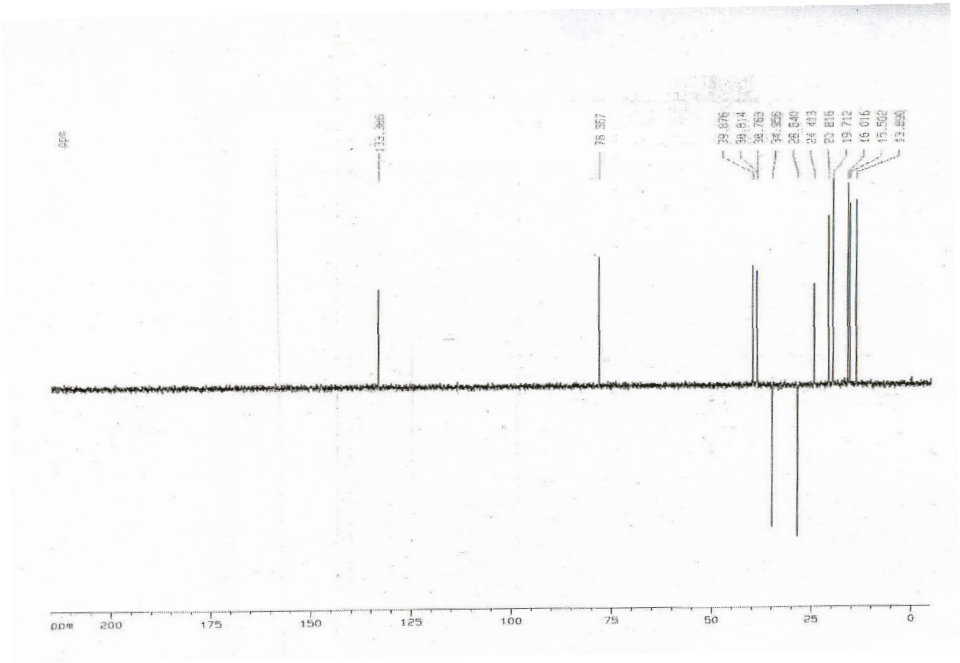


Figure 2.20: Dept 135 spectrum of steenkrotin A.

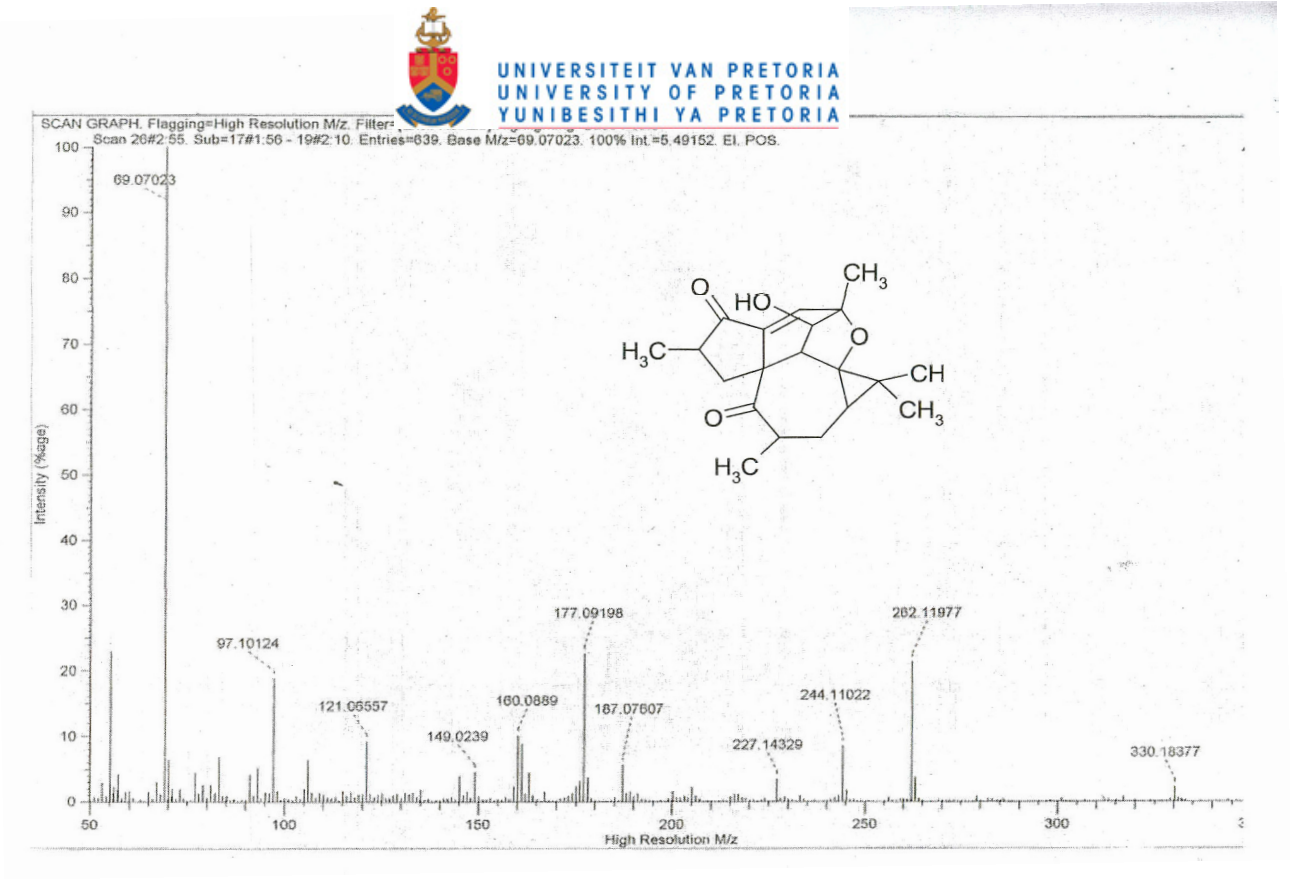


Figure 2.21: MS data of steenkrotin A.

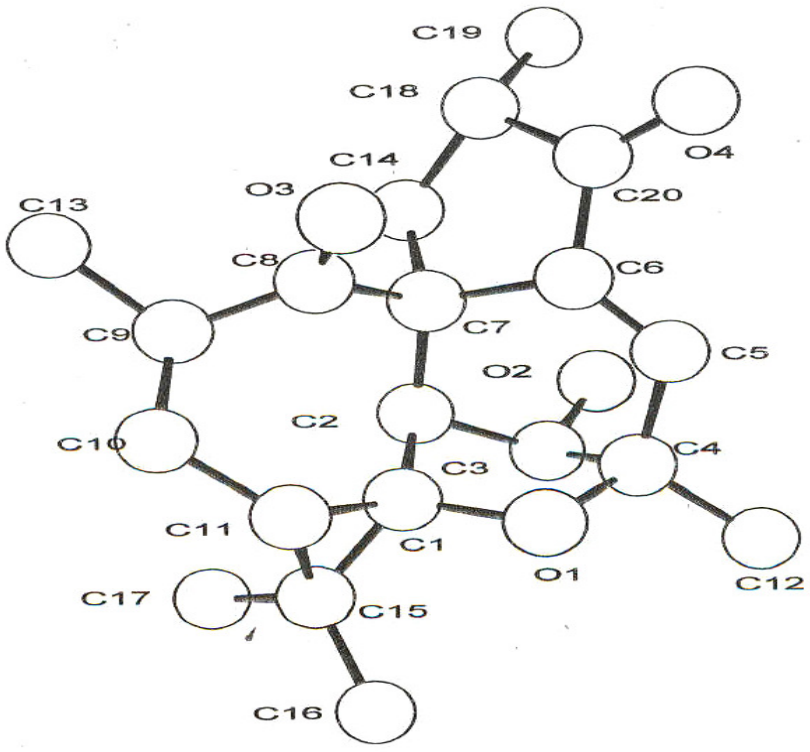


Figure 2.22: X-ray structure of steenkrotin A.

Steenkrotin B (3): colourless fine needles (MeOH), mp 130-132 ºC (decomp.); $[\alpha]_D^{20}$ +24.4 (*c* 0.119, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 233 (3.98) nm; IR (KBr) ν_{max} 3435, 2976, 2932, 1731, 1691, 1676, 1457, 1372, 1194, 1078, 901 cm^{-1}; ^1H and ^{13}C NMR: see Table 2.2; (ESI)MS positive mode *m/z* 403 $[M+Na]^+$, 783 $[2M+Na]^+$; (ESI)MS negative mode *m/z* 379 $[M-H]^-$; EIMS *m/z* 380 $[M]^+$ (0.3), 362 (1), 348 (1), 338 (25), 305 (19), 264 (27), 245 (21), 219 (27), 203 (38), 187 (45), 177 (56), 161 (55), 152 (39), 141 (43), 121 (40), 95 (31), 91 (41), 85 (41), 83 (45), 71 (57), 69 (54), 55 (100); *anal.* C 63.31%, H 7.36%, calcd for $C_{20}H_{28}O_7$, C 63.14%, H 7.42%. Steenkrotin B (**3**, $C_{20}H_{28}O_7$) showed ^1H, ^{13}C, COSY, HSQC, and HMBC NMR spectra (Fig. 2.23-2.28) very similar to those of **2** (Table 2.2). The observed differences were consistent with the presence in **3** of a hydroxyisopropyl group [δ_H 1.45 and 1.34 (Me-16 and Me-17); δ_C 22.3 and 25.7 (C-16 and C-17), and 87.6, qC, C-15] [δ_H 1.45 and 1.34 (Me-16 and Me-17); δ_C 22.3 and 25.7 (C-16 and C-17), and 87.6, qC, C-15] and a hemiacetal carbon (δ 110.1, qC, C-14) instead of the pentasubstituted cyclopropane of **2**. This structural difference was also supported by the HMBC spectra, because **2** showed correlations through three bonds between C-15 and H-8, and between C-14 and Me-16 and Me-17, that were not observed for **3** (Table 2.2). Three of the seven oxygen atoms of **3** were involved in two ketones [δ 206.5 (C-3) and 212.4 (C-10)] and in a secondary hydroxyl group [δ_H 4.30; δ_C 75.6, CH (C-7)] like in **2**, and two other oxygens must be placed at C-14 and C-15 as one of the hemiacetal oxygens and as a tertiary hydroxyl group, respectively (see above). The remaining two oxygens of **3** must be part of an endoperoxide moiety between C-6 (δ 68.6, qC) and the hemiacetalic C-14 carbon. The presence of this endoperoxide was also in agreement with molecular formula requirements (7 unsaturations) and with the observed loss of O_2 (ion at *m/z* 348) from the molecular ion (*m/z* 380) in the EI mass spectrum of **3** (Perales, 1983).

Treatment of **3** with acetic anhydride-pyridine yielded the triacetyl derivative **4** ($C_{26}H_{34}O_{10}$), the IR spectrum of which was devoid of hydroxyl absorptions. In the ^{13}C NMR spectrum of **4**, the methyl carbon of one of the acetates (δ_C 167.0, qC and 17.3, CH_3; δ_H 2.08) appeared unusually shifted ((δ_C 17.3) indicating the presence of a peroxyacetate function (Olah *et al.,*1976; Baj and Chrobok*,* 2000), which is also in agreement with molecular formula requirements. Thus, apart from the esterification of the hydroxyl groups at C-7 and C-15, the acetylation of **3** caused the hydrolysis of the 14-hemiacetal and subsequent acetylation of the resulting 6-hydroperoxide, as was

also evidenced by the appearance of an additional ketone at C-14 ((δ_C 208.2, HMBC correlated with H-7, H-8, H_2-12, and H-13, Table 2.2) in the ^{13}C NMR spectrum of **4**.

The relative stereochemistry of **3** and **4** must be identical to that of **2** for the following reasons: NOE experiments (Table 2.3) established that H-8 and H-11 are in a *cis* spatial relationship in **2** and **4**, because a strong NOE enhancement was observed in H-11 when H-8 was irradiated and vice versa. Consequently, Me-18 must be *trans* (β-oriented) with respect to H-8. Moreover, the NOE observed between H-13 and Me-18 in the 1D NOESY spectra of **2** – **4** was in agreement with a β-orientation of H-13. In the case of **2**, a *cis* arrangement between H-8α and the 1-methylene protons was supported by weak NOE enhancements, but no NOEs between these protons were observed for **3** and **4**. However, the similar ^{1}H NMR chemical shifts and the almost identical coupling constant values for H_2-1, H-2, and Me-19 in **2** – **4** (Table 2.2), together with the absence of NOE between Me-18 and Me-19, support an α-orientation for the 1-methylene and Me-19 groups in **3** and **4**, as it was established for **2** by an X-ray analysis. In addition, one of the H_2-1 protons of **3** (δ2.63) showed NOE with the close Me-18 group, and this proton must be the β-oriented one, which in turn displayed a strong NOE with H-2 and no NOE with Me-19, thus establishing an α-orientation for Me-19. This conclusion was also supported by the 1D NOESY behavior of **4**, that showed a weak NOE in H-2 (+1%) and a medium NOE in Me-19 (+3.4%) when H-1α (δ 1.72) was irradiated. For structural requirements, the α-orientation of H-8 in **2** forces to a 6β,14β closure of its cyclic ether, and **3** must also have the same stereochemistry for its endoperoxide, because steenkrotin B (**3**) also has its H-8 proton α-oriented (see above). Consequently, the 6-peroxyacetate of **4** must be β-oriented.

The cyclohexene ring of **3** and **4** adopts an envelope conformation with the flap at C-7, like in **2** (Fig. 2.4), and H-7β and 7α-OH (or 7α-OAc in **4**) are equatorial and axial substituents, respectively, thus explaining the similar $J_{7\beta,8\alpha}$values (Table 2.2) and the NOE behaviour of these compounds (Table 2.3). NOE experiments also allowed the assignment of the Me-16 and Me-17 groups in **2**, as it is shown in the formula and Table 2.2. Irradiation at δ 1.43 (Me-20) caused NOE enhancement only in one of the two C-15 methyls (δ 1.03, Me-16), whereas the signal of the other one (δ 1.07, Me-17) was enhanced when H-8α was irradiated. The assignment of the configuration for both

12-methylene protons in **2** – **4** (Table 2.2) was also supported by NOE results (Table 2.3).

To the best of our knowledge, diterpenoids **2** and **3** possess new carbon skeleta that may be derived from the tigliane (**5**) and daphnane (**6**) types, respectively, by an 8(9→10) *abeo* rearrangement. It is of interest that diterpenes belonging to **5** and **6** carbon frameworks have repeatedly been found in several *Croton* species and in other Euphorbiaceae genera (Connolly and Hill, 1991).

Transformation of Steenkrotin B (3) into Compound 4. Treatment of **3** (15.0 mg) with Ac_2O-pyridine (1:1, 4.0 ml) for 24 h at room temperature, followed with evaporation of the volatiles at 50 ºC under reduced pressure, yielded a residue which was chromatographed on a silica gel column using 10% ethyl acetate in hexane to give **4** (8 mg, 53.0 % yield): amorphous white solid; $[\alpha]_D$ (Farruggia, 1997) –99.3 (*c* 0.293, $CHCl_3$); IR (KBr) ν_{max} 2929, 1745, 1736, 1706, 1694, 1457, 1369, 1225, 1102, 1076, 1039, 945, 922, 849 cm^{-1}; ^1H and ^{13}C NMR: see Table 2.2; (ESI) MS positive mode *m/z* 529 $[M+Na]^+$, 1035 $[2M+Na]^+$; *anal.* C 61.83%, H 6.80%, calcd for $C_{26}H_{34}O_{10}$, C 61.65%, H 6.77% (Fig. 2.29-2.34).

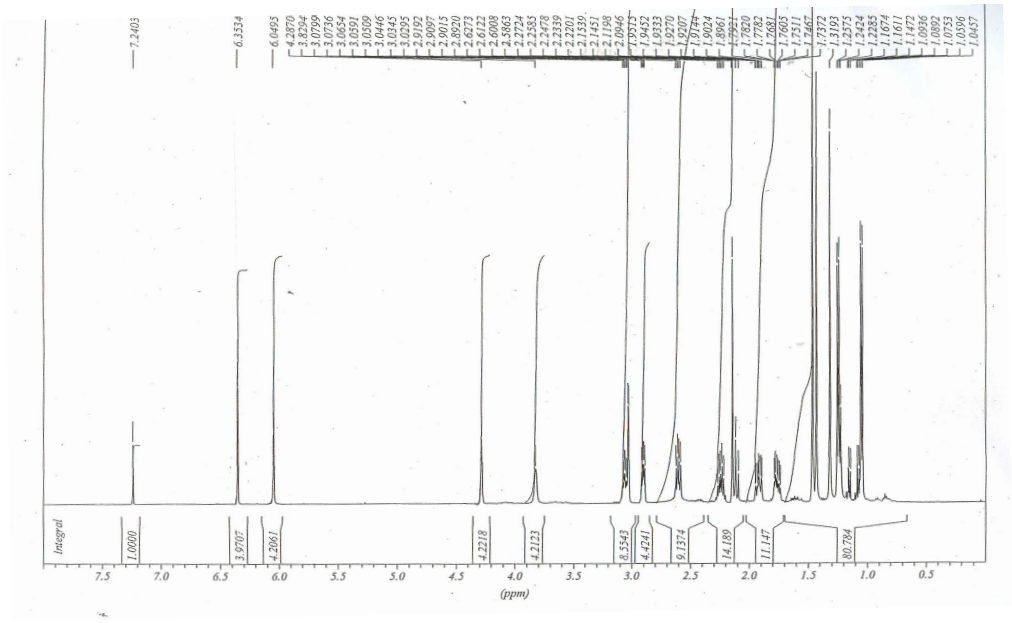


Figure 2.23: ^1H-NMR spectrum of steenkrotin B.

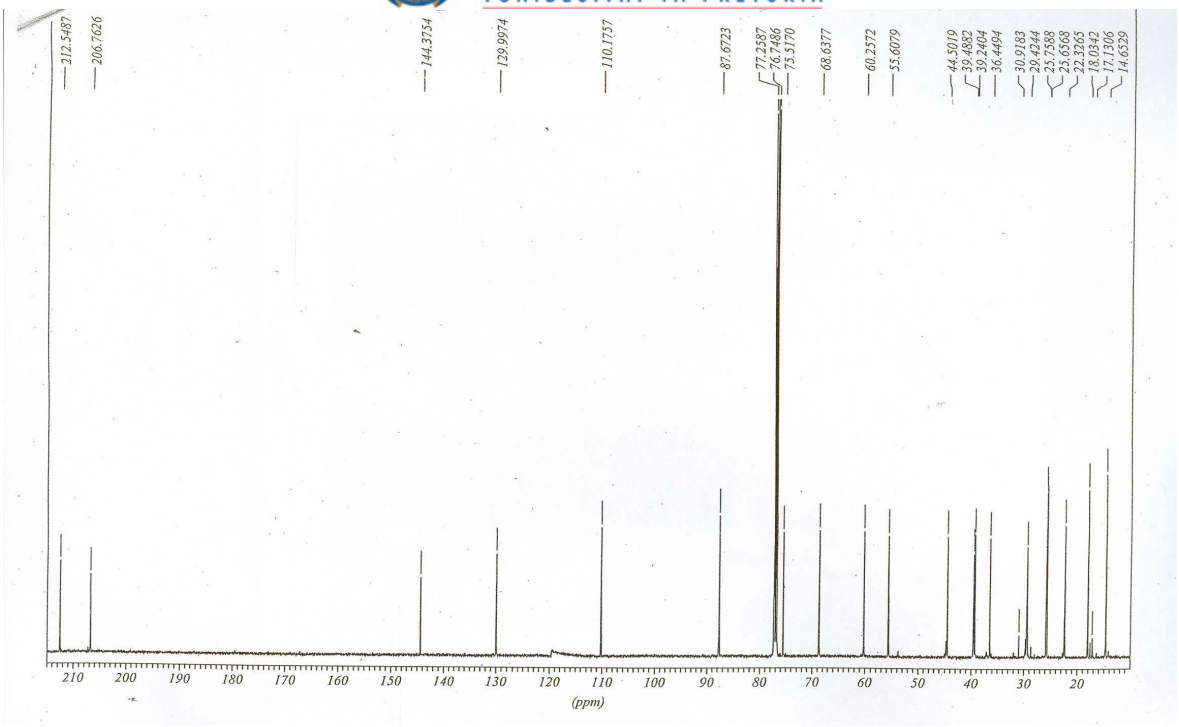


Figure 2.24: ^{13}C-NMR spectrum of steenkrotin B.

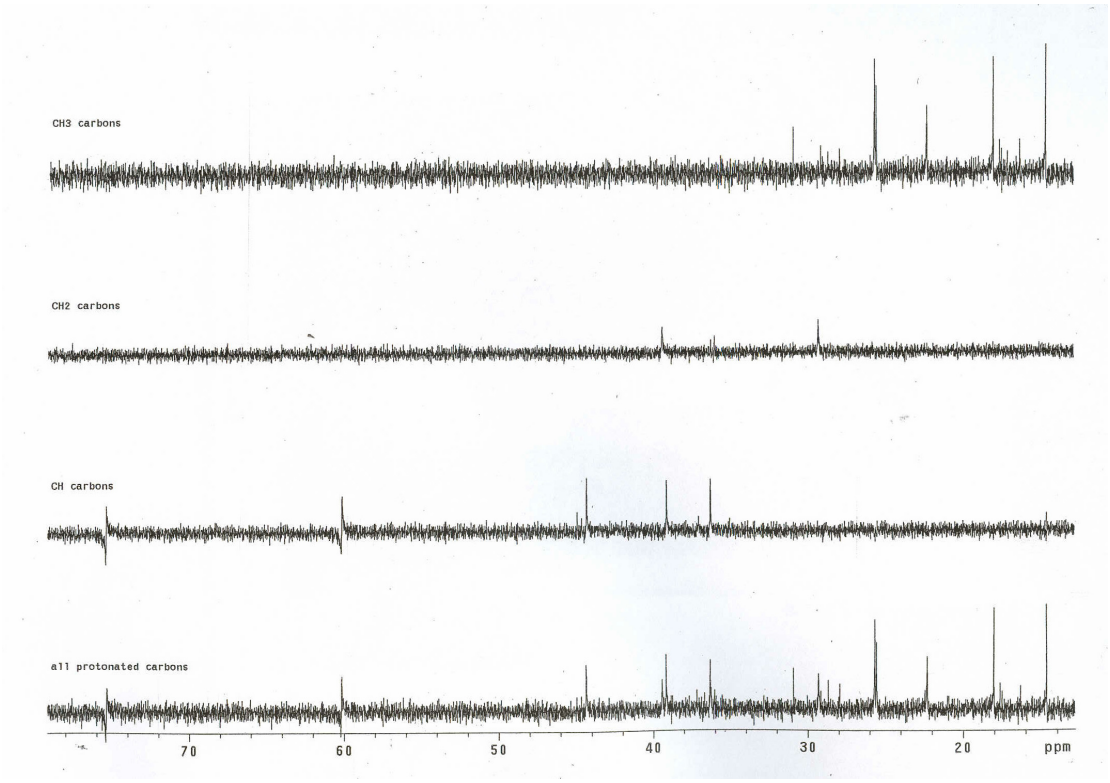


Figure 2.25: Dept spectrum of steenkrotin B.

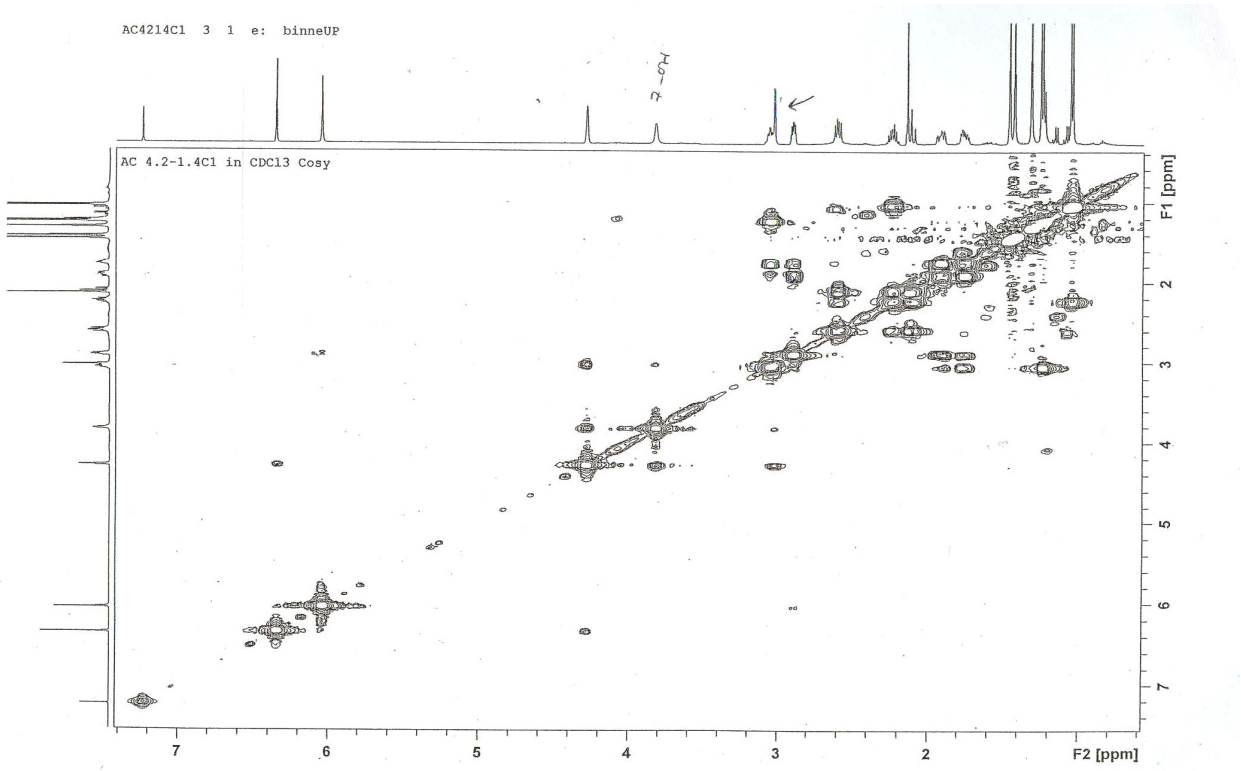


Figure 2.26: COSY spectrum of steenkrotin B.

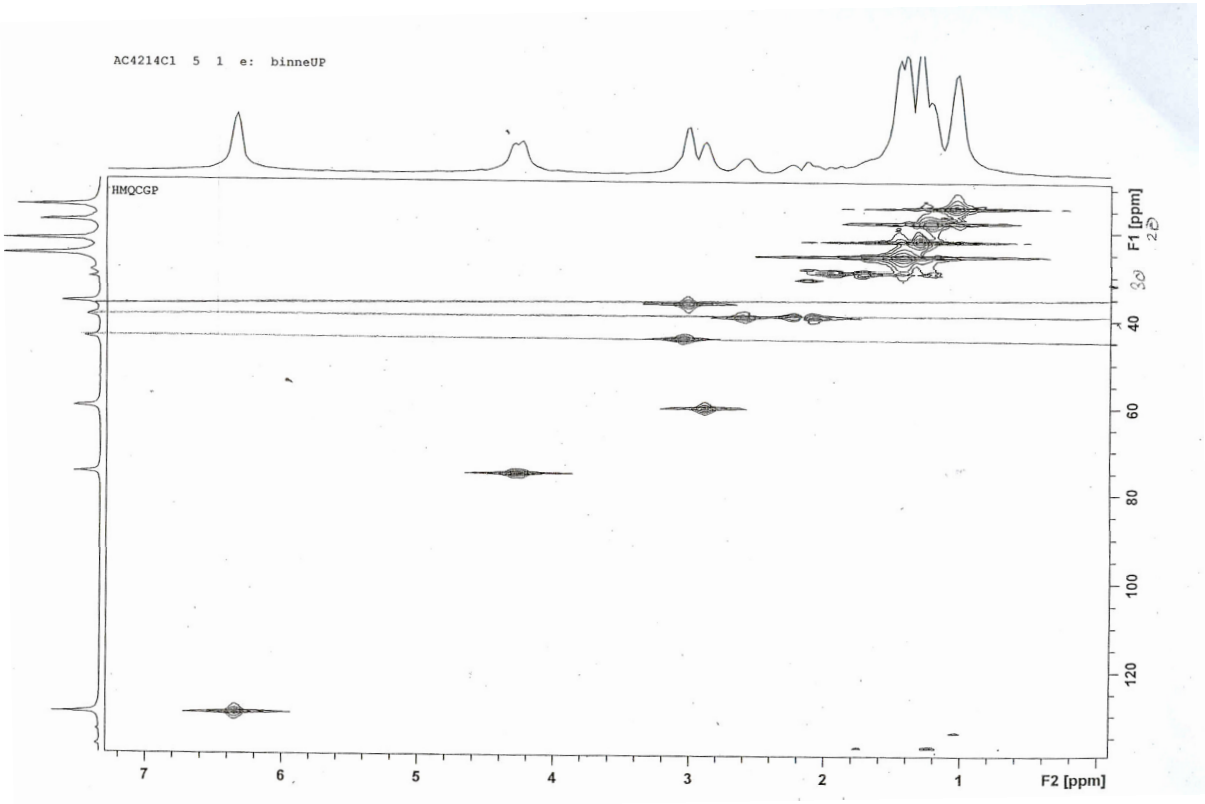


Figure 2.27: HMQC spectrum of steenkrotin B.

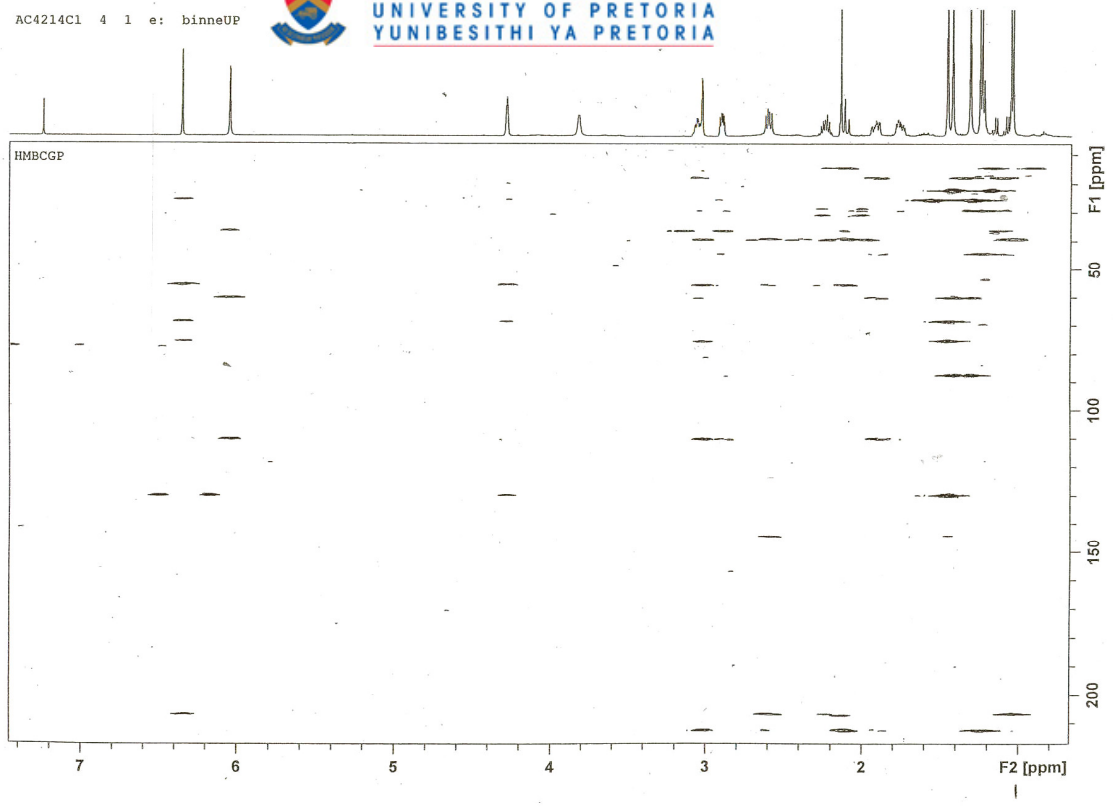


Figure 2.28: HMBC spectrum of steenkrotin B.

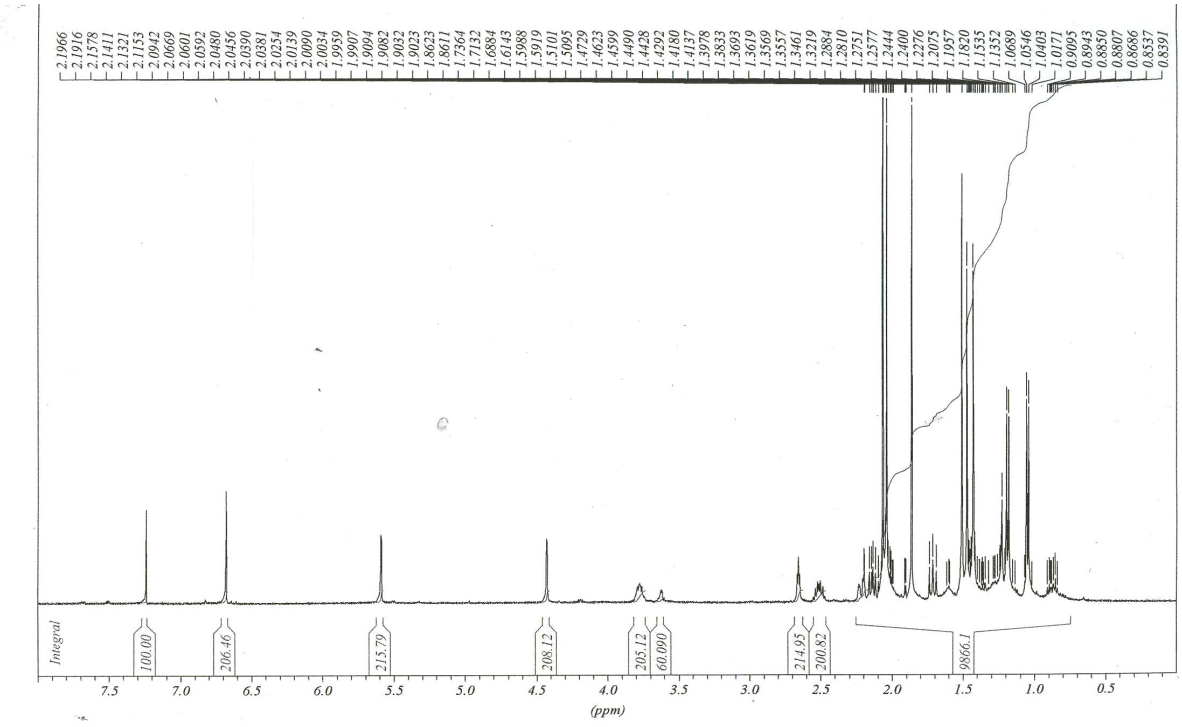


Figure 2.29: ^{1}H-NMR spectrum of steenkrotin B acetate.

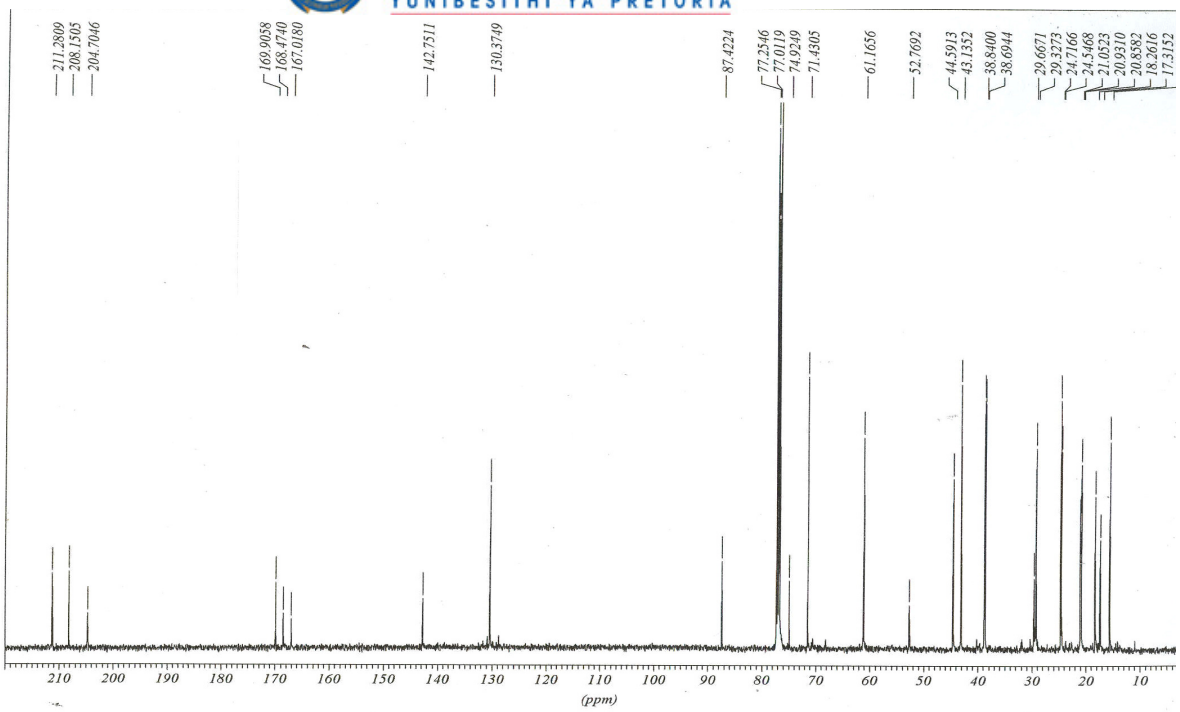


Figure 2.30: ^{13}C-NMR spectrum of steenkrotin B acetate.

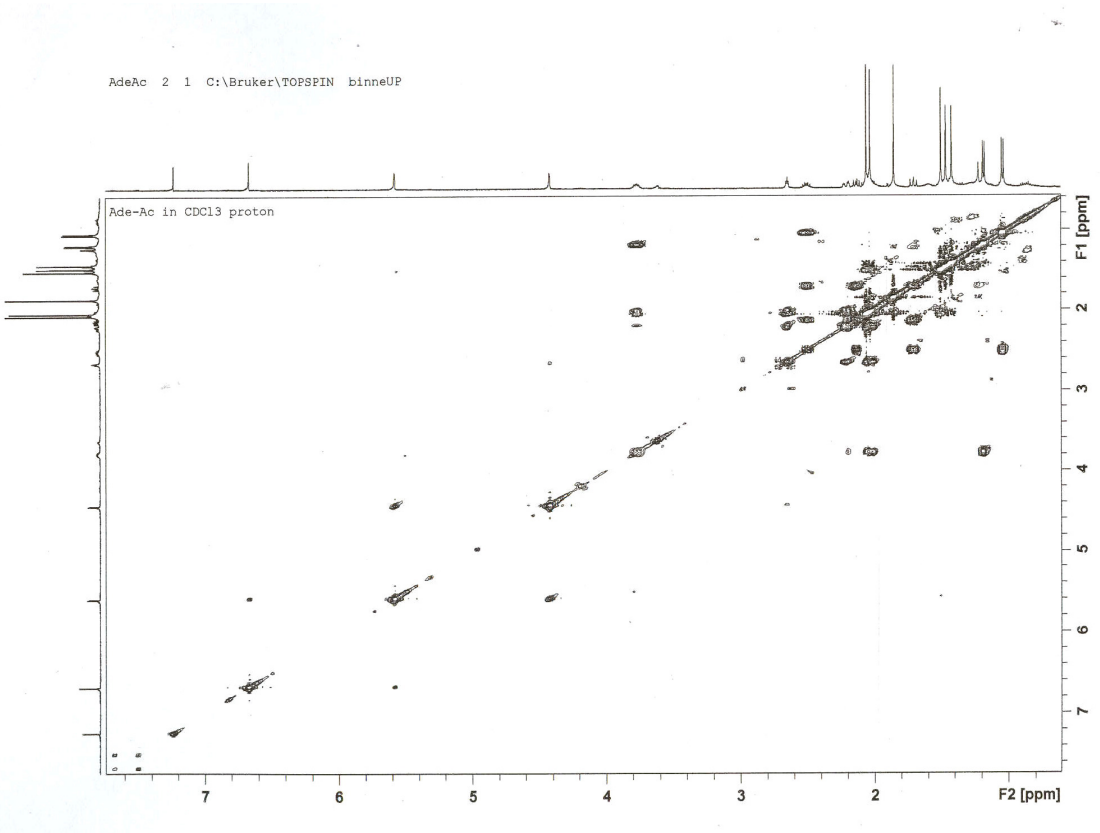


Figure 2.31: COSY spectrum of steenkrotin B acetate.

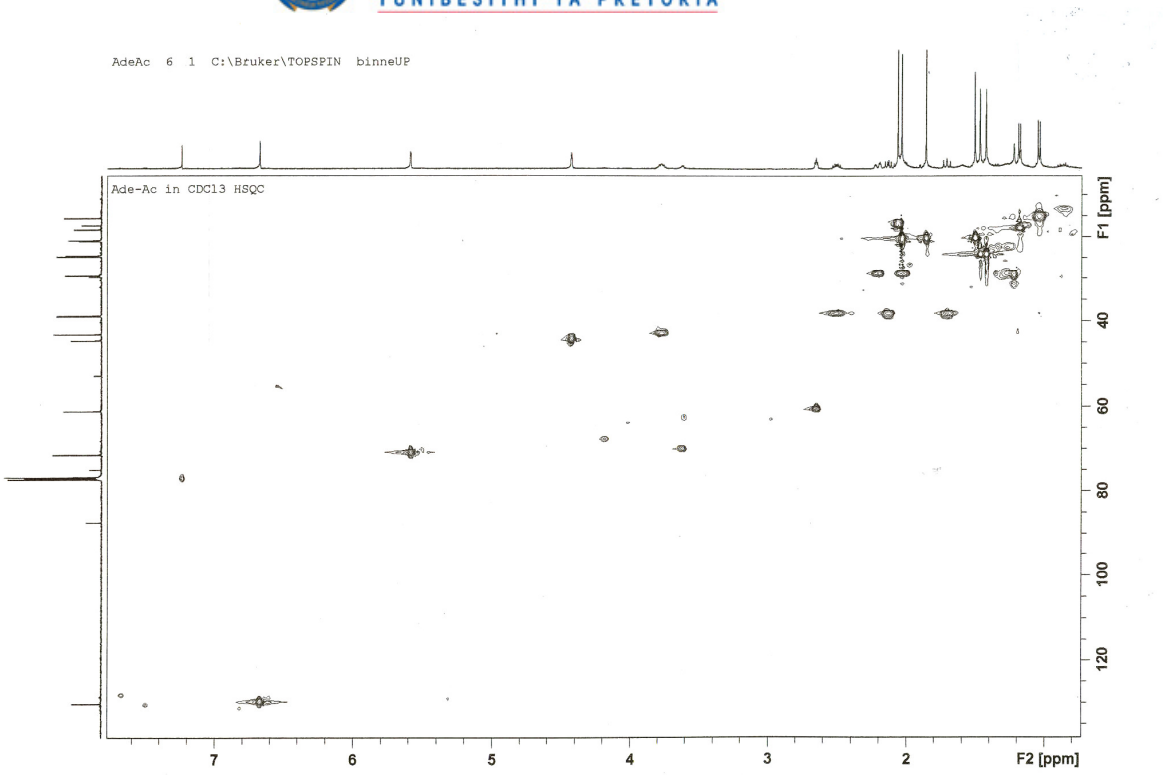


Figure 2.32: HSQC spectrum of steenkrotin B acetate.

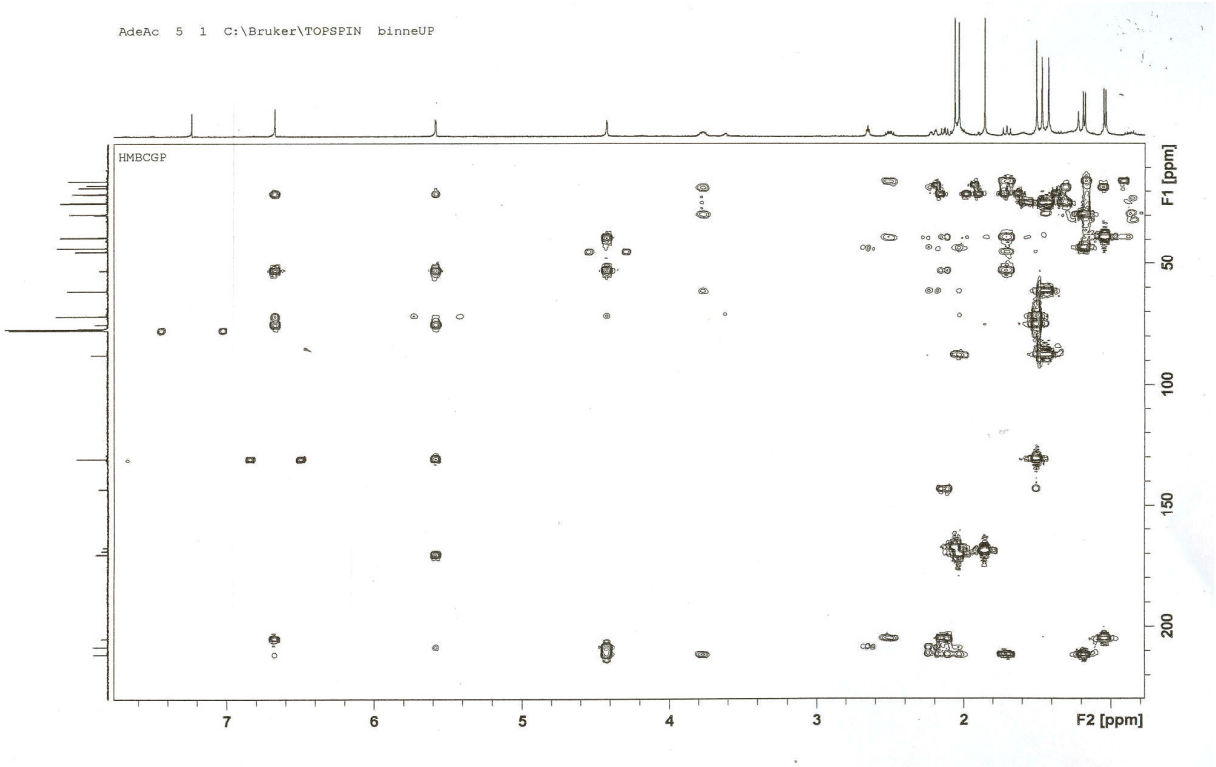


Figure 2.33: HMBC spectrum of steenkrotin B acetate.

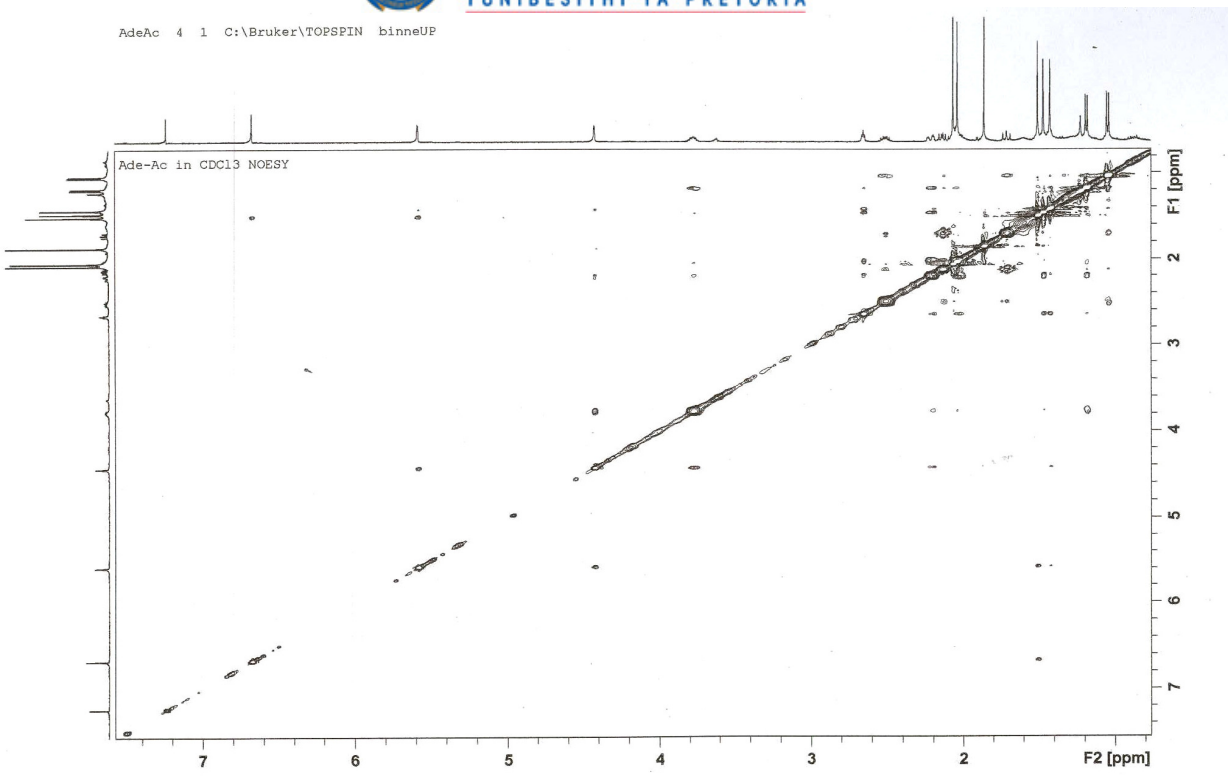


Figure 2.34: NEOSY spectrum of steenkrotin B acetate.

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CHAPTER 3

ANTIPLASMODIAL BIOACTIVITY OF CRUDE EXTRACT AND ISOLATED COMPOUNDS

CHAPTER 3

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3.1 Introduction

Among many and different maladies that afflict mankind, malaria appears to be making a strong comeback (Van der Westhuyzen; Parkinson, 2005). Cases of malaria are on the increase in the world, more especially, in the tropical and sub-tropical regions. The World Health Organization estimates that 300-500 million new cases of malaria are reported annually, causing the death of about 2.5 million people (Hyde, 2002; Van der Westhuyzen; Parkinson, 2005). The increasing prevalence and distribution of malaria has been attributed to a number of factors, one of them being the emergence and spread of drug resistant parasites. The search for new methods to combat the disease has become imperative as commonly available cheap drugs are no longer effective for treatment. Efforts are now being directed towards the discovery and development of new chemically diverse antimalarial agents (Clarkson *et al*., 2004). The success of artemisinin and its derivatives has encouraged and supported the continued investigation of plant resources for novel antimalaria drugs (Willcox, 2004).

Before 1976 drug sensitivity testing was done only *in vivo*, making the procedure very expensive and difficult. Since the development of *in vitro* assays, drug sensitivity testing has become easier, faster and more efficient (Noedl *et al.,* 2003). Although *in vitro* antiplasmodial testing is a lot easier than *in vivo* methods, bio-guided isolation of antiplasmodial compounds using *in vitro* methods remains a long and difficult process. The antibacterial screening test (direct bioautography) is the easiest and might be successful in isolating antiplasmodial compounds (Prozesky, 2004; Boonphong, 2007; Zdzislawa, 2007).

3.2 Methods

3.2.1 Culture medium and washed human erythrocytes

The wash medium was made up of 10.4 g RPMI 1640 (containing L-glutamine), 5.94 g HEPES, 4.0 g D-glucose, 44 mg hypoxanthine, 5% $NaHCO_3$ and 4 mg of gentamycin dissolved in 900 ml deionised sterile water. The culture medium was prepared by supplementing the wash medium with 5% Albumex II.

Erythrocytes for maintenance of the parasite cultures were obtained from the whole blood of a O^+ blood group donor, and then centrifuged in a Hermle Z 320 bench centrifuge at 500 g for 5 minutes. The plasma and buffy coat was removed. The erythrocytes were then re-suspended in wash medium and centrifuged at 500 g for 5 minutes. The supernatant was removed and the procedure was repeated three times. The washed erythrocytes were then stored in 10 ml wash medium at 4°C for up to 2 weeks (Trager and Jensen, 1976; Stoltz, 1992; Hoppe, 1993).

3.2.2 *In vitro* culturing of malaria parasites

Two chloroquine sensitive (D6 and D10) and two chloroquine resistant (Dd2 and W2) strains of *Plasmodium falciparum* was used in the bioassay (Stoltz, 1992). For continuous *in vitro* culturing a slightly modified version of the Trager and Jansen method was employed (Trager and Jensen, 1976; Hoppe, 1993).

The frozen malaria-isolates (~5% parasitemia) in cryotubes stored in liquid nitrogen, was quickly thawed in a water bath at 37°C. The content of the croytube (~1 ml) was transferred under sterile conditions to a 10 ml centrifuge tube and 0.2 ml sterile 12% NaCl was slowly added to the thawed culture. It was then mixed well for (10-20) seconds after which a sterile 1.6% NaCl solution was slowly added and mixed for (10-20) seconds. The solution was centrifuged at 2 500 g for 5 min and the supernatant was removed. The parasite was then re-suspended in a 75 ml culture flask (Sterilin) containing

10 ml culture medium and supplemented with fresh uninfected human erythrocytes from the O^+ blood group.

The hematocrit in the parasite culture was adjusted and maintained at 5% by adding washed erythrocytes to the culture medium. The culture flask was then filled with a special gas mixture made up of 5% oxygen, 5% carbon dioxide and 90% nitrogen before being incubated in a Forma incubator at 37C. Daily the parasite culture was checked, with the thin blood smear (reported in section 3.2.3). The culture medium was changed, flask filled with the gas mixture from above and returned to the incubator. The hematocrit of the cultured parasites was continually maintained at 5% by adding fresh cells at least every 2-3 days.

3.2.3 Giemsa stained thin blood smear preparation

A smear of parasitic culture was made by placing a drop of about 12 µl parasitic culture at one end of a slide and by using a second slide at an angle, the drop was evenly spread along the first slide and then allowed to dry. Methanol (analytical grade) was used to fixate the blood smear by allowing it to stand for 1 minute, after which the methanol was removed by decanting. The DNA intercalator Giemsa (Sigma) was used to stain the parasitic DNA (Wright, 1984). The Giemsa stain was formulated in glycerol and methanol as indicated by the supplier. A phosphate buffer, containing 9.5 g/l sodium dihydrogen phosphate at a pH of between 6.8 and 7.2 was used to dilute the Giemsa solution. Two drops of Giemsa solution were added for every 1 ml of phosphate buffer. The slide was covered with this solution for 5 minutes, rinsed in water and then allowed to dry in the open air. A drop of microscope oil immersion was placed on the slide and it was evaluated using a 100x oil objective of a Nikon phase contrast microscope**.**

3.2.4 *In vitro* synchronisation of malaria parasites

The method described by Vernes *et al.* (1984) and modified by Hoppe (1993) was used for the *in vitro* synchronization of malaria parasites. Synchronisations were performed on malaria cultures consisting of 80% ring-phase parasites. The cultures were transferred from the culture flask to a 50

ml centrifuge tube, centrifuged at 500 g for 5 minutes and the supernatant removed. The pellet volume was about 0.5 ml when 10 ml of a parasitic culture (5% hematocrit) was centrifuged. Then 4 ml of a 15% D-sorbitol solution was added to every 0.5 ml of parasite pellet. After careful mixing, by inversion, the solution was incubated at 37°C for 5 minutes, where after, 8 ml of a 0.1% D-glucose solution was added per 0.5 ml of parasite pellet, followed by mixing by tube inversion. The parasite solution was again incubated at 37°C for 5 minutes, centrifuged at 500 g for 5 minutes and the supernatant containing lysate erythrocytes, was removed. The pellet ring-phase infected and uninfected erythrocytes were re-suspended in 10 ml of the culture medium and returned to the culture flasks. The hematocrite was adjusted to about 5% by adding washed erythrocytes, filled with the special gas mixture described in 3.2.2 and returned to the incubator. This procedure was repeated until the cultures consisted of about 95% ring-phase parasites.

3.2.5 Preparation of microculture plates

Microculture plates were prepared in the same way for all the antiplasmodial bioassays. Final concentrations of the extracts were made by appropriate dilutions of stock solutions with the culture medium. 20 µl extract/compound in duplicate and at different concentrations were added to the flat-bottom wells of a 96-wells microculture plate, as well as 80 µl of a 5% hematocrit of 0.5-1.0% parasitized cells (95-100% rings) in supplemented RPMI 1640 medium. Microcultures were incubated for 48 hours in a modular incubator chamber at 37°C in a gas mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen for the assessment of antiplasmodial activity. After 24 hours, 50µl of the medium from each well was removed and replaced by 10 µl of the extract/compound and 40 µl of the fresh culture medium (Makler *et al.,* 1995).

3.2.6 Determination of antiplasmodial activity with the Malstat method

The method used was a slightly modified version of the lactate dehydrogenase assay (Makler *et al.,* 1995). The experiment was done in duplicate at 1% parasitemia and 5% hematocrite as described in section 3.2.5 After 48 hours, the 96-well microculture plate was prepared by adding 100 µl

Malstat reagent (133 ml Triton X-100, 1.33 g lactate, 0.44 g Tris buffer and 44 mg 3-actylpyrimidine adenine dinucleotide (APAD) made up to 200 ml) to each well together with 25 µl developing dye solution (160 mg nitroblue tetrazolium (NBT) and 8 mg phenazine ethosulphate (PES) to 100 ml Millipore water) and 10 µl from the incubated plate. The plate was then incubated for 20 min in the dark and read with an ELISA plate reader at 620 nm.

3.2.7 Determination of antiplasmodial activity with the microfluorimetric method

The method used was based on the detection of parasitic DNA by the intercalation of PicoGreen (Corbett *et al.*, 2004). Synchronized ring form cultures (2% hematocrit and 1% parasitemia) were used to determine the activity of pure compounds in 96-well microculture culture plates. The cultures of *P. falciparium* were grown and synchronized as described in section 3.2.2 and 3.2.4. The microculture plates were prepared as described in section 3.2.5. Compounds were dissolved in DMSO and tested in duplicate at the final concentration of 10.0, 2.0 and 0.4 µg/ml and re-evaluated at higher or lower concentration when necessary. The final dilution in the wells contains less than 0.1% DMSO, which had no measurable effect on the parasite survival in this system. The negative control was 0.1% DMSO in RPMI 1640 culture media which represent 100% parasitic viability. The positive control consisted of chloroquine at concentrations of 100.0, 10.0, and 1.0 nM that provided a measure of susceptibility of the parasite to known antimalarial drugs (Corbett *et al.,* 2004).

3.2.7.1 Fluorimetric susceptibility test

After 48 hours, 150 µl of the culture described in 3.2.7 was transferred to a new 96 well microculture flat bottom plate. Fifty micro-litres of the fluorochrome mixture, which consisted of PicoGreen (Molecular Probes, Inc., EUGENE, OR), 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE buffer), and a 2% Triton X-100 dilution with double distilled DNAse-free water, was then added to liberate and label the parasitic DNA. The plates were then incubated for 5-30 minutes in the dark. The fluorescence signal, measured as relative

fluorescence units (RFU) was quantitated with a fluorescence microplate reader (FL_X 800; Bio-Tek Instruments, Inc., Winooski, VT) at 485/20 nm excitations and 528/20 nm emissions. Simultaneously, the RFU from the samples (compounds, negative and positive control) were obtained, stored, and analysed (Corbett *et al*., 2004).

The data analyses were performed with a programmed calculus sheet on Microsoft (Redmond, WA) Excel 2000 that processes the relative fluorescence units exported through the KC junior software from the microplate fluorimeter. The calculus sheet consists of 1) a formula that calculates the mean of the two replicates per sample condition, 2) the subtraction of the respective colour background of each dilution of the plant extract, 3) the conversion of the mean RFU value to a percentage of the response, taking as 100% the mean of the negative control, and 4) the conversion of the percentage to the 50% inhibitory concentration (IC_{50}) by log regression. To adjust for the potential contribution of the haemoglobin pigments from the erythrocytes and the possible fluorescence from the intrinsic pigments present in some plant extracts, control wells were used that consisted of non-infected erythrocytes alone, samples of the diluted drugs or extracts with non-infected erythrocytes.

3.2.7.2 Synergistic activity

The chloroquine reversal effect of compounds with chloroquine was determined by measuring the activity of each of the compounds alone and in combination with chloroquine. Two strains of *P. falciparum* were used, a sensitive strain (D6) and resistant strain (W2). They were cultured and synchronised as described in sections 3.2.2 and 3.2.4. The *In vitro* antiplasmodial test was done with the microfluorimetric method described in section 3.2.7. The compounds at concentrations of 10.0, 2.0 and 0.4 µg/ml were combined with chloroquine at concentrations of 100.0, 10.0 and 1.0 nM respectively.

3.3 Results and Discussion

The antiplasmodial activity of the compounds isolated are shown in Table 3.1. Two of the flavonoids (tamarixetin and eriodictyol) isolated were not tested because they were obtained in insufficient quantities. Quercetin showed a very good antiplasmodial activity against D6 (0.34 µg/ml) and Dd2 (0.73 µg/ml). Antimalarial activity of quercetin was also reported by Bylka *et al.* (2004), Tona *et al.,* (2004) and Prozesky (2004). The activity shown by steenkrotin A is moderate and promising. This is because it is more active against resistant strains (3.00 and 3.10 µg/ml for W2 and Dd2 respectively) than the sensitive strain D10 (5.20 µg/ml). It did not show any activity against D6 at the concentration in which it was tested. This compound was first isolated and described as an antiplasmodial agent by Prozesky (2004).

Table 3.1: Antiplasmodial activity of compounds and extract.

Extract/Compounds	IC_{50} (µg/ml)			
	Sensitive strains		Resistant strains	
	D6	D10	W2	Dd2
Ethanol extract	ND	8.60	ND	5.80
Eriodictyol	ND	ND	ND	ND
Indane	>10.00	>10.00	>10.00	>10.00
Quercetin	>10.00	0.34	>10.00	0.73
Steenkrotin A	>10.00	5.20	3.00	3.10
Steenkrotin B	>10.00	>10.00	>10.00	>10.00
Steenkrotin B acetate	ND	>10.00	ND	>10.00
Tamarixetin	ND	ND	ND	ND
Chloroquine (Q)	0.01	0.01	0.03	0.07
Indane + Q	>10.00	ND	2.00	ND
Quercetin + Q	8.00	ND	3.00	ND
Steenkrotin A + Q	12.00	ND	2.00	ND
Steenkrotin B + Q	3.00	ND	3.00	ND

ND = not done

Q = chloroquine

Steenkrotin B, steenkrotin B acetate and the indane were not active (compounds with IC_{50}<10 µg/ml is said to be active) against the strains of *P. falciparum* at the concentrations at which they were tested. Steenkrotin B and its acetate were expected to be active because they contain an endoperoxide which has been linked to antiplasmodial activity of artemisinin, a potent antimalarial agent (van der Westhuyzen; Parkinson, 2005). The inactivity of these two compounds against *P. falciparum* seem to indicate that there are severe structural constraints involved in them that prevent either the access of the radicals to the membranes of the parasite, or are involved in some mode of deactivation of the material itself. Studies on structural modifications of these compounds are needed to determine the activity of the endoperoxide in them. Furthermore, there is no report on the antimalarial activity of an indane in literature.

When the indane, steenkrotin A and steenkrotin B were spiked with chloroquine and tested against D6 and W2, the IC_{50} of the compounds improved a little bit. However, Prozesky (2004) reported synergistic activity of steenkrotin A (called crotrene A) with chloroquine on *P. falciparum* (RB1). He also reported a similar observation that the compound is more potent against a resistant strain than a sensitive one.

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CHAPTER 4

ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF ISOLATED COMPOUNDS

CHAPTER 4

Antibacterial and Antioxidant Activity of Isolated Compounds

4.1 Introduction

Bacterial diseases continue to present a major threat to human health. Tuberculosis, for instance, rank among the world's leading causes of death. *Streptococcus*, another bacterium, continues to be a frequent cause of life threatening infections during the first two months of life. Food-borne and water-borne bacteria such as *Samonella* and *Campylobacter* are responsible for a recent troubling increase in diarrhoea related diseases. Meanwhile, during the last decade, scientists have discovered many new organisms and new strains of many familiar bacteria, such as *Escherichia coli*. Emerging bacterial diseases present a clear challenge to biomedical researchers (NIAID, 2007; Mandell *et al.,* 2005). Suffering caused by these pathogens is worsened by their development of resistance to antibiotics.

The complexity of this challenge is becoming even clearer as researchers begin to appreciate the many unsuspecting mechanisms that bacteria have for causing trouble to human beings. For example, gene transfer among different strains of bacteria, and even between different species of bacteria, is now understood to be a common means whereby these organisms acquire resistance to antibiotics. Basic research has also discovered that some bacteria may play a major role in certain chronic diseases not formerly associated with bacterial infection. The bacterium *Helicobacter pylori*, for example, have been found to cause stomach ulcers and may contribute to stomach cancer; Guillain Barré syndrome has been associated with prior diarrhoea related diseases caused by *Campylobacter jejuni* (NIAID, 2007; Mandell *et al.,* 2005).

In addition, the frequency of serious nosocomial bacterial and fungal infections is rising due to the use of newer and more powerful antimicrobial agents. As additional new antimicrobial agents are being found, micro-organisms become more resistant to existing chemotherapies. Thus, there is continuous need to develop novel antimicrobial compounds that would be effective against these and other pathogens (Zgoda and Porter, 2001).

4.2 Materials and Methods

4.2.1 Qualitative determination of antibacterial activity

The direct bioautography method described by Begue and Kline (1972) was used. TLC plates were spotted with compounds or fractions and developed in a solvent system: 3-10% methanol in chloroform. The developed plates were allowed to dry overnight under a stream of air to remove residual solvent, which might inhibit bacterial growth. The two cultured bacteria (*Escherichia coli* and *Bacillus cereus*) were then sprayed on the TLC plates (i.e. *E. coli* on one plate and *B. cereus* on another) and incubated at 37℃ in humid conditions. After incubation, plates were sprayed with 2 mg/ml solution of p-iodonitrotetrazolium violet [INT] (Sigma) (Section 2.2.2.2). Clear zones on the chromatograms indicated inhibition of growth after incubation (Fig. 4.1).

4.2.2 Quantitative determination of antibacterial activity

Round bottom sterile 96-well microplates (0.5 ml volume, Fisher Scientific) were used to determine minimal inhibitory concentration (MIC) of the compounds isolated (Eloff, 1998). The stock solution of positive control (streptomycin) and the compounds were dissolved in 5% dimethylsulfoxide (DMSO) (Sigma) so that the concentration of DMSO in the microwells was less than 1% (Langfield *et al*., 2004). The stock solution was kept on ice until used. The concentrations of compounds in the micro-wells ranged from 1.56 to 200.00 µg/ml. The control and compounds were tested in duplicate.

A multipipettor was used to dispense 100 µl of nutrient broth (Merck Chemicals) into all the wells on the microplate. Hundred micro-litres of control were dispensed into each of the first two wells in role A. The same amount of solution containing each of the compounds was introduced into the subsequent two wells on the row. Using a multipipettor set at 100 µl, the control and compounds in the wells were mixed by sucking up and down 6-8 times without splashing. Serial dilutions of the compounds and the control in row A were made by withdrawing 100 µl of the mixture from the wells and transferring these to wells in row B. Similarly, 100 µl of solution in wells on row B were transferred to row C after mixing them for 6-8 times. This procedure

was repeated down the columns to row H. 100 µl of the solutions were withdrawn and discarded from the wells in the last row (H).

One hundred microlitres of cultured bacteria (Section 2.2.2.2) were dispensed into all the wells except the ones in the last two rows (G and H) which were for sterility control and incubated overnight at 37°C. To indicate bacterial growth 40 µl of p-INT solution (0.20 mg/ml) was then added to each well and the plates incubated for an additional ½ hour to 1 hour.

4.2.3 Antimycobacterial testing

The radiometric BACTEC method was used to determine the antimycobacterial activity of the isolated compounds on the H37Rv (reference, drug susceptible) strain of *Mycobacterium tuberculosis* (Lall and Meyer, 2001; Lall *et al.,* 2003). The H37Rv strain was obtained from the American Type Collection (Rockville, MD, USA). The principle of the method is based on the metabolism of the ^{14}C-labelled substrate (palmitic acid) present in the BACTEC 12B broth (7H12 medium) by viable bacteria to produce ^{14}C-labelled carbon dioxide. The amount of $^{14}CO_2$ detected which is reflected by the rate and amount of growth occurring in the sealed vial, is expressed in terms of the growth index (GI) (Mativandlela *et al.*, 2005).

A homogenized culture (0.1 ml) of the strain of *M. tuberculosis* (H37Rv), yielding 1 x 10^4 – 1 x 10^5 colony forming U/ml (CFU per ml), were inoculated in the vials containing the compound, as well as in the control vial. Two compound free vials were used as controls: one vial was inoculated in the same way as the vials containing the compound, and the other was inoculated with a 1:100 dilution of the inoculum (1:100 control), to produce an initial concentration representing 1% of the bacteria population (1 x 10^2 – 1 x 10^3 CFU per ml) found in the vials containing the compound. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compound that inhibited more than 99% of the bacterial population.

Inoculated bottles were incubated at 38ºC and each bottle was assayed everyday at about the same time until the cumulative results were interpretable. The difference in the GI values of the last 2 days is designated

as ΔGI. The reading of the vials containing the compound was compared with the control vial, containing a 1:100 dilution of the inoculum. Readings were taken until the control vials containing a 100 times lower dilution of the inoculum, than the vial with the compound, reached a GI of 30 or more. If the ΔGI value of the vial containing the compound was less than the control the population was reported to be susceptible to the compound. All compounds (steenkrotin A, steenkrotin B and the indane) were tested in triplicate (Lall and Meyer, 2001).

4.2.4. Antioxidant testing

The antioxidant activity was assessed by measurement of the scavenging ability of the isolated compounds on the free radical 2, 2'-diphenyl-1-picryldrazyl (DPPH) ($C_{18}H_{12}N_5O_6$). The radical DPPH is reduced to the corresponding colourless hydrazine upon reaction with hydrogen donors (Pandey et al. 2005). Both qualitative and quantitative assays were performed. DPPH was obtained from Fluka Chemie AG, Bucks. Ascorbia acid (Sigma) was used as the control. DPPH (4 mg) was dissolved in MeOH (50 ml) to obtain a concentration of 80 µg/ml.

4.2.4.1 Qualitative assay

The compounds tested were applied on a TLC plate and sprayed with DPPH solution using an atomiser. It was allowed to develop for 30 min. The colour changes (purple on white) were then noted (Kumarasamy *et al.,* 2002) (Fig. 4.2).

4.2.4.2 Quantitative assay

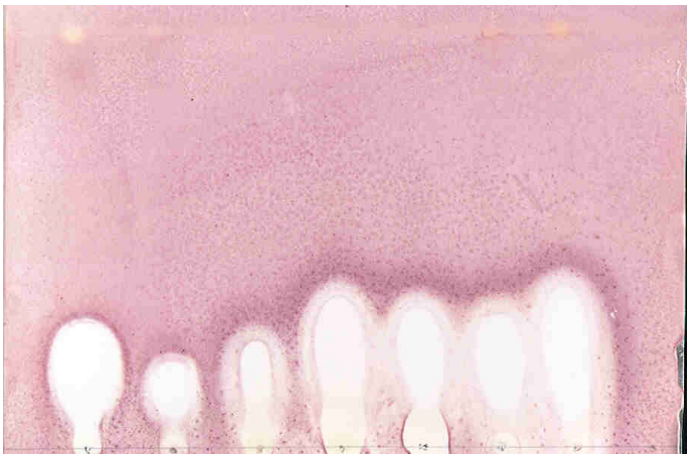
The ethanol extract and compounds were dissolved in MeOH to obtain a concentration 3 mg/ml and 1 mg/ml respectively. Serial dilution of the extract, compounds and control were made on a 96-welled plate. The extract was diluted to obtain final concentrations of 1 000.0, 500.0, 250.0, 125.0, 62.5, 31.3, 15.6 and 7.8 µg/ml. The compounds (except quercetin), ethanol extract and control (ascorbic acid) (Table 4.2) were diluted to obtain final concentrations of 500.0, 250.0, 125.0, 62.5, 31.3, 15.6, 7.8 and 3.9 µg/ml. The quercetin was tested at a final concentration of 0.05 µg/ml. The diluted

solutions (100 µl) were mixed with DPPH (100 µl) and allowed to stand for half an hour to allow for any reaction to occur. A negative control (blank) was also included in the assay. The wells for the negative control contain solvent, MeOH (100 µl) and DPPH (100 µl) (Kumarasamy *et al.,* 2002) (Fig.4.3).

The program (KC Junior) was used to read the plate on an ELISA plate reader at 550 nm. The experiment was done in triplicate and the absorption was noted for each concentration. The percentage decrease of the absorbance was calculated by the formula: $I = [(A_B-A_A)/A_B] \times 100$, where I = % inhibition, A_B = absorbance of the blank sample (t = 0), A_A = absorbance of the test sample at the end of the reaction (t = 30 min). The IC_{50} was extrapolated from the standard curve of the percentage inhibition against concentration (Paixão *et al.,* 2007).

4.3 Results and Discussion

All compounds tested with bioautography on TLC plates (Fig. 4.1) showed interesting activity against Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*B. cereus*). Therefore, the MIC of the compounds on these pathogens was determined with a serial dilution microplate assay (Table 4.1).



1 2 3 4 5 6 7

Figure 4.1: Bioautogram of the indane in lanes 1-7. A TLC plate was developed and sprayed with *B. cereus*, incubated overnight then sprayed with INT. Growth inhibition is indicated by the lighter zones on the lanes.

Table 4.1: MIC of compounds against *B. cereus* and *E. coli*.

Compound	**MIC (µg/ml)** ***E. coli***	**MIC (µg/ml)** ***B. cereus***
Eriodictyol	100.0	100.0
Quercetin	50.0	50.0
Steenkrotin A	50.0	50.0
Steenkrotin B	50.0	50.0
The indane	100.0	50.0
Streptomycin	10.0	10.0

The MIC of the compounds on both pathogens was promising against Gram negative and positive bacteria. The observed activity of quercetin corresponds to the findings of Bylka *et al.* (2004). The possibility of synergistic activity of the compounds need further investigation, this is because the bioautography of the fractions indicated more intense inhibition of the bacteria than that obtained for pure compounds (Fig. 2.2b and 2.3).

In literature, derivatives of the indane and diterpenes have been reported to be active against *M. tuberculosis* (Turan-Zitouni *et al.,* 2008; Berrue *et al.,* 2007; Dettrakul *et al.,* 2003). However, the IC_{50} for the three compounds: steenkrotin A, the indane (2,6-dimethyl-1-oxo-4-indanecarboxylic acid) and steenkrotin B tested for antimycobacterial activity was greater than 10 µg/ml (highest concentration in well) while that of the control RMP was 0.12 µg/ml. Since the compounds tested did not show activity at highest concentration, they are classified as inactive against *M. tuberculosis*. Although the activity of RMP used as positive control against *M. tuberculosis* (H37Rv) was much higher than those for the novel compounds tested, it would be interesting to study the synergistic effect, if any, when these compounds are tested in combination with established antitubercular drugs.

The crude extract and all the isolated compounds tested showed DPPH radical scavenging activity (Fig. 4.2, 4.3 and 4.4).

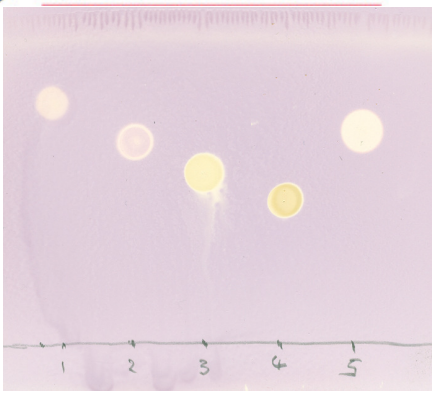
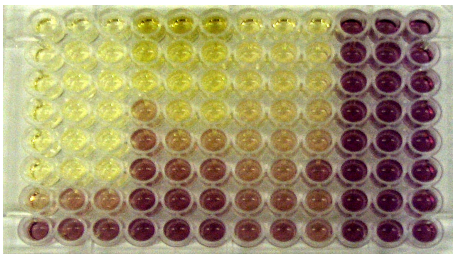


Figure 4:2 Qualitative antioxidant assay. All compounds tested showed antioxidant activity. The indane in lane 1, steenkrotin A in lane 2, tamarixetin in lane 3, quercetin in lane 4 and steenkrotin B in lane 5.



1 2 3 4 5 6 7 9 10 11 12 13

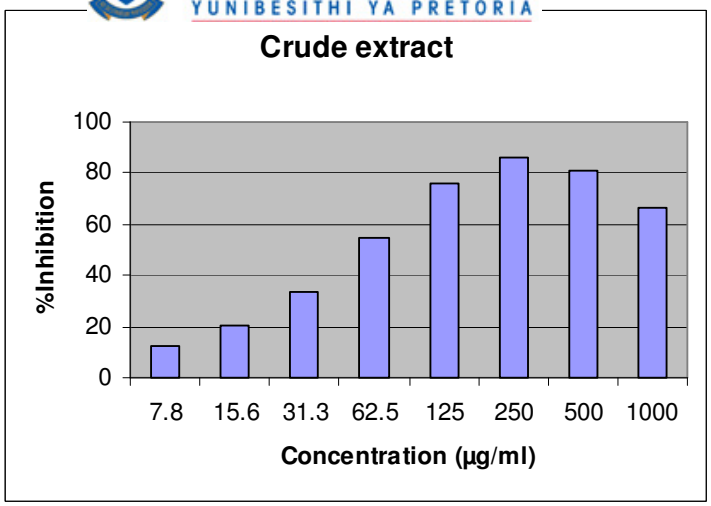
Figure 4.3 Quantitative antioxidant assay. Lane 1-3: ascorbic acid, lane 4-6: crude extract, lane 7-10: steenkrotin B and lane 11-12: negative control (blank).

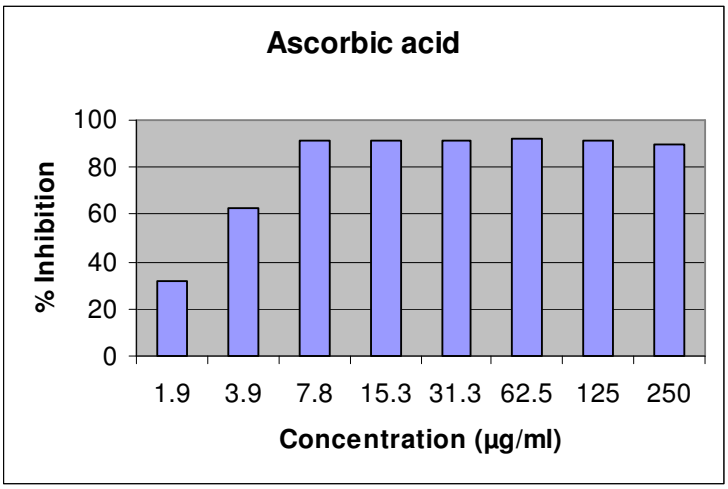
Quercetin was the most active of all the substances tested (Table 4.2). This can be attributed to the presence of the phenolic group in its molecule (Kumarasamy *et al.*, 2002). From the IC_{50} values, it can be seen that the

Table 4.2 Quantitative antioxidant activities of the ethanol crude extract and the isolated compounds.

Substance	**IC_{50} (µg/ml)**	**Ascorbic equivalents**
Ethanol crude extract	76.01	0.04
Indane	> 280.00	> 0.01
Quercetin	0.05	66.00
Steenkrotin A	> 280.00	> 0.01
Steenkrotin B	274.00	0.01
Ascorbic acid	3.30	1.00

increasing order of activity was quercetin > ascorbic acid > crude extract > steenkrotin B > steenkrotin A and indane. It was established that the DPPH scavenging activity of quercetin was 66 times more than that of ascorbic acid, 1 535 times more than that of the crude extract, 5 500 times more than steenkrotin B and over 5 500 times more active than that of steenkrotin A and indane. The moderate antioxidant activity of the ethanol extract was probably due to its rich phenolic compounds content (Narasimhan *et al*. 2005).





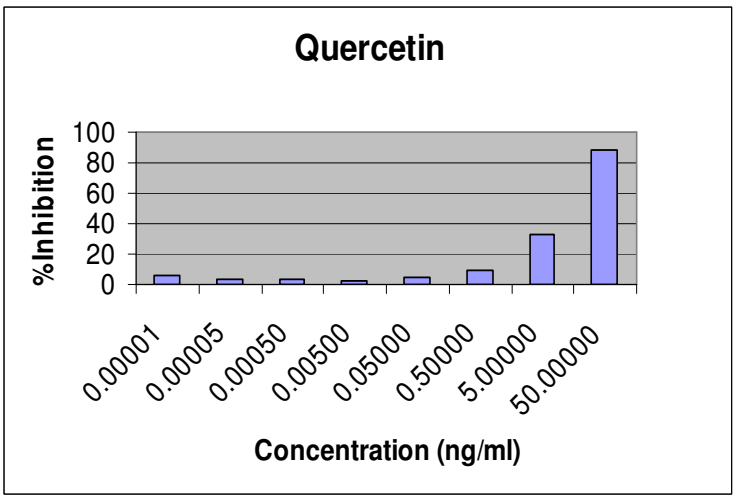


Figure 4.4 Antioxidant activities of the crude extract and compounds.

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CHAPTER 5

ANTI-HIV ACTIVITY OF THE ISOLATED COMPOUNDS

CHAPTER 5

ANTI-HIV ACTIVITY OF THE ISOLATED COMPOUNDS

5.1 Introduction

Globally, millions of people are infected and are still being infected with the human immunodeficiency virus (HIV) (Fig 5.1), the pathogen that causes Acquired Immunodeficiency Syndrome (AIDS) (Gurib-Fakim, 2006). AIDS is a collection of symptoms and infections in humans resulting from the specific damage to the immune system by the virus. The late stage of the condition leaves individuals prone to opportunistic infections and tumors (Marx, 1982). HIV uses cells of the immune system (macrophages and helper T cells) as sites for reproduction. Multiple copies of the viral genetic material (RNA) are made and packaged into new viral particles ready for dispersal into a new viral host. More and more cells of the immune system are killed or damaged with each round of infection, while millions of viral particles may be produced each day. Despite the production of antibodies and helper T cells that fight the disease, eventually the virus prevails and the infections and cancer associated with AIDS begins to appear (Gurib-Fakim, 2006). Although treatments for AIDS and HIV exist to slow the virus's progression, there is no known cure. HIV is transmitted through direct contact of a mucous membrane or the bloodstream with a bodily fluid containing HIV, such as blood, semen, vaginal fluid, pre-seminal fluid and breast milk. This transmission can come in the form of anal, vaginal or oral sex, blood transfusions, contaminated needles, exchange between mother and baby during pregnancy, childbirth or breast feeding, or other exposure to bodily fluids (Mandell *et al*., 2005).

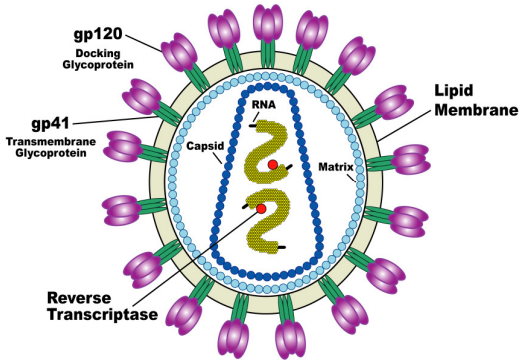

Figure 5.1: Human immunodeficiency virus (NIAID, 1998).

Most researchers believe that HIV originated in sub-Saharan Africa during the twentieth century (Gao *et al.*,1999); it is now a pandemic with an estimated 38.6 million people living with the disease worldwide. As of January 2006, the joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organisation (WHO) estimated that AIDS has killed more than 25 million people since it was first recognised on June 5, 1981, making it one of the most destructive epidemics in recorded history. In 2005 alone, AIDS claimed an estimated 2.4-3.3 million lives, of which more than 570,000 were children. A third of these deaths are occurring in sub-Saharan Africa, retarding economic growth and destroying human life (UNAIDS, 2006).

5.1.1 HIV in South Africa

A number of documents have described the seriousness of HIV/AIDS in the southern Africa region with particular emphasis on South Africa being the most affected (UNAIDS, 2000). The prevalent rate for South Africa is estimated to be 12.5%, which is one of the highest national prevalent rates in the world (James *et al.,* 2006). Women are the worst hit by the epidemic of HIV/AIDS. Of the 5.54 million people living with HIV in South Africa in 2005, 18.8% are adults aged 15-49 of which women account for approximately 55%. The infection is more pronounced in the age group 20-24 and 25-29 years where the HIV prevalence rates are 23.9% for women, 6% for men and 33.3% for women, 12.2% for men respectively (NSP, 2007). HIV was around 3% among children aged 2-14 year and nearly 4% for people in their sixties (Dinkelman *et al.*, 2005).

Children under the age of 18 years comprise 40% of the population of South Africa. In 2004, it was reported that 13% of them have lost either mother or father, half of which was due to AIDS. Children from deeply impoverished household were worst affected by the impact of AIDS (UNAIDS, 2004).

The severe effect of HIV/AIDS has led to a dramatic increase in the probability of death in South Africa's adult population. The latest forecast from the Actuarial Society of South Africa showed the likelihood of death before 60th birthday among men jumping from 36% in 1990 to 61% in 2008, whereas the likelihood of death among women increases from 21% in 1990 to 53% in 2008 (Collins and Leibbrandt, 2007).

5.1.2 Anti-HIV compounds

Currently, there is no cure for HIV/AIDS. Antiretroviral treatments reduce both the mortality and the morbidity of HIV infection, but routine access to antiretroviral medication is not available in all countries. Antiretroviral therapy (ART) consists of four major treatment modalities, nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and fusion or entry inhibitors (Grinspoon, 2005). The HIV/AIDS stigma is more severe than that associated with other life-threatening conditions and extends beyond the disease itself to providers and even volunteers involved with the care of people living with HIV (UNAIDS, 2006).

New anti-HIV compounds from natural sources are reported almost daily, some essentially unproven and others with distinct promise based on *in vitro* research. Some of these compounds are at different stages in clinical trials while further studies on others have stopped because they are found not to be suitable for use as drugs (Gurib-Fakim, 2006). The search for molecules from natural resources with anti-HIV activity or the ability to treat AIDS related infections needs to be intensified and accelerated so that the possibility of developing a drug will soon be realised.

5.1.3 Reverse transcriptase (RT)

Reverse transcriptase is a DNA polymerase that will either use an RNA or DNA strand as a primer. It is responsible for the production of a double stranded DNA copy of the single stranded RNA genome that is contained in the HIV virus particle. RT from HIV-1 is of tremendous medical interest as it is the target enzyme for the best known anti-AIDS drug, AZT, which acts by causing chain termination of the polymerase reaction. Knowledge of its detailed three dimensional structures will greatly assist the development of new anti-AIDS drugs (Arnold *et al.*, 1995).

5.1.4 Replication of HIV

Infection typically begins when an HIV particle, which contains two copies of the HIV RNA, encounters a cell with a surface molecule called cluster designation 4 (CD4). Cells with this molecule are known as CD4 positive (CD4+) cells. One or more of the virus's glycoprotein (gp120) molecules binds tightly to the CD4 molecule(s) on the cell's surface (Fig. 5.2). The membrane of the virus and the cell fuse, a process that probably involves the envelope protein of the HIV (NIAID, 1998).

Although CD4+ T cells appear to be the HIV's main target, other immune system cells with CD4 molecules on their surfaces are infected as well. Among these are the long-lived cells called monocytes and macrophages, which apparently can harbour large quantities of the virus without being killed, thus acting as reservoirs of HIV. CD4+ T cells also serve as important reservoirs of HIV: a small proportion of these cells harbour HIV in a stable, inactive form. Normal immune processes may activate these cells, resulting in the production of new virions (NIAID, 1998).

In the cytoplasm of the cell, HIV reverse transcriptase converts viral RNA into DNA, the nucleic acid form in which the cell carries its genes. Seven of the 11 antiviral drugs approved in the United States for the treatment of people with HIV infection namely zidovudine (AZT), zalcitabine (ddC), didanosine (ddI), stavudine (d4T), lamivudine (3TC), neviraine (NVP) and delavirdine (DLV), work by interfering with this stage of the viral life cycle.

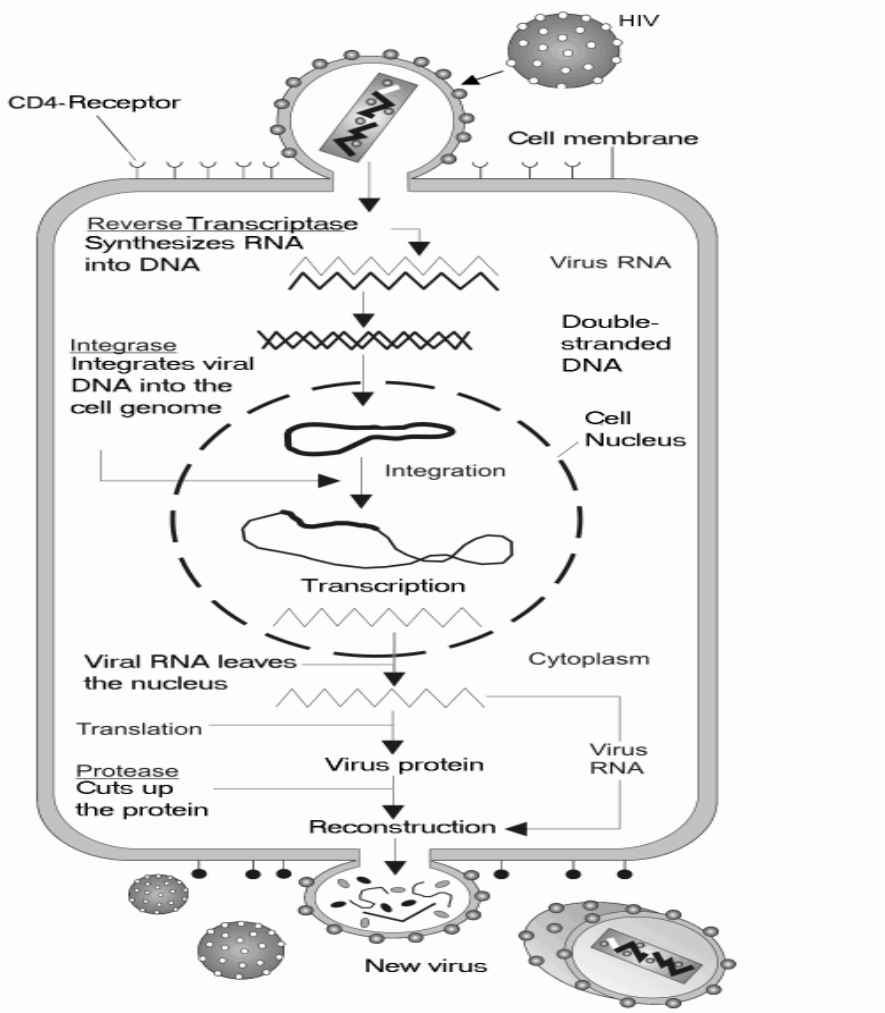

Figure 5.2: The HIV replication cycle (NIAID, 1998).

The newly made HIV DNA moves to the cell's nucleus, where it is spliced into the host's DNA with the help of HIV integrase. Once incorporated into the cell's genes, HIV DNA is called a "provirus." Integrase is an important target for the development of new drugs. For a "provirus" to produce new viruses, RNA copies must be made that can be read by the host cell's protein making machinery. These copies are called messenger RNA (mRNA). Production of mRNA is called transcription, a process that involves the host cell's own enzymes. Transcription requires the presence of transcription factors, the most important of which is NF kappa B (NF-$_K$B). Proteins, cytokines, that are involved in the normal regulation of the immune response also may regulate transcription. Molecules such as necrosis factor (TNF)-alpha and interleukin (IL)-6 that are secreted in elevated levels by the cells of HIV-infected people

may help to activate HIV "proviruses". Early efforts at stopping HIV replication focused on these chemicals. Other infections, by organism such as *Mycobacterium tuberculosis*, also may enhance transcription (NIAID, 1998; Hopkins, 1999).

After HIV mRNA is processed in the nucleus, it is transported into the cytoplasm. HIV proteins are critical to this process: for example, a protein encoded by HIV's *rev* gene allows HIV structural proteins encoding mRNA to be transferred from the nucleus to the cytoplasm. Without the rev protein, structural proteins are not made. In the cytoplasm, the virus co-opts the cell's protein-making machinery including structures called ribosomes to make long chains of viral proteins and enzymes, using HIV mRNA as a template. This process is called translation result in immature viral particles which at this stage are not yet infectious. The long chains of proteins and enzymes that make up the immature viral core are now cleaved into smaller pieces by a viral enzyme called protease. This step results in infectious mature viral particles. Drugs called protease inhibitors (saquinavir, ritonavir, indinavir and nelfinavir) interfere with this step of the viral life cycle (NIAID, 1998).

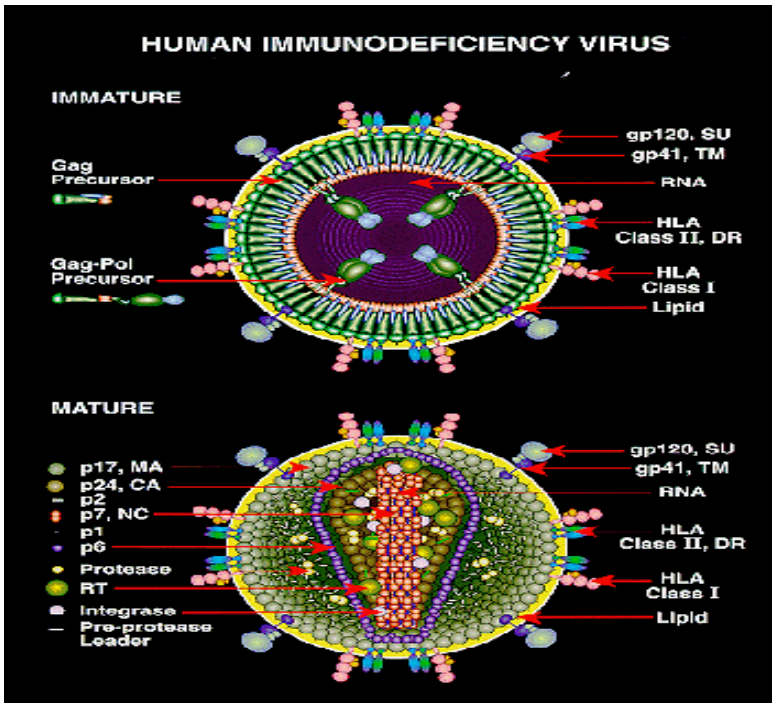

Figure 5.3: The immature and mature forms of the HIV (NIAID, 1998).

Several reports have linked human immunodeficiency virus (HIV) infection and prolong usage of highly active antiretroviral therapy (HAART) with increased risk of cardiovascular disease (CVD), premature atherosclerosis and development of metabolic syndrome (dyslipidemia, insulin resistance, fat redistribution and hypertension) (Maggi *et al.,* 2007; Friis-Møller *et al*., 2003). The relative risk rate of myocardial infarction is said to increase by 26% per year of HAART exposure (Grinspoon, 2005).

The fact that currently there is no cure in sight and that available treatment predisposes patients to risky side effects, is reason enough for desperate and radical search for new remedy with curative or preventive properties. Hence, the compounds isolated were assessed for anti-HIV activities.

5.2 Materials and Methods

5.2.1 Materials

The preparation of the crude extract and the isolation of compounds were as described in Chapter 2. The reverse transcriptase assay kit was purchased from Roche Applied Science, the dimethylsufoxide (DMSO) from Sigma-Aldrich and the sterile 96-well microplates from Fishers Scientific.

5.2.2 Method

The anti-HIV activity of the compounds isolated was determined with the reverse transcriptase colorimetric assay. The protocol outlined in the kit was followed. The pure compounds were tested in triplicate at 50 µg/ml final concentration. Sterile 96-well microplates were used for the experiment. One positive controls and one negative control were included in each assay. The well for the positive control contained Doxirubicin at 100 µg/ml with 3.3% DMSO, 20 µl of the 83.33 ng/ml enzyme, 20 µl lysis buffer and 20 µl reaction mixture. The negative control was made of 40 µl lysis buffer with 20 µl reaction mixture without the enzyme. All other wells contained 20 µl of the 83.33 ng/ml enzyme, 50 µg/ml of the compounds with 3.3 % DMSO and 20 µl reaction mixture. The plate was then incubated at 37℃ for an hour after which it was washed five times with 250 µl washing buffer per well per washing

cycle. Two hundred micro-litres antibody solution was added to each well and the plate was incubated at 37℃ for an hour. The plate was washed again five times with 250 µl washing buffer per well per washing cycle. After 200 µl of the ABT substrate solution was added to each well, the plate was incubated for 10 minutes before being measured at 412 nm wavelength in an ELISA plate reader.

5.3 Results

Steenkrotin A, steenkrotin B, the indane and quercetin were each tested against reverse transcriptase *in vitro.* Indane showed 6% activity against the enzyme at 50 µg/ml. Eriodictyol and tamarixetin was not tested because the quantities isolated were very small. Quercetin, steenkrotin A and steenkrotin B were not active at the concentration (50 µg/ml) at which they were tested.

5.4 Discussion

Many natural products have been found to be inhibitors of HIV-1 RT. These compounds belong to diverse structural classes which include coumarins, flavonoids, tannins, alkaloids, lignans, terpenes and quinines (Mahidol *et al.*, 2002). For this reason the possible anti-HIV activity of the isolated compounds was explored. Mahidol *et al.* (2002) reported anti-HIV activity of quercetin-3-*O*-α-L-rhamnoside. The fact that quercetin had no anti-HIV activity can only be attributed to structural change (absence of glycosidic side chain attached to the molecule). The two diterpenes (steenkrotin A and steenkrotin B) also did not show anti-HIV activity at 50 µg/ml concentration. This can also be linked to differences in molecular structure of the compounds as a furanoid labdane diterpene was reported to exhibit antiviral activity (Kittakoop *et al.*, 2001). Of the compounds tested only the indane showed weak activity of inhibiting reverse transcriptase. Its observed cytotoxicity was very low (chapter 6). However, the mechanism of action leading to the observed activities is not clear at the moment. It could be due to the acidic property of the compound (presence of the carboxylic acid side chain in the molecule).

Furthermore, in order to fully explore the possible anti-HIV activity of the compounds isolated, they may have to be tested at higher concentrations and

different assays methods may have to be used. Possible derivatives of the compound could be made and tested for anti-HIV activity.

5.5 References

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CHAPTER 6

CYTOTOXICITY OF THE ISOLATED COMPOUNDS

CHAPTER 6

CYTOTOXICITY OF ISOLATED COMPOUNDS

6.1 Introduction

Drug development is a very expensive and time consuming process and only 1 in 5 000 drug candidates that enter pre-clinical testing makes it through the development process (Kraljevic *et al.,* 2004). One-third of these failures are due to unacceptable toxicity levels. An *in vitro* cytotoxicity assay is a rapid and cost effective tool to identify likely failures before a compound is entered into the costly development process and also helps to choose the optimal candidate.

Cytotoxicity can be measured by the following assays: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole); XTT (sodium 3-[1(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate); Trypan blue (TB); Sulforhodamine B (SRB); WST and the clonogenic assay (Mosmann, 1983; Wilson, 2000).

XTT and MTT assays operate on the same principle. They are standard colorimetric assays (that is assays which measure changes in colour) used for measuring cellular proliferation (cell growth). The principle of the MTT or XTT assays are based on the cleavage of the yellow tetrazolium salts MTT or XTT to purple or orange formazan (Figure 6.1), respectively in the mitochondria of living cells. A solubilization solution (usually either dimethyl sufoxide or a solution of the detergent sodium dodecyl sulphate in dilute hydrochloric acid) is added to dissolve the insoluble formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at a wavelength of 400 nm using a spectrophotometer (ELISA reader) (Mosmann, 1983; Wilson, 2000).

This cleavage takes place only when mitochondria reductase enzymes are active, and therefore conversion is directly related to the number of viable (living) cells. When the amount of formazan produced by the cells treated with an agent is compared with the amount of formazan produced by the untreated control cells, the effectiveness of the agent in causing death of cells can be

deduced, by calculations using a dose-response curve (Mosmann, 1983; Roche, 2005). The cell proliferation kit II (XTT) was used for the cytotoxicity measurement in this study.

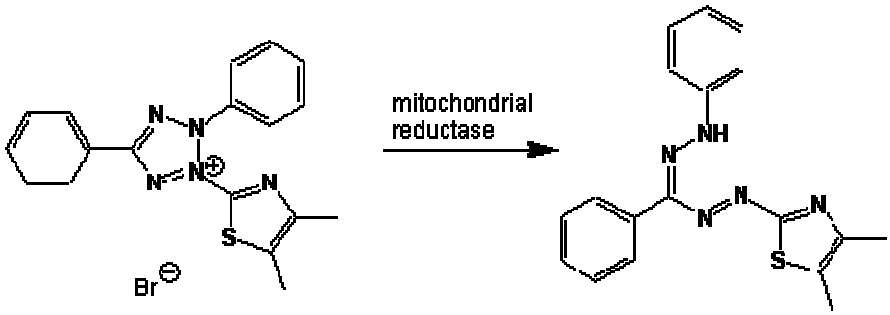

Figure 6.1: The reduction of yellow tetrazolium salt MTT to purple formazan (Roche, 2005).

6.2 Materials and Methods

6.2.1 Plant material

The leaves of *Croton steenkampianus* were collected at Thembe Elephant Park in northern KwaZulu-Natal as described in section 2.2.1.

6.2.2 Preparation of extract and isolation of compounds

The crude ethanol extract preparation was carried out as described in 2.2.2.1 and the isolation of the compounds as described in section 2.2.2.3.

6.2.3 Cell culture

The cytotoxicity of the crude extract and the isolated compounds were tested against Vero cell lines. Cells were cultured in Eagle's minimal essential media (MEM) supplemented with 1.5 g/l sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/ml penicillium, 10 µg/ml streptomycin, 0.25 µg/ml fungizone, and 10 % fetal bovin serum at 37 ºC in a humidified incubator set at 5 % CO_2.

6.2.4 Toxicity screening (XTT viability assay)

The XTT colourimetric assay was used to measure the cytotoxicity of the crude extract and the isolated compounds (Roche, 2005). On day one of the experiment, the outer wells of the 96- well plate (Figure 6.2) were filled with 200 µl of incomplete medium while the inner wells were filled with cell suspension. The plate was then incubated overnight at 37 ºC in a humidified incubator set at 5 % CO_2. The 100 µl of the crude extract/pure compound was dispensed into the cell-containing wells of the sample plate in triplicate. The final concentrations of the crude extract in the wells were 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00 µg/ml. The final concentrations of the pure compounds in the wells were 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 µg/ml. Control wells received a final concentration of 1 % DMSO in complete medium. Doxorubicin and zelaralenone were used as positive controls. The plate was then incubated for 3 days.

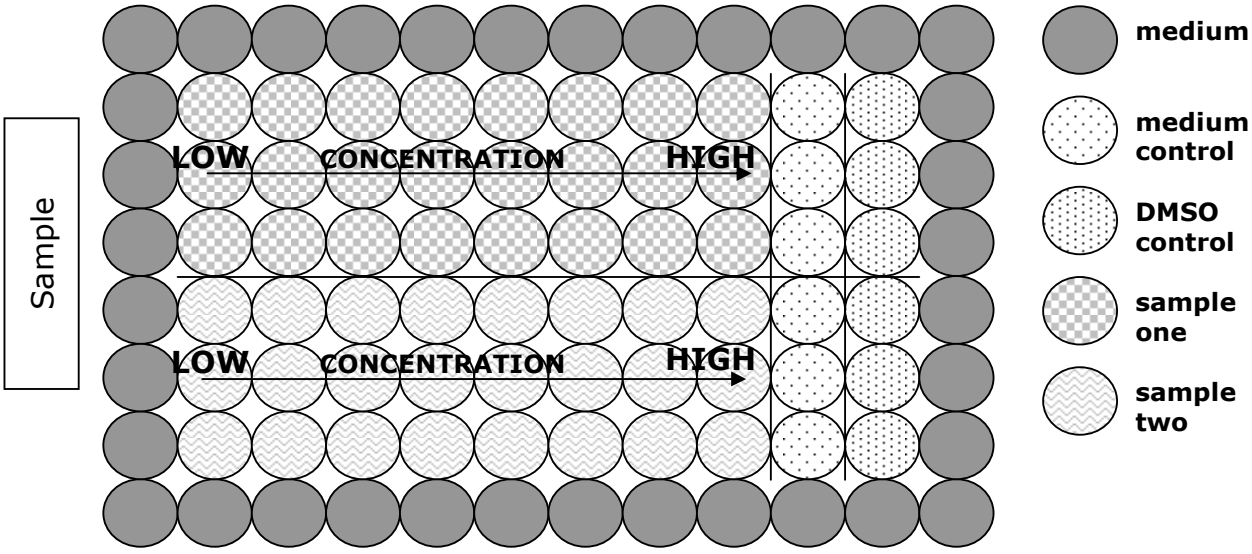

Figure 6.2: Sample plate design.

Reference plates (without cells), containing 100 µl of medium and 100 µl of diluted extract/compound, were also prepared in triplicate. These plates were also incubated at 37 ºC in a humidified incubator set at 5 % CO_2 for 3 days. On the 4th day, 50 µl of sodium 3-[1(phenylaminocarbonyl)-3,4-tetrazolium]-bis

(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) reagent was added to the wells and incubated for 1.4 hours. The colour changes were measured at 450 nm (690 nm reference wavelength). The program (KC Junior) used to read the plates automatically subtracted the values at 690 nm from the corresponding ones at 450 nm. Reference plate values were then subtracted from their corresponding sample values. Cell viabilities were assessed by comparing the sample values to the control values.

6.3 Results and Discussion

The results obtained for the cytotoxicity of the crude extract and pure compounds showed that they have little or no toxicity (Table 6.1 and Fig. 6.3).

Table 6.1: Cytotoxicity of the crude ethanol extract and compounds isolated from *C. steenkampianus* on Vero cells.

Plant extract/compound	**ID_{50} µg/ml**
Ethanol extract	45.0
Eriodictyol	Nt
Indane	248.2
Quercetin	33.6
Steenkrotin A	35.3
Steenkrotin B	305.9
Steenkrotin B acetate	Nt
Tamarixetin	53.8
Chloroquine	25.0

Nt = not done

All the isolated compounds as well as the extract had lower toxicity than chloroquine. The indane and steenkrotin B showed the lowest toxicity in this *in vitro* assay.

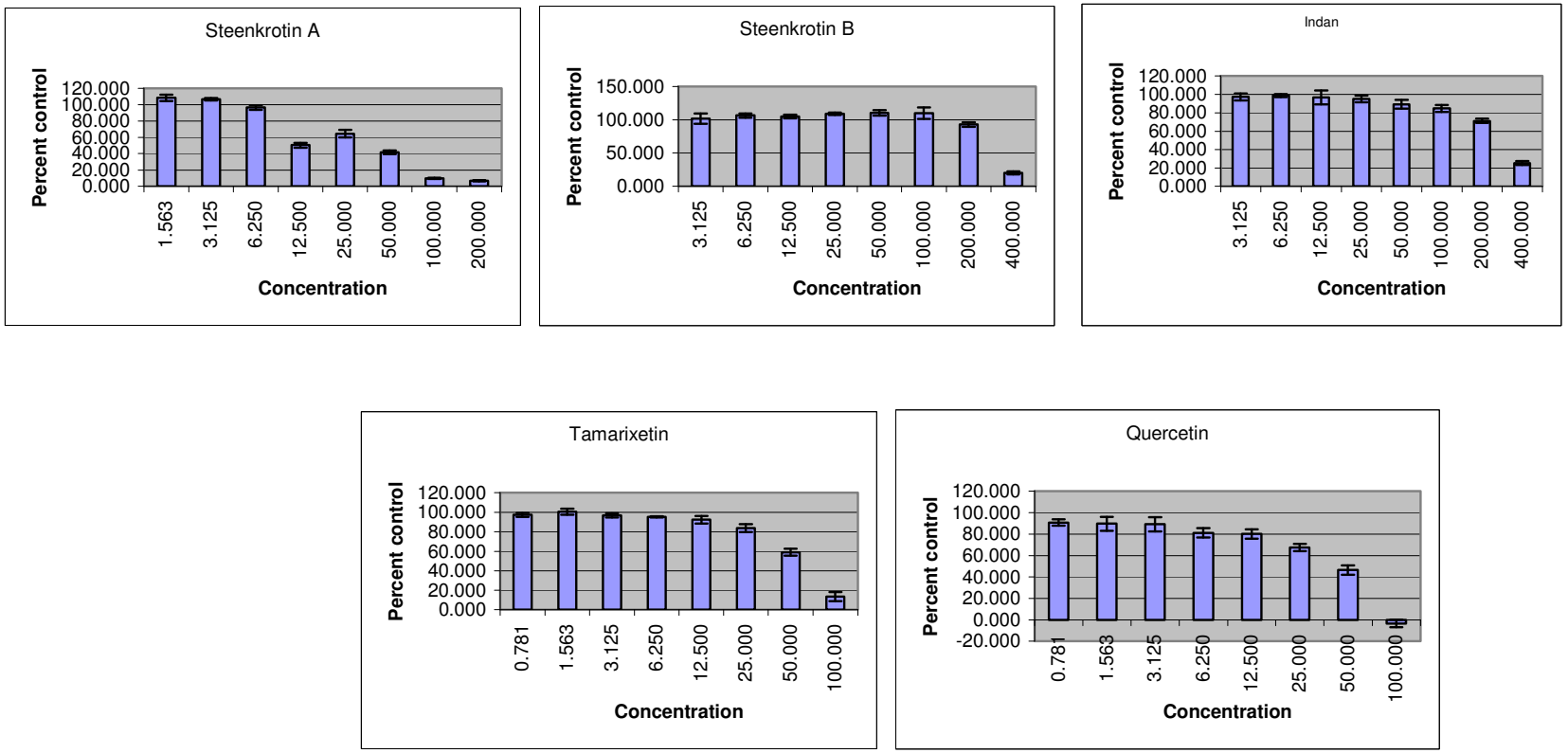

Figure 6.3: Activity of the isolated compounds on the growth of Vero cells in µg/ml.

Quercetin is the most toxic when its ID_{50} is compared to those of the crude extract and compounds isolated. However, It showed the highest antiplasmodial activity (0.34 and 0.73 µg/ml) at concentrations where its toxicity on Vero cells cause between 0% and 10% inhibition. Its therapeutic index for antimalarial activity is 46.0 to 98.8, which are not as high as the proposed index for a good remedy (Prozesky, 2004). However, it is being used as an important drug agent with very broad biological activities (Terao *et al.*, 1994; Hollman *et al.,* 1995).

Steenkrotin A showed no toxicity on the Vero cells at concentration of 1.5 to 6.3 µg/ml. This finding is very interesting and promising in that at this range it showed antiplasmodial activity (3.0 to 5.2 µg/ml) with corresponding therapeutic index of 7.0 to 11.8. Just as in the case of quercetin, its low therapeutic index based on this *in vitro* study may not mean that it will not be suitable for *in vivo* use. Nevertheless, Its suitability as a drug candidate requires further investigation *in vivo*.

Steenkrotin B did not show toxicity on Vero cells at concentration of 3.1 to 200 µg/ml. It is the least toxic of compounds isolated. However, its percentage inhibition increases from 0 to 80% at 400 µg/ml. Its antibacterial activities were at the concentrations where Vero cells' growth was not inhibited. Again, its therapeutic index (6.1) is very low compared to what is expected of a good drug. However, its very low toxicity properties might make it a promising therapeutic agent.

The indane did not inhibit the growth of Vero cells at concentrations between 3.1 and 25 µg/ml while between 50 and 200 µg/ml, the growth of cells dropped from 90 to 75%. The concentrations (50 to 100 µg/ml) where it has shown biological activities (anti-HIV and antibacterial) correspond to concentrations where Vero cells' growth was inhibited. Further evaluation needs to be performed to establish whether the observed biological activities are due to toxicity of the compound at these concentrations.

The crude extract showed lower toxicity (45.0 µg/ml) compared to the known drugs (chloroquine and quercetin). Its rich diverse component compounds which include flavonoids and terpenes known for their medicinal properties as well as its relatively low toxicity makes it a good candidate to be considered as medicine just like other species of the genus *Croton* being used traditionally for treatment of ailments (Pooley, 1993; Ngadjui *et al.,* 2002; Suarez *et al.,* 2006).

In conclusion, *in vivo* studies are needed to determine and establish the overall potential (safety, efficacy, suitability etc) of the crude extract and compounds as potential drug agents.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

CHAPTER 7

General discussion and conclusion

7.1 Introduction

New and re-emerging infectious diseases for which no effective therapy is available and the development of resistance of many pathogens to currently used drugs are of utmost concern. In the developing countries, malaria, tuberculosis (TB) and human immunodeficiency virus (HIV) are the three major infectious diseases. They account for approximately half of the mortality caused by infectious diseases, which is almost half of the mortality in the developing countries (Mahidol *et al.,* 2002). Malaria has been responsible for much of human suffering and misery. Every year there are more than 300 million cases of malaria in the world and malaria kills more than one million people. Over the last ten years, the malaria situation has been worsening in many areas of the world. The need to find new antimalarials is pressing, due to the resistance of the human malarial parasite, *Plasmodium falciparum* to the presently available common antimalarial drugs. Treatment has thus become both less effective and much more expensive. The problem is further aggravated by the resistance of the vector anopheline mosquitoes to the most effective and least toxic insecticides, which had been used to kill them.

The potential of natural products as therapeutic agents in the treatment of malaria is enormous and the research work in this area has been the subject of some recent reviews.

7.2 Bioassay guided fractionation of the ethanol crude extract and isolated compounds

The crude extract of the leaves of *C. steenkampianus* was prepared in ethanol. The isolation of the compounds was guided with antibacterial and antimalarial bioassays. The correlation between the active antibacterial and active antimalarial fractions reported by Prozesky, 2004, Boonphong, 2007 and Zdzislawa, 2007 was verified. The compounds were isolated with silica and Sephadex chromatography. Six compounds were isolated: an indane,

steenkrotin A, steenkrotin B, quercetin, tamarixetin and eriodictyol. These compounds were identified with NMR, LCMS, IR and X-ray crystallography.

7.3 Biological evaluation of the compounds

Quercetin showed the highest antiplasmodial activity among the compounds isolated. Its activity was the best against D10 and Dd2 strains of the parasite. It also displayed moderate antibacterial activity and best antioxidant activity. It showed no anti-HIV activity. Quercetin is an established medicine of value with increasing application in health care. Its biological activities (antibacterial and antioxidant) reported in this work were as documented in the literature (Kumarasamy *et al.,* 2002; Brahmachari and Gorai, 2006). Of the compounds isolated, quercetin showed the highest toxicity value but less toxicity than that of chloroquine.

The antiplasmodial activity shown by steenkrotin A is very interesting and promising. Even though its activity is less than that of quercetin against the parasite, it is more active against resistant strains than sensitive ones. The potential of steenkrotin A as an antiplasmodial agent needs further studies and development. Several derivatives of the compound needs to be synthesised and tested for improved activity. Steenkrotin A showed moderate antibacterial activity and weak antioxidant activity. No activity was observed against reverse transcriptase. Its toxicity value is similar to that of quercetin.

Steenkrotin B did not show antiplasmodial activity on the strains of *P. falciparum* used. However, it showed moderate antibacterial activity and moderate antioxidant activity, and no anti-HIV activity at the concentration in which it was tested. The activity of steenkrotin A and steenkrotin B on bacteria is promising and indicated antibiotic activity against Gram negative and positive bacteria. The antiplasmodial activity of steenkrotin B needs to be researched further because of the presence of an endoperoxide in its molecule. The high activity of artemisinin against malarial parasites has been linked to the endoperoxide in its structure. In order to optimise the possible antimalarial activity of steenkrotin B, many derivatives need to be made and tested for activity. The fact that artemisinin is active against *P. falciparum*

while steenkrotin B is not, may mean that even though both contain endoperoxides they are in different chemical environments. Steenkrotin B showed the best overall toxicity value, being the lowest of all the compounds isolated. However, more studies are required to identify other biological properties of this compound.

The indane did not show antiplasmodial activity at the concentrations in which it was tested. However, it showed moderate antibacterial activity, slight anti-HIV activity and weak antioxidant activity. The indane showed the second best toxicity value in this study. It had low cytotoxicity but moderate antibacterial and weak anti-HIV activity.

The biological study of the activities of tamarixetin and eriodictyol were not done because their quantities isolated were very small. However, their antioxidant, antimalarial and antibacterial activities are well documented (Prozesky, 2004; Mahidol *et al.,* 2002; Newman *et al.,* 2003). The toxicity (ID_{50}) of tamarixetin was 53.8, which is relatively low.

Only three compounds (indane, steenkrotin A and steenkrotin B) were tested against *Mycobacterium tuberculosis*. None of them show antimycobacterial activity at the concentration at which they were tested.

The crude ethanol extract showed moderate antimalarial, antioxidant and antibacterial activity. These bioactivities can be linked to the compounds (flavonoids, diterpenes, triterpenes etc) of the extract. Without doubt *C. steenkampinus* like other species of *Croton* (Pooley, 1993) possesses medicinal properties. Its low cytotoxicity unlike others species (Mahidol *et al.,* 2002) makes it worthy of further studies *in vivo*.

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APPENDIX 1: PAPER PUBLISHED FROM THESIS

Bioactive Diterpenes and Other Constituents of *Croton steenkampianus*

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A new indanone derivative (**1**) and two new diterpenoids (**2** and **3**), together with three known flavonoids, have been isolated from an ethanol extract of the leaves of *Croton steenkampianus*. The structure of **2** was solved by single-crystal X-ray diffraction analysis, whereas those of **1** and **3** were established mainly by 1D and 2D NMR spectroscopic methods. The isolated compounds were tested for their antiplasmodial activity and cytotoxicity. Antiplasmodial assays against chloroquine-susceptible strains (D10 and D6) and the chloroquine-resistant strains (Dd2 and W2) of *Plasmodium falciparum* showed that compound **2** gave moderate activities at 9.1−15.8 μM, while none of the compounds were cytotoxic against Vero cells.

Croton steenkampianus Gerstner (Euphorbiaceae), commonly known as "Marsh Fever-berry" and "Tonga Croton", is a shrub or tree endemic to restricted areas of central Africa and eastern parts of southern Africa.[1] Various medicinal uses of the genus *Croton* are reported in countries all over the world, and many species are used to treat bleeding, bleeding gums, chest complaints, coughs, fever, indigestion, malaria, and rheumatism.[1] Chemically, the genus contains very diverse compound types including alkaloids, flavonoids, and terpenoids.[2,3] However, no phytochemical reports have appeared for the species *C. steenkampianus*. Medicinally, the vapor from the fresh leaves is used to relieve aches.[1] In this paper, we describe the isolation of six compounds, namely, three known flavonoids, quercetin (**5**), taxmarixetin, and eriodictyol,[4–6] a new indane derivative (**1**), and two new diterpenoids (**2** and **3**), from an ethanol extract of leaves of *C. steenkampianus*. The structures of **1**−**3** were elucidated mainly by 1D and 2D NMR spectroscopic means and, in the case of **2**, by single-crystal X-ray diffraction analysis. In addition, we also report on antiplasmodial activity and cytotoxicity of the isolated compounds.

Repeated chromatographic processes on the EtOH extract of the dried leaves of *C. steenkampianus* allowed the isolation of three flavonoids (quercetin (**5**), taxmarixetin, and eriodictyol) together with the indane derivative **1** and the diterpenoids **2** and **3** (steenkrotins A and B, respectively). The flavonoids were identified by comparison of their data with published values.[4–6]

Low-resolution mass spectrometry and combustion analysis indicated a molecular formula of $C_{12}H_{12}O_3$ for **1**, and its NMR spectroscopic data were in agreement with the structure of 2,6-dimethyl-1-oxo-4-indanecarboxylic acid (**1**). In particular, the HMBC correlations observed for **1** (C-1 with H-2, H_2-3, H-7, and Me-10; C-12 with H-5 and H-7; C-9 with H-2, H_2-3, H-5, and H-7; C-11 with H-5; and C-12 with H-5 and H-7) established a 1-indanone skeleton and a 2,6-dimethyl-4-carboxyl substitution pattern for this new substance. Since **1** did not show any optical rotation between 589 and 365 nm, it was evident that it is a racemic mixture of the two enantiomers at the C-2 asymmetric center, which can be easily racemized through the enolic form of the 1-indanone.

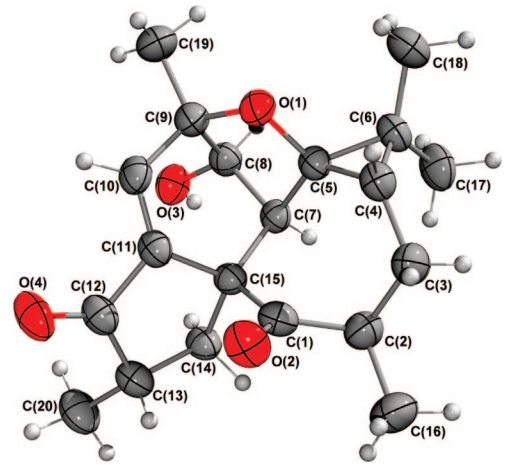

Figure 1. ORTEP diagram for compound **2**.

The structure of steenkrotin A (**2**, $C_{20}H_{26}O_4$) was established from X-ray diffraction studies. Figure 1 is a perspective view of the molecule of **2** showing its relative configuration. Moreover, the 1D and 2D NMR spectroscopic data of **2** (Table 1 and Tables S1 and S2, Supporting Information) were in complete agreement with the proposed structure.

Steenkrotin B (**3**, $C_{20}H_{28}O_7$) showed ^{1}H and ^{13}C NMR spectra very similar to those of **2** (Table 1). The observed differences were consistent with the presence in **3** of a hydroxyisopropyl group [δ_H 1.45 and 1.34 (Me-16 and Me-17); δ_C 22.3 and 25.7 (C-16 and C-17), and 87.6, qC, C-15] and a hemiacetal carbon (δ 110.1, qC, C-14) instead of the pentasubstituted cyclopropane of **2**. This structural difference was also supported by the HMBC spectra (Table S1, Supporting Information), because **2** showed correlations through three bonds between C-15 and H-8 and between C-14 and Me-16 and Me-17 that were not observed for **3**. Three of the seven oxygen atoms of **3** were involved in two ketones [δ 206.5 (C-3) and 212.4 (C-10)] and in a secondary hydroxyl group [δ_H 4.30; $\delta\mu_C$ 75.6, CH (C-7)], as in **2**, and two other oxygens must be placed at C-14 and C-15 as one of the hemiacetal oxygens and as a tertiary hydroxyl group, respectively (see above). The remaining two oxygens of **3** must be part of an endoperoxide moiety between C-6 (δ 68.6, qC) and the hemiacetalic C-14 carbon. The presence of this endoperoxide was also in agreement with molecular formula

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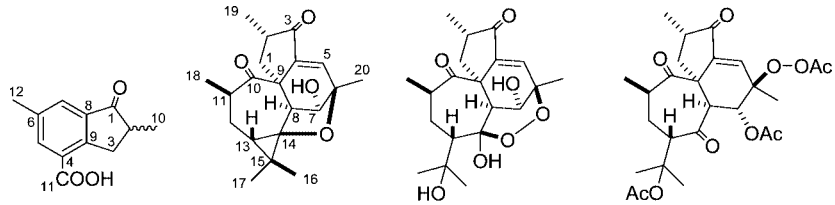
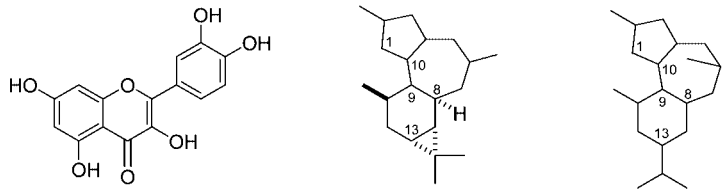
Table 1. NMR Spectroscopic Data [400 (^{1}H) and 100 (^{13}C) MHz, $CDCl_3$] for Compounds **2**−**4**[a]

	2		**3**		**4**	
position	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1[b]	35.9, CH_2	1.98, dd (12.5, 11.4) 2.20, dd (12.5, 8.4)	39.5, CH_2	2.13, dd (13.1, 12.5) 2.63, dd (13.1, 7.2)	38.8, CH_2	1.72, dd (12.8, 11.3) 2.15, dd (12.8, 8.4)
2	39.73, CH	2.31, ddq (11.4, 8.4, 7.0)	39.2, CH	2.27, ddq (12.5, 7.2, 7.0)	38.7, CH	2.52, ddq (11.3, 8.4, 7.0)
3	206.5 qC		206.5, qC		204.7, qC	
4	143.7, qC		144.6, qC		142.7, qC	
5	134.5, CH	6.50, s	129.7, CH	6.36, d (0.8)	130.4, CH	6.69, d (1.0)
6	75.1, qC		68.6, qC		74.9, qC	
7	79.2, CH	4.22, d (3.9)	75.6, CH	4.30, dd (2.5, 0.8)	71.4, CH	5.60, dd (2.3, 1.0)
8	40.8, CH	2.69, d (3.9)	36.5, CH	3.05, d (2.5)	44.6, CH	4.44, dd (2.3, 0.8)
9	62.6, qC		55.6, qC		52.8, qC	
10	213.1, qC		212.4, qC		211.3, qC	
11	39.73, CH	2.95, ddq (12.5, 6.8, 6.4)	44.5, CH	3.08, ddq (7.4, 7.1, 3.4)	43.1, CH	3.79, ddq (10.7, 2.4, 6.7)
12[b]	29.6, CH_2	1.67, ddd (14.3, 6.8, 2.4) 1.83, ddd (14.3, 12.5, 10.1)	29.4, CH_2	1.93, ddd (15.7, 8.9, 3.4) 1.79, ddd (15.7, 7.1, 4.9)	29.3, CH_2	2.22, ddd (16.3, 4.5, 2.4) 2.07, ddd (16.3, 6.7, 4.5)
13	25.3, CH	0.68, dd (10.1, 2.4)	60.3, CH	2.93, dd (8.9, 4.9)	61.2, CH	2.66, td (4.5, 0.8)
14	74.1, qC		110.1, qC		208.2, qC	
15	22.8, qC		87.6, qC		87.4, qC	
16	20.7, CH_3	1.03, s	22.3, CH_3[c]	1.45, s[c]	24.4, CH_3[c]	1.48, s[c]
17	16.5, CH_3	1.07, s	25.74, CH_3[c]	1.34, s[c]	24.7, CH_3[c]	1.44, s[c]
18	17.0, CH_3	1.06, d (6.4)	18.0, CH_3	1.27, d (7.4)	18.3, CH_3	1.20, d (6.7)
19	14.9, CH_3	1.04, d (7.0)	14.7, CH_3	1.07, d (7.0)	15.6, CH_3	1.06, d (7.0)
20	21.8, CH_3	1.43, s	25.70, CH_3	1.47, s	20.9, CH_3	1.52, s
OOAc-6β				[d]	167.0, qC 17.3, CH_3	2.08, s
OAc-7α				[d]	169.9, qC 21.1, CH_3	2.05, s
OAc-15				[d]	168.5, qC 21.0, CH_3	1.87, s

[a] Assignments were in agreement with COSY and HSQC spectra and with 1D NOESY experiments. [b] For methylene groups, the first reported δ value belongs to the α-proton and the second δ value is assigned to the β-proton. [c] Interchangeable assignments. [d] Hydroxyl protons at δ 5.89 br s, 3.56 br, and 2.16 s.



1 **2** **3** **4**



5 **6** **7**

requirements (seven unsaturations) and with the observed loss of O_2 (ion at m/z 348) from the molecular ion (m/z 380) in the EIMS of **3**.[7]

Treatment of **3** with acetic anhydride−pyridine yielded the triacetyl derivative, **4** ($C_{26}H_{34}O_{10}$), the IR spectrum of which was devoid of hydroxyl absorptions. In its ^{13}C NMR spectrum, the methyl carbon of one of the acetates (δ_C 167.0, qC and 17.3, CH_3; δ_H 2.08) appeared unusually shifted (δ_C 17.3), indicating the presence of a peroxyacetate function,[8,9] which is also in agreement with molecular formula requirements. Thus, apart from the esterification of the hydroxyl groups at C-7 and C-15, the acetylation of **3** caused the hydrolysis of the 14-hemiacetal and subsequent acetylation of the resulting 6-hydroperoxide, as was also evidenced by the appearance of an additional ketone at C-14 (δ_C 208.2, HMBC correlated with H-7, H-8, H_2-12, and H-13, Table S1, Supporting Information) in the ^{13}C NMR spectrum of **4**.

The relative stereochemistry of **3** and **4** must be identical to that of **2** for the following reasons: NOE experiments (Table S2, Supporting Information) established that H-8 and H-11 are in a *cis* spatial relationship in **2** and **4**, because a strong NOE enhancement was observed in H-11 when H-8 was irradiated and vice versa. Consequently, Me-18 must be *trans* (β-oriented) with respect to H-8. Moreover, the NOE observed between H-13 and Me-18 in the 1D NOESY spectra of **2**−**4** was in agreement with a β-orientation of H-13. In the case of **2**, a *cis* arrangement between H-8α and the 1-methylene protons was supported by weak NOE enhancements, but no NOEs between these protons were observed for **3** and **4**. However, the similar ^{1}H NMR chemical shifts and the almost identical coupling constant values for H_2-1, H-2, and Me-19 in **2**−**4** (Table 1), together with the absence of NOE between Me-18 and Me-19, supported an α-orientation for the 1-methylene and Me-19 groups in **3** and **4**, as was established for **2** by X-ray crystallographic

Table 2. Antiplasmodial Activities and Cytotoxicity (μM) of Isolates against Chloroquine-Susceptible Strains (D10 and D6) and the Chloroquine-Resistant Strains (Dd2 and W2) of *Plasmodium falciparum*

	antiplasmodial activity (IC_{50})				
sample	D6	W2	D10	Dd2	cytotoxicity (IC_{50})
ethanol extract[a]	nt[b]	nt[b]	8.60	5.80	45.0
1	>49.0	>49.0	>49.0	>49.0	1216.7
2	>30.0			9.40	107.0
3	>26.3	9.1	15.8	>26.30	805.0
quercetin (**5**)	nt[b]	>26.3	>26.3		110.0
tamarixetin	nt[b]			2.40	170.3
eriodictyol	nt[b]	nt[b]	1.10	nt[b]	nt[b]
chloroquine[c]		nt[b]	nt[b]	nt[b]	
	0.03	0.06	0.02	0.14	48.5

[a] Activity recorded in μg/mL. [b] nt = not tested. [c] Positive control substance.

analysis. In addition, one of the H_2-1 protons of **3** (δ 2.63) showed a NOE with the proximal Me-18 group, and this proton must be the β-oriented one, which in turn displayed a strong NOE with H-2 and no NOE with Me-19, thus establishing an α-orientation for Me-19. This conclusion was also supported by the 1D NOESY behavior of **4**, which showed a weak NOE in H-2 (+1%) and a medium NOE in Me-19 (+3.4%) when H-1α (δ 1.72) was irradiated. For structural requirements, the α-orientation of H-8 in **2** requires a 6β, 14β closure of its cyclic ether, and **3** must also have the same stereochemistry for its endoperoxide, because steenkrotin B (**3**) also has its H-8 proton α-oriented (see above). Consequently, the 6-peroxyacetate of **4** must be β-oriented.[10]

The cyclohexene ring of **3** and **4** adopts an envelope conformation with the flap at C-7, like in **2** (Figure 1), and H-7β and OH-7α (or OAc-7α in **4**) are equatorial and axial substituents, respectively, thus explaining the similar $J_{7\beta,8\alpha}$ values (Table 1) and the NOE behavior of these compounds (Table S2, Supporting Information).

NOE experiments also allowed the assignment of the Me-16 and Me-17 groups in **2**, as it is shown in the formula and Table S2 (Supporting Information). Irradiation at δ 1.43 (Me-20) caused NOE enhancement only in one of the two C-15 methyls (δ 1.03, Me-16), whereas the signal of the other one (δ 1.07, Me-17) was enhanced when H-8α was irradiated. The assignment of the configuration for both methylene protons at C-12 in **2**−**4** (Table 1) was also supported by NOE results (Table S2, Supporting Information).

To the best of our knowledge, diterpenoids **2** and **3** possess new carbon skeletons that may be derived from the tigliane (**6**) and daphnane (**7**) types, respectively, by an 8(9→10)-*abeo* rearrangement.[11] It is of interest that diterpenes belonging to **6** and **7** carbon frameworks have repeatedly been found in several *Croton* species and in other Euphorbiaceae genera.[12]

Antiplasmodial testing (Table 2) showed that the ethanolic extract of *C. steenkampianus* demonstrated an IC_{50} activity against the chloroquine-susceptible strain D10 (8.6 μg/mL) and the chloroquine-resistant strain Dd2 (5.8 μg/mL) of *Plasmodium falciparum*. Activity was more pronounced against the resistant strain. Compound **2** displayed antiplasmodial activities of 15.8 (D10), 9.1 (W2), and 9.4 (Dd2) μM. Quercetin (**5**) was active against the D10 and Dd2 strains. The cytotoxicity values for the isolated compounds on Vero cells using MTT assays were all >30 μM, showing that the substances tested (Table 2) are not potent cytotoxic agents.

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler block and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 MC polarimeter. UV spectra were recorded on a Perkin-Elmer Lambda 2 UV/vis spectrophotometer. IR spectra were obtained on a Perkin-Elmer Spectrum One spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution on a Varian INOVA 400 spectrometer at 400 and 100 MHz, respectively. Chemical shifts are reported relative to the residual $CHCl_3$ (δ 7.25) for protons and to the solvent (δ_{CDCl3} 77.00) for carbons. All the assignments for protons and carbons were in agreement with 2D COSY, gHSQC, gHMBC, and 1D NOESY spectra. Mass spectra were registered in the positive EI mode on a Hewlett-Packard 5973 instrument (70 eV) and in the positive or negative ESI mode on a Hewlett-Packard Series 1100 MSD spectrometer. Elemental analyses were conducted on a Leco CHNS-932 apparatus. Merck Si gel (70−230 and 230−400 mesh, for gravity flow and flash chromatography, respectively) was used for column chromatography. Merck 5554 Kieselgel 60 F254 sheets were used for TLC analysis. Petroleum ether (bp 50−70 °C) was used for column chromatography.

Plant Material. Leaves of *Croton steenkampianus* were collected in April 2003, at Tember Elephant Park in northern KwaZulu-Natal, South Africa, and voucher specimens (registry number 92520) are identified and preserved at the H.G.W.J. Schweickerdt Herbarium (PRU) at the University of Pretoria.

Extraction and Isolation. The dried leaves (2 kg) were extracted with ethanol (0.2 kg leaves/L solvent) for 3 days at room temperature. The extract was filtered and concentrated with a rotary evaporator at 40 °C. The extraction procedures were repeated twice. The total extract (80.0 g) was applied to a flash silica gel column, developed with a solvent gradient of hexane−ethyl acetate mixtures of increasing polarity. Similar fractions were combined together, to yield four main fractions, A1−A4. Fraction A2 (10 g) was applied to a silica gel column developed with hexane− ethyl acetate (95:5) to yield pure compounds **1** (148 mg) and **2** (250 mg) and eriodictyol (5 mg). Fraction A4 (3.0 mg) was applied to a silica gel column developed with a solvent gradient of hexane−ethyl acetate (90:10). Subfraction 2 (350 mg) was further chromatographed over Sephadex LH-20, using ethanol as eluent, to give quercetin (**5**, 10 mg), tamarixetin (5 mg), and compound **3** (50 mg).

2,6-Dimethyl-1-oxo-4-indanecarboxylic acid (1): amorphous, white solid; $[\alpha]_D^{20}$ 0, $[\alpha]_{578}^{20}$ 0, $[\alpha]_{546}^{20}$ 0, $[\alpha]_{436}^{20}$ 0, $[\alpha]_{365}^{20}$ 0 (*c* 0.26, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (5.56), 248 (4.02), 310 (3.66) nm; IR (KBr) ν_{max} 3400−2300, 2924, 1709, 1580, 1459, 1423, 1250, 1182, 1133, 927, 836, 693 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.31, (3H, d, J = 7.0 Hz, H-10), 2.45, (3H, s, H-12), 2.72, (1H, ddq, J = 7.9, 7.0, 3.7 Hz, H-2), 3.02, () (H, dd, J = 18.6, 3.7 Hz, H_2-3), 3.77 (1H, dd J = 18.6, 7.9 Hz, H_1-3), 7.79 (1H, d, J = 2.0 Hz, H-7), 8.18 (1H, d J = 2.0 Hz, H-5), 8.60 (1H, br, H-11); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.2 (CH_3, C-10), 20.9 (CH_3, C-12), 36.1 (CH_2, C-3), 42.0 (CH, C-2), 127.1 (qC, C-4), 129.4 (CH, C-7), 137.9 (qC, C-8), 138.0 (CH, C-5), 138.1 (qC, C-6), 153.1 (qC, C-9), 171.3 (qC, C-11), 209.1 (qC, C-1); EIMS *m/z* 204 $[M]^+$ (100), 189 (86), 176 (21), 161 (44), 159 (19), 148 (44), 131 (26), 115 (37), 91 (21), 77 (18), 63 (13), 51 (14); *anal.* C 70.67%, H 5.81%, calcd for $C_{12}H_{12}O_3$, C 70.58%, H 5.92%.

Steenkrotin A (2): colorless needles (EtOAc−petroleum ether), mp 219−221 °C; $[\alpha]_D^{20}$ +188.6 (*c* 0.32, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 227 (3.96) 250 shoulder (3.68) nm; IR (KBr) ν_{max} 3359, 3050, 3020, 2971, 2940, 1702, 1649, 1454, 1375, 1286, 1231, 1112, 1055, 1019, 905, 743, 650 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 330 $[M]^+$ (3), 315 (0.5), 312 (2), 297 (0.5), 262 (60), 244 (27), 227 (14), 187 (18), 177 (62), 161 (30), 160 (26), 145 (16), 121 (29), 97 (35), 91 (14), 83 (13), 77 (14), 69 (100), 55 (29); *anal.* C 72.43%, H 8.25%, calcd for $C_{20}H_{26}O_4$, C 72.70%, H 7.93%.

X-ray Structure Determination of 2.[13] The crystallographic data set was collected at 20 °C on a Siemens P4 diffractometer fitted with a Bruker 1K CCD detector and SMART control software[14] using graphite-monochromated Mo Kα radiation by means of a combination of phi and omega scans. Data reduction was performed using SAINT+,[18] and the intensities were corrected for absorption using SADABS.[18] The structure was solved by direct methods using SHELXTS[14] and refined by full-matrix least-squares using SHELXTL[14] and SHELXL-97.[15] All hydrogen atoms for the structure of **2** were located experimentally. In the refinement, the hydrogen atoms were refined without any positional constraints. Isotropic displacement parameters for the non-methyl hydrogen atoms were calculated as 1.2 × U_{eq} of the atom to which they were attached, and the corresponding value for methyl hydrogen atoms was 1.5 × U_{eq} of the carbon atom to which they were attached. All non-hydrogen atoms were refined with anisotropic displacement parameters. Drawings of the structure were

produced using ORTEP-3 for Windows,[16] Mercury,[17] and POV-Ray for Windows.[18]

Steenkrotin B (3): colorless fine needles (MeOH), mp 130−132 °C (dec); $[\alpha]_D^{20}$ +24.4 (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 233 (3.98) nm; IR (KBr) ν_{max} 3435, 2976, 2932, 1731, 1691, 1676, 1457, 1372, 1194, 1078, 901 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive mode ESIMS *m/z* 403 $[M + Na]^+$, 783 $[2M + Na]^+$; negative mode ESIMS *m/z* 379 $[M - H]^-$; EIMS *m/z* 380 $[M]^+$ (0.3), 362 (1), 348 (1), 338 (25), 305 (19), 264 (27), 245 (21), 219 (27), 203 (38), 187 (45), 177 (56), 161 (55), 152 (39), 141 (43), 121 (40), 95 (31), 91 (41), 85 (41), 83 (45), 71 (57), 69 (54), 55 (100); *anal.* C 63.31%, H 7.36%, calcd for $C_{20}H_{28}O_7$, C 63.14%, H 7.42%.

Acetylation of Steenkrotin B (3). Treatment of **3** (15.0 mg) with Ac_2O−pyridine (1:1, 4.0 mL) for 24 h at room temperature, followed with evaporation of the volatiles at 50 °C under reduced pressure, yielded a residue, which was chromatographed on a silica gel column using 10% ethyl acetate in hexane to give **4** (8 mg, 53.0% yield): amorphous, white solid; $[\alpha]_D^{20}$ −99.3 (*c* 0.29, $CHCl_3$); IR (KBr) ν_{max} 2929, 1745, 1736, 1706, 1694, 1457, 1369, 1225, 1102, 1076, 1039, 945, 922, 849 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; positive mode ESIMS *m/z* 529 $[M + Na]^+$, 1035 $[2M + Na]^+$; *anal.* C 61.83%, H 6.80%, calcd for $C_{26}H_{34}O_{10}$, C 61.65%, H 6.77%.

Antiplasmodial Bioassay. The antiplasmodial activity against strains of *Plasmodium falciparum* (Table 2) was determined as previously described.[19–21]

Cytotoxicity. The cytotoxicity was determined as previously described.[22]

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Supporting Information Available: Tables of 2D NMR data for **1**−**4** and crystallographic data of **2**. This information is available free of charge via the Internet at http://pubs.acs.org.

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